Summer 2021

Effects of Land-based Sources of Pollution on Coral Thermotolerance

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EFFECTS OF LAND-BASED SOURCES OF POLLUTION ON CORAL THERMOTOLERANCE

A Thesis

Presented to the

Faculty of

Moss Landing Marine Laboratories

California State University, Monterey

Bay

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Marine Science

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by

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Summer 2021

CALIFORNIA STATE UNIVERSITY MONTEREY BAY

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ACKNOWLEDGEMENTS

This thesis project would not have been possible without the collaboration, support, and advising of many others. Foremost, I would like to thank my primary research advisor Dr. Cheryl Logan, who has shaped my scientific approach and guided me in overcoming every obstacle on this project. She always delivers insightful feedback with kindness and has helped me gain confidence in my research skills and presentations. I thank her for introducing me to the wonders of coral physiology, open data science, effective science communication and the many other skills I have learned throughout my Master's. Additionally, I would like to thank my MLML-advisor Dr. Amanda Kahn for her enthusiasm about my project and her helpful guidance on statistics and in organizing my thesis. She always makes the research process fun and I thank her for showing me all the wonderful intertidal invertebrates around Monterey Bay. I also would like to thank my thesis committee member Dr. Maxime Grand for his insight into the role of pollution in this project and his suggestions for my water quality measurements in American Samoa. Thank you to all three of my committee members for their support, guidance, and excellent feedback on this thesis project.

Thank you to the amazing undergraduates who have helped both in the field and lab components of this project. First, thanks to Jennifer Grossman, without who I would never have been able to collect the field data needed for this project. I thank her for spending long hours testing and debugging the CBASS assay, and always encouraging us to take a break and eat ice cream afterwards. Another big thank you to Casey Juliussen, who was also integral to our field work in American Samoa. Thanks to both Jenny and Casey for traveling with me to American Samoa, lugging around over ten suitcases, exploring the most polluted waters we could find, and spending long hours watching corals bleach. Another big thanks to Dr. Taylor Sale, who helped me learn to extract DNA from coral samples and boosted my confidence in a molecular lab. Thanks to the MSCI430/530 class at CSUMB for their help with the RNAseq portions of this project. And thank you to Silvia Vasquez, Bella Gutierrez, and Amalia Siegal for assisting with DNA extractions and qPCR for the 2014 symbiont analysis that motivated this project.

I would also like to thank everyone else that made the field work in American Samoa possible. Thank you to Hideyo Hattori, Hanae Spathias, Meagan Curtis, and Georgia Coward for answering questions about research on Tutuila and for help with coordination. Thanks to Dr. Christopher Schuler for advice on site selection in Tutila and to Marieke Sudek and Eric Brown for assistance with permitting. Finally, thanks to Sonny and Ruth for a lovely homestay in American Samoa that made us feel at home.

I would also like to thank funding sources that made travel to Tutuila possible including the Myers Trust, CSUPERB, and the Explorer's Club. Another thank you to other funding including UROC, COAST, the Women's Leadership Council, and the Evolving Seas RCN, which helped support me, my research, and travel during my graduate career.

A big thank you to my fellow Logan Lab members for all their support throughout this program. Thanks to Holly Doerr for assisting my RNA extractions and to Jacoby Baker, Juliana Cornett and Arie Dash for their help with cDNA library preparation. I'd also like to thank Caroline Rodriguez for always being available to talk through research questions and problems. I'd also like to thank Steve Ryan for assistance with CBASS debugging and teaching me to solder. Thanks to all the Logan Lab members for their guidance, feedback, and friendship over the years.

Finally, I would like to thank my family and friends who helped support me throughout my time in this program. My father, Jonathan Naugle, grandfather, Dr. Kurt Kohn, and of my friends for their endless support. Thank you to my partner, Bryan van Orman, for always encouraging me and supporting me. And a special thank you to my mom, Julia Robinson, who would have been so proud to watch me chasing my dreams.

ABSTRACT

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California State University Monterey Bay, 2021

Phenotypic plasticity is one way that species may cope with stressful environmental changes associated with climate change. Reef building corals present a good model for studying phenotypic plasticity because they have experienced rapid climate-driven declines in the past thirty years, often with differential survival among individuals during heat stress. One potential reason for underlying differences in thermotolerance may be due to differences in baseline levels of environmental stress. Stress associated with pollution has been shown to produce synergistic effects with heat stress, exacerbating the physiological damage of heat stress. Conversely, it is possible that mild pollution stress could prepare corals to better cope with heat stress via cross tolerance mechanisms. Cross tolerance occurs when a mild stressor prepares an organism for more extreme, subsequent stress, reducing the impact of that stressor on the organism. To examine these two possibilities, acute heat stress experiments were conducted on *Acropora hyacinthus* from five sites around Tutuila, American Samoa with differing pollution impact. Bleaching responses were measured visually, using photographic assessment to estimate chlorophyll content, and using pulse amplitude fluorometry to measure photosynthetic efficiency. Endosymbiont community composition was assessed at each site using quantitative PCR. RNA sequencing was used to compare differences in genes expression patterns prior to and during heat stress. Symbiont communities differed among sites, with heat tolerant *Durusdinium* dominating in areas with higher pollution impact and heat sensitive *Cladocopium* relatively more common in pristine areas. Pollution stress may induce a shift towards *Durusdinium* thereby enhancing resistance to subsequent heat stress in the near term. Gene expression patterns showed few differences correlating to site or pollution level. Thermotolerance, however, did correlate with gene expression patterns, both during heat stress and under control conditions. In this thesis, I present potential mechanisms underlying coral thermal tolerance in pollution-impacted areas. Our results highlight the importance of measuring pollution impacts on thermotolerance and identifying heat tolerant corals in "non-pristine" areas and their potential to seed nearby reefs following bleaching events.

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INTRODUCTION

Anthropogenic climate change presents a bleak future for many species with major declines in global biodiversity predicted (Bellard et al. 2012). Rising global temperatures and other changing environmental conditions are predicted to push many species past their physiological limits (Tomanek 2008). To avoid extinction, species may respond to this environmental stress in three ways: 1. Range shifts to more favorable environmental conditions 2. Natural selection and subsequent evolution towards more suitable genotypes, or 3. Plastic responses that allow changes in physiology without changes in genotype (Holt 1990; Davis et al. 2005; Gienapp et al. 2008; Hofmann and Todgham 2010; Nogués-Bravo et al. 2018; Catullo et al. 2019). Some combination of these three responses is necessary for species persistence when environmental changes exceed their physiological limits (Davis et al. 2005; Gienapp et al. 2008).

Species range shifts have now been well documented (Chen et al. 2011). However, if species cannot adjust their range to keep up with changing climate, they must adjust their physiology to persist. Species may adaptively evolve via changes in allele frequencies towards more suitable genotypes to tolerate their changing climate (Hoffmann and Sgrò 2011). Yet, since climate change is occurring at unprecedented rates (IPCC 2018), phenotypic plasticity may be especially important for longer-lived organisms since it can occur over remarkably short time scales compared to genetic adaptation (Hendry et al. 2008). Plasticity has been found to be particularly important in studies that attempt to disentangle it from adaptation, though this has proven an especially difficult question to test (Gienapp et al. 2008; Hendry et al. 2008; Merilä and Hendry 2014). Plasticity refers to the change in phenotype in response to a change in environment without a change in genotype (Scheiner 1993). Though plasticity may occur over multiple generations (termed trans-generational plasticity), plasticity in the context of this thesis focuses on within-generation plasticity (Jablonka and Raz 2009). It is also worth noting that plasticity itself can evolve and may be adaptive or maladaptive and therefore should also be considered in the context of evolution (Scheiner 1993). This study examines plasticity within one generation, though future work should also incorporate multi-generational processes.

Reef building corals present a good model for studying phenotypic plasticity because they have experienced rapid climate-driven declines in the past twenty years, often with

differential survival among individuals (Marshall and Baird 2000; West and Salm 2003; Hughes et al. 2017). Since corals are tropical animals that already live close to their thermal maximum global climate change is expected to exacerbate these effects, leading to the continued decline of corals around the world (Hughes et al. 2017; Tewksbury et al. 2008). However, some coral communities, species, and genotypes have been shown to be 'winners' and others 'losers' in this battle to tolerate a changing environment. Some corals exhibit greater thermotolerance: the ability to tolerate physiologically stressful temperatures. More thermotolerant corals are more likely to resist bleaching: the expulsion of the coral's endosymbionts. For example, some reefs in Hawaii bleached more than others during mass bleaching events (Jokiel and Brown 2004). This was likely at least partially due to differences in environment variables such as cloud cover and water depth, but differences in plasticity likely also played a role in determining thermotolerance (Jokiel and Brown 2004). Some coral species or populations have greater thermotolerance or greater capacity for plasticity, allowing them and the communities they make up to persist in changing climate better than others (Barshis et al. 2013; Grottoli et al. 2014; Kenkel and Matz 2016; Thomas et al. 2019). Finally, corals of the same species occupying the same reef have been shown to differ in their thermotolerance, which may be due to plasticity, genetic differences or variations in microenvironments (Jokiel and Brown 2004; Cornwell et al. 2020). Corals are a good candidate for studies investigating plastic responses to climate change because they have high species-level and individual-level differences in thermotolerance. Corals are stationary and have limited dispersal capabilities to escape climate change stress, so they must adaptively respond in order to persist, through adaptation or plasticity (Catullo et al. 2019).

Plasticity can occur at multiple levels within a coral 'holobiont.' A coral holobiont is the coral organism plus all the associated micro-organisms (e.g., bacteria and viruses) and symbiotic algae that live within the coral tissue. For example, plasticity can occur within the coral animal itself (e.g., gene expression shifts to increase heat shock proteins or antioxidants during thermal stress; Dixon et al. 2020), or within the members of the coral holobiont community that contribute to coral thermotolerance (e.g., endosymbiont shifts towards more heat tolerant species or 'symbiont shuffling'; Berkelmans and van Oppen 2006). While other plasticity processes exist (e.g. transgenerational plasticity; Jablonka and Raz 2009), gene expression shifts within the coral host and symbiont shuffling are well-studied mechanisms by which coral are known to adjust

their thermotolerance within a lifetime of an individual and will be the focus of this study (Berkelmans and van Oppen 2006; Thomas et al. 2018; Thomas et al. 2019).

Below, I review what is known about how these plastic mechanisms enable corals to respond to temperature and pollution stress and what is known about how they might respond to combined heat and pollution stress.

Plastic Responses to Heat Stress

During environmental stress, organisms can counteract macromolecular damage through a cellular stress response (CSR), inducing a suite of gene and protein expression changes (Hochachka and Somero 2002; Kültz 2005; Evans and Hofmann 2012). The conserved CSR is triggered when environmental stress begins to damage macromolecules, including proteins, nucleic acids, and membranes, which can impair physiological function and disrupt cellular homeostasis (Somero 2020; Kültz 2020). To maintain cellular homeostasis and mitigate damage, the CSR is critical to repair macromolecules, modify energy metabolism, regulate cell proliferation, and initiate cell death in cells with excessive damage (Kültz 2005; Evans and Hofmann 2012; Somero 2020). In corals, as well as virtually all other organisms, the CSR is highly conserved and has diverged little throughout history (Kültz 2005; Kültz 2020). Since different stressors often produce similar types of macromolecular damage, the CSR often induces highly similar suites of responsive genes (Evans and Hofmann 2012; Dixon et al. 2020). These CSR gene suites occur in tiers, depending on the timing and intensity of the stress (Evans and Hofmann 2012). For heat stress specifically, the early CSR is deemed the 'heat shock response' which is characterized by the production of molecular chaperones, especially heat shock proteins, along with other proteins to mitigate thermal damage (Lindquist 1986; Hochachka and Somero 2002; Somero 2020). This often comes at the cost of downregulating genes involved in growth, typically to maintain the organism's energy budget (Zakrzewska et al. 2011). In corals, this upregulation of stress-related genes and down-regulation of growth-related genes has been repeatedly shown to be triggered by heat stress and results in an increase in thermotolerance (DeSalvo et al. 2010; Thomas et al. 2019; Dixon et al. 2020).

Another plastic process known as symbiont shuffling also contributes to coral thermotolerance. Symbiont shuffling is the process whereby a coral's symbiont community composition shifts to change the proportions of each symbiont species, which can boost

thermotolerance (Buddemeier and Fautin 1993; Baker 2003; Baker et al. 2004). Under mild heat stress, corals may experience acute bleaching, allowing the opportunity for a shift in their symbiont community (Buddemeier and Fautin 1993). There are at least nine distinct lineages of *Symbiodiniaceae*, the family of coral-dwelling symbionts, with certain species conferring greater host thermotolerance than others (Baker 2003; Tchernov et al. 2004; Ladner et al. 2012; LaJeunesse et al. 2018). However, there are limits to the increase in thermotolerance due to symbiont shuffling and not all coral species can "shuffle" (Goulet 2006). Once symbiont shuffling has occurred and corals host entirely one species of symbiont, they may have maximized their ability to increase symbiont-driven thermotolerance (Howells et al., 2020) or they may revert back to less heat tolerant symbiont communities once the heat stress has diminished (Thornhill et al. 2006). As with gene expression shifts, symbiont shifts also are thought to be accompanied by tradeoffs with growth (Cunning et al. 2015). Corals that host more thermotolerant symbiont species exhibit lower growth rates than those that host less tolerant symbionts (Jones and Berkelmans 2010). Corals may employ both gene expression shifts and symbiont shuffling simultaneously to tolerate heat stress, and by utilizing both of these plasticity strategies, may improve their thermotolerance (Thomas et al. 2019). Symbiont shuffling alone may improve thermotolerance by up to 1-1.5°C, but the improved thermotolerance due to a combination of symbiont shuffling and changes in gene expression is not yet known (Berkelmans and van Oppen 2006).

Responses to Pollution Stress

While gene expression shifts and symbiont shuffling have been well studied in response to temperature stress, fewer studies have investigated how these processes respond when temperature stress interacts with local stressors such as pollution. Land-based pollution can bring nutrients and sediments into marine environments, affecting corals and their symbionts (Silbiger et al. 2018). This pollution can have a variety of direct and indirect effects on the coral holobiont, ranging from the organismal scale (e.g. changes in growth rate, calcification rate, increased symbiont densities) to the ecological (e.g. higher prevalence of disease, outbreaks of corallivorous starfish, and increased competition with macroalgae) (Stambler et al. 1991; Koop et al. 2001; McCook et al. 2001; Voss and Richardson 2006; Fabricius et al. 2010; D′Angelo and Wiedenmann 2014).

Nutrient and sediment pollution may also affect holobiont thermotolerance. Sediment pollution may reduce light stress and thus increase thermotolerance, or nutrient pollution may cause symbiont densities to spike which would increase light absorption and thus reduce thermotolerance (Cunning and Baker 2013; D′Angelo and Wiedenmann 2014). There have been multiple accounts of elevated nutrients lowering thermotolerance (Wooldridge 2009; Wiedenmann et al. 2013; Donovan et al. 2020) or improving thermotolerance (Béraud et al. 2013; Zhou et al. 2017; Morris et al. 2019). Since 'pollution' is a broad term that encompasses highly variable environments, its effects on coral thermotolerance may be context dependent. These discrepancies in how pollution affects thermotolerance may be partially explained by differences in nutrient levels and ratios among nutrient concentrations (Wiedenmann et al. 2013; D′Angelo and Wiedenmann 2014; Morris et al. 2019). Additionally, differences in the source of nitrogen can affect how corals respond (Burkepile et al. 2020; Fernandes de Barros Marangoni et al. 2020). Laboratory studies have shown that thermotolerance is increased by moderate levels of ammonium (~0.3 μM) but is decreased by nitrate unless accompanied by phosphate (Rosset et al. 2017; Morris et al. 2019). These moderate nutrient additions can increase symbiont densities, which can increase holobiont health and thermotolerance, but imbalanced N:P ratios can disrupt the symbiosis and lead to bleaching (Rosset et al. 2017). While the effects of nutrient pollution have been well-studied in lab-controlled studies, there are fewer instances of field-based assessments of pollution and thermotolerance (Wooldridge 2009). Further, there are fewer accounts of mechanistic explanations for field-based studies of pollution affecting thermotolerance.

Responses to Heat and Pollution Stress

Long-term pollution stress may either increase or decrease coral thermotolerance to acute heat stress. When corals are exposed to multiple stressors, many studies have shown synergistic effects, whereby the cumulative effect of two stressors is greater than each stressor independently (Kersting et al. 2015; Towle et al. 2016; Ellis et al. 2019). A recent meta-analysis showed that local stressors including pollution exacerbate coral loss due to heat stress (Donovan et al. 2021). More specifically, one study found that pre-exposure to nutrient stress can exacerbate mortality due to heat stress (Zaneveld et al. 2016). However, other studies have shown that multiple stressors can produce antagonistic effects in corals, whereby the cumulative

effect of two stressors is less than each stressor independently (Zhou et al. 2017; Marangoni et al. 2019; Darling et al. 2020). Since the CSR is similar even for different types of environmental stress, it is possible that if mild long-term pollution stress triggers macromolecular damage and increases constitutive expression of CSR genes, this could lead to higher thermotolerance during heat stress (**Figure 1**). This may be due to the 'frontloading' of stress response genes in polluted waters, whereby the baseline gene expression more closely resembles CSR gene expression, better preparing the coral to tolerate acute stress (Barshis et al. 2013; Thomas et al. 2018). It is also possible that long-term pollution stress has induced symbiont community shifts in favor of more stress-tolerant symbionts, leading to higher thermotolerance. This concept of increased tolerance to one stressor due to exposure to a different stressor is termed "cross-tolerance" and has been demonstrated in many species (Li and Hahn 1978; Sabehat Adnan et al. 1998; Ely et al. 2014; Gunderson et al. 2016). A similar concept, "pre-conditioning" or "stress-hardening," can also be applied when mild exposure to an environmental stressor results in more tolerance to a subsequent stronger exposure to the same stressor compared with no exposure at all.

Pre-exposure has been well explored in corals (e.g. inducing mild heat stress improves thermotolerance during later, more significant heat stress; Maynard et al. 2008; Thompson and van Woesik 2009; Bellantuono, Hoegh-Guldberg, et al. 2012). The mechanisms underlying preconditioning could be symbiont shuffling or gene expression shifts (e.g. increasing basal levels of CSR expression), though gene expression shifts tend to act over shorter time scales than symbiont shuffling (Bellantuono, Granados-Cifuentes, et al. 2012; Silverstein et al. 2015). Fewer studies have examined these mechanisms during two different sources of environmental stress in corals, where they would be categorized as 'cross-tolerance' rather than 'pre-conditioning' (Towle et al. 2016). A recent meta-analysis showed that *Acropora* corals exhibit a generalized stress response, expressing similar genes regardless of the source of environmental stress, as long as the intensity of the stress was similar (Dixon et al. 2020). A study that specifically measured gene expression during heat and nutrient stress found a number of stress response genes in common between the two stressors (Rosic et al. 2014). Zhou et al. (2017) also measured heat and nutrient stress simultaneously and found that elevated ammonium concentrations buffered gene expression shifts during heat stress, especially genes involved in tumor necrosis factor signaling, cell death, and apoptosis, which are all typically involved in the cellular stress response. This

evidence suggests the possibility of a cross-tolerance effect that may buffer nutrient-stressed corals from heat stress.

If pollution helps to induce thermotolerance in corals, this may present a "protection paradox" for conservation managers who typically focus on reducing local stressors on coral reefs (Bates et al. 2019). This paradox arises when a protected area with reduced human impact is more affected by perturbation (e.g., a storm or bleaching event) compared to a higher impact area. Higher impacted areas may favor hardier genotypes or species that are more likely to persist during perturbation. Lower impacted areas may have a higher proportion of vulnerable species or genotypes that will be lost during perturbation. This might suggest a portfolio approach to coral marine conservation, where both pristine and polluted sites are targeted for protection and for selecting corals used in nurseries and outplanting programs (Bates et al. 2019; Walsworth et al. 2019). The strategy for seeking resilient corals has historically been to search in pristine, less impacted reefs, but there may be potential resilience value in more impacted reefs as well. It is important to consider adaptive potential when determining coral management plans to protect heat-resistant corals, cooler refuge habitat, and the habitat connectivity between these areas (Walsworth et al. 2019). If interventions such as selective breeding or coral nurseries are considered, identification of the most heat resistant corals is vital, since heat-tolerant parents generate more resilient nursery stock (Morikawa and Palumbi 2019). These management needs further support the motivations for this study of mechanisms of pollution-induced plasticity that build thermotolerance.

Research Questions

This thesis investigates the interaction between *in situ* exposure to chronic land-based sources of pollution and a short-term heat stress event in setting coral thermal tolerance. My study site is the main island of Tutuila in American Samoa where a mosaic of land-based pollution impacts is situated near relatively pristine reefs (Houk et al. 2005; Comeros-Raynal et al. 2019; Shuler et al. 2019). Tutuila is an ideal location to test plastic responses to nutrient pollution and thermotolerance in a field setting. I chose to conduct this study on *Acropora hyacinthus,* a common 'weedy' coral species that is faster growing but more vulnerable to bleaching than other coral species (Linares et al. 2011). *A. hyacinthus* is well-studied ecologically with abundant phenotypic and genomic data (Barshis et al. 2013). While the

remarkably thermotolerant *A. hyacinthus* on nearby islands such as Ofu have been well studied, there are fewer data assessing thermotolerance among Tutuila's *A. hyacinthus* (Craig et al. 2001; Schumacher et al. 2018; Thomas et al. 2019). This study provides updated assessments of differences in thermotolerance among *A. hyacinthus* on Tutuila and investigates potential mechanisms of plasticity that may account for those differences.

If chronic exposure to pollution stress induces cross-tolerance to acute thermal stress in a field setting, corals dwelling on more polluted reefs will likely exhibit greater thermotolerance than corals on pristine reefs. High thermotolerance on polluted reefs may be accompanied by differences in gene expression shifts consistent with cross-tolerance and/or symbiont communities with higher proportions of more thermotolerant species. Alternatively, corals on pristine reefs may exhibit greater thermotolerance, due to the reduction of other stressors (Donovan et al. 2021). High thermotolerance on pristine reefs may occur if different genes are needed to respond to pollution stress and heat stress, or if pollution induces symbiont shifts to symbiont species that are less thermotolerant. Finally, there may be no difference in thermotolerance among differently polluted reefs, with similar symbiont communities and gene expression patterns across sites. I expect that gene expression profiles and/or symbiont community composition will partially explain variation in thermotolerance around the island of Tutuila, and that other factors such as thermal history, oceanography, and reef type may also play a role. I tested these hypotheses through an acute heat stress assay on corals from differently polluted reefs around Tutuila, followed by an assessment of how symbiont communities and gene expression during heat stress vary by pollution level.

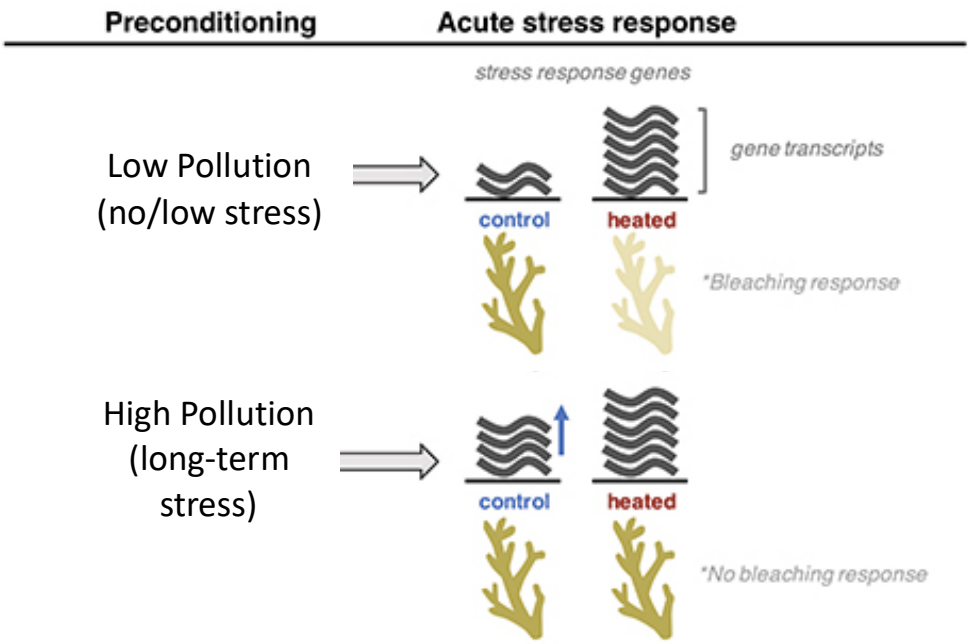

Figure 1. Hypothesized acute heat stress response to low and high pollution preconditioning. Corals previously exposed to high pollution may display 'frontloading' of stress response genes and could be better suited to tolerate acute heat stress compared to those in a low pollution treatment. This could result in a bleaching response in corals exposed to low pollution and not in corals exposed to higher chronic levels of pollution. Adapted from Thomas et al. (2018).

METHODS

Study System

Inhabited islands across the Indo-Pacific experience pollution from human populations, and this is generally thought to negatively affect coral reefs. Potential interactions between pollution and temperature stress can be studied using a representative island with mosaics of pollution impact. Tutuila, the largest island in American Samoa provides an ideal study site, with areas that are relatively pristine nearby to areas that have been polluted for decades. Pollution in Tutuila stems from three main sources: agriculture runoff, piggeries, and on-site disposal systems. These sources bring nutrients and sediments into the seawater and affect water quality, particularly affecting the concentrations of nitrogen and phosphorus (Shuler et al. 2017).

Tutuila reefs have been assessed for their climate change resilience potential by accounting for characteristics such as pollution, sedimentation, herbivory, macroalgae cover, coral diversity, coral recruitment, disease prevalence, bleaching resistance, physical impacts, fishing pressure, and sea surface temperature variability (**Figure 2,** Schumacher et al. 2018). This assessment found that the reefs most resilient to climate change were found on the Northeast section of Tutuila and in areas of lower population density (Schumacher et al. 2018). However, this assessment did not include any experimental heat stress assays to quantify thermotolerance, nor any measurement of gene expression during heat stress or symbiont community differences. Tutuila's coral cover, one indicator of general coral health, varies by reef and ranges from less than 10% to almost 40% (Schumacher et al. 2018).

To determine sampling sites, I began by compiling resources from the American Samoa Environmental Protection Agency (Tuitele et al. 2019) and dissolved inorganic nitrogen (DIN) measurements from Schuler and Comeros-Raynal (2020) from seven sites that were previously studied in a similar set of heat stress assays in 2014 (Oliver and Logan, unpublished) in order to compare changes in thermal physiology over time (**Table 1**). DIN load (**Figure 3**) in each watershed was determined to be a useful indicator of overall watershed pollution level, with higher DIN loads indicating watersheds that were most affected by land-based pollution (Shuler and Comeros-Raynal 2020). DIN concentrations are composed of concentrations of nitrate, nitrite, and ammonium (Shuler and Comeros-Raynal 2020). I further assessed the seven potential sites by collecting data on the specific location of *Acropora hyacinthus* colonies as well as basic

nutrient and water quality characteristics and temperature (**Table 2**). I sampled water quality at a single timepoint at each site at approximately the same location and depth of the proposed sampling site (< 5 meters depth) prior to sampling. A Hach Colorimeter was used to measure turbidity, nitrate (working range: 0.4-30.0 mg/L NO_3-N) and phosphorus (working range: 0.02-3.00 mg/L PO_4). Nitrate measurements were consistently below the working range of the Hach colorimeter (**Table 2**). pH was measured using an EcoTestr portable handheld pH probe. It should be noted that pH values were below expected seawater pH levels, and the probe may have been incorrectly calibrated (**Table 2**). *In situ* temperatures were measured every 30 minutes using HOBO loggers which were deployed on the reef for four to ten days prior to sampling (**Table 2**). One site, Faga'malo was not included in the study as there were too few *A. hyacinthus* colonies on the reef. The Aoa site was sampled and *A. hyacinthus* were exposed to the heat stress assay, but data from this site were discarded due to technical malfunctions during the experiment. The final five study sites used in this experiment were chosen for their environmental characteristics, pollution level, level of available pollution data, and abundance of *A. hyacinthus* (**Figure 4**). These sites included Cannery and Coconut Point as high pollution, Faga'alu as moderate pollution, and Vatia and Faga'tele as low pollution. It should be noted that Vatia has been classified in this study as low pollution but has previously been classified as moderate pollution. We classify Vatia as low pollution in this study due to the low human population, lower DIN load, its protection status as a U.S. National Park, and decreases in nutrient concentrations over time due to increased management efforts (Whitall et al. 2019).

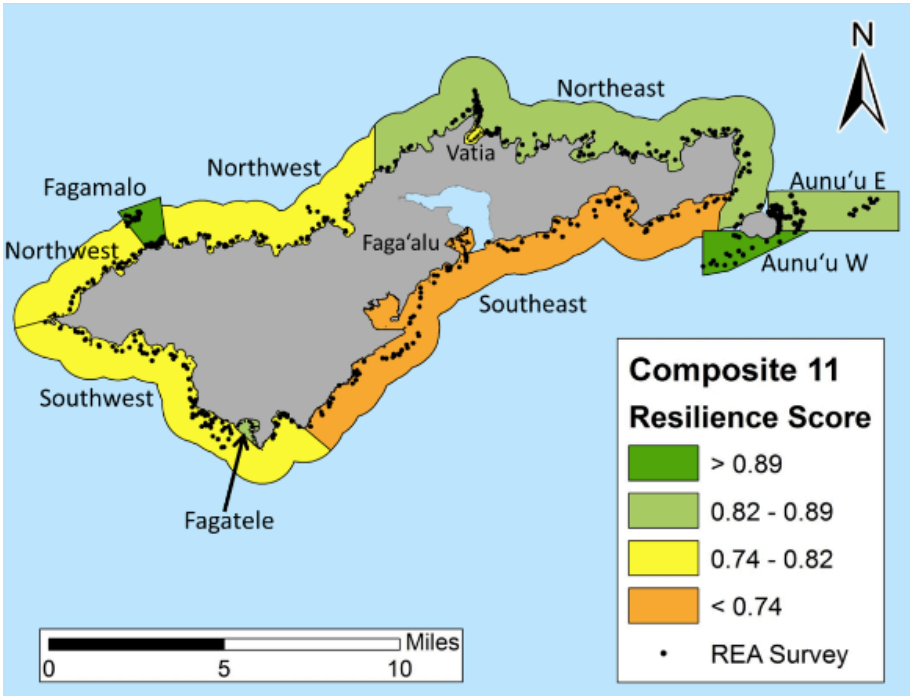

Figure 2. Coral resilience potential based on measurements of pollution, sedimentation, herbivory, macroalgae cover, coral diversity, coral recruitment, disease prevalence, bleaching resistance, physical impacts, fishing pressure, and sea surface temperature variability (from Schumacher et al. 2018). Green represents reefs with the highest resilience scores (where corals are predicted to be most resilient to climate change and human impacts), while orange represents reefs with lower resilience scores.

Table 1. Summary of Study Site Differences Including Population Data from the American Samoa Environmental Protection Agency (ASEPA) FY18 Watershed Report (Tuitele et al. 2019), Dissolved Inorganic Nitrogen Loading (kg/day) as reported by (Shuler and Comeros-Raynal 2020) and Relevant Protection Status.

Site	ASEPA Watershed Human Impact Classification (2016)	Human Population (2016)	DIN Load (kg/day/km^2)	Protection Status
Cannery	Extensive	9276	4.6	No protection; in Pago Pago Harbor
Coconut Point	Extensive	6707	4.3	No protection; next to airport
Aoa	Extensive	855	4.0	Marine Protected Area
Faga'alu	Extensive	910	2.8	No protection
Vatia	Intermediate	640	2.5	National Park
Faga'tele	Pristine	0	0.3	NOAA Sanctuary
Faga'malo	Pristine	47	0.9	Marine Protected Area

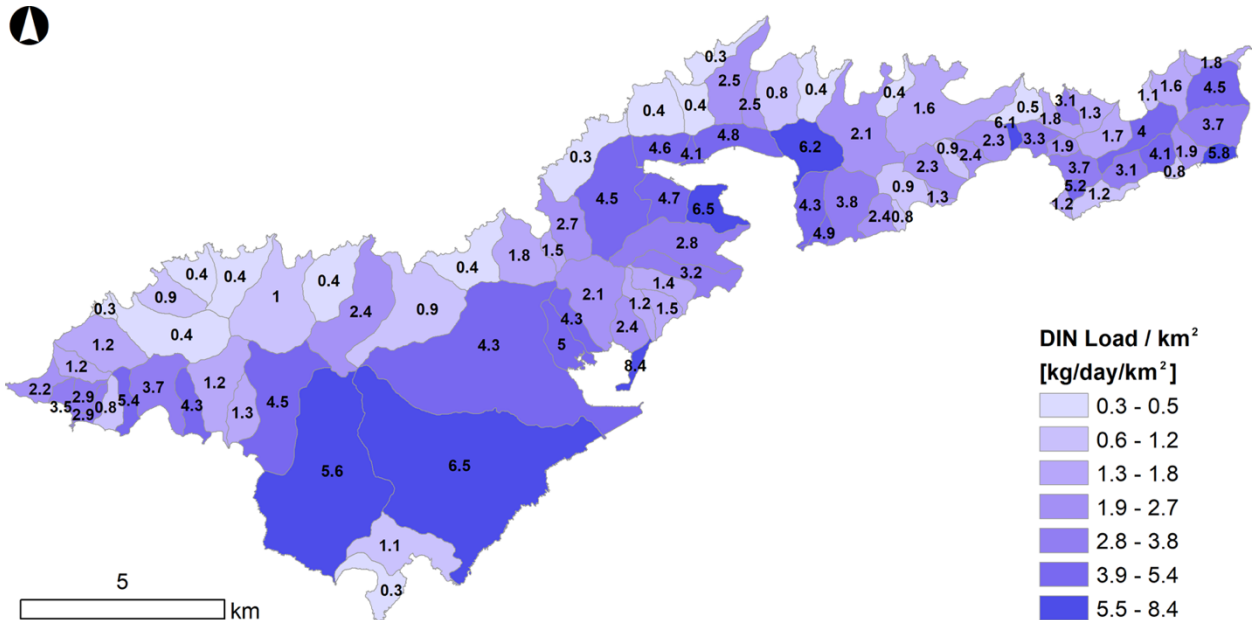

Figure 3. Tutuila watersheds classified by dissolved inorganic nitrogen (DIN) load scaled for each watershed area (from Shuler and Comeros-Raynal 2020). Darker watersheds had higher DIN loads and are considered most affected by land-based pollution. DIN loads are reported in kg/day/km^2.

Table 2. Study Site Locations and Water Quality Measurements Taken August 2019 (this study). Mean and maximum temperatures as measured at sampling reefs over 4-10 days.

Site (pollution level rating)	Date and Time Sampled	GPS Location	Mean Temp. (°C) (SD)	Max Temp. (°C)	pH	Turbidity (FAU)	NO_3^--N (mg/L)	PO_4 (mg/L)
Cannery (extensive)	8/7/2019 13:00	S 14 16.250 W 170 41.043	28.3 (0.304)	29.3	7.5	3	0.04	0.33
Coconut Point (extensive)	8/9/2019 14:10	S 14 19.476 W 170 42.004	NA	NA	7.5	1	0.02	> 3
Aoa (intermediate)	8/7/2019 17:06	S 14 16.178 W 170 41.071	28.8 (0.606)	30.7	7.4	0	0.02	0.23
Faga'alu (intermediate)	8/13/2019 17:03	S 14 17.496 W 170 40.846	28.7 (0.777)	30.7	7.5	0	0.03	1.19
Vatia (pristine)	8/13/2019 14:20	S 14 16.625 W 170 42.393	29.0 (0.621)	30.7	7.5	0	0.02	> 3
Faga'tele (pristine)	8/15/2019 8:17	S 14 17.494 W 170 40.848	28.7 (0.504)	30.5	7.5	0	0.03	2.61
Faga'malo (pristine)	8/9/2019 17:00	S 14 17.880 W 170 48.641	28.9 (0.569)	30.8	7.4	1	0.02	3.00

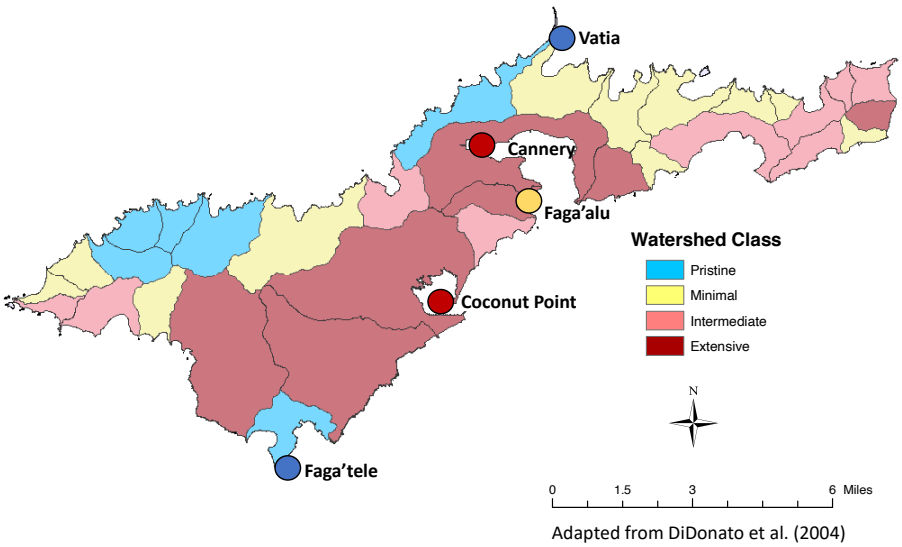

Figure 4. Watershed human-impact classifications on the island of Tutuila, American Samoa (adapted from Didonato 2004 with study site locations labeled). Blue represents most pristine, yellow represents intermediately polluted, and red represents most polluted. Watershed class distinctions were determined by DiDonato (2004) using human population size in each watershed circa 2004.

Study Species

This study tests the effects of acute thermal stress on *Acropora hyacinthus* (Dana 1846), a stony coral species that spans the Indo-Pacific. Also known as table coral, *A. hyacinthus* has a morphology that forms large, tiered tables made up of branchlets. Colonies can grow over three meters in diameter, with branchlets 3-7 mm in diameter and up to 20 mm long. Growth is determinate, and colonies can take 3-8 years to reach maturation (Wallace 1999). This species is one of the most common *Acropora* species and is cosmopolitan across the Indo-Pacific, primarily preferring upper reef slopes and outer reef flats (Wallace 1999). *A. hyacinthus* comprises multiple cryptic species, with differences in morphology (Suzuki et al. 2016). *Acropora* corals tend to be faster growing and more heat sensitive than massive colonies. They are also often early colonizers of disturbed sites (Didonato 2004). *A. hyacinthus* can reproduce either sexually or asexually. Sexual reproduction occurs through broadcast spawning with external fertilization while asexual reproduction occurs through fission or fragmentation (Ayre

and Hughes 2000). Stony corals can host seven different genera of Symbiodinicidae (LaJeunesse et al. 2018), but *A. hyacinthus* in American Samoa tends to host only two genera, *Cladocopium* and *Durusdinium* (Ladner et al. 2012). *Durusdinium* has been shown to increase coral thermotolerance, while *Cladocopium* has been shown to be more heat sensitive, but may be associated with increased coral growth rates (Cunning et al. 2015).

Thermotolerance Measurements

Field Collections

In August 2019, eight colonies of *Acropora hyacinthus* were sampled at each study site between the hours of 07:00 hr to 11:00 hr on a rising tide. 17 fragments of approximately two cm^3 were collected haphazardly using stainless steel coral cutters from each colony. Four replicates for each of four temperature treatments (n = 16) were needed as well as one field control (n = 1) which was never placed in the temperature stress assay. Colonies larger than 30 cm in diameter were sampled at least 10 meters apart to reduce the likelihood of sampling clones. All fragments were collected on snorkel and were less than two meters in depth.

Temperature Stress Assay

Thermal resistance to temperature stress was measured using a standardized short-term acute heat stress assay (Klepac and Barshis 2020; Voolstra et al. 2020). This portable heat stress system has been shown to determine relative differences in coral thermotolerance similarly to a classic long-term heat stress assay (Voolstra et al. 2020). This assay is termed the Coral Bleaching Autonomous Stress System (CBASS) and consists of four replicate tanks to test three experimental temperature treatments and one control. The temperature stress assay begun at 13:00 hr for each study site and continued until the following morning at 06:00 hr (**Figure 5**). Replicate coral branches (n = 16 per tank, with 2 replicates of each of 8 colonies) were allowed to acclimate to tank conditions at 28°C for one hour. 28˚C was chosen as the acclimation temperature and control temperature based on *in situ* temperatures at the sample sites (**Table 2**) as well as August monthly water temperature buoy data from Aunu'u, American Samoa (http://www.pacioos.hawaii.edu/water/buoy-aunuu/). At 14:00 hr corals were exposed to a ramp-hold temperature profile: control (28°C), 33°C, 34°C or 35°C during a 2-hour ramp, 3-hour hold,

and overnight recovery at 28°C (**Figure 5**). Light levels were measured using an Apogee underwater quantum meter twice during the assay and maintained between 210 – 250 μmol m^{-2} s^{-1}. To mimic natural field conditions, lights were turned off at 19:00 hr and turned on in the morning at 06:00 hr (Roleadro LED Aquarium Light). Partial water changes (~2 L) using water from the sampling site were performed 4-5 times over the course of the assay to maintain water quality and minimize changes in nutrient concentrations over the course of the assay. Water temperatures were controlled using a custom-built Arduino controller linked to aquarium heaters (Finnex HMA-200S Titanium Aquarium Heater) and custom-built cooling loops connected to a Hamilton Technology Aqua Euro Max Aquarium Chiller. Water temperatures were measured every minute using HOBO UA-002-64 temperature and light loggers. Temperatures were kept to within 0.5°C of the desired temperature.

Quantification of Thermal Tolerance

Thermotolerance was measured through changes in visual color paling using the CoralWatch® Coral Health Chart (Siebeck et al. 2006) using the same observer for all trials. Coral Health Chart scores were taken during acclimation (~13:00), during heat stress (~18:00) and after recovery (~07:00). Color paling was also measured through colorimetric analysis using an Olympus TG-5 taken by the same photographer in the same location for all measurements. Photos for colorimetric analysis were taken during heat stress (~18:30) and after recovery (07:00). These images were normalized using a greyscale and assessed for intensity within the red channel, which has been shown to correlate with bleaching (Winters et al. 2009). Red intensity was quantified by taking the average value from ten haphazardly selected points on each coral fragment. All normalization and colorimetric analysis were performed in MATLAB. Symbiont photochemical efficiency (Fv/Fm) was measured using a Walz Junior Pulse Amplitude Modulation (PAM) Fluorometer after 30 minutes of dark acclimation at 19:30 (Jones et al. 1999). An initial temperature stress assay (for n = 4 from the high-pollution Cannery site) was conducted on temperatures ranging from 32°C to 39°C to determine suitable temperatures to use in the assay. While PAM measurements indicated a decline of symbiont photoefficiency at 38°C, I chose to select temperatures that were more consistent with the literature and with predictions of future warming in the next 100 years (Heron et al. 2016). A subset of coral fragments (n = 4

per temperature treatment) were collected during heat stress (at 19:00) and after overnight recovery (at 07:00 the following day) and preserved in RNAlater for further analysis at California State University, Monterey Bay. Samples were collected at two time points to determine differences in bleaching, gene expression, and symbiont community during heat stress and after overnight recovery.

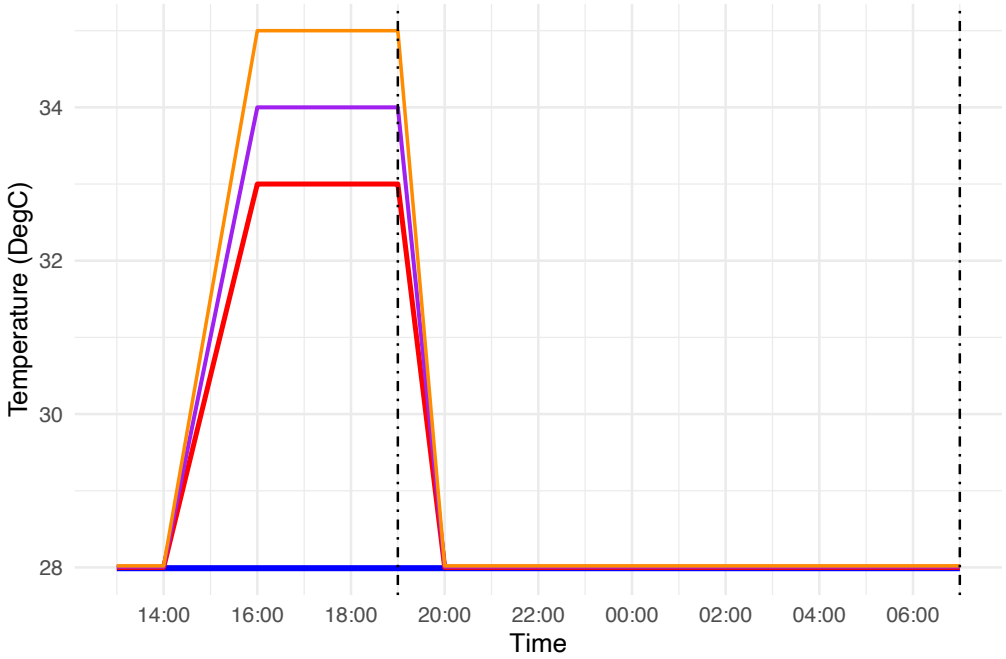

Figure 5. Visualization of the heat ramp of the acute temperature assay (CBASS) beginning at 13:00 with a 1-hr hold at 28°C, 2-hr ramp to 33-35°C, 1-hr ramp down to 28°C, and overnight recovery. Colored lines show the four temperature treatments (blue = control (28°C), red = 33°C, purple = 34°C, orange = 35°C). Dashed lines indicate when samples were collected and stored in RNAlater, at 19:00 and 07:00.

Statistical Analysis of Thermotolerance

Color paling (CoralWatch Color Card and red intensity) and photochemical efficiency (Fv/Fm) data from 33°C, 34°C, and 35°C treatments were normalized to controls at 28˚C. CoralWatch Color Card data was calculated as the change in color from the initial timepoint (13:00) to the recovery timepoint (at 07:00). All data were assessed for normality using the Shapiro test and equality of variance using the Levene test. Data were analyzed using a two-way ANOVA with site, treatment, and site x treatment interaction as factors. If the interaction term was not significant ($p > 0.05$), the two-way ANOVA was repeated with only site and treatment

as factors. Tukey post-hoc tests were performed to determine differences among treatments and among sites. All analyses were conducted in R version 4.1.0 using the `car` package for ANOVA analysis and the `agricolae` package for the Tukey HSD test. All data and code are available at: https://github.com/melissanaugle/CBASS_bleachingdata.

Generating a Single Bleaching Metric

Color paling data from CoralWatch Color Card and red intensity measurements was used to generate a single metric for thermotolerance for each colony. These two metrics were used since they both measure color paling (bleaching) while photochemical efficiency (Fv/Fm) measures symbiont photoefficiency rather than bleaching. Additionally, logistic and beta regression models that included photochemical efficiency (Fv/Fm) did generate models with effective fit. Raw CoralWatch and red intensity values were normalized in an open interval from 0-1 across temperature treatments from 28˚C to 35˚C, with a maximum of 36.5˚C if curves did not reach the midpoint by 35˚C. Logistic curves were fit to the data across temperatures and the midpoint of the curves was used as an indication of temperature at which bleaching occurred. The mean of the two midpoints (CoralWatch and red intensity) was used to generate a two-variable mean for each coral colony. This two-variable mean was used to determine the most and least thermotolerant corals. The highest and lowest 10, 20, and 30% of the two-variable means were used to classify the 10, 20, and 30% least and most thermotolerant corals (used for WGCNA analysis described below).

Quantification of Symbiont Communities

DNA Extraction and Quantitative PCR

Field-collected coral fragments were stored in RNAlater and transported back to California State University, Monterey Bay where they were held at -20°C. Total genomic DNA were extracted from preserved fragments using the Qiagen DNeasy® Blood and Tissue Kit (Cat. No. 69504). Samples were prepared by selecting a portion of the coral nubbin, removing excess RNAlater and homogenization in a Qiagen TissueLyser LT for 5 minutes at 50 hz. Total DNA was assessed for quality and concentration using a Nanodrop spectrophotometer and a Qubit fluorometer. All DNA extractions met the following criteria: > 2 ng/ul (on Qubit), 260:280 > 1.8,

260:230 > 1.59. Total DNA were prepared for qPCR using methods described in Cunning and Baker (2013) to quantify symbiont communities within each coral colony. All samples were run in triplicate with a no-template control on a Biorad CFX96 Touch Real-Time PCR Detection System. Reaction volumes were 10 µl, with 5 µl Taqman Genotyping Master Mix and 1 µl genomic DNA template. qPCR analysis uses genera-specific tags to identify *Cladocopium* and *Durusdinium*, which comprise the majority of the *Symbiodinium* in American Samoan *Acropora hyacinthus* (Ladner et al. 2012). Since *Durusdinium* are more thermally tolerant than *Cladocopium*, ratios between the two species provide a metric to understand coral thermotolerance contributed by the symbiont community (Cunning and Baker 2013).

Statistical Analysis of Symbiont Communities

Ratios of *Cladocopium* to *Durusdinium* cycle threshold (Ct) values were calculated using results from qPCR. Baseline thresholds were chosen for each run to remove background noise. Ct values were recorded for samples that amplified past the threshold in fewer than 40 cycles. Ct values were averaged across triplicate and cell numbers of *Cladocopium* and *Durusdinium* were calculated using the following formula: $2^{(40-Ct)}$ / cell copy number (where cell copy number = 9 for *Cladocopium* and 1 for *Durusdinium* (Cunning and Baker 2013). Proportions of *Cladocopium* and *Durusdinium* were calculated using the following formula: Proportion *Durusdinium* = Cell Number *Durusdinium* / (Cell Number *Durusdinium* + Cell Number *Cladocopium*), or vice versa for *Cladocopium*. Data did not pass assumptions for normality nor equality of variances, so colonies were categorized into either *Durusdinium* only or *Cladocopium* and *Durusdinium* and were compared to null expected numbers in a contingency table analysis using Fisher's Exact Test. All analyses were conducted in R version 4.1.0. All data and code are available at: https://github.com/melissanaugle/Symbiont_qPCR.

Gene Expression Analysis

RNA Extraction and Sequencing

A subset of samples from each site in control (28˚C) and heat stress (35˚C) treatments, sacrificed during peak heat stress (~19:00, **Figure 5**), were used for RNAseq analysis (n=4 per site per treatment). Control and the highesst temperature were chosen because physiological effects differed most between these two treatments. Coral samples were selected for sequencing

based on availability (not all samples were preserved in RNAlater due to restrictions on space and reagents) and RNA quantity and quality. Coral samples were stored in RNAlater and held at -20˚C until RNA extraction. RNA extraction and sequencing were performed in two batches. RNAseq on samples from Faga'tele, Faga'alu and Coconut Point was performed in February/March 2020, and RNAseq on samples from Cannery and Vatia was performed in March/April 2021. Total RNA was extracted using the Qiagen RNeasy® Plus Mini Kit (Cat. No. 74034), following the manufacturer's protocol. Samples were homogenized using a TissueLyser LT for 10 minutes at 50 Hz in 2020 and for 3 minutes at 50 Hz in 2021. RNA was assessed using a NanoDrop™ (Thermo Scientific), a Qubit fluorometer and a BioAnalyzer. 38 cDNA libraries were constructed from 300 ng of total RNA using the NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina with Sample Purification Beads® (Cat. No. E7765) with the NEBNext® Poly(A) mRNA Magnetic Isolation Module (Cat. No. E7490). Paired-end libraries were sequenced on a HiSeq4000 150 bp lane at NovoGene in Davis, CA.

Differential Gene Expression Analysis and WGCNA

Low quality reads and adapter sequences were discarded using Trimmomatic (Bolger et al. 2014). Trimmomatic parameters were set to remove reads below 25 bp long, leading and trailing bases below quality "5," and reads that did not meet quality standards for a sliding window where in a four base sliding window, the average quality per base drops below a 5. Sequences were also trimmed of adapter sequences including standard Illumina adapters and polyT sequences. Quality of trimmed reads were assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequenced reads were aligned to the reference *A. hyacinthus* transcriptome described in Barshis et al. (2013). Reads were aligned to the transcriptome assembly using bowtie2 and counted using RSEM in UNIX (Li and Dewey 2011; Langmead and Salzberg 2012). Differential gene expression statistical analyses were conducted using `edgeR` with a four fold-change cutoff and a false discovery rate (FDR) of FDR < 0.001 and FDR < 0.05. Annotations were obtained from the Dryad Repository associated with Barshis et al. (2013) (https://datadryad.org/stash/dataset/doi:10.5061/dryad.bc0v0). Annotations were obtained from BLASTx matches to the NCBI NR, Uniprot, Swissprot, and TrEMBL databases (Barshis et al. 2013).

Gene expression analyses were conducted in three ways: 1. On all samples from both temperature treatments (28˚C and 35˚C) to measure the impact of heat stress, 2. On all samples at control (28˚C) to measure differences among sites prior to heat stress, and 3. On all samples at heat stress (35˚C) to measure differences among sites during heat stress. For each of these three analyses, an MDS plot was created using the `edgeR` package in R and a heatmap was created using the `ggplot2` package in R. For the analysis of all samples at control and heat stress, a Venn diagram was created using the `VennDiagram` package in R. For each of these three analyses, a weighted gene co-expression network analysis (WGCNA) was conducted to compare co-regulated gene networks (called modules) and their association with temperature treatment, pollution level, symbiont community, and thermotolerance (Langfelder and Horvath 2008). WGCNA identifies co-expressed gene modules using hierarchical clustering of expression data and relates those modules to sample traits. Select modules with significant correlations to traits ($p < 0.05$) were statistically analyzed for gene ontology enrichment using the `GO_MWU` package in R, which uses a Mann-Whitney U-Test (Wright et al. 2015; Huerta-Cepas et al. 2017). The `GO_MWU` package tests the kME (module membership score or eigengene-base connectivity) in among-module genes compared to other genes in the transcriptome outside the module to test if genes in the module of interest are significantly enriched. Gene ontology enrichment analysis was used to identify enriched gene ontology terms relating to biological processes and molecular function. GO terms and gene names from genes differentially expressed under heat stress and from significant WGCNA modules, were compared to published gene lists in Barshis et al. (2013a) and Dixon et al. (2020) in R. All data and code are available at: https://github.com/melissanaugle/RNAseq_allsites_Barshisreference. All analyses were conducted in R version 4.1.0.

RESULTS

Thermotolerance Results

Colorimetric Analysis of Bleaching

No significant differences in color occurred at the heat stress timepoint (**Figure 6a**). The interaction term of site x treatment was not significant ($F_{(6,180)} = 0.52$, $p = 0.79$). When the interaction term was removed from the model, red intensity did not vary with site ($F_{(3,186)} = 0.73$, $p = 0.54$) or temperature ($F_{(2,186)} = 2.23$, $p = 0.11$). Data were assessed for normality using the Shapiro test and equality of variance using the Levene test. Coconut Point was not sampled at the stress time point due to an experimental error.

After an overnight recovery, a colorimetric analysis of bleaching showed that when normalized to controls at 28°C, corals in the higher temperature treatments bleached more than at lower temperatures ($F_{(2,230)} = 10.33$, $p = 5.04e\text{-}05$, **Figure 6b**). A Tukey post-hoc test showed that corals at 33°C had lower average red intensity (i.e., bleached less) than those at 34°C and 35°C ($p < 0.001$). Site also impacted average normalized red intensity ($F_{(4,230)} = 2.93$, $p = 0.02$). A Tukey post-hoc test showed that Vatia (low pollution) bleached less than Cannery (high pollution) and Faga'tele (low pollution; $p < 0.05$). No other site comparisons were significantly different. There was no significant interaction between temperature treatment and site ($F_{(8,222)} = 1.06$, $p = 0.39$), so the final model did not include the interaction term of site x treatment. Data passed assumptions for normality using the Shapiro test and equality of variance using the Levene test.

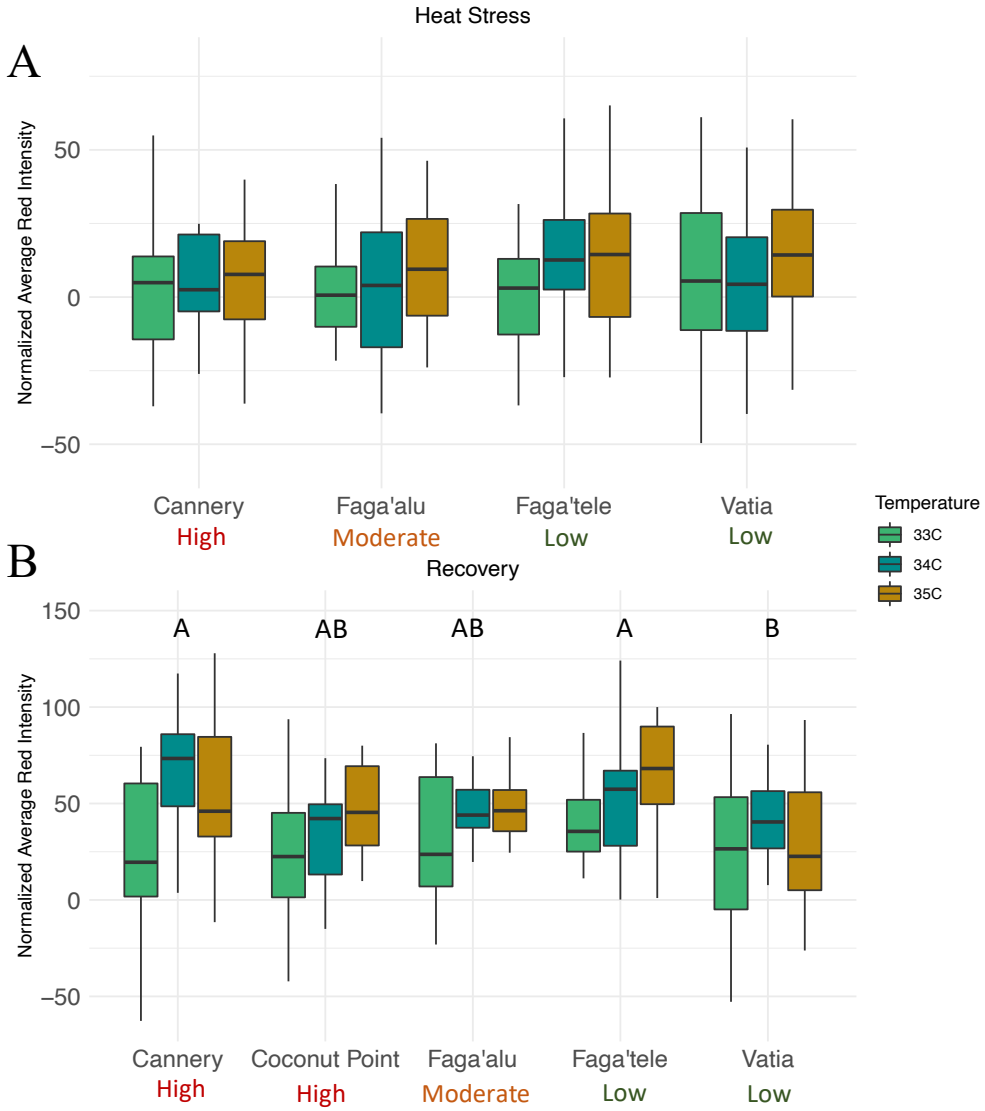

Figure 6. Average red intensity as a proxy for bleaching at two time points: A.) 'heat stress' time point (two hours into heat stress) and B.) 'recovery' time point (after three hours heat stress and overnight recovery). Three temperature treatments are shown as normalized to controls at 28°C. Letters above bars indicate significant differences between sites determined by Tukey post-hoc test. Higher average red intensity indicates corals that are paler in color and more bleached. Coconut Point corals were not assessed for red intensity at the stress time point. Outliers not shown in figures.

CoralWatch Color Card Health Score

The change in color card health score from acclimation to the heat stress sampling time point showed that bleaching did not vary among temperatures but did vary among sites (**Figure 7a**; $F_{(4,470)} = 11.66$, $p < 0.001$). Corals from Coconut Point (high pollution) and Faga'alu

(moderate pollution) showed the greatest signs of bleaching and Faga'tele (low pollution) showed the least.

The change in color card health score from when the coral were collected to after an overnight recovery showed that higher temperatures at 35˚C and 34˚C bleached more than 33˚C (**Figure 7b**; $F_{(2,222)}= 13.39$, $p < 0.001$). Site was not significant, though was approaching significance ($F_{(4,222)}= 2.18$, $p = 0.07$). There was a significant interaction between temperature treatment and site ($F_{(8,222)} = 1.22$, $p = 0.03$). A Tukey post-hoc test also showed that Vatia (low pollution) bleached less than Faga'alu (moderate pollution) and Coconut Point (high pollution). Faga'alu (moderate pollution) also bleached more than Cannery (high pollution). Data passed assumptions for normality using the Shapiro test and equality of variance using the Levene test.

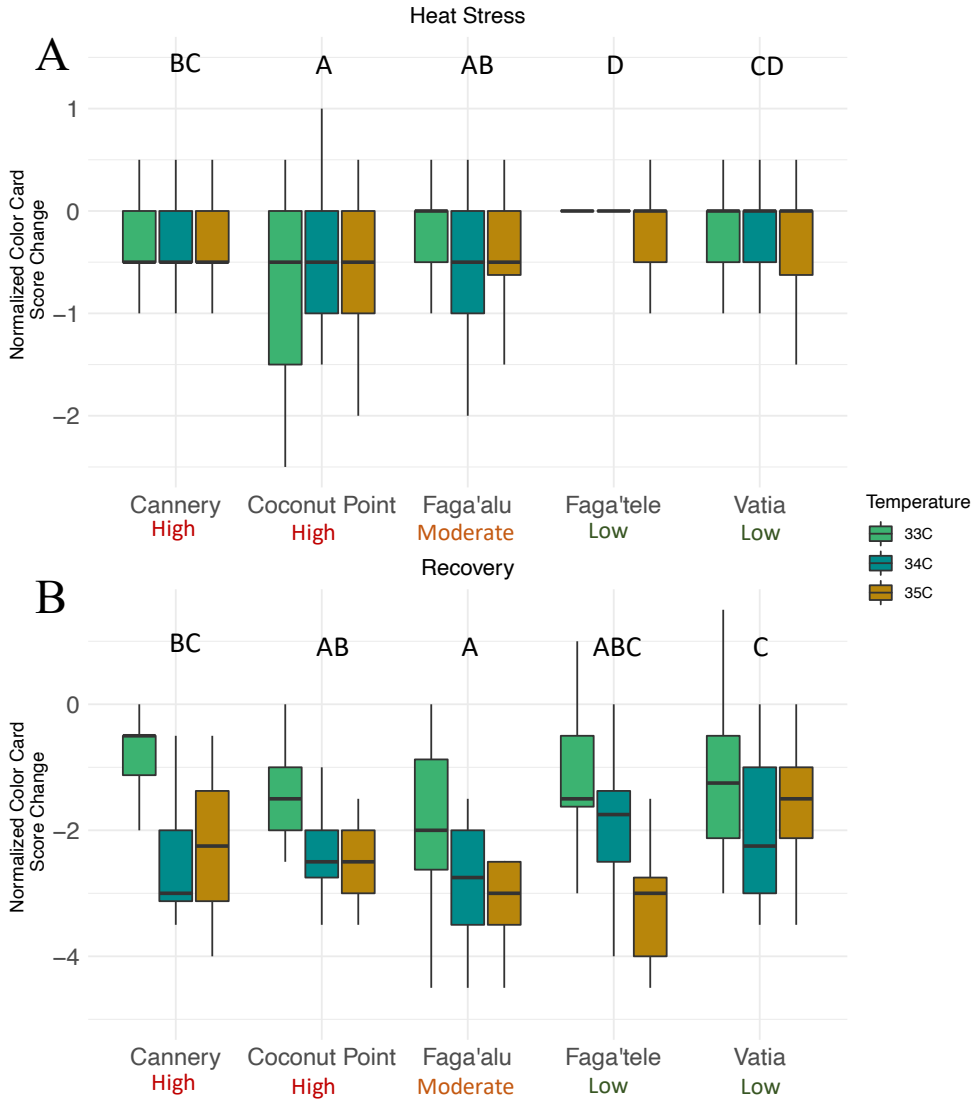

Figure 7. Coral Watch Color Card Health Score difference between A.) baseline score and at 'recovery' time point and between baseline score and 'heat stress' time point. Three temperature treatments are shown as normalized to controls at 28°C. Letters above bars indicate significant differences between sites determined by Tukey post-hoc test. Higher color card score change (lower on y-axis) indicates corals bleached more.

Photochemical Efficiency

Photochemical efficiency measurements showed no differences among temperature treatments ($F_{2,346}$) = 1.80, p = 0.17) but did show differences among sites ($F_{(4,346)}$ = 3.08, p = 0.02, **Figure 8**). Tukey's post-hoc comparisons showed that Faga'tele (low pollution) corals had lower photochemical efficiency than Faga'alu (moderate pollution). Data passed assumptions for equality of variances using the Levene test and were approximately normal (p = 0.02, Shapiro test). Data were unimodal but slightly negatively skewed. This violation of normality is justified by the large sample size, balanced experimental design, unimodal distribution, and robustness of ANOVA tests. Four fragments produced Fv/Fm values of 0 due to total bleaching or coral death and were not included in the analysis.

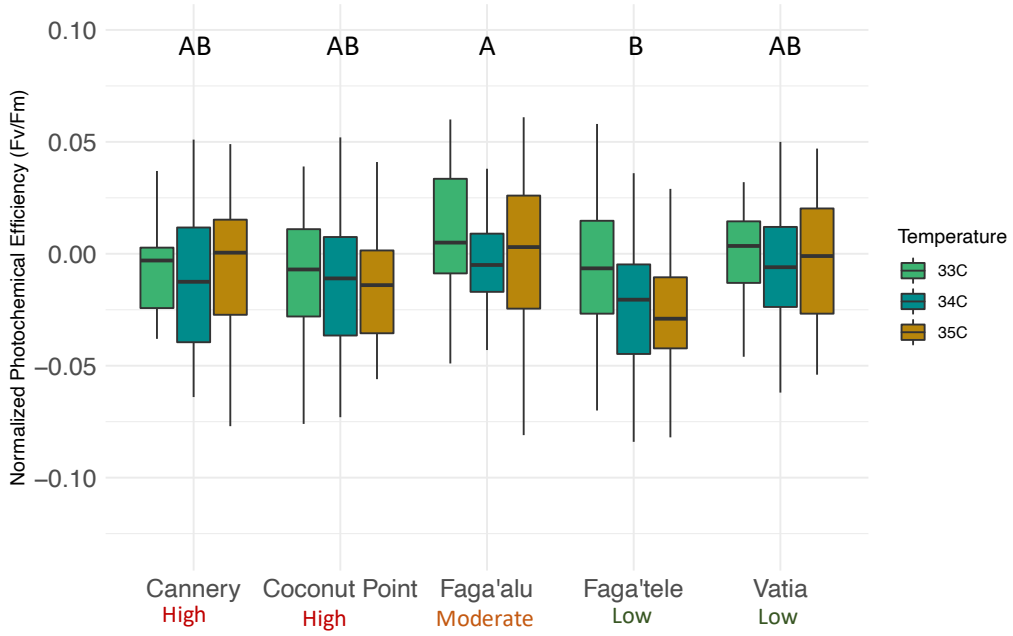

Figure 8. Photochemical efficiency of 30-minute dark-acclimated corals as measured by Fv/Fm. Three temperature treatments are shown as normalized to controls at 28°C. Letters above bars indicate significant differences between sites determined by Tukey post-hoc test. Outliers not shown on figure.

Summary of Thermotolerance Metrics

We measured physiological responses to heat stress using three different metrics to determine site-level differences in the heat stress response. Among the five sample sites, the heat stress response did not correlate with pollution level nor did the three metrics consistently correspond with each other (**Table 3**). When taken together these three measurements show that in two of three metrics Vatia (low pollution) was least affected by heat stress and Faga'tele (low pollution) was most affected by heat stress. Cannery (high pollution), Coconut Point (high pollution), and Faga'alu (moderate pollution) showed more variation among the three metrics.

Midpoints based on logistic models of red intensity and color card data showed some variation by site, with some of the most thermotolerant corals belonging to Vatia and Coconut Point and some of the least thermotolerant corals belonging to Faga'tele and Faga'alu (**Figure 9**).

Site level differences in thermotolerance also did not appear to relate to thermal history measurements taken during this study (**Figure 10**). Faga'tele, Faga'alu, and Vatia showed similar temperature trends while Cannery showed less variation and consistently lower temperatures. Yet, Cannery corals were equally thermotolerant in two of three thermotolerance metrics. More significant differences in thermotolerance occurred between Vatia and Faga'tele, which had similar thermal maximums and variation. We do not have temperature data from Coconut Point due to a lost HOBO logger.

Table 3. Summary of three bleaching metrics among sites. Entries are shown for metrics where sites were significantly different in Tukey post-hoc comparison ($p < 0.05$) or denotes n.s. for not significant (no significant Tukey post-hoc pairwise comparisons).

		Color Paling		Symbiont Performance
Site	Pollution Level	Red Intensity (Recovery)	CoralWatch Color Card Health Score (Change from Initial to Recovery)	Photochemical Efficiency
Cannery	High	More bleached	n.s	n.s.
Coconut Point	High	n.s.	More bleached	n.s.
Faga'alu	Moderate	n.s.	More bleached	Less bleached
Faga'tele	Low	More bleached	n.s	More bleached
Vatia	Low	Less bleached	Less bleached	n.s.

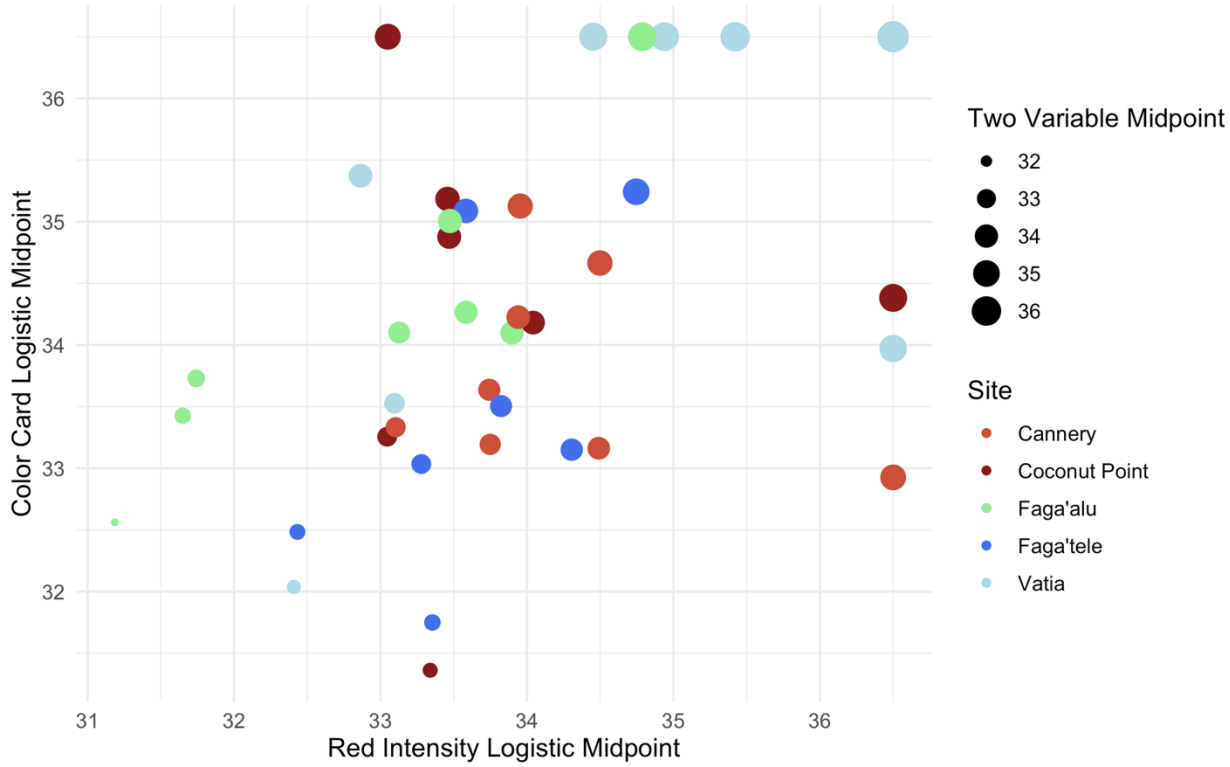

Figure 9. Red intensity and color card logistic midpoints shown by site. The color of the point represents the site, with red and orange as high pollution sites, green as moderate pollution and blue and light blue as low pollution. The size of the points corresponds to the two-variable metric of thermotolerance (mean of logistic model midpoint of color card score and of red intensity) with larger symbols indicating higher thermotolerance.

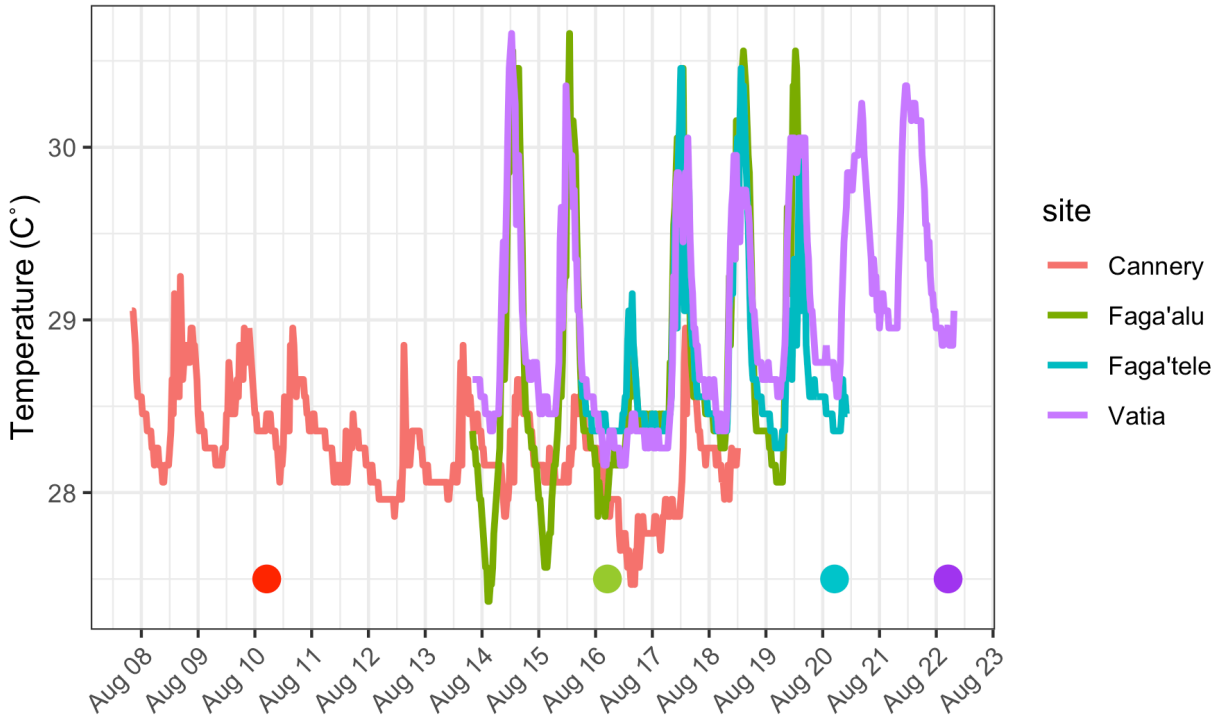

Figure 10. HOBO logger temperature profiles at five sampling sites in August 2019. Circles indicate the day on which the corals were collected, and the heat stress assay was performed. Temperature measurements were taken every 30 minutes.

Symbiont Community Results

Measurements of the relative levels of heat-sensitive *Cladocopium* and heat-tolerant *Durisdinium* in field-collected coral fragments at each site yielded differences among sites. Corals from high pollution sites (Cannery and Coconut Point) hosted entirely *Durisdinium.* Corals from moderate and low pollution sites hosted a combination of *Cladocopium* and *Durisdinium,* with increasing proportions of corals hosting *Cladocopium* as pollution level decreased. Moderate pollution site Faga'alu held only one of eight coral fragments that hosted any *Cladocopium,* while low pollution site Vatia held two of seven coral fragments that hosted *Cladocopium,* and lowest pollution site Faga'tele held six of eight coral fragments that hosted *Cladocopium* (**Figure 11**). A contingency table analysis showed that the numbers of colonies hosting either *Durisdinium* only or a combination or *Cladocopium* and *Durisdinium* differed among sites (**Table 4**, Fisher's exact, $p = 0.001$). While the proportion of *Cladocopium* increased with decreasing pollution level, all coral fragments primarily hosted *Durisdinium* (**Figure 12**).

When measured against two metrics of thermotolerance, the proportion of *Cladocopium* did not show any trend with thermotolerance (**Figure 13**). A Spearman correlation test showed no significant relationship between the proportion of *Cladocopium* and red intensity ($p = 0.40$) nor between the proportion of *Cladocopium* and photochemical efficiency ($p = 0.45$).

Table 4. Contingency table showing number of field-collected colonies at each site containing either *Durisdinium* only, or a combination or *Cladocopium* and *Durisdinium*.

Site	*Cladocopium* and *Durisdinium*	*Durisdinium* only
Coconut Point (high pollution)	0	8
Cannery (high pollution)	0	7
Faga'alu (moderate pollution)	1	7
Vatia (low pollution)	2	5
Faga'tele (low pollution)	6	2

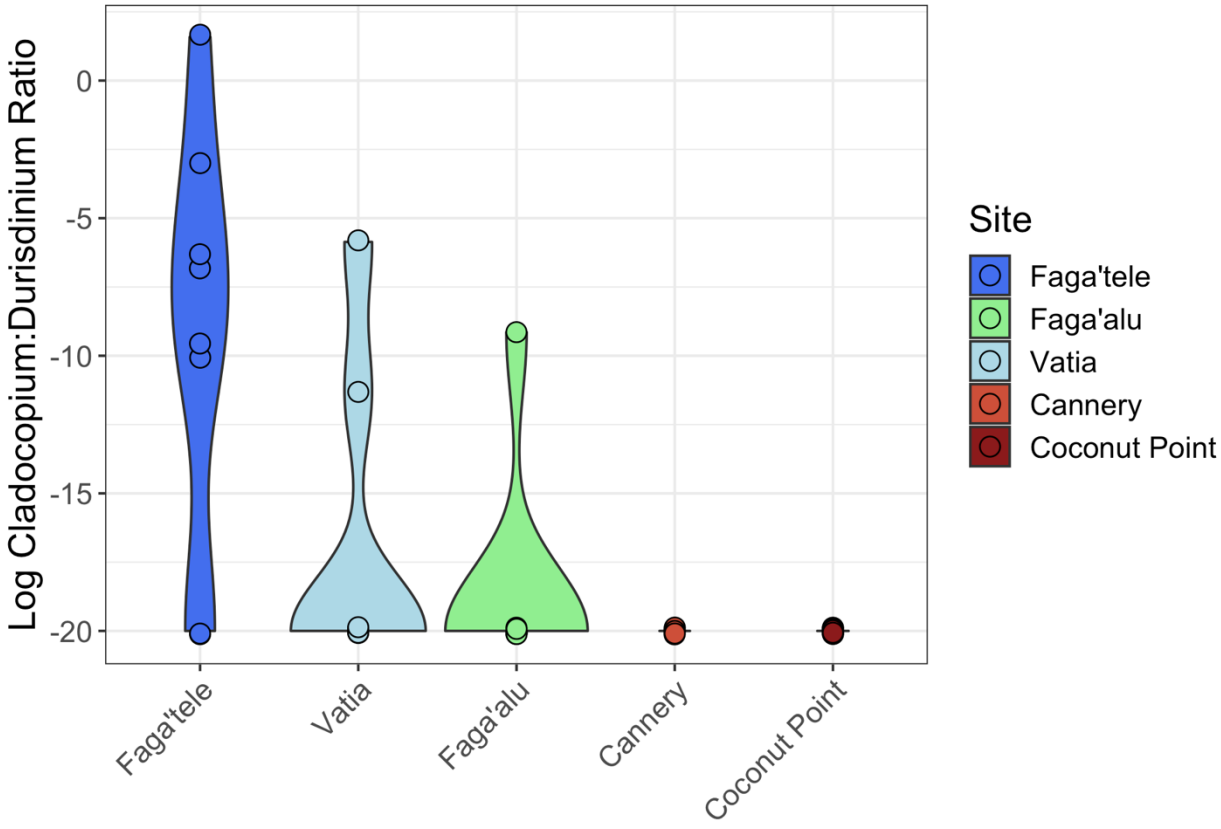

Figure 11. Ratios of *Cladocopium* to *Durisdinium* symbiont types in field-collected coral fragments collected at each site (n = 8/site). Lowest pollution sites are shown to the left and highest pollution sites are shown to the right. Points with a value > 0 hosted more *Cladocopium* than *Durisdinium.* Each point represents a unique coral colony. Points at -20 hosted only *Durisdinium*.

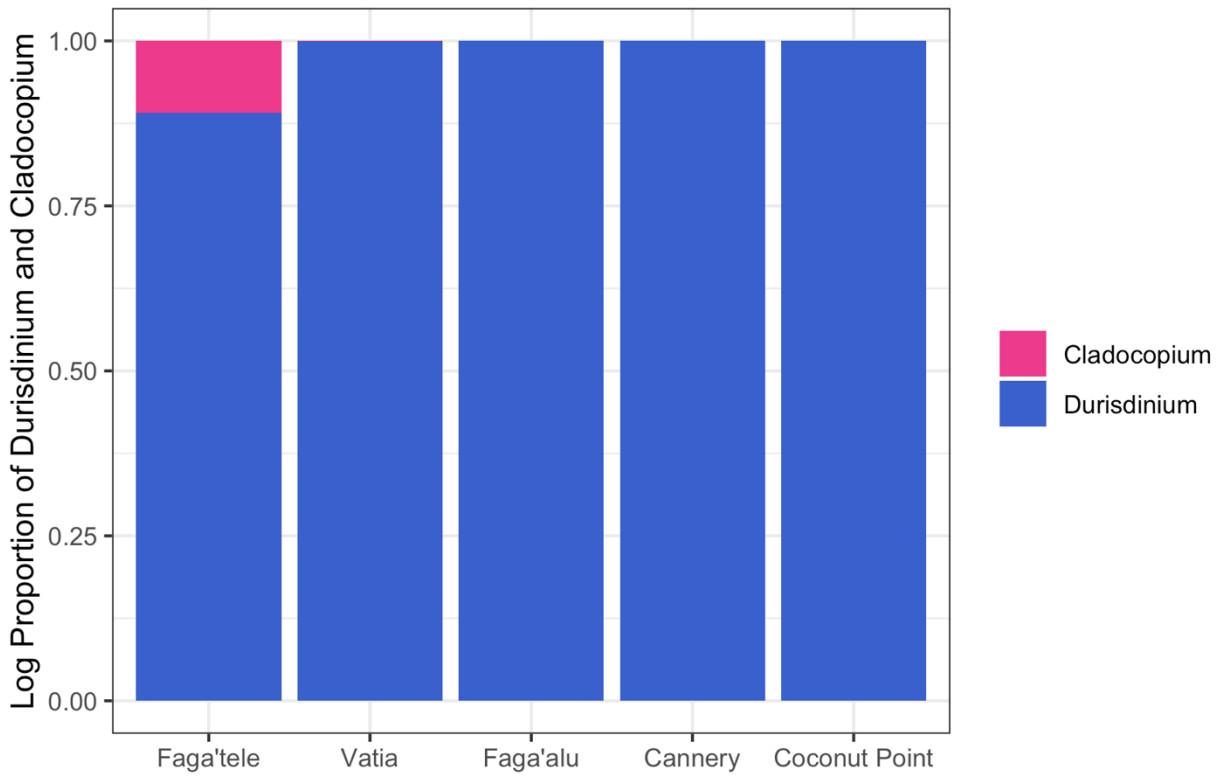

Figure 12. Mean proportions of *Cladocopium* and *Durisdinium* in field-collected coral samples at each site. Lowest pollution sites are shown to the left and highest pollution sites are shown to the right.

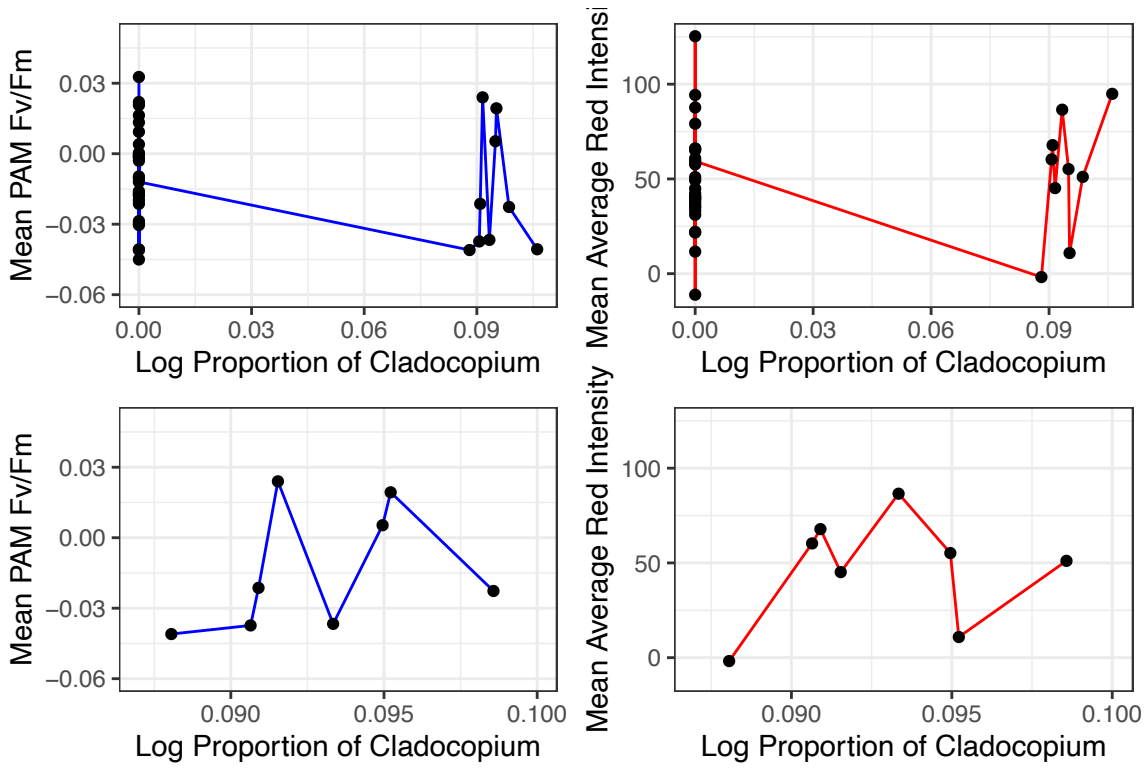

Figure 13. PAM photosynthetic efficiency (Fv/Fm) and average red intensity shown as a function of the proportion of *Cladocopium* for all colonies (top row) and colonies with proportion of *Cladocopium* > 0 (bottom row). No correlation is observed between symbiont community and either thermotolerance metric.

Gene Expression Results

RNA sequencing was performed on five sites of differing pollution level at control and heat stress treatments. Gene expression patterns were compared: 1. Among all sites and treatments to determine the influence of heat stress across sites 2. Among sites at control conditions to determine the influence of pollution stress alone and 3. Among sites at heat stress to determine the influence of pollution on heat stress responses. In each comparison, gene expression patterns are shown in a MDS plot (**Figures 14, 19, 22**) and differential gene expression is visualized using a heatmap (**Figures 15, 20, 23**). A WGCNA was performed in each comparison and Gene Ontology analysis identified significantly enriched gene functions in significant modules (**Figures 18, 21, 24**).

Differential Gene Expression Between Heat and Control Treatments

Differential gene expression analysis was performed on coral fragments from the five sample sites and a subset of four colonies samples from two treatments per site (heat stress at 35˚C and control at 28˚C). Gene expression patterns of all 15,109 genes that were mapped clustered strongly by temperature treatment (**Figure 14**). The first principal component (PC1) explained 33.58% of the variation in gene expression, and PC2 and PC3 explained 9.53% and 4.93% of the variation, respectively. Gene expression profiles were driven strongly by temperature treatment, with 6,020 genes differentially expressed between heat and control treatments across all sites (edgeR, FDR < 0.001, **Figure 15**).

The number of genes differentially expressed between heat and control treatments at each site varied by pollution level, with more genes differentially expressed with increasing pollution level (**Figure 16**). A common core set of 476 genes were differentially expressed between control and heat stress at all five sites (**Figure 16**). We searched this list of 476 genes for known heat response gene functions and found 35 genes that were annotated as heat shock proteins or involved in heat shock protein binding (GO:0031072). 13 genes were involved in apoptosis (GO:0006915), eight in response to stress (GO:0006950), and 11 in protein folding (GO:0006457). A gene ontology analysis was used to find significantly enriched gene ontology categories in the common core set of 476 genes. Genes upregulated during heat stress compared to control included those involved in DNA binding, neurotransmitter transporter, cell-cell adhesion, immune response, and the MAPK cascade (**Figure 17**). Genes downregulated during heat stress included those involved in oxidoreductase, aromatase, mRNA binding, and hormone biosynthetic processes (**Figure 17**). GO terms associated with this common core set of genes were compared to GO terms from Dixon et al. (2020), where a meta-analysis was performed to create a list of GO terms common to the general stress response in *Acropora* corals. Of the 3446 GO terms described as ‘stress response terms’ by Dixon et al. (2020), seven matched our list of 476 common core heat stress genes. These genes included genes involved in DNA binding, zinc ion binding, metal ion binding, collagen, mitochondrial inner membrane, muscle organ development, and lipid catabolic processes. Genes in our common core set of heat response genes were also compared to heat stress response genes from Barshis et al. (2013), where gene expression during heat stress was measured in *A. hyacinthus* from pools in Ofu, American Samoa. Of the 1636 genes that Barshis et al. (2013) found differentially expressed during heat

stress, 59 matched our list of 476 common core heat stress genes. These 59 genes were involved in ATP binding, calcium ion binding, DNA binding, G-protein coupled receptor activity, response to stress, and neuropeptide signaling.

A weighted gene co-expression network analysis was conducted to investigate how gene networks (called modules) correlate to temperature treatment and pollution level, both when corals were exposed to each stressor separately and in combination. 12 modules correlated with heat stress including exceptionally strong negative correlation with the blue module and positive correlation with the darkgrey module (Pearson's $R > 0.9$, **Figure 18).** Of the 6036 genes in the blue module, 157 contained GO terms that matched *Acropora* general stress response expression from Dixon et al. (2020). These GO terms were primarily involved in intracellular signal transduction, ATP binding, calcium ion binding, oxidoreductase activity, RNA binding, and zinc binding. Of the 3969 genes in the darkgrey module, 84 contained GO terms that matched *Acropora* general stress response expression (Dixon et al. 2020), including GO terms involved in apoptosis, protein transport, ATP binding, calcium ion binding, DNA binding, metal ion binding, and zinc ion binding. Gene ontology analysis on the blue module revealed enrichment for tRNA processing, citrate metabolic process, carbohydrate catabolic process, nuclear transport, protein-cofactor linkage, cilium or flagellum-dependent cell motility, mitochondrion organization, regulation of chromosome organization, and folic acid-containing metabolic processes ($p < 0.001$**, Table 5**). These processes were therefore downregulated during heat stress compared to control conditions. Gene ontology analysis on the darkgrey module revealed enrichment for immune response, metabolic process, cellular process, and response to stimulus ($p < 0.001$, **Table 5).** These processes were therefore upregulated during heat stress compared to control conditions.

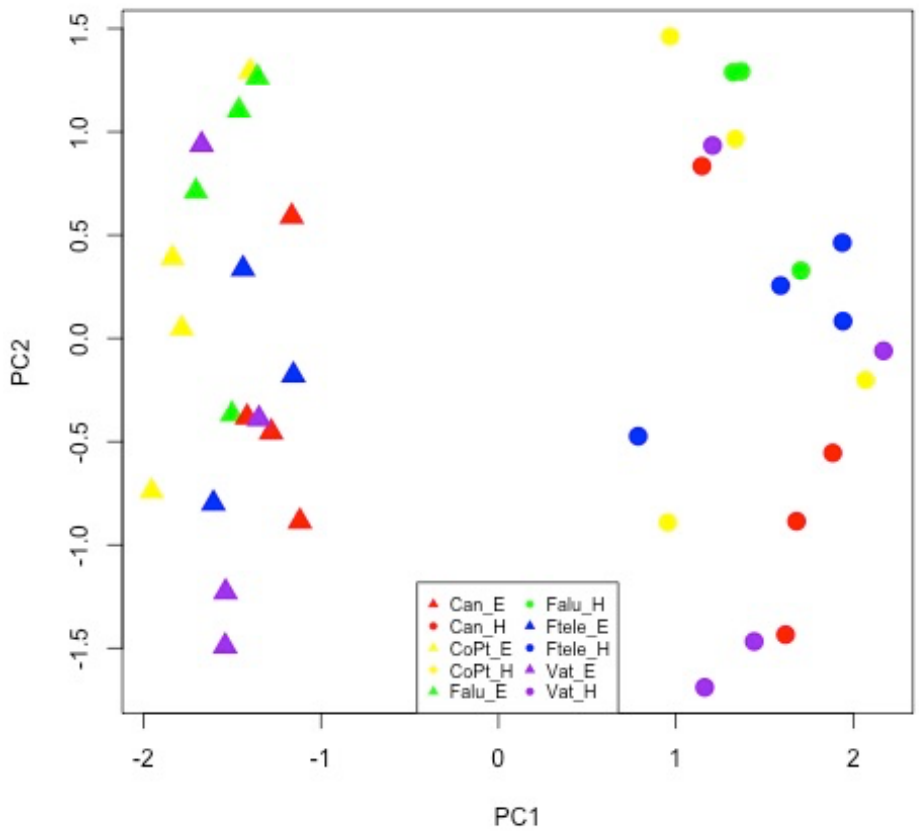

Figure 14. MDS plot showing differences in fold-change of 15,109 genes on all genes (prior to differential expression analysis). Circles represent coral fragments that underwent heat stress (35˚C) and triangles represent those from control (28˚C). Red and yellow denote the high pollution sites (Cannery and Coconut Point, respectively). Green denotes the moderate pollution site (Faga'alu). Blue and purple denote the low pollution sites (Faga'tele and Vatia, respectively).

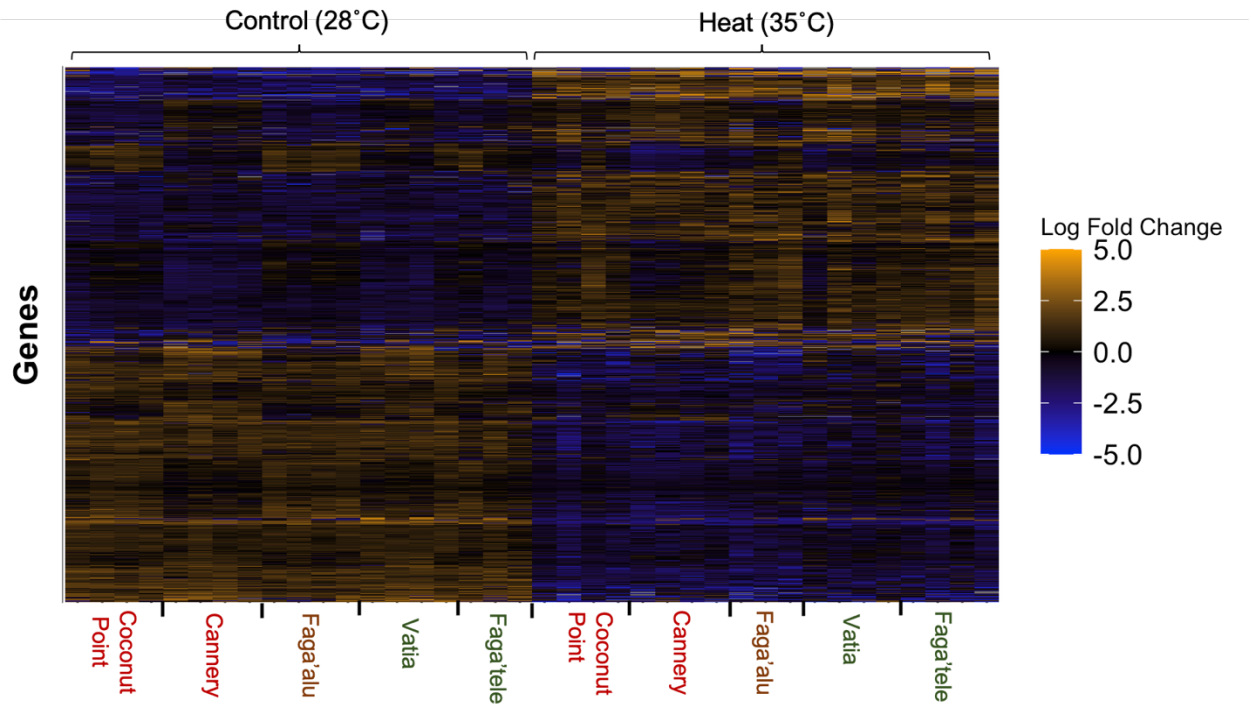

Figure 15. Heatmap showing log2 fold change to visualize gene expression differences between heat and control treatments for 6020 genes (edgeR, FDR < 0.001). Green labels denote low pollution sites (Faga'tele and Vatia), orange denotes moderate pollution (Faga'alu) and red denotes high pollution (Coconut Point and Cannery). Control corals are shown on the left and heat stressed corals are shown on the right.

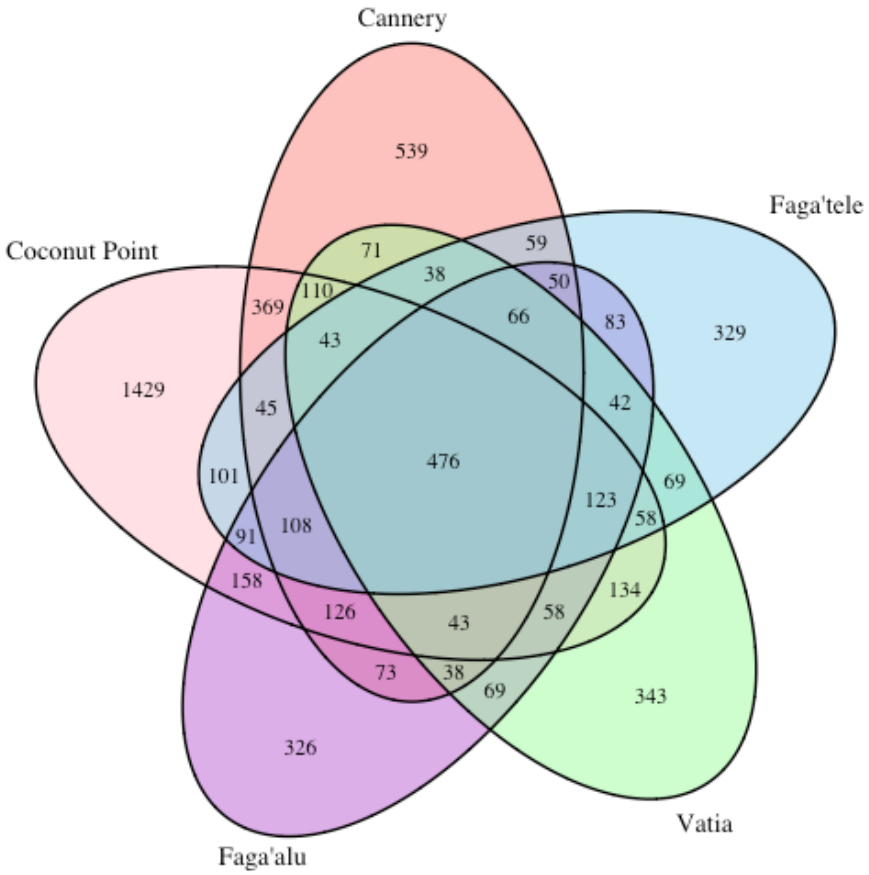

Figure 16. Venn Diagram showing number of genes differentially expressed between coral fragments that underwent heat stress (35˚C) and those from controls conditions (28˚C) across five sites (edgeR, FDR < 0.05).

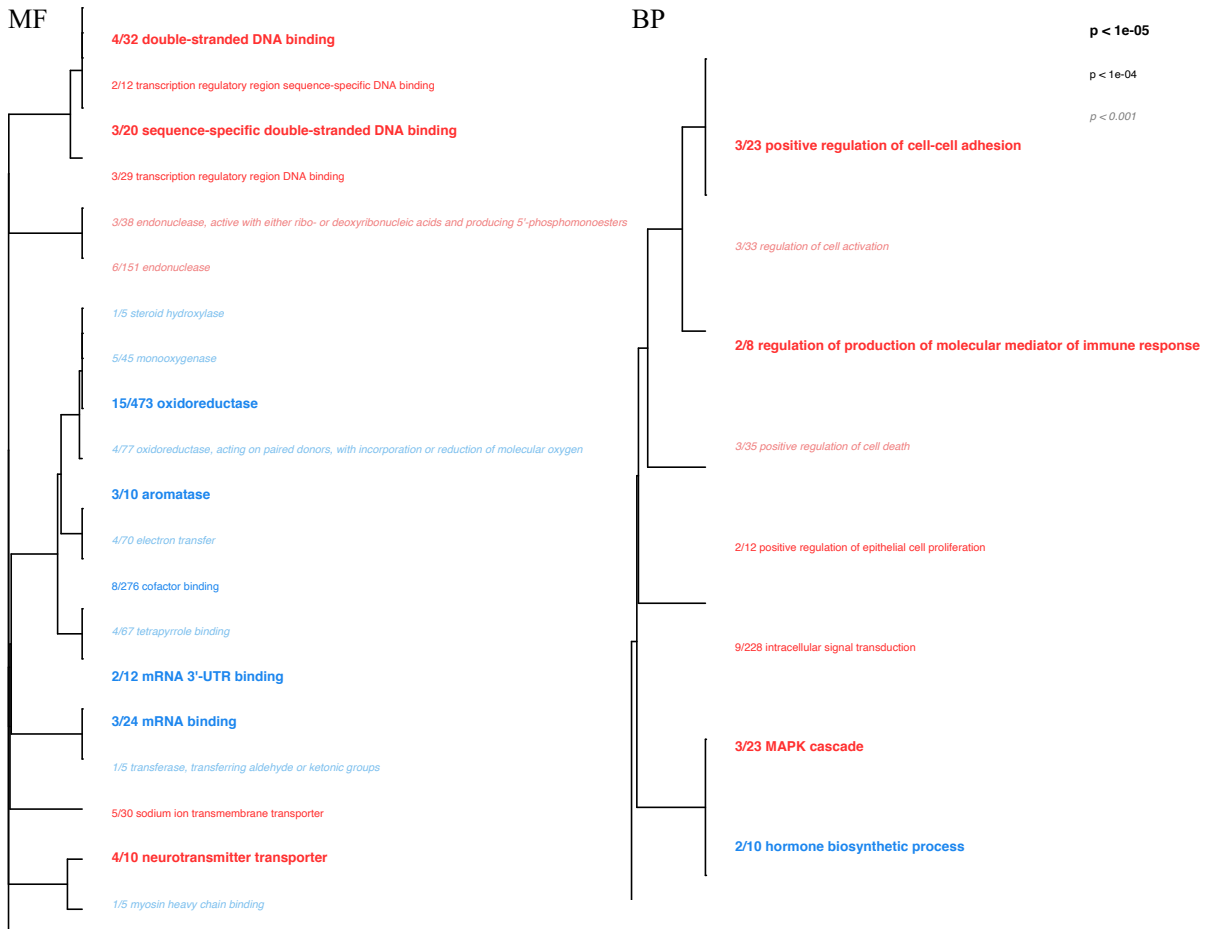

Figure 17. Hierarchical clustering of significantly enriched molecular function (MF) and biological process (BP) gene ontology terms up-regulated (red) or down-regulated (blue) in the common core set of 476 genes differentially expressed between heat stress and control corals across all sites. The fraction preceding the GO term indicates the number of genes annotated with the term within an unadjusted p-value threshold of 0.05. Font size indicates the significance of the term and hierarchical clustering indicates sharing of genes among GO categories.

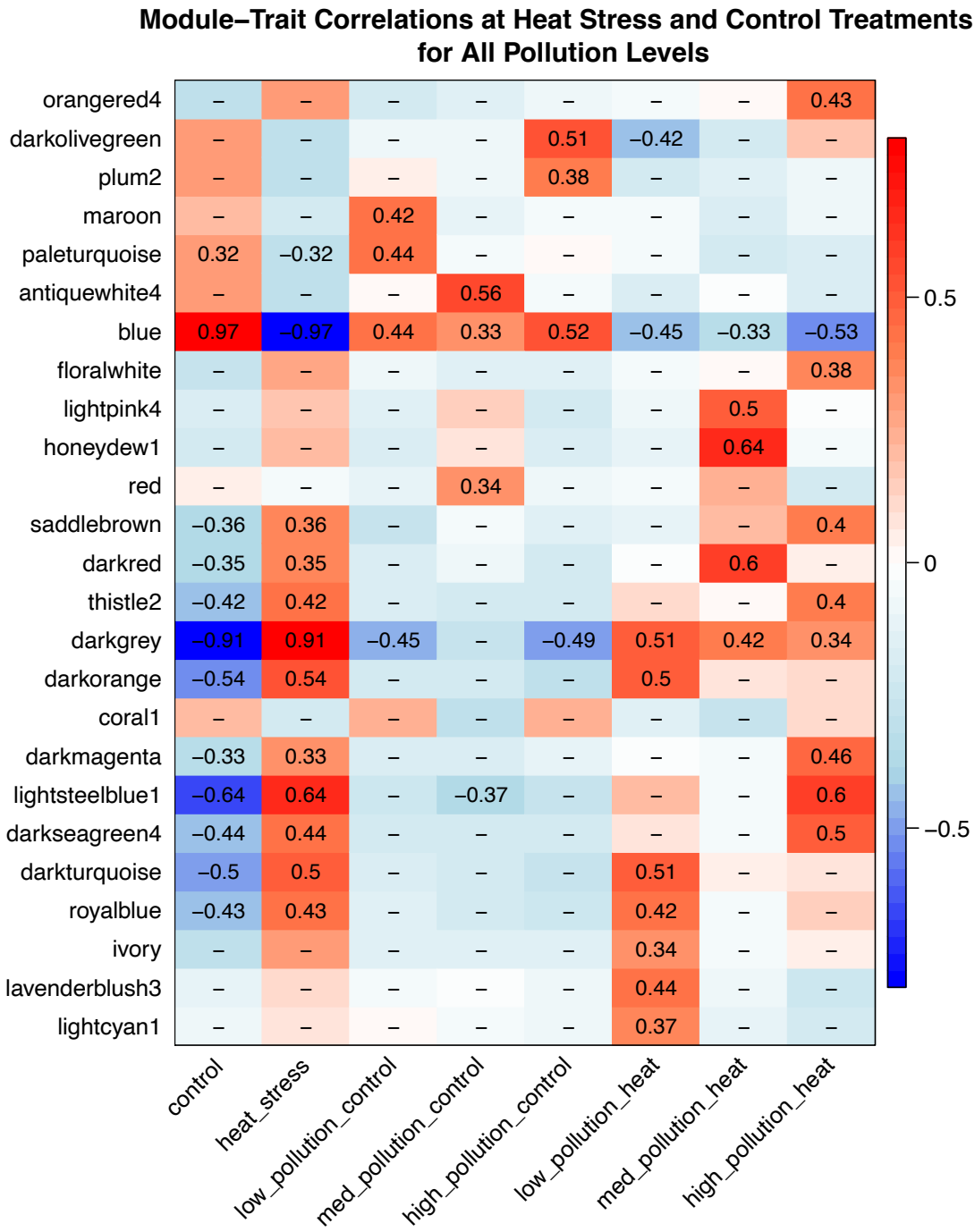

Figure 18. Heatmap showing module-trait correlations for 15,109 genes with treatments including control, heat stress, each pollution level at control, and each pollution level at heat stress. Pearson's R for significant correlations ($p < 0.05$) are reported with red indicating a positive correlation and blue indicating a negative correlation.

Table 5. Significantly enriched Biological Processes (BP) GO terms for the blue and darkgrey modules associated with different correlation patterns in control versus heat stress. GO terms were included if adjusted p-value ≤ 0.001 and were selected by the `GO_MWU` package to best represent independent groups of GOs. GO terms associated with the blue modules were

downregulated under heat stress and those associated with the darkgrey module were upregulated under heat stress.

GO term(s)	Description	adj p. value	Module
Cellular component organization			
GO:0007005	mitochondrion organization	<1e-5	blue
GO:0033044	regulation of chromosome organization	<1e-5	blue
GO:0045104;GO:0045103	intermediate filament cytoskeleton organization	<1e-5	darkgrey
Cellular process			
GO:0030011	maintenance of cell polarity	<1e-5	darkgrey
GO:0030029;GO:0030036	actin filament-based process	<1e-5	darkgrey
Developmental process			
GO:0010001	glial cell differentiation	0.009	blue
GO:0040024	dauer larval development	0.010	darkgrey
Immune response			
GO:0002822;GO:0002819	regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	<1e-5	darkgrey
GO:1902105	regulation of leukocyte differentiation	0.004	darkgrey
Localization			
GO:0006913;GO:0051169	nuclear transport	<1e-5	blue
GO:0001539	cilium or flagellum-dependent cell motility	<1e-5	blue
GO:0006839	mitochondrial transport	0.007	blue
Metabolic process			
GO:0008033	tRNA processing	<1e-5	blue
GO:0006099;GO:0006101;GO:0072350	citrate metabolic process	<1e-5	blue
GO:0016052	carbohydrate catabolic process	<1e-5	blue
GO:0018065	protein-cofactor linkage	<1e-5	blue
GO:0006760;GO:0046653	folic acid-containing compound metabolic process	<1e-5	blue
GO:0043112	receptor metabolic process	<1e-5	darkgrey
GO:0045892;GO:1903507; GO:2000113; GO:1902679;GO:0010558;GO:0031327; GO:0051253;GO:0009890;GO:0045934	negative regulation of biosynthetic process	<1e-5	darkgrey
GO:0030163	protein catabolic process	0.008	darkgrey
Response to chemical			
GO:0034097	response to cytokine	0.005	blue
GO:0009636	response to toxic substance	0.006	blue
Response to stimulus			
GO:0009611	response to wounding	<1e-5	darkgrey
GO:0019722	calcium-mediated signaling	<1e-5	darkgrey

Differential Gene Expression at Control

At control conditions, gene expression patterns did not cluster strongly by site (**Figure 19**). However, a heatmap of the 2,155 genes that were differentially expressed among control samples showed stronger patterns by sampling site (**Figure 20**). Gene expression patterns also appeared to relate to the batch in which they were sequenced: Faga'tele, Faga'alu, and Coconut Point were sequenced in 2020 while Cannery and Vatia were sequenced in 2021.

A weighted gene co-expression network analysis was used to measure how gene modules in control samples were correlated with pollution level, symbiont community, and the top 10-30% most and least thermotolerant corals. This analysis showed that the grey60 module correlated to both the high and low pollution treatments, with opposite effects in the low versus high pollution treatments (**Table 6**). Genes associated with organic acid metabolic processes, metabolic processes, and response to chemical were upregulated in the corals from high pollution sites and downregulated in the corals from low pollution sites.

Four gene modules correlated with the most thermotolerant corals. Purple and darkmagenta modules correlated with the top 10% most thermotolerant corals, and the dark orange and paleturquoise modules correlated with the top 20-30%. An analysis in GO_MWU of the modules associated with high thermotolerance showed overrepresentation of genes associated with cytokine production, immune responses, and multi-organism process, which were upregulated in more thermotolerant corals ($p < 0.05$, **Table 7**). Two gene modules correlated with the least thermotolerant corals. Thistle1 and honeydew1 correlated with the 10-20% least thermotolerant corals. An analysis in GO_MWU of the modules associated with high thermotolerance showed overrepresentation of genes associated with apoptosis, protein catabolic process, protein localization, ion transport, RNA processing, and developmental processes, which were upregulated in the least thermotolerant corals ($p < 0.05$, **Table 8**).

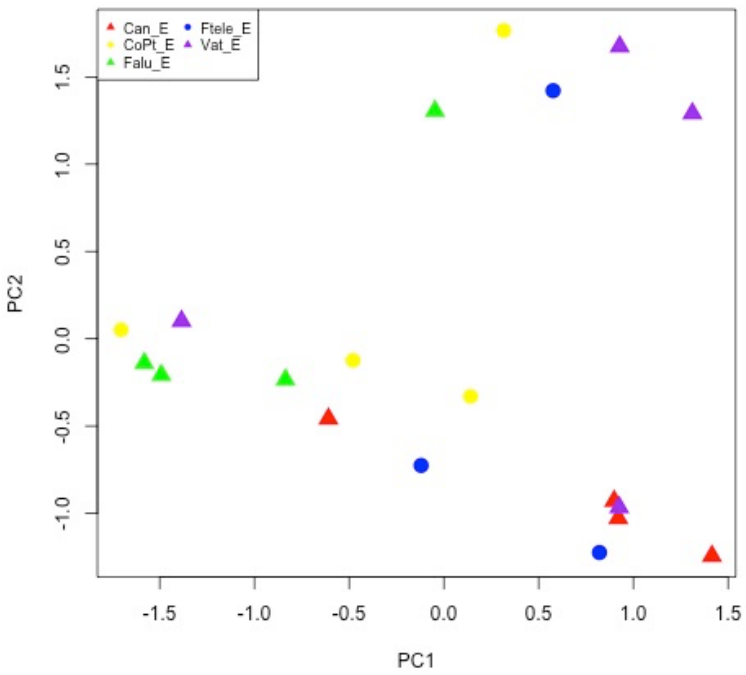

Figure 19. MDS plot showing differences in fold-change of 14,148 genes in corals from control treatments only (prior to differential expression analysis). Red and yellow denote the high pollution sites (Cannery and Coconut Point, respectively). Green denotes the medium pollution site (Faga'alu). Blue and purple denote the low pollution sites (Faga'tele and Vatia, respectively).

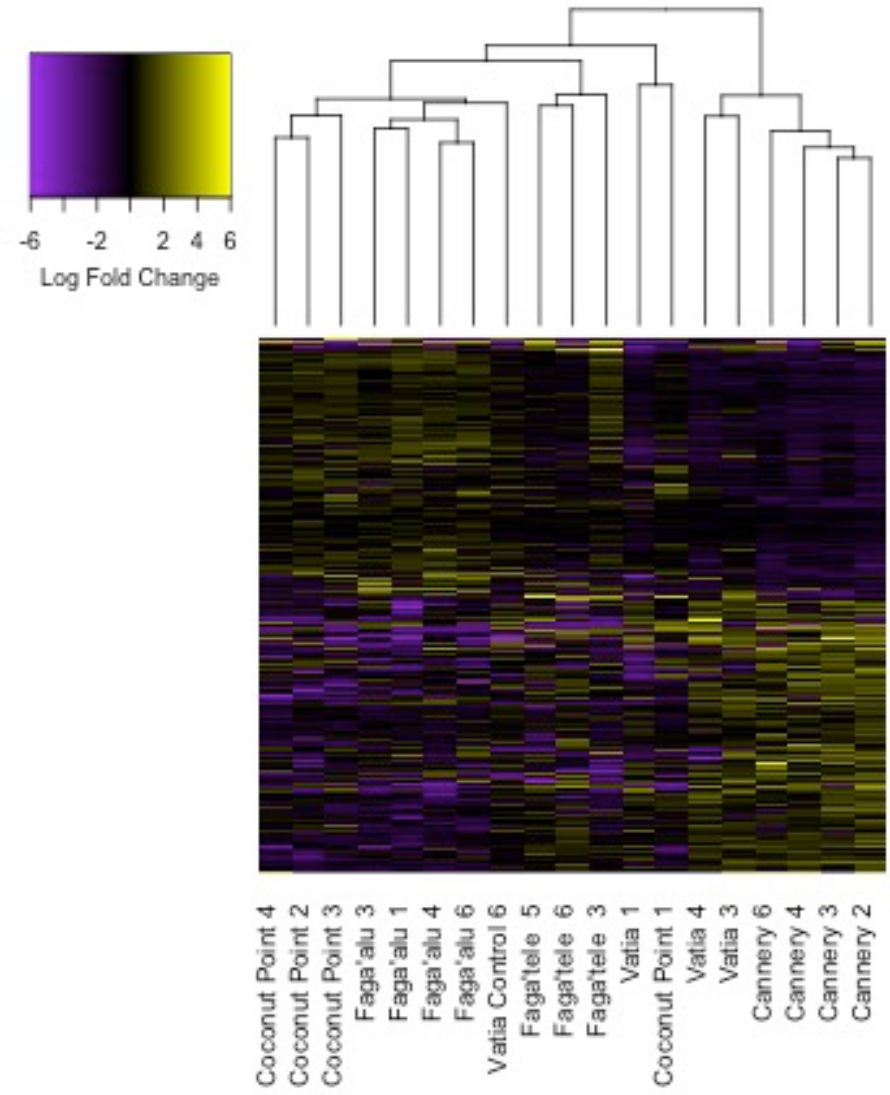

Figure 20. Heatmap showing log2 fold change to visualize gene expression differences among sites in control treatments for 2155 genes (FDR < 0.05) from differential gene expression analysis run in classic edgeR on control (28˚C) samples only.

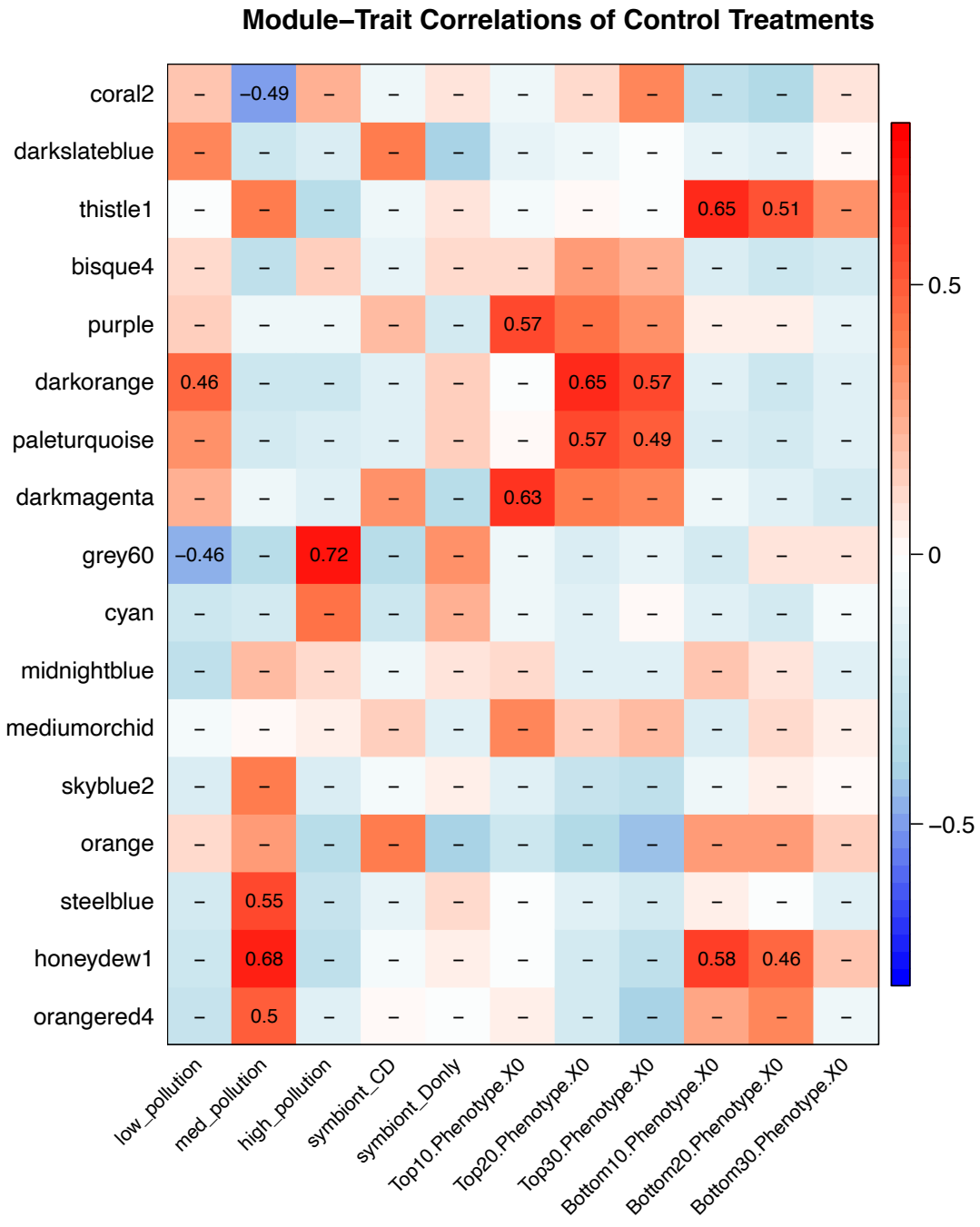

Figure 21. Heatmap showing module-trait correlations for 14,148 genes for control (28˚C) treatments with three pollution level categories, sites hosting a combination of *Cladocopium* and *Durisdinium*, sites hosting entirely *Durisdinium,* and 10, 20 and 30% most/least thermotolerant colonies. Pearson's R for significant correlations ($p < 0.05$) are reported with red indicating a positive correlation and blue indicating a negative correlation.

Table 6. Significantly enriched Biological Processes GO terms for the grey60 module associated with high and low pollution in control only treatments (28˚C) by site. GO terms were included if adjusted p-value ≤ 0.05. All GO terms listed were upregulated in high pollution and downregulated in low pollution corals at control conditions.

GO term(s)	Description	Adj. p value
Cellular component organization		
GO:0071555;GO:0045229; GO:0071554	external encapsulating structure organization	<1e-5
Metabolic process		
GO:0009164;GO:0034656; GO:1901658	nucleobase-containing small molecule catabolic process	0.01
GO:0043094	cellular metabolic compound salvage	0.021
GO:0009116;GO:1901657	glycosyl compound metabolic process	0.023
Organic acid metabolic process		
GO:0000096	sulfur amino acid metabolic process	<1e-5
GO:0019509;GO:0071267; GO:0043102;GO:0071265	L-methionine salvage	<1e-5
GO:0006555;GO:0009086; GO:0000097;GO:0009067	methionine metabolic process	0.013
GO:0046394;GO:0016053	organic acid biosynthetic process	0.017
GO:0009066	aspartate family amino acid metabolic process	0.019
GO:0006520	cellular amino acid metabolic process	0.025
GO:1901607;GO:0008652	cellular amino acid biosynthetic process	0.028
Response to chemical		
GO:0009737	response to abscisic acid	0.025
GO:0009751	response to salicylic acid	0.025

Table 7. Significantly enriched Biological Processes GO terms for modules associated with the most thermotolerant corals (purple, darkmagenta, darkorange, and paleturquoise) as measured prior to heat stress (baseline gene expression at 28˚C). GO terms were included if adjusted p-value ≤ 0.05. All GO terms listed were upregulated in the most thermotolerant corals in control conditions compared to less thermotolerant corals.

GO term(s)	Description	Adj. p value	Module
Cytokine production			
GO:0001819	positive regulation of cytokine production	0.033	darkmagenta
GO:0001816	cytokine production	0.038	darkmagenta
GO:0071345	cellular response to cytokine stimulus	0.045	darkmagenta
GO:0001817	regulation of cytokine production	0.05	darkmagenta
Immune response			
GO:0006955	immune response	0.025	darkmagenta
GO:0045088;GO:0045089; GO:0031349	regulation of innate immune response	0.05	darkmagenta
Miscellaneous			
GO:0043900	regulation of multi-organism process	0.025	darkmagenta
GO:0048525	negative regulation of viral process	0.04	darkmagenta
GO:0043901	negative regulation of multi-organism process	0.05	darkmagenta
GO:0006412	translation	0.05	purple

Table 8. Significantly enriched Biological Processes GO terms for modules associated with the least thermotolerant corals (honeydew1 and thistle1) as measured prior to heat stress (baseline gene expression at 28˚C). GO terms were included if adjusted p-value ≤ 0.05. All GO terms listed were upregulated in the least thermotolerant corals in control conditions compared to more thermotolerant corals.

GO term(s)	Description	Adj. p value	Module
Apoptosis			
GO:0042771;GO:0072332	intrinsic apoptotic signaling pathway by p53 class mediator	<1e-5	honeydew1
GO:0070059	intrinsic apoptotic signaling pathway in response to ER stress	<1e-5	honeydew1
GO:0008630	intrinsic apoptotic signaling pathway in response to DNA damage	0.007	honeydew1
GO:0097193;GO:0097190	intrinsic apoptotic signaling pathway	0.008	honeydew1
Developmental process			
GO:0030324	lung development	0.008	honeydew1
GO:0010771;GO:0010977; GO:0031345	negative regulation of neuron projection development	0.007	honeydew1
GO:0061002;GO:0050774; GO:0061000; GO:2000171	negative regulation of dendritic spine morphogenesis	0.027	honeydew1
GO:0050768;GO:0010721; GO:0051961	negative regulation of nervous system development	0.034	honeydew1
GO:0007420	brain development	0.035	honeydew1
GO:0045665	negative regulation of neuron differentiation	<1e-5	honeydew1
Ion transport			
GO:0070838;GO:0072511	divalent inorganic cation transport	<1e-5	thistle1
GO:0006812	cation transport	0.05	thistle1
GO:0006811	ion transport	0.05	thistle1
Protein catabolic process			
GO:0010508	positive regulation of autophagy	0.037	honeydew1
GO:0042177	negative regulation of protein catabolic process	<1e-5	honeydew1
GO:0032435;GO:1901799; GO:2000059;GO:1903051; GO:1903363;GO:0007130; GO:0070193	negative regulation of proteasomal protein catabolic process	<1e-5	honeydew1
GO:0032434;GO:2000058	regulation of ubiquitin-dependent protein catabolic process	<1e-5	honeydew1
GO:0042176	regulation of protein catabolic process	<1e-5	honeydew1
GO:0061136;GO:1903050; GO:1903362	regulation of cellular protein catabolic process	<1e-5	honeydew1
GO:0031330;GO:0009895	negative regulation of catabolic process	0.004	honeydew1
GO:0045861	negative regulation of proteolysis	0.007	honeydew1
GO:0031329;GO:0009894	regulation of catabolic process	0.009	honeydew1
GO:0016573;GO:0018393; GO:0018394	internal peptidyl-lysine acetylation	0.036	honeydew1

Protein localization			
GO:0071816;GO:0045048	tail-anchored membrane protein insertion into ER membrane	<1e-5	honeydew1
GO:0090150	establishment of protein localization to membrane	<1e-5	honeydew1
GO:0051205	protein insertion into membrane	0.008	honeydew1
GO:0072657	protein localization to membrane	0.009	honeydew1
GO:0006620	posttranslational protein targeting to ER membrane	0.037	honeydew1
Reproductive process			
GO:0070192	chromosome organization involved in meiotic cell cycle	0.004	honeydew1
GO:1903046	meiotic cell cycle process	0.009	honeydew1
RNA processing			
GO:0000381	regulation of alternative mRNA splicing	<1e-5	honeydew1
GO:0000245	spliceosomal complex assembly	0.03	honeydew1
Miscellaneous			
GO:0022402	cell cycle process	0.036	honeydew1
GO:0007033	vacuole organization	0.036	honeydew1
GO:0072331	signal transduction by p53 class mediator	0.026	honeydew1
GO:0032269;GO:0051248	negative regulation of protein metabolic process	0.033	honeydew1
GO:0070628	proteasome binding	0.05	honeydew1

Differential Gene Expression at Heat Stress

At heat stress, gene expression patterns did not appear to cluster strongly by site (**Figure 22**). However, a heatmap of the 332 genes that were differentially expressed among heat stress samples showed patterns by sampling site (**Figure 23**). Gene expression patterns again appear to relate to the batch in which they were sequenced: Faga'tele, Faga'alu, and Coconut Point were sequenced in 2020 while Cannery and Vatia were sequenced in 2021.

A WGCNA was performed to examine gene modules in heat stressed corals that correlated to polluted level, symbiont community, and high or low performing thermotolerance (**Figure 24**). No modules showed strong trends with pollution level. Four modules correlated to symbiont community type (either hosting a combination of *Cladocopium* and *Durisdinium* or hosting entirely *Durisdinium*). These modules included bisque4, palevioletred3, cyan and darkolivegreen. The darkolivegreen and cyan modules also correlated to the most thermotolerant corals. Additionally, four other modules correlated with at least one category of high thermotolerance (either top 10, 20, or 30% of the most thermotolerant corals). One module, saddlebrown, correlated to the 10 and 20% least thermotolerant corals. Gene ontology analysis

for the four modules correlated to symbiont community type showed enrichment for cellular component organization, metabolic process, and nucleic acid metabolic process (**Table 9**). Gene ontology analysis for the four modules correlated to high thermotolerance showed enrichment for nucleic acid metabolic process, metabolic process, cellular component organization and cellular process (**Table 10**). Gene ontology analysis for the two modules correlated to low thermotolerance showed enrichment for MAPK activity and vesicle-mediated transport, which were upregulated in the least thermotolerant corals.

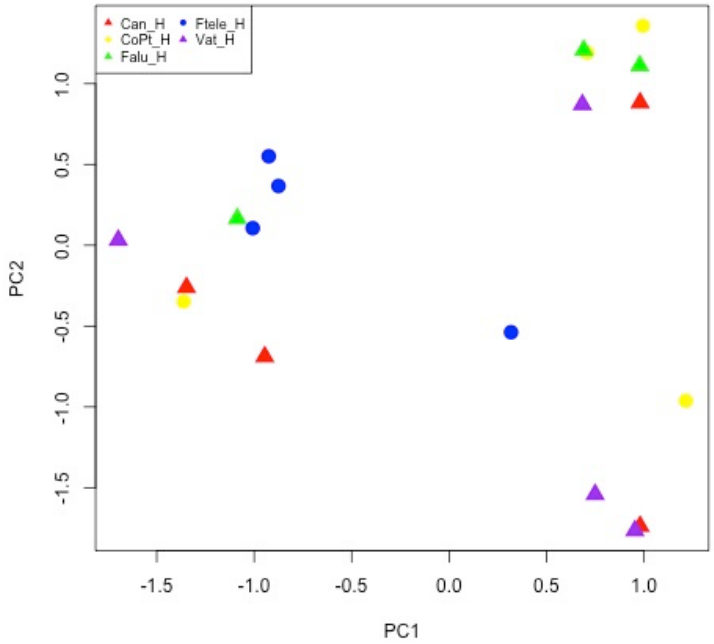

Figure 22. MDS plot showing differences in fold-change of 11,331 genes in corals from heat stress treatment at 35˚C only (prior to differential expression analysis). Red and yellow denote the high pollution sites (Cannery and Coconut Point, respectively). Green denotes the medium pollution site (Faga'alu). Blue and purple denote the low pollution sites (Faga'tele and Vatia, respectively).

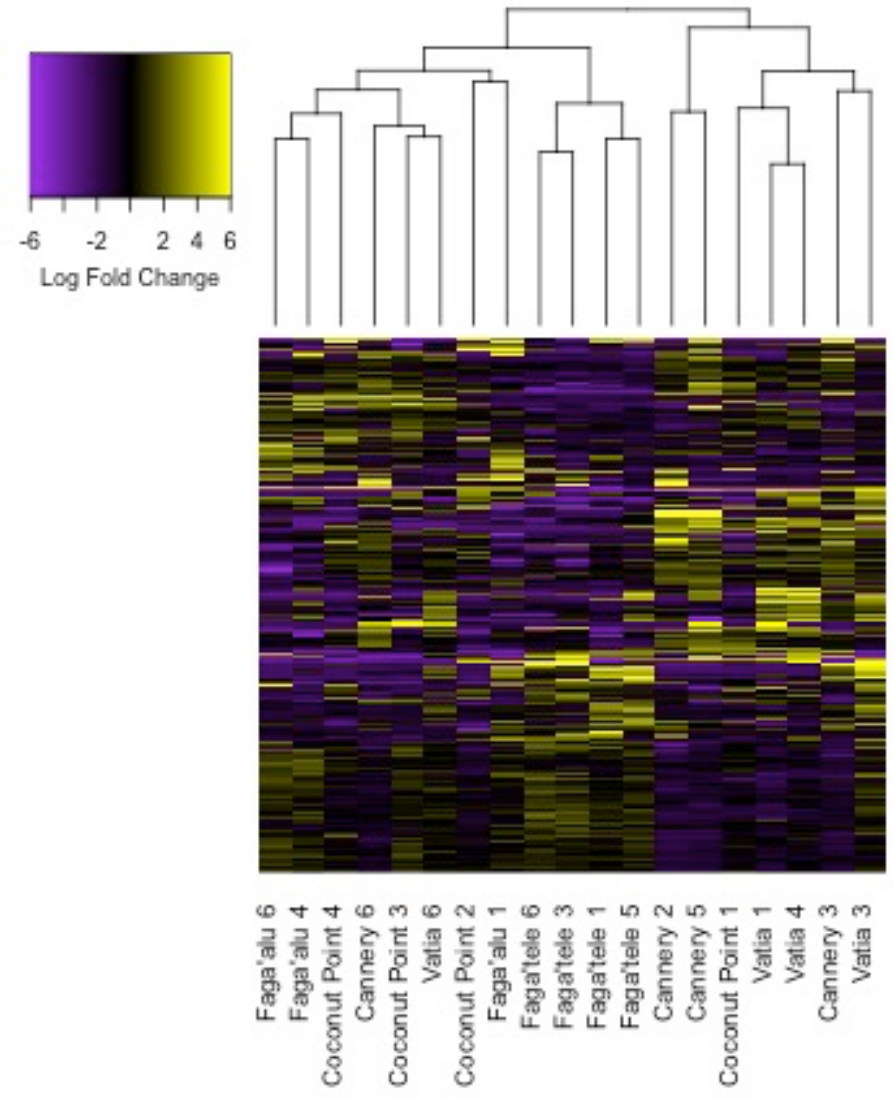

Figure 23. Heatmap showing log2 fold change to visualize gene expression differences among sites in the heat treatment at 35˚C for 332 genes (FDR < 0.05) from differential gene expression analysis run in classic edgeR on only heat stress samples.

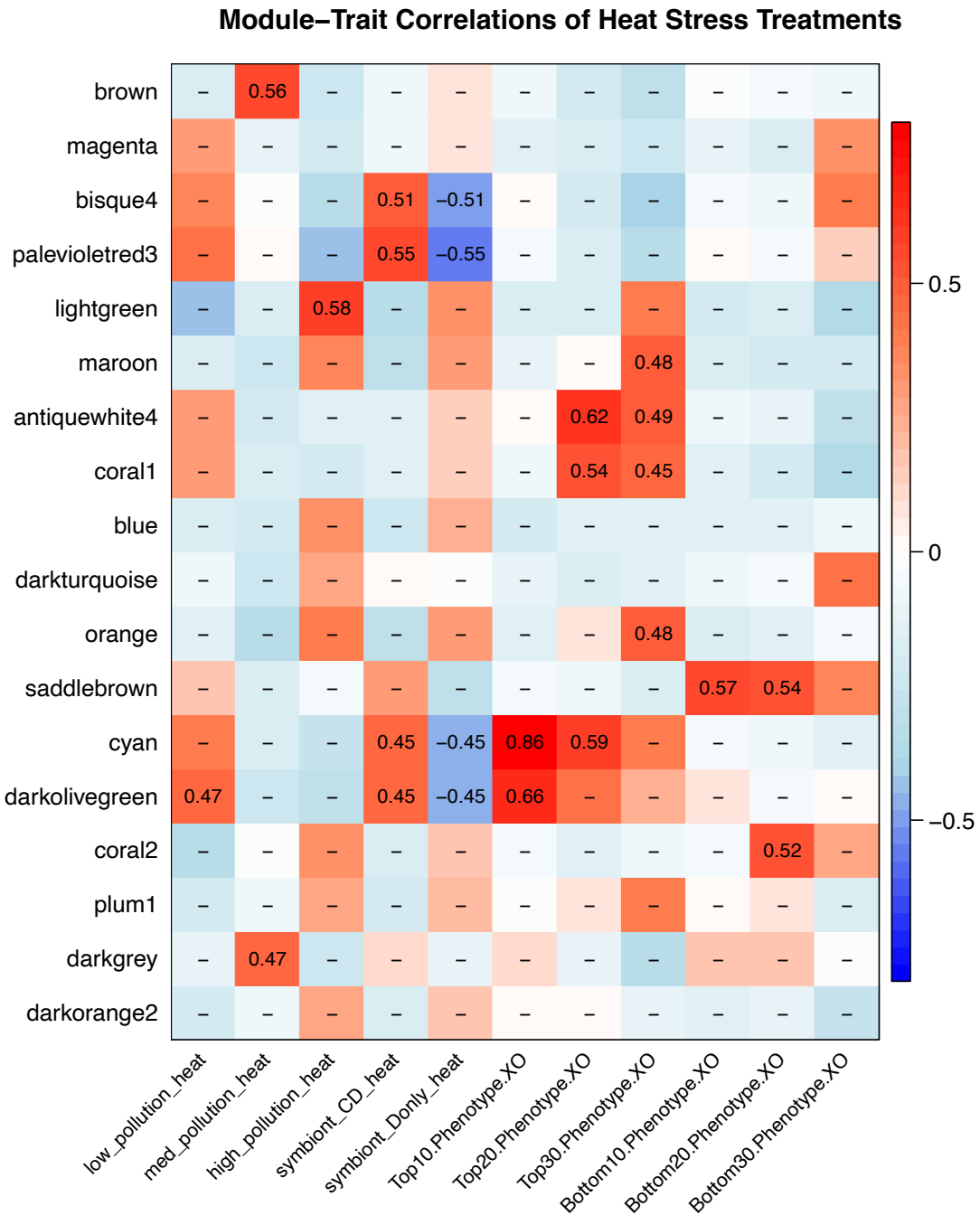

Figure 24. Heatmap showing module-trait correlations for 11,331 genes for heat stress treatment at 35˚C with three pollution level categories, sites hosting a combination of *Cladocopium* and *Durisdinium*, sites hosting entirely *Durisdinium,* and 10, 20, and 30% most and least thermotolerant colonies. Pearson's R for significant correlations ($p < 0.05$) are reported with red indicating a positive correlation and blue indicating a negative correlation.

Table 9. Significantly enriched Biological Processes (BP) GO terms for the darkolivegreen, bisque4, cyan and palevioletred3 modules associated with different correlation patterns in colonies hosting entirely *Durisdinium* versus those hosting *Durisdinium* and *Cladocopium* under heat stress at 35˚C. GO terms were included if adjusted p-value ≤ 0.001 and were selected by the `GO_MWU` package to best represent independent groups of GOs. GO terms associated with all four modules were upregulated in *Durisdinium* and *Cladocopium* colonies and downregulated in *Durisdinium* only colonies.

GO term(s)	Description	adj p value	Module
Cell cycle			
GO:0010564	regulation of cell cycle process	<1e-5	darkolivegreen
Cellular component organization			
GO:0007020	microtubule nucleation	<1e-5	bisque4
GO:0022613;GO:0044085; GO:0042254	cellular component biogenesis	0.004	darkolivegreen
GO:0022618;GO:0071826	ribonucleoprotein complex assembly	0.005	darkolivegreen
Immune response			
GO:0006958	complement activation, classical pathway	<1e-5	bisque4
Ion Transport			
GO:0006816	calcium ion transport	<1e-5	bisque4
Metabolic process			
GO:0006520	cellular amino acid metabolic process	<1e-5	darkolivegreen
GO:0009116;GO:1901657	nucleoside metabolic process	0.008	cyan
Nucleic acid metabolic process			
GO:0006281	DNA repair	<1e-5	darkolivegreen
GO:0008380	RNA splicing	<1e-5	darkolivegreen
GO:0043046;GO:0034587	piRNA metabolic process	<1e-5	cyan
GO:0006281	DNA repair	0.007	cyan

Table 10. Significantly enriched Biological Processes (BP) GO terms for the darkolivegreen, cyan, coral1 and antiquewhite4 modules associated with the top 10-20% thermotolerant corals under heat stress at 35˚C. GO terms were included if adjusted p-value ≤ 0.001 and were selected by the `GO_MWU` package to best represent independent groups of GOs. GO terms associated with all four modules were upregulated in the most thermotolerant corals.

GO term(s)	Description	adj p value	Module
Cellular process			
GO:0048278;GO:0140056; GO:0022406	Membrane docking	<1e-5	coral1
GO:0010564	regulation of cell cycle process	<1e-5	darkolivegreen
Cellular component organization			
GO:0022613;GO:0044085; GO:0042254	cellular component biogenesis	0.004	darkolivegreen
GO:0022618;GO:0071826	ribonucleoprotein complex assembly	0.005	darkolivegreen
Metabolic process			
GO:0001817	regulation of cytokine production	<1e-5	coral1
GO:0006520	cellular amino acid metabolic process	<1e-5	darkolivegreen
GO:0009116;GO:1901657	nucleoside metabolic process	0.008	cyan
Nucleic acid metabolic process			
GO:0006281	DNA repair	<1e-5	darkolivegreen
GO:0008380	RNA splicing	<1e-5	darkolivegreen
GO:0043046;GO:0034587	piRNA metabolic process	<1e-5	cyan
GO:0006281	DNA repair	0.007	cyan

Summary of WGCNA Results

Three analyses were performed to compare gene expression patterns: control and heat stress samples, control samples only, and heat stress samples only. 12 modules correlated to differences between control and heat stress treatments (**Table 11**). Ten modules containing genes involved in reactive oxygen species and signaling were upregulated under heat stress while two modules containing metabolic process genes were downregulated. Among control samples, only one module correlated to differences between high and low pollution, and contained genes involved in external encapsulating structure organization (**Table 11**). The control sample comparison also showed four modules upregulated in the most thermotolerant corals and two modules upregulated in the least thermotolerant corals. The most thermotolerant corals showed

higher expression of external encapsulating cytokine production and immune response genes at control conditions compared to less successful corals. Among heat stress samples, four modules related to symbiont community and were involved in gene silencing and RNA processing (**Table 11**). Heat stress samples also showed gene expression differences between the most and least thermotolerant corals, with four modules upregulated in the top performers and two modules upregulated in the bottom performers. Top performers upregulated genes involved in gene silencing and RNA processing, and bottom performers upregulated genes involved in transport and signaling.

Table 11. Summary of all three WGCNA analyses (control and heat stress samples, control samples, and heat stress samples). Analysis is listed along with significantly correlated modules ($p < 0.05$) to heat-stress related phenotypes. Number of genes in module, Pearson's R, and expression pattern (upregulated or downregulated) are also listed. Top Biological Processes (BP) and Molecular Function (MF) Gene Ontology categories are also reported. Under phenotype, "D only" refers to colonies hosting entirely *Durisdinium,* and "top/bottom 10%" refers to thermotolerance performance.

Analysis	Figure	Module	Num. genes	Phenotype/ Treatment	Correlation	Expression Pattern	Top GO term (BP)
Control v heat	18	paleturquoise	117	heat stress	-0.32	down	
Control v heat	18	blue	6036	heat stress	-0.97	down	Small molecule metabolic process
Control v heat	18	saddlebrown	125	heat stress	0.36	up	Amino acid activation
Control v heat	18	darkred	172	heat stress	0.35	up	DNA metabolic process
Control v heat	18	thistle2	391	heat stress	0.42	up	DNA integration
Control v heat	18	darkgrey	3969	heat stress	0.91	up	Signaling
Control v heat	18	darkorange	291	heat stress	0.54	up	
Control v heat	18	darkmagenta	112	heat stress	0.33	up	Reactive oxygen species
Control v heat	18	lightsteelblue 1	691	heat stress	0.64	up	
Control v heat	18	darkseagreen 4	150	heat stress	0.44	up	Cellular metabolic process
Control v heat	18	darkturquoise	267	heat stress	0.5	up	Downregulation of cytokine production
Control v heat	18	royalblue	174	heat stress	0.43	up	DNA biosynthetic process
Control	21	grey60	190	low pollution	-0.46	down	External encapsulating structure organization
Control	21	grey60	190	high pollution	0.72	up	External encapsulating structure organization
Control	21	purple	495	top 10%	0.57	up	Translation
Control	21	darkmagenta	117	top 10%	0.63	up	Immune response
Control	21	darkorange	267	top 20%	0.65	up	
Control	21	paleturquoise	406	top 20%	0.57	up	
Control	21	thistle1	69	bottom 10%	0.65	up	Inorganic cation transport
Control	21	honeydew1	280	bottom 10%	0.58	up	Downregulation of catabolic process
Heat stress	24	bisque4	412	D only	-0.51	down	Calcium ion transport
Heat stress	24	palevioletred 3	762	D only	-0.55	down	Developmental processes
Heat stress	24	cyan	256	D only	-0.45	down	Gene silencing by RNA
Heat stress	24	darkolivegree n	600	D only	-0.45	down	RNA processing
Heat stress	24	cyan	256	top 10%	0.86	up	Gene silencing by RNA
Heat stress	24	darkolivegree n	600	top 10%	0.66	up	RNA processing
Heat stress	24	antiquewhite4	627	top 20%	0.62	up	RNA splicing
Heat stress	24	coral1	472	top 20%	0.54	up	Membrane docking
Heat stress	24	saddlebrown	143	bottom 10%	0.57	up	Transport
Heat stress	24	coral2	210	bottom 20%	0.52	up	Activation of MAPKK activity

DISCUSSION

Our study represents one of few field-based assessments of how pollution affects coral thermotolerance and attempts to uncover two potential thermotolerance mechanisms that may be influenced by pollution. We exposed corals from a gradient of pollution levels to a heat stress assay to determine how thermotolerance varies with pollution level, and how thermotolerance and pollution level vary with symbiont community and gene expression patterns. We found that the thermotolerance phenotype did not correlate with pollution level, but that symbiont community and gene expression patterns were related to pollution level. Corals at polluted sites hosted entirely heat-tolerant *Durisdinium* while corals at low and moderately polluted sites hosted a combination of *Durisdinium* and *Cladocopium.* Gene expression patterns were driven primarily by heat stress but also correlated with pollution level. At control conditions, expressional levels of some genes were correlated with pollution level, indicating some differences in baseline gene expression at polluted sites. Additionally, expression of gene networks in control corals were correlated with their subsequent performance under heat stress, indicating differences in baseline gene expression in corals that may dictate heat stress responses regardless of site or pollution level.

Thermotolerance

Thermotolerance around Tutuila did not vary by pollution

We measured thermotolerance across five sites of different pollution levels in Tutuila, American Samoa using three different metrics: red intensity via colorimetric analysis, CoralWatch color health score, and photochemical efficiency. Red intensity and coral health scores both measure color paling while photochemical efficiency measures endosymbiont function. There was variation in these three metrics, but Faga'tele was consistently more sensitive to heat stress than Vatia. Both of these sites were characterized as low pollution, since both sites have watersheds that support a low human population, and both have shown low DIN loading (Tuitele et al. 2019; Shuler and Comeros-Raynal 2020). Faga'tele was found to have the lowest dissolved inorganic nitrogen of 25 sites sampled across Tutuila (Comeros-Raynal et al. 2019). Faga'tele is also a NOAA National Marine Sanctuary and is the southernmost site on Tutuila that was sampled in this study. These factors combined may justify Faga'tele as the

'lowest' pollution site of the five sampled. However, Faga'tele was most affected by the heat stress assay, showing significant bleaching over Vatia in two of three metrics. Vatia is also characterized as a low pollution site with modest dissolved inorganic nitrogen and is also a U.S. National Park (Comeros-Raynal et al. 2019). However, when comparing Vatia and Faga'tele, the pollution impact at Vatia is higher that Faga'tele. Vatia's watershed hosts a modest human population as well as some piggeries while Faga'tele's watershed hosts virtually zero humans or other land-based activities that would impact the water quality there. Yet, these two sites remain the two lowest pollution sites compared with the other sites we examined. Since the two lowest pollution sites showed different responses to heat stress, environmental differences other than pollution level appear to be driving heat stress responses.

One potential environmental difference at these two sites is wave energy. Vatia and Faga'tele both are exposed reefs (not wave sheltered) but differ in their wave energy intensity. Faga'tele has stronger wave energy than Vatia, though both sites have stronger wave energy than sheltered sites such as Cannery (Comeros-Raynal et al. 2019). Higher water flow associated with higher wave energy has been shown to reduce photoinhibition, which can buffer the effects of higher temperatures and reduce bleaching during heat stress (Nakamura et al. 2003; Nakamura et al. 2005). However, Faga'tele was the site with the highest wave energy, yet it bleached the most during heat stress.

Another important difference to note is that Vatia is on the north side of Tutuila and is classified as a North reeftype, which has been shown to be distinct from the Southern reeftype (Houk et al. 2010; Comeros-Raynal et al. 2019). The Northern reeftype, typically seen on the North side of Tutuila, tends to have less interstitial space in the reef matrix and a well-cemented reef basement while the Southern reeftype, typically seen on the South side of Tutuila, tends to have more interstitial porosity (Houk et al. 2010). These geomorphological differences have been shown to relate to biological differences between the northern and southern reefs on Tutuila, including distinct coral, fish, and benthic assemblages (Comeros-Raynal et al. 2019). Since Vatia was the only Northern reeftype and the most consistently different site, it is possible that Northern reeftype corals are more thermotolerant than the Southern reeftype corals, though additional Northern reeftype site replicates would be needed to support this claim.

Thermotolerance did not relate to thermal history

Physiological measurements did not appear to relate to variation in thermal history across the sites. Temperature data collected in August 2019 showed that similar temperature profiles at Faga'alu, Faga'tele, and Vatia, while Cannery was cooler. Additionally, Cannery and Vatia showed less variability in temperature compared to the other sites. Cooler and less variable temperatures at Cannery would indicate that Cannery might be more susceptible to bleaching than other warmer sites since previous work has shown that corals living in warmer and more variable environments tend to be less susceptible to bleaching (Oliver and Palumbi 2011a; Barshis et al. 2013). However, Cannery corals did not consistently bleach more than other sites. Additionally, variation in bleaching metrics did not appear to correlate strongly with average, maximum, minimum or standard deviation temperature. Hence, it appears that thermotolerance was influenced more strongly by factors other than recent thermal history at these five sites. Temperature data over longer time intervals could provide additional insight since temperature data collected during this study is limited to a 4 to10 day period and may be overlooking longer-scale differences in thermal history among sites.

Limitations to our assessments of thermotolerance

To further isolate the effects of pollution on coral thermotolerance, future work should include additional site replicates, especially of the Northern reeftype. This may determine if reeftype influences thermotolerance. Additionally, a similar experiment could be repeated during summer months to determine if similar trends are seen during time periods when corals are more likely to experience bleaching. This study was conducted in August, during winter in Tutuila when corals are unlikely to bleach. Bleaching differences may be more apparent and more relevant during summer seasons, when natural bleaching events are more likely to occur. Future studies should also investigate the potential for variation in cryptic species *Acropora hyacinthus*. Cryptic *A. hyacinthus* may vary in their thermotolerance and may be identified using genetic methods (Ladner and Palumbi 2012). Finally, this study was a field-based study attempting to uncover how pollution impacts thermotolerance; but factors other than pollution may be influencing our results. To study impacts of pollution (e.g., elevated nutrient levels) on thermotolerance without confounding factors, a lab-based study manipulating one variable (e.g., elevated nutrient levels) would be useful. These studies have been conducted (and have been

described in the introduction) but they may overlook how pollution impacts corals *in situ* (Rosic et al. 2014; Rosset et al. 2017). Recent field-based studies have investigated effects of nutrients on coral along natural gradients or through field-based nutrient enrichment experiments but have not also investigated plastic processes that may account for thermotolerance differences (Becker and Silbiger 2020; Becker et al. 2021). Our field-based study does not include extensive water quality sampling in the time leading up to the study nor does it include fine scale differences in water quality at each reef, therefore our estimations of pollution at each site are a rough estimation that may be difficult to compare to lab-based studies.

Symbiont Communities

Polluted sites hosted entirely heat-tolerant Durisdinium

At all sites, coral fragments contained primarily *Durisdinium*, with the proportion of *Cladocopium* increasing with lower pollution level. This pattern of higher proportions of *Durisdinium* at high pollution sites follows previous work showing that more variable or stressful regions tend to favor *Durisdinium* (Fabricius et al. 2004; Oliver and Palumbi 2009; Carballo-Bolaños et al. 2019). Some work has also linked increased proportions of *Durisdinium* to areas with higher pollution level or human impact (LaJeunesse et al. 2010). Our findings support the idea that corals that undergo pollution stress, similarly to heat stress, favor *Durisdinium*. This preference for *Durisdinium* in more stressful regions may be due to symbiont shuffling: whereby higher stress in polluted areas has induced a shift in symbiont communities towards *Durisdinium.* Symbiont community data from 2014 showed that symbiont communities across all sites hosted higher levels of *Cladocopium* (Oliver et al., unpublished) compared with data presented here, collected in 2019. This supports the idea that a shift in symbionts may have occurred between 2014 and 2019, perhaps due to bleaching events in 2015 and 2017 (Morikawa and Palumbi 2019; Witze 2015).

Interestingly, in our study, the proportions of *Cladocopium* to *Durisdinium* did not appear to relate to thermal history differences among the sites. This contrasts with other work showing that mean maximum temperatures tend to correlate with the percentage of *Durisdinium* (Oliver and Palumbi 2009; Cooper et al. 2011; Oliver and Palumbi 2011b). Though it should be noted that our measurements of symbiont communities represent a snapshot in time and our thermal history measurements also are limited in scope. Additionally, the differences in the percentages

of *Durisdinium* were minimal, meaning that all sites had similar symbiont communities. Therefore, it may be more difficult to correlate these small differences in symbiont community to other environmental factors like thermal history. However, since thermal history did not correlate to symbiont community differences while pollution level did correlate to symbiont community, our results may suggest that pollution is equally or more important than temperature in determining symbiont community.

The results presented here show that less polluted sites tend to host a higher proportion of *Cladocopium* while highly polluted sites tend to host only *Durusdinium.* This larger proportion of *Durusdinium* at higher polluted sites may confer increased thermal tolerance. *Durusdinium* has been shown to tolerate 1.0 to 1.5°C higher than *Cladocopium* (Berkelmans and van Oppen 2006). Therefore, it is possible that more polluted sites hold more thermotolerant corals – though our results show that the most thermotolerant site, Vatia, had the second highest proportion of *Cladocopium.* Differences in proportions of *Cladocopium* may relate to other physiological differences that were not the focus of this study, including growth rate. Corals that host primarily *Cladocopium* exhibit faster growth rates than those hosting primarily *Durusdinium* (Stat and Gates 2011). Additionally, hosting a single symbiont species may offer the coral host less flexibility to react to changes in their environment. Corals that hosted a single symbiont species did not see a change in symbiont composition when exposed to environmental stress, including heat stress (Goulet 2006). This reduction in symbiont diversity at polluted sites may improve thermal tolerance (though not shown in this study) but at the expense of possible tradeoffs and reduced flexibility to respond to environmental change. Though it should also be noted that differences in symbiont communities among sites in our study were minimal.

High levels of Durisdinium in 2019 indicate a shift from prior levels

When compared to previous data from *A. hyacinthus* symbiont communities from the same sampling sites in Tutuila, our results indicate a shift in the symbiont community towards *Durisdinium* (Oliver et al. unpublished). In 2014, all corals sampled around Tutuila hosted *Cladocopium* and *Durisdinium,* with a higher proportion of *Cladocopium* at all sites (Oliver et al. unpublished). By 2019, coral fragments at all sites hosted almost entirely *Durisdinium*. This shift over time may have occurred due to symbiont shuffling after bleaching, such as after the bleaching event in 2015 and/or 2017 (Morikawa and Palumbi 2019; Witze 2015). Since

Durisdinium outcompete other symbiont species in stressed corals, they are predicted to continue to overtake coral symbiont communities over time, especially after continual bleaching events (Stat and Gates 2011; Howells et al. 2020). Shifts to *Durisdinium* typically increase coral thermotolerance, though this trend was not seen in this study. In 2014, differences in the proportion of *Durisdinium* among corals was more apparent, and these differences explained variation in thermotolerance (Oliver et al. unpublished). By 2019, *Durisdinium* essentially dominates symbiont communities, and thermotolerance variation can no longer be explained by symbiont community. Additionally, shifts to *Durisdinium* may be accompanied by tradeoffs, including to growth rate (Stat and Gates 2011). While growth rate was not included in this study, it is possible that corals in 2019 had lower growth rates or other physiological differences compared to 2014.

Limitations to symbiont community analysis

There are a few limitations of this symbiont community analysis that should be noted here. In this study, we measured two symbiont genera, *Cladocopium* and *Durisdinium,* since they are the most prevalent on Tutuila, but it is possible that other symbiont species are present that we did not measure. For example, *Symbiodinium* (formerly clade A) has been detected at low levels on Tutuila but was not measured as a part of this study (Oliver and Palumbi 2009). Additionally, there was variation among colonies within each site, with multiple coral fragments at each site that hosted entirely *Durisdinium.* There may be within-site differences among coral colonies that account for these differences in symbiont community, including depth, light exposure, or other environmental variables that we did not measure within sites (Frade et al. 2008; Innis et al. 2018). For example, distance from shore has been shown to affect symbiont community in American Samoa, with *Durisdinium* dominating back-reef habitats and a combination of *Durisdinium* and *Cladocopium* in fore-reef habitats (Oliver and Palumbi 2009). This study did not measure distance from shore (either within or among-sites), which may also influence symbiont community. There may also be variation within the fragments that were sampled, as it has been shown that symbiont proportions can vary over different portions of a single colony (Goulet and Coffroth 2003; Rowan et al. 1997). Future work encompassing broader field sampling and a larger sample size per site could address some of these limitations.

Gene Expression

Gene expression patterns were best explained by heat stress

When examining all sites and treatments, gene expression patterns were driven primarily by heat stress. As seen in numerous other studies, heat stress is a strong driver of gene expression patterns (Li and Dewey 2011; Barshis et al. 2013). Heat stress appeared to induce gene expression shifts in known heat shock response genes including Heat Shock Protein 70 (hsp70). Hsp70 has been proposed as a biomarker of environmental stress in corals since it tends to upregulate under thermal stress, as well as under general stress (Louis et al. 2017; Wiens et al.) Hsp70 is a part of a broader group of heat shock proteins, which are molecular chaperones responsible for maintaining the integrity of proteins and protein complexes that may be damaged during stress (Louis et al. 2017). HSPs have been observed to upregulate in a variety of organisms under heat stress, including corals (Fangue et al. 2006; Kenkel et al. 2011; Bentley et al. 2017). The 'common core' set of heat stress response genes shared across all sites included 35 genes annotated as associated with 'heat shock proteins' (**Figure 16**). Other cellular stress response genes found to be differentially regulated under heat stress include those involved in apoptosis, protein folding, metal ion binding, and DNA binding.

A weighted gene co-expression network analysis (WGCNA) indicated numerous gene modules that related to the heat stress response (**Table 5**). Two modules were negatively correlated with the heat stress treatment and ten modules were positively correlated with the heat stress treatment, indicating that multiple groups of genes are involved in the heat stress response. The blue WGCNA module contained a large suite of genes that were downregulated under heat stress. Some of these gene groups included metabolic and catabolic process genes, which typically downregulate when an organism is under stress in order to reduce energetic cost (Hand and Hardewig 1996). The darkgrey module was the most significantly correlated upregulated module associate with heat stress. This module included some expected heat stress response genes, including GO terms falling under 'response to stress,' 'signaling,' and 'immune response' categories (Kültz 2005; Palmer et al. 2008). Notably, enrichment for the 'response to wounding' category was upregulated under heat stress. Taken together, these patterns indicate that heat-stressed corals in our study are responding to macromolecular damage via gene expression.

In examining the heat stress response among sites, 476 genes were commonly found to be involved in the heat stress response among all sites (**Figure 16**). This indicates that a common core set of genes are differentially regulated under heat stress regardless of site-specific differences. When comparing how genes are differentially expressed between heat stress and control among sites, it appears that higher pollution sites differentially express more genes compared to lower pollution sites (**Figure 16**). Coconut Point (high pollution) showed the largest number of differentially expressed genes between control and heat stress. Since gene expression shifts can be energetically expensive, the considerable shift in gene expression at Coconut Point may be disadvantageous to coral health at that site. Coconut Point did bleach more than other sites in one out of three physiology metrics measured in this study, but this is not strong evidence that greater gene expression shifts at that site affected bleaching.

When gene expression differences were compared between low and high pollution sites, it appeared that gene expression profiles were very different in control treatments but converged under heat stress. While there were hundreds to thousands of differentially expressed genes between low pollution sites and between high pollution sites at control, there were only tens of differentially expressed genes between low pollution sites and between high pollution sites during heat stress. This lack of gene expression diversity under heat stress suggests that these corals are expressing conserved genes needed to tolerate heat stress and cannot afford to express their normal site-specific variation in gene expression. This trend is again seen when comparing gene expression differences among all sites at control and among all sites at heat stress. There were 2155 genes differentially expressed among controls and only 332 genes differentially expressed under heat stress (**Figure 20, Figure 23**). Under high heat stress, corals typically express a conserved response (DeSalvo et al. 2010; Barshis et al. 2013; Thomas et al. 2019; Dixon et al. 2020). Yet, under mild stress, corals have been shown to have more variable gene expression patterns (Dixon et al. 2020). This also suggests that 35˚C represents a high heat stress temperature for *A. hyacinthus.*

Baseline gene expression patterns related to pollution level

While gene expression profiles among controls did not group by site on an MDS plot (**Figure 19**), some grouping by site was seen on a heatmap (**Figure 20**). However, gene expression appeared to group by the batch in which sites were sequenced: Coconut Point (high

pollution), Faga'alu (moderate pollution), and Faga'tele (low pollution) in 2020 and Cannery (high pollution) and Vatia (low pollution) in 2021. Batch effects in RNA sequencing data have been reported in previous work and may be underlying these groupings (Liu and Markatou 2016). However, each sequencing batch included one low and one high pollution site, so by grouping these pollution treatments together, we can attempt to filter out these potential batch effects.

At control conditions, gene expression patterns were compared among sites to determine if pollution affects gene expression prior to heat stress, and to examine the possibility of "front-loading (*sensu* Bashis et al. 2013). In a WGCNA analysis of gene expression patterns among controls, the grey60 module showed opposing patterns in the low pollution versus the high pollution sites (**Figure 21**). This module contained genes relating to 'response to chemical,' indicating that chemical detection and response genes are upregulated in corals from higher pollution sites. These genes included categories of 'response to abscisic acid' and 'response to salicylic acid,' both of which are plant hormones that may counter oxidative stress (Larkindale and Knight 2002). Additionally, this module contained multiple GO categories relating to various metabolic and biosynthetic processes, suggesting that corals exposed to higher pollution may be focusing more energy on metabolism compare to those exposed to lower pollution. This supports other research showing that low to moderately elevated nutrient levels improve coral growth and metabolism (Bongiorni et al. 2003; Sawall et al. 2011; Morris et al. 2019). When compared to GO categories upregulated under nutrient stress in Rosic et al. (2014), none of the categories matched GO terms in the grey60 module. We also compared the GO terms of genes in the grey60 module to the generalized *Acropora* stress response GO terms from Dixon et al. (2020) and found five of the 190 genes shared GO terms. These five genes were primarily mini-collagen and calcium-binding proteins. While few of the GO terms in the grey60 module matched previous studies, many of the GO terms appears to be involved in production of methionine, which is an amino acid that has been shown to mitigate oxidative stress (Luo and Levine 2009; Aguilar et al. 2017). Therefore, it is possible that pollution is inducing stress response genes, just different genes than those from two previous studies. This may be due to the highly context-dependent nature of pollution, whereby differences in pollution may induce different stress response genes.

Baseline gene expression correlated with thermotolerance

Gene expression at control conditions was linked to thermotolerance during heat stress in six gene modules (**Figure 21**). Interestingly, those gene expression patterns did not appear to be dictated by sample site, meaning that regardless of site, some corals express genes that correlate with future heat stress tolerance. This suggests that baseline gene expression may dictate how corals will respond to heat stress. In the most thermotolerant corals, baseline gene expression including upregulation of cytokine production and immune response genes (**Table 7**).

To examine the possibility that these genes are frontloaded in the most thermotolerant corals, we compared the genes in the four modules where baseline expression correlated to high thermotolerance against frontloaded genes identified in Barshis et al. (2013). The four modules correlating to high thermotolerance matched three of the 135 genes identified as frontloaded in Barshis et al. (2013). These three genes were annotated as a large repetitive protein, a non-collagenous (NC) domain protein, and a protein kinase family protein (Barshis et al. 2013). In the least thermotolerant corals, baseline gene expression included upregulation of apoptosis and ion transport, which are characteristic stress response genes (Kültz 2005). These patterns indicate that corals with baseline gene expression patterns characteristic of the cellular stress response perform worse during heat stress. Our results suggest that expression of certain stress response genes can hinder cellular stress response effectiveness, perhaps due to the severity of the stress. Expression of apoptotic or programmed cell death related genes could be indicative of severe or chronic stress. This idea has been proposed previously: constitutive expression of stress response genes may not benefit organisms if 1) overexpression of these genes is costly or 2) these genes drive tradeoffs in the stress response (Rivera et al. 2021). The most thermotolerant corals may use other gene pathways to protect against macromolecular damage without triggering apoptosis (Rivera et al. 2021). Baseline levels of thermotolerant corals in our study expressed higher levels of cytokine production and immune response genes. One study found that disease-tolerant corals upregulated cytokine-related pathways under stress while disease-susceptible corals upregulated apoptotic-related pathways (Fuess et al. 2017). Taken together, our results indicate that cytokine production and immune response genes at baseline conditions benefit corals during heat stress while apoptosis-related genes hinder thermotolerance. These differences in baseline gene expression may be due to variables that were not measured in this study, including environmental, ecological, or evolutionary variation.

Gene expression during heat stress correlated with thermotolerance and symbiont community

During heat stress, gene expression did not correlate with pollution, but did correlate with symbiont community (**Figure 24**). This follows previous work showing that symbiont community can affect gene expression in the coral host (Yuyama et al. 2012; Barfield et al. 2018; Helmkampf et al. 2019). Two gene modules showed opposite patterns in corals hosting entirely *Durisdinium* comparing to those hosting *Cladocopium* and *Durisdinium*. These four modules were upregulated in *Cladocopium*-containing corals during heat stress. These modules contained genes involved in nucleic acid metabolic process and cellular component organization (**Table 9**). Interestingly, two of these modules that were upregulated in *Cladocopium*-containing corals were also upregulated in the most thermotolerant corals. This is unexpected since previous work shows that colonies hosting entirely *Durisdinium* are typically more thermotolerant (Berkelmans and van Oppen 2006; Stat and Gates 2011; Howells et al. 2020). This indicates that our results suggest that a small fraction of *Cladocopium* supports gene expression patterns that correlate to higher thermotolerance, though we note that levels of *Cladocopium* in our study were extremely low.

Gene expression at heat stress was also related to high or low thermotolerance (**Figure 24**). Four modules were related to the top 10-20% most thermotolerant corals, and these modules contained genes relating to RNA splicing, processing, and gene silencing. This suggests that the most thermotolerant corals, or corals that host trace amounts of *Cladocopium,* are regulating their RNA in different ways than less thermotolerant corals. RNA processing and modification genes have been shown to upregulate in corals hosting *Cladocopium* compared to those hosting *Durisdinium* (Barfield et al. 2018). This suggests that maintaining symbiosis with *Cladocopium* may require post-transcriptional modifications (Baumgarten et al. 2017; Barfield et al. 2018). The least thermotolerant corals correlated to expression of two gene modules during heat stress, including one that was enriched for MAPK signaling. Mitogen-activated protein kinases (MAPK) are signaling proteins involved in repairing oxidative damage that occurs during stress (Kültz 2005). The least thermotolerant corals are expressing stress response genes during heat stress, perhaps because they are encountering greater macromolecular damage than the more thermotolerant corals.

Limitations in gene expression analysis

There are some important caveats to this gene expression study that should be discussed. As mentioned above, we saw some evidence of batch effects, since Vatia and Cannery corals underwent RNA extraction and sequencing in 2021 while Faga'tele, Faga'alu, and Coconut Point corals underwent RNA extraction and sequencing in 2020. These batch effects may lead to some additional variation in gene expression patterns that is not due to environmental variables, but rather due to differences in sequencing preparation. Additionally, the samples taken for RNAseq during heat stress were taken after a two hour ramp up to 35˚C followed by a two and a half hour hold at 35˚C. Since the heat stress response is known to occur in tiers (e.g., different stress response genes are expressed during the initial hour of heat stress compared to later heat stress), our single timepoint may not capture a complete picture of how heat stress affects corals from different pollution levels (Seneca and Palumbi 2015; Traylor-Knowles et al. 2017). Lastly, our sample size for RNAseq analysis was relatively low (n= 3-4 per site per treatment), but this is not uncommon for gene expression studies where the cost of sequencing is high (Ching et al. 2014). Our results should be interpreted while acknowledging these limitations.

CONCLUSION

This thesis explored the impact of pollution on coral thermotolerance, symbiont communities and gene expression in a field-based experiment. Symbiont communities showed trends with pollution level with more polluted sites hosting higher proportions of heat tolerant *Durisdinium*. Yet, all sites overwhelmingly hosted *Durisdinium,* demonstrating a noticeable shift in symbiont communities from 2014, which contained much higher levels of heat sensitive *Cladocopium*. Thermotolerance was not determined by symbiont communities nor pollution level, but did relate to gene expression patterns, even at control conditions. This suggests that differences in baseline gene expression may allow some corals to better tolerate subsequent heat stress. We found that baseline expression of apoptotic genes resulted in lower coral thermotolerance, and that thermotolerance was improved in corals that upregulated cytokine production genes prior to heat stress and RNA processing genes during heat stress. Future work should investigate what triggers these differences in baseline gene expression to better understand how management efforts can manipulate them to improve coral thermotolerance. This study highlights how gene expression patterns will be especially important in a future where most corals are dominated by *Durisdinium* and symbiont-driven thermotolerance has reached an upper limit.

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