# CHARACTERIZING THE ROLE OF COLOR IN HEAT STRESS RESISTANCE IN THE SCLERACTINIAN CORAL *Acropora surculosa*

**BY**

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**Title: Characterizing the role of color in heat stress resistance in the Scleractinian coral *Acropora surculosa***

Anthropogenic impacts have increased ocean temperatures, causing large-scale bleaching events across the world's coral reefs. Emerging studies suggest that coral color plays a role in bleaching resilience, however the molecular patterns behind coral color and response to heat stress have not yet been characterized. In this study, we characterize the relationship between environmental stress and color phenotypes in *Acropora surculosa* in their natural environment and in controlled experiments at both physiological and transcriptomic levels. We surveyed the distribution of *A. surculosa* color morphs along a depth gradient (2m to 8m) in Pago Bay, Guam for two years. Higher abundances of brown phenotypes were found at shallow depths, in more variable environments. Red phenotypes were more abundant at deeper and more stable environments. We then sequenced 36 transcriptomes of *A. surculosa* to explore gene expression patterns of red and brown color phenotypes during two sequential, acute heat stress assays. We found that red phenotypes are more sensitive to heat stress, require more energy to mitigate the stress, show significant signs of cell damage, and exhibit limited signs of recovery. In contrast, brown phenotypes seem better prepared to cope with thermal stress, utilize more oxidative and immune response pathways to mitigate stress, and rapidly recover. The striking disparity between red and brown colonies in the activation of molecular pathways to respond to thermal stress matches their distribution on the reef and might be due to differences in fluorescent protein

type and abundance. Altogether we highlighted new molecular mechanisms to explain differences between stress resilience in color morphs, affecting bleaching mitigation in an important reef coral on Guam.

**Keywords:** *coral color, bleaching, transcriptomics, molecular biology*

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# Chapter 1

## Impact of Study

Scleractinian corals are the main infrastructure for one of the world's most diverse and productive ecosystems—coral reefs. Coral reefs provide resources that support the livelihood of millions of people, especially those living in coastal ecosystems (Cinner et al., 2012). Worldwide, coral reefs are vital to economic growth in the form of fisheries, tourism, and habitat for valuable species, and serve critical roles in coastal protection, recreation, and cultural or traditional usage (Hicks, 2011). In Guam, coral reefs are the main source of food and income for local fishing families, and are responsible for the economic well-being of the entire community due to the prominence of the tourism industry (Guam Visitors Bureau, 2016). There is a rich cultural history of fishing and spiritual practices that pre-date modern society on the island (Cunningham, 1992). Thus, the success and vitality of coral reefs are crucial.

Global bleaching events have put these delicate ecosystems at risk. From 2013 to 2017, Guam coral reefs experienced nearly five consecutive years of bleaching events (Raymundo, personal communication). This follows global trends, in which bleaching events have become more frequent over time and are expected to continue increasing in frequency and geographic scale (Hoegh-Guldberg, 1998; Hughes et al., 2017). Some locations have experienced extreme losses, with a 50% decrease of living coral cover reported in the Caribbean since the start of reef monitoring in the 1970s (Jackson et al., 2014), and reports of 48-86% declines of large coral colony abundance in the northern half of the Great Barrier Reef since historical baselines from 1995 (Dietzel et al., 2020). While Guam has avoided this massive scale of coral reef decline, the recent bleaching events have significantly disturbed live coral cover at an overall rate of 34% around the island from 2013-2017 (Raymundo et al., 2019). In response on the global scale, there

has been a call for informed management to restore and manage the livelihood of coral reefs (Obura & Grimsditch, 2009).

Understanding coral resilience mechanisms and causes of coral degradation is key to the success of restoration programs (Edwards et al., 2010; Johnson et al., 2011), and provides the evidence needed to shape expectations for future coral community compositions (Darling et al., 2012). As bleaching susceptibility is typically not equal among habitats (Golbuu et al., 2007), coral species (Darling et al., 2012), or even conspecific colonies (Marshall & Baird, 2000), there are a slew of different mechanisms that promote coral resilience to stressors. Currently, a comprehensive understanding of the factors that contribute to differences in bleaching susceptibility is yet to be achieved. This lack of information, combined with the urgency of coral reef decline both worldwide and in Guam, calls for more research questions that address resilience in the face of a warming climate.

**Coral Bleaching**

Coral bleaching causes a breakdown of the relationship with the symbiotic algae, of family Symbiodiniaceae, that the coral host depends on for nutrients, metabolic processes, and disposal of waste (Davy, Allemand, & Weis, 2012; Weis, 2008). Symbiosis can be disrupted by a number of conditions including increased heat, irradiance, or sedimentation, which leads to accumulation of toxic reactive oxygen species (ROS) in the coral tissues, promoting expulsion of symbionts from the coral (Michael P. Lesser, 2006; V. M. Weis, 2008). Another proposed disruption of the coral-symbiont relationship is that stress drives the holobiont to shift into a parasitic relationship, in which increased heat and light drive the photosynthetic symbionts to overproduce and hoard resources at a deficit to the coral host (D. M. Baker et al., 2018). This

ultimately results in the host expelling the "parasitic" symbionts. Either way, without symbionts, corals are vulnerable to starvation and death (A. C. Baker et al., 2008).

As previously mentioned, not all corals exposed to the same environmental stressors experience bleaching and subsequent mortality. Among conspecific coral colonies, phenotypic plasticity is one of the observed mechanisms that provides resilience in changing environments. Morphological variation in colony and corallite shape is common depending on light environment, sedimentation, and food availability (Todd, 2008) in numerous species including *Porites sillimaniani* (Muko et al., 2000) *Montastraea annularis* (Foster, 1979), *Acropora formosa* (Oliver et al., 1983), and *Stylophora pistillata* (Shaish et al, 2006). Varying symbiont assemblages can change bleaching outcomes for corals, in which switching to the genus *Durusdinium* reduces bleaching and promotes survival in *Acropora millepora* (Bay et al., 2016; Berkelmans & Van Oppen, 2006) and *Montastraea cavernosa* (Silverstein et al., 2015). Acclimatization to stress due to differences in habitat type are thought to be another type of phenotypic plasticity, in which corals from highly variable habitats bleach less than those from more stable habitats in *Acropora hyacinthus* (Barshis et al., 2013). Finally, variation in color morphology in conspecific colonies is thought to be an adaptive tool for mitigating environmental stressors, as described in more details in the next paragraph.

**Coral Color and its role in resilience**

A handful of studies suggest that different color morphs of corals may have different bleaching susceptibility. During a thermal bleaching event on the northern Great Barrier Reef, green color morphs of *Acropora millepora* displayed less bleaching and mortality than their yellow and red neighbors; this was then confirmed in lab experiments (Paley & Bay, 2012; Paley

et al., 2014). The same study also noted colony color changes, in which switching from a green morph to a red morph corresponded to declining health (Paley et al., 2014). Recently, yellow-green color morphs of *Acropora tenuis* in Okinawa were observed to experience minimal bleaching, while purple morphs bleached and recovered, and brown morphs bleached in higher numbers and experienced higher mortality (Satoh et al., 2020). In both studies, all compared color morphs hosted similar symbiont types, which eliminates the possibility of symbiont assemblages as the driver of differences in thermotolerance of specific color morphs (Paley et al., 2014; Satoh et al., 2020).

Holobiont color is a combination of symbiont density in the coral host cells, and the presence of fluorescent proteins (FPs) and non-fluorescent chromoproteins (CPs) in the coral host tissue (Dove et al., 2001). FPs and CPs are homologous to green fluorescent proteins (Kelmanson & Matz, 2003; Takahashi-Kariyazono et al., 2016), synthesized by the coral host (Dove et al., 2001; Kawaguti, 1969; Matz et al., 2002), and exist in a majority of Scleractinian coral genera. Chromoproteins are generally purple/blue, whereas FPs are characterized in four different colors: green, cyan, red, and yellow (Dove et al., 2001; Kelmanson & Matz, 2003; Matz et al., 2006). In *Montastraea cavernosa,* different color morphs possess the same genes coding for three colors (cyan, green, and red) that are differentially expressed from one another to cause distinct visible color morphs (Kelmanson & Matz, 2003). In *Acropora millepora*, tissue pigment concentration is strongly correlated with gene copy number, indicating that color polymorphism is due to differential expression of multicopy genes (Gittins et al., 2015). Ultimately, abundance of FPs and CPs within coral tissue appear to drive the color polymorphisms observed in corals.

There are likely multiple significant functions of FPs and CPs, as they comprise up to 14% of the soluble cellular proteins in coral tissues (Oswald et al., 2007) and phylogenetic

analyses reveal that they arose through adaptively significant processes, signaling that they have persisted in corals by way of positive or balancing selection (Field et al., 2005; Kelmanson & Matz, 2003). Some of the proposed functions of FPs and CPs include light manipulation within the coral host tissue (Kawaguti, 1969; Salih et al., 1998; Schlichter et al., 1985), immunity (Palmer et al., 2009), growth (D'Angelo et al., 2012; Melissa S. Roth & Deheyn, 2013), attraction of symbionts (Aihara et al., 2018), photoprotection (Gittins et al., 2015; Salih et al., 2000), antioxidant activity (Bou-Abdallah et al., 2006; Palmer et al., 2009), and heat mitigation (Smith et al., 2013).

Light manipulation and photoprotection are the most studied function of FPs and CPs due to their ability to change and scatter the wavelengths of incoming light into less energetic photons in the coral host tissue (Kawaguti, 1969; Salih et al., 1998). The scattering of light results in lower irradiance in deeper coral tissues when FPs and CPs are found in the upper layers of coral tissue as they essentially block the light (Salih et al., 2000; Schlichter et al., 1985). Conversely, irradiance is increased within coral tissue when the proteins are found in lower layers, under or amongst the symbionts (Kawaguti, 1969; Schlichter et al., 1985). Pre-existing differences in FPs (Quick et al., 2018), as well as changing abundances of FPs (Gittins et al., 2015; M. S. Roth et al., 2010) impact how corals respond to different light regimes.

Additionally, type of FP and CP contributes to photoprotective qualities that mediate coral bleaching. Green, and red FPs, as well as CPs, have been identified as possessing beneficial photoprotection (Gittins, 2015; M. S. Roth et al., 2010; Smith et al., 2013). Despite this evidence, it is still unclear whether or not FP and CP type or concentration resulting in distinct color polymorphisms is correlated with the amount of light found in different habitats. For example, while there are some observed relationships between depth and FP/CP concentrations in

*Acropora millepora* and *Acropora valida* by researchers (Gittins et al., 2015; Smith et al., 2013), other studies deliberately describe no such correlation in *Montastraea favelota* and *Montastraea cavernosa* (Mazel et al., 2003). Clarifying the interaction between color polymorphisms and depth may help to elucidate the benefits and role of specific FP types in photoprotection.

The role of coral tissue color in heat stress is not as clear. Though there is evidence that corals downregulate expression of FPs and CPs in response to thermal stress (Knochel, 2017; Rodriguez-Lanetty et al., 2009) as they reduce synthesis of energetically costly proteins (Gittins et al., 2015), higher abundances of fluorescent proteins before and during thermal stress are associated with less bleaching and mortality (Paley & Bay, 2012; Smith et al., 2013). The ability of FPs to scavenge a large amount of harmful ROS without any alteration of the proteins (Bou-Abdallah et al., 2006) likely contributes to this, and again FP/CP type plays a role. The amount of ROS scavenged in several species (*Montastraea annularis, M. faveolata, M. cavernosa, Diploria strigosa, Dichocoenia, Sideastrea siderea, Porites asteroides* and *Acropora millepora*) reportedly differs between FP variants, in which red fluorescent protein and CPs scavenge the highest amount of ROS *in vivo,* followed by green fluorescent protein, and finally cyan fluorescent protein (Palmer et al., 2009). While trends between coral color and bleaching susceptibility have been observed, the physiological and molecular mechanisms underlying the trends have yet to be understood.

**The Use of Transcriptomics to Identify Differences Between Color Morphs**

Examining molecular thermal stress response pathways remains a valuable tool for elucidating coral host mitigation strategies that may not be measurable otherwise or are less obvious than visual phenotypic or other quantifiable physiological reactions (Goff & Dubinsky,

2016; Louis et al., 2017). Recently, coral transcriptomic studies have focused on identifying molecular markers of stress (review in Louis et al., 2017), in order to characterize coral stress responses. Upregulation of stress genes before the onset of thermal stress, otherwise known as "transcriptional frontloading," has been identified as a marker of acclimatization or thermotolerance in corals (Barshis et al., 2013). Furthermore, transcriptomics have enabled identification of molecular cascades underlying coral responses to CO2 stress (Moya et al., 2015), disease stress (Closek et al., 2014), the process of bleaching (Pinzón et al., 2015b), and thermal tolerance (Bellantuono et al., 2012). The onset of transcriptional change is thought to play a role in coral bleaching resistance, in which fast return of expression to pre-bleaching baselines is associated with less bleaching (Seneca & Palumbi, 2015).

The use of coexpression networks and functional enrichment to examine gene expression data has helped to provide context and understanding of complex patterns in transcriptional stress responses (Dixon et al., 2020a; Drury, 2020). Different color phenotypes in corals may regulate different functional pathways that promote survival during thermal stress.

**Study Organism**

*Acropora surculosa* (Dana, 1846) is a branching coral, found throughout the Pacific Ocean. It is found mainly on central and Indo-Pacific reefs such as the Great Barrier Reef and reefs in the Coral Triangle. In Guam, they are distinguishable from other *Acropora* species thanks to their constantly extended long tentacles that produce a "hairy" appearance of the coral. *Acropora surculosa* are hermaphroditic broadcast spawners, and typically spawn during the last quarter of lunar cycles in July (Richmond & Hunter, 1990). They are generally categorized as a

"competitive" coral, meaning they grow quickly in ideal environments but are more susceptible to stress than other more hardy and tolerant genera (Darling et al., 2012).

During bleaching events, *Acriporidae* and other branching corals are disproportionately impacted compared to other genera (Baker et al., 2008; Paulay, 1999). This is concerning because *Acropora* corals are widely considered as valuable reef builders and provide habitat for reef fishes. Additionally, *Acriporidae* may also be especially susceptible to mortality by bleaching (Loya et al., 2001; Marshall & Baird, 2000). This may be due to a number of reasons, including tissue thickness (Loya et al., 2001), the lack of genes coding for some essential metabolic enzymes (Shinzato et al., 2011), and general differences in morphology or physiology (McClanahan et al., 2004).

Colonies of *Acropora surculosa* are found all around the island of Guam and have mostly survived the consecutive years of bleaching events that have impacted the island. A diverse range of color morphs are observed, including green, brown, tan, orange, pink, and mixtures of those colors. This study focuses on *Acropora surculosa* due to the abundance and availability of color phenotypes present. Additionally, a reference genome for the closely related species *Acropora digitifera* exists (Shinzato et al., 2011), which was used to facilitate transcriptome assembly and annotation. A reference genome guided approach utilizing a related species improves the assembly of a reference transcriptome for the target species, which is vital to all downstream analyses (Lischer & Shimizu, 2017).

**Objectives of this study**

This study seeks to examine the underlying molecular and physiological processes associated with color polymorphism and heat stress resistance in *Acropora surculosa*. Here we

(1) monitor *A. surculosa* along a depth transect to characterize the distribution of color morphs in relation with light intensity, and (2) investigate the physiological and molecular responses of two specific color morphs in controlled acute heat shock tank experiments. We tested two main hypotheses:

H1o Color morphs of *Acropora surculosa* are distributed randomly across a depth gradient.

H1a Color morphs of *Acropora surculosa* are not distributed randomly across a depth gradient.

H2o There are no differences in physiological or molecular responses to thermal stress between *Acropora surculosa* color morphs

H2a Physiological and molecular responses to thermal stress differ between *Acropora surculosa* color morphs.

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**Chapter 2**

# CHARACTERIZING THE ROLE OF COLOR IN HEAT STRESS RESISTANCE IN THE SCLERACTINIAN CORAL *Acropora surculosa*

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## Abstract

Anthropogenic impacts have increased ocean temperatures, causing large-scale bleaching events across the world's coral reefs. Emerging studies suggest that coral color plays a role in bleaching resilience, however the molecular patterns behind coral color and response to heat stress have not yet been characterized. In this study, we characterize the relationship between environmental stress and color phenotypes in *Acropora surculosa* in their natural environment and in controlled experiments at both physiological and transcriptomic levels. We surveyed the distribution of *A. surculosa* color morphs along a depth gradient (2m to 8m) in Pago Bay, Guam for two years. Higher abundances of brown phenotypes were found at shallow depths, in more variable environments. Red phenotypes were more abundant at deeper and more stable environments. We then sequenced 36 transcriptomes of *A. surculosa* to explore gene expression patterns of red and brown color phenotypes during two sequential, acute heat stress assays. We found that red phenotypes are more sensitive to heat stress, require more energy to mitigate the stress, show significant signs of cell damage, and exhibit limited signs of recovery. In contrast, brown phenotypes seem better prepared to cope with thermal stress, utilize more oxidative and immune response pathways to mitigate stress, and rapidly recover. The striking disparity

between red and brown colonies in the activation of molecular pathways to respond to thermal stress matches their distribution on the reef and might be due to differences in fluorescent protein type and abundance. Altogether we highlighted new molecular mechanisms to explain differences between stress resilience in color morphs, affecting bleaching mitigation in an important reef coral on Guam.

## Introduction

Scleractinian corals are the main ecosystem engineers for one of the world's most diverse and productive ecosystems—coral reefs. Coral reefs support the livelihood of millions of people economically, act as a source of coastal protection and recreation, serve as habitat to other valuable species, and possess vital cultural value (Hicks, 2011), especially for people living in coastal environments (Cinner et al., 2012). However, reefs around the world have experienced severe declines in health and diversity due to rising sea surface temperatures (Spalding & Brown, 2015). Increased temperatures can disrupt the symbioses between coral hosts and the endosymbiotic algae that live within their tissues (Vidal-Dupiol et al., 2009). A major source of disruption is the accumulation of toxic reactive oxygen species (ROS) in coral tissues, which ultimately causes corals to expel their symbionts (Lesser, 1997). Because coral hosts depend on their symbionts for nutrients, metabolic processes, and disposal of waste (Weis, 2008), symbiont expulsion from host tissue eventually leads to coral mortality (A. C. Baker et al., 2008). Symbiont populations can recover if the coral host is no longer stressed, but this must occur before the coral starves or contracts a disease (A. C. Baker et al., 2008).

Understanding coral resilience to thermal stress is key to the success of restoration programs (Edwards et al., 2010; Johnson et al., 2011; Obura & Grimsditch, 2009). As bleaching

susceptibility is typically not equal among habitats (Golbuu et al., 2007), coral species (Darling et al., 2012), or even conspecific colonies (Marshall & Baird, 2000), there are a slew of different mechanisms that promote coral resilience to stressors (see Carballo-Bolaños et al., 2020 for a summary). For example, phenotypic plasticity is a well-studied mechanism observed in corals that promotes survival in changing environments (Torda et al., 2017). Morphological variation in colony and corallite shape is common depending on light environment, sedimentation, and food availability (Todd, 2008) in numerous species including *Porites sillimaniani* (Muko et al., 2000) *Montastraea annularis* (Foster, 1979), *Acropora formosa* (Oliver et al., 1983), and *Stylophora pistillata* (Shaish et al, 2006). Varying symbiont assemblages can change bleaching outcomes for corals, in which switching to the genus *Durusdinium* reduces bleaching and promotes survival in *A. millepora* (Bay et al., 2016; Berkelmans et al., 2006) and *M. cavernosa* (Silverstein et al., 2015). Acclimatization to stress due to differences in habitat type are thought to be another type of phenotypic plasticity, in which corals from highly variable habitats better withstand stress than those from more stable habitats in *A. hyacinthus* (Barshis et al., 2013). Finally, variation in tissue color in conspecific colonies is also thought to be an adaptive tool for mitigating environmental stressors.

A handful of studies suggest that different color morphs of corals may have different bleaching susceptibility. During a thermal bleaching event on the northern Great Barrier Reef, green color morphs of *A. millepora* displayed higher bleaching resilience, and as a result less mortality, than their yellow and red neighbors; this was then confirmed in lab experiments (Paley et al., 2012; Paley, 2014). The same study also noted colony color changes, in which switching from a green morph to a red morph corresponded to declining health (Paley, 2014). Recently, yellow-green color morphs of *A. tenuis* in Okinawa were observed to experience minimal

bleaching, while purple morphs bleached and eventually recovered, and brown morphs bleached in higher numbers and experienced higher mortality (Satoh et al., 2020). In both studies, all compared color morphs hosted similar symbiont types, which eliminates the possibility of symbiont assemblages as the driver of differences in thermotolerance of specific color morphs (Paley, 2014; Satoh et al., 2020).

The holobiont color, which is an indicator of health, is a combination of symbiont density in the coral host cells, and the presence of fluorescent proteins (FPs) and non-fluorescent chromoproteins (CPs) in the coral host tissue. FPs and CPs are homologous to green fluorescent proteins (Kelmanson & Matz, 2003; Takahashi-Kariyazono et al., 2016), synthesized by the coral host (Dove et al., 2001; Kawaguti, 1969; Matz et al., 2002), and exist in a majority of scleractinian coral genera. Chromoproteins are generally purple/blue, whereas FPs are characterized in four different colors: green, cyan, red, and yellow (Dove et al., 2001; Kelmanson & Matz, 2003; Matz et al., 2006). In *M. cavernosa*, different color morphs possess the same genes coding for three colors (cyan, green, and red) that are differentially expressed from one another to cause distinct visible color morphs (Kelmanson & Matz, 2003). In *A. millepora*, tissue color and FP abundance is strongly correlated with gene copy number, indicating that color polymorphism is due to differential expression of multicopy genes (Gittins et al., 2015). Ultimately, abundance of FPs and CPs within coral tissue appear to drive the color polymorphisms observed in corals.

The actual role of coral tissue color in heat stress is not clear. Despite the evidence that corals downregulate expression of FPs and CPs in response to thermal stress (Knochel, 2017; Rodriguez-Lanetty et al., 2009) as they reduce synthesis of energetically costly proteins (Gittins, 2015), higher abundances of fluorescent proteins before and during thermal stress are associated

with less bleaching and mortality (Paley er al., 2012; Smith et al., 2013). Additionally, FPs are powerful antioxidants with the ability to scavenge harmful ROS without any damage to or alteration of the proteins (Bou-Abdallah et al., 2006). The amount of ROS scavenged in several species (*Montastrea annularis, M. faveolata, M. cavernosa, Diploria strigosa, Dichocoenia, Sideastrea siderea, Porites asteroides* and *A. millepora*) reportedly differs between FP variants, in which red fluorescent protein and CPs scavenge the highest amount of ROS *in vivo*, followed by green fluorescent protein, and finally cyan fluorescent protein (Palmer et al., 2009).

To characterize the underlying molecular processes linking heat stress/resistance to color morphs in scleractinian corals, we examined different color morphs of *Acropora surculosa* utilizing a transcriptomic framework. Coral reefs surrounding the island of Guam are abundant in a diverse range of color morphs of *A. surculosa,* including green, brown, tan, orange, pink, and mixtures of those colors (Figure 1). Examining molecular thermal stress response pathways remains a valuable tool for elucidating coral host mitigation strategies that may not be measurable otherwise or are less obvious than visual phenotypic or other quantifiable physiological reactions (Anderson et al., 2016; Louis et al., 2017). To comprehensively characterize the role of color in thermal stress resistance, we (*a*) monitored *A. surculosa* color morphs among a depth transect to elucidate whether color polymorphism is correlated with light and temperature variations associated with depth; and (*b*) we further investigated the divergent transcriptomic responses to heat stress in two specific color morphs in controlled heat shock experiments.

**Figure 1**. A few of the different color morphs of *A. surculosa* found in Pago Bay, Guam. Pictures: Moscato, Victoria

## Methods

### Transect survey

In order to characterize whether abundances of different *A. surculosa* color morphs are correlated to depth, coral colonies were tagged on a transect in Pago Bay (Figure 2) and monitored for color changes for two consecutive years from 2018-2019. The transect was 90 meters long, perpendicular to shore, and marked by intervals at depths of 2m, 4m, 6m, and 8m. Each interval was equipped with Onset HOBO (Bourne, MA) temperature and irradiance

loggers. Twenty-two colonies of *A. surculosa* were tagged on the transect for monitoring in 2017 during a thermal bleaching event in October; six at 2m, nine at 4m, four at 6m, and three at 8m. Each colony was photographed to record their color. The CoralWatch (Brisbane, Australia) color chart was used to record the saturation level (0-5) and "health status" of each colony as in Siebeck et al., (2006). During the dry season of 2018, in April, 10 additional colonies were tagged (2, 3, and 4 at 2m, 6m, and 8m respectively). More colonies were added to the transect in July (2 at 4m and 1 at 8m), and October (1 at 2m and 2 at 8m), resulting in a final total of 38 marked colonies (Figure 2). Every month, the transect was surveyed to identify any changes in color or health such as bleaching, mortality, and recovery.

Temperature (°C) and irradiance (lux) measurements were tested for normality using a Shapiro Wilk test and the significance of differences between depths were determined with an ANOVA. Colony CoralWatch values were tested for normality using a Shapiro Wilk test before grouping colonies by depth and color to test for significant differences in health between depth and seasons using a Kruskal Wallace test.

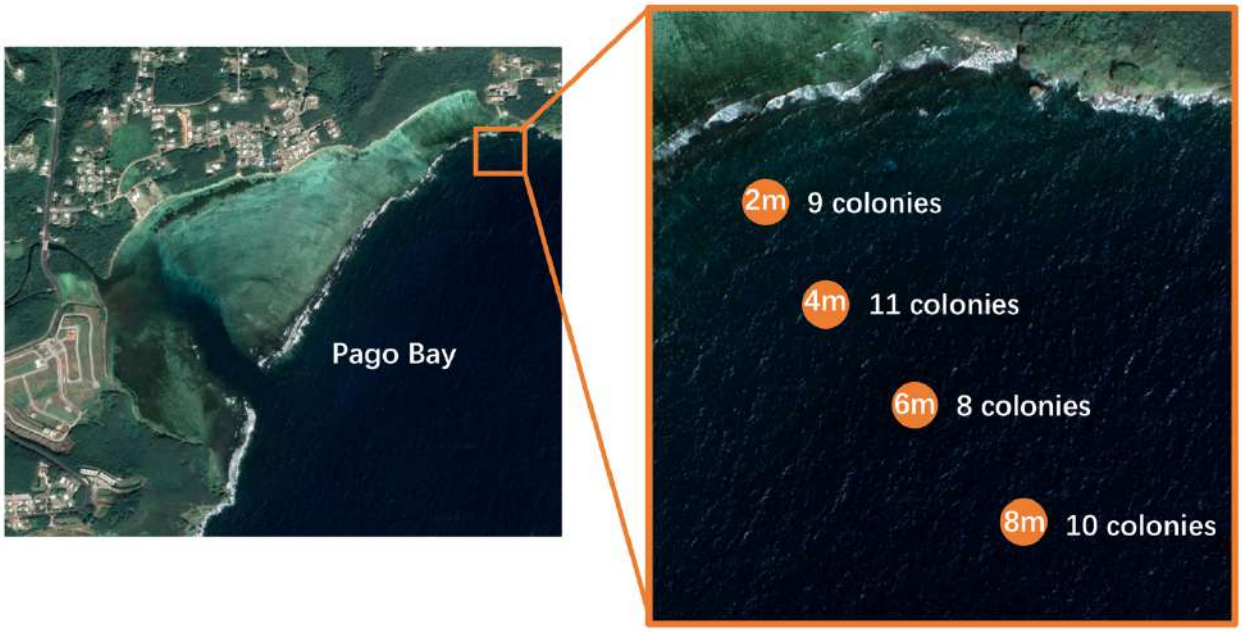


**Figure 2**. Satellite Image from Google 2012—modified. Location of the transect and HOBO markers in Pago Bay, Guam. The total length of the transect was 90 meters. HOBO marker locations are approximated by the orange circles. Number of colonies reflects the total number of colonies tagged at each site over the course of the survey.

**Tank experimental design and sampling**

We used a controlled tank experiment to closely monitor gene expression and physiological changes in the two most contrasting *A. surculosa* color morphs: red and brown. In February of 2019, untagged red and brown corals were identified visually at the 6m marker of our transect, and five colonies of each color were randomly selected. Suitable colonies were either uniformly red or uniformly brown in color, paler or varied color morphs were not included (Figure 3a). Five nubbins were broken of each colony, placed individually into sea water filled zip lock bags, and transported to the wet lab (~200 meters). All nubbins (n=50) were mounted in cut PVC tubes with putty (Figure 3b) and placed randomly into three opaque tanks with free-flowing sea water. Nubbins were left to acclimate for 10 days at ambient temperature. Over the

course of a five-week period, two sequential heat stress assays were performed. In all tanks, seawater was heated to 32°C to reflect the highest temperatures corals experienced during the 2017 bleaching event, using Finnex® 800-watt titanium heaters with temperature control (one per tank, HC-0810M), and additional individual digital thermometers (Risepro®). Tanks were gradually heated from 28°C to 32°C over 1 hour and were kept at 32°C (+/- 1°C) for 5 hours. After 5 hours the heaters were turned off and the seawater gradually cooled back to 28°C over ~2 hours. The coral nubbins were given two weeks to recover between heat shocks.

Tissue samples were collected for gene expression and symbiont density analysis from one nubbin of each colony before each heat shock (n=10), one hour into each heat shock (n=10), and 20 hours after the start of each heat shock (n=10) by cutting away a portion of tissue. This resulted in 60 total tissue samples. When possible, nubbins were only partially sampled (i.e. halved) so that additional samples could be taken during the subsequent heat stress, in order to observe their reaction to subsequent stresses. All tissue samples were immediately placed in RNALater (Sigma-Aldrich R0901) and stored at -80°C on site. We utilized all 60 samples for symbiont density calculations. For RNA Sequencing, samples from only 3 colonies per color morph were selected at random, resulting in 36 samples.

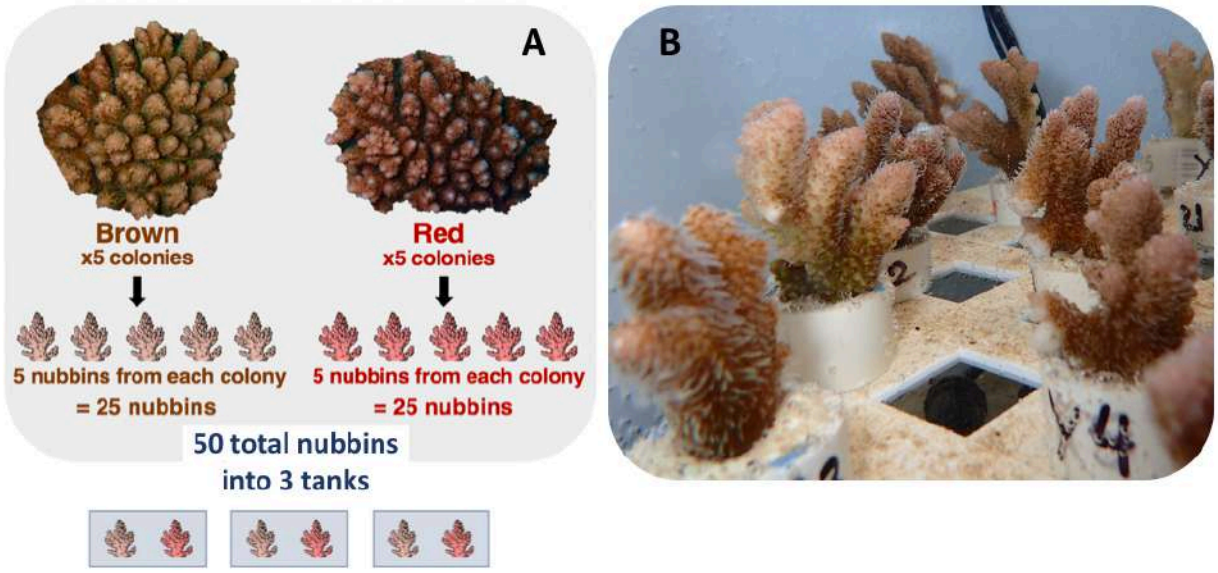


**Figure 3:** Examples of colonies selected in the field, and breakdown of coral nubbins used for tank experiment (**A**). Within the tanks, coral nubbins were placed in marked PVC stands (**B**). Pictures: Moscato, Victoria.

**Physiological Measurements**

For the duration of the experiment, every 24 hours CoralWatch color chart readings were taken for each coral nubbin. CoralWatch values were grouped by color group and the significance of differences were tested using a Kruskal Wallace test after normality testing with a Shapiro Wilke test. Differences between red and brown color morphs were examined throughout the entirety of the experiment as well as focusing on before, during, and after heat shock time points. A Junior PAM (Walz, Germany) and a diving PAM (Walz, Germany) were used to collect the maximum quantum yield (Fv/Fm) of symbiont photosystem II (PSII), i.e. photosynthetic efficiency, every morning before sunrise by placing the measuring probe on the nubbin. Due to the change of equipment between heat shock 1 and heat shock 2 (from Junior PAM to Diving PAM), we did not directly compare Fv/Fm measurements between them. We

examined differences in Fv/Fm first by testing for normality using the Shapiro Wilke test, and then a Kruskal Wallace test. Comparisons were performed between red and brown nubbins throughout the first heat shock, and then throughout the second heat shock.

**Symbiont density**

Symbiont densities were calculated for each sample (n=60) as established by Fitt et al., (2000). In brief, coral tissue was removed from the coral nubbins with an air gun in 5-10 mL of filtered seawater (FSW) and vortexed to separate the coral tissue from the algae cells. A 2mL aliquot of the slurry was centrifuged at 1500 rpm for five minutes to pellet the algae. Pellets were resuspended in FSW and centrifuged again at 1500 rpm for five minutes two more times, decreasing the FSW content to a final aliquot of 1 mL. Densities of Symbiodiniaceae were calculated from 3 replicate hemocytometer counts from aliquots. We grouped all samples by their color morph and sampling point into 12 different treatment groups comprising both the first and second heat stress experiments: Red Before Heat 1 (BH1), Red During Heat 1 (DH1), Red After Heat 1 (AH1), Red Before Heat 2 (BH2), Red During Heat 2 (DH2), Red After Heat 2 (AH2), Brown Before Heat 1 (BH1), Brown During Heat 1 (DH1), Brown After Heat 1 (AH1), Brown Before Heat 2 (BH2), Brown During Heat 2 (DH2), Brown After Heat 2 (AH2). Comparisons were then made between and within red and brown samples at each time point. Symbiont densities were screened for normality using a Shapiro-Wilk test. The significance of differences between samples were determined by a Kruskal-Wallace test.

**RNA Sequencing Library Preparation, transcriptome assembly and annotation**

To identify genes involved in the differential response to thermal stress between color morphs, we used RNA sequencing. Total RNA was extracted from 3 colonies per color morph per time point (see previous section for list of time points), using a Qiagen RNeasy kit (Hildenheim, Germany) on a QIAcube extraction robot (Qiagen, Hildenheim, Germany), following the manufacturer's instructions. After extraction, total RNA per sample was quantified using a Qubit (Life Technologies, Carlsbad, CA) and qualified using a picoRNA assay on a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA). In total 36 cDNA libraries were constructed using the NEBNext Ultra II RNA Library Prep Kit for Illumina (New England Biolabs). Libraries were barcoded with individual indices from NEBNext Multiplex Oligos for Illumina (New England Biolabs). The final concentrations of cDNA libraries were assessed using a Qubit HSDNA kit (Life Technologies, Carlsbad, CA). Individual library qualities were assessed with HSDNA BioAnalyzer assays before being pooling them into two sets of 18 samples and re-quantifying them with a Qubit HSDNA kit. Both library pools were sequenced separately, using an Illumina NextSeq 500 (San Diego, CA) with 75bp paired-end reads in the Molecular Lab at the University of Guam Marine Lab. An additional field sample of *A. surculosa* was prepared as described above and sequenced with paired end reads of 150bp to facilitate the assembly of our high-quality reference transcriptome.

The resulting reads were demultiplexed and each sample was filtered for low quality reads and adapter-trimmed using Trim Galore (Martin, 2011). All reads with an average quality score lower than 30 and shorter than 25bp, were discarded. Quality of filtered and trimmed reads was assessed with FastQC (Andrews, 2010). One sample was determined to have too low reads and was subsequently re-sequenced. Quality filtered reads were then cleaned of rRNA and

symbionts contamination by using Bowtie v2.1.0 to align them to SILVA LSU and SSU rRNA databases (https://www.arb-silva.de/), and three concatenated symbiont genomes (*Symbiodinium kawagutii, Symbiodinium microadriaticum,* and *Breviolum minutum,* reads available at NCBI under accession numbers GCA_001939145.1, GCA_003297005.1, and GCA_009767595.1, respectively). Sequences that aligned to the symbiont genomes were retained for genera identification. Our reference transcriptome was assembled using all 36 samples from our heat stress experiments and the additional field sample.

In order to perform Trinity genome-guided assembly (Haas et al. 2013), we aligned all cleaned reads to the *Acropora digitifera* genome (Shinzato et al., 2011), using Bowtie v2.1.0 (Langmead & Salzberg, 2012). The resulting BAM files were used to generate our reference transcriptome with the default Trinity parameters. We checked the quality of the resulting reference using Trinity's Transcriptome Assembly Quality Assessment (Haas et al., 2013), and determined our reference was too fragmented (329,563 total transcripts). To reduce sequence redundancy and improve performance of downstream analyses, we filtered the reference transcriptome by clustering highly homologous sequences using cd-hit-est (Fu et al., 2012) with default settings, resulting in a final transcriptome size of 183,544 total transcripts. Finally, our assembled reference transcriptome for *A. surculosa* was annotated by performing BLASTX searches of reads against cnidarian sequences from the uniprot database (www.uniprot.org) with an e-value cutoff of 1e-5. Gene ontology (GO) terms were obtained from the uniprot website.

**Symbiont Clade Identification**

Methods for identifying Symbiodiniaceae genera were adopted from Barfield et al (2018). Symbiont sequences separated from our coral sequences (as described above) were

aligned with Bowtie v2.1.0 to transcriptomes of Symbiodiniaceae genera *Symbiodinium* and *Breviolum* from Bayer et al. (2012) and genera *Cladocopium* and *Durusdinium* from Ladner et al. (2012), simultaneously. The resulting SAM files were used to count relative proportions of reads producing highly unique matches, determined by a mapping quality of 40 or higher, to each Symbiodniaceae transcriptome, using a custom perl script zooxType.pl (https://github.com/z0on/).

**Gene expression analyses**

From here on, we refer to each experimental sample using the following code: BH for before the heat shock, DH for during the heat shock and AH for after the heat shock; followed by 1 or 2 to indicate stress assays 1 or 2.

Differential expression analyses were conducted for both genes and isoforms but are presented here only for the gene level. Here, we are using the Trinity definition of "isoform" and "gene", in which isoforms are unique assemblies of transcripts, while a gene is the sum of all isoforms that share transcripts. Reads from each sample were mapped against our annotated *A. surculosa* reference transcriptome using Bowtie v2.1.0. Read counts per gene were generated with RSEM (Li & Dewey, 2011) using the Trinity script align_and_estimate.py (Grabherr et al., 2011; Haas et al., 2013) with default parameters. Gene counts were imported into R v4.0.2 (R Development Core Team, 2018) using the *tximport* package (Soneson et al., 2015). For all downstream analyses, a minimum of 10 read counts across all samples was used as the threshold for retaining a gene. Significant differentially expressed genes (DEGs) across multiple comparisons were determined with a cut-off of log fold change > 2 and an adjusted p value (padj) < 0.01 using the *DESeq2* package (Love et al., 2014).

Principle component analysis of variance stabilized gene expression was performed using the *plotPCA* function from the *DESeq2* package in R to visualize clustering of data for significant DEGs (padj < 0.01, log fold change >2). We also used discriminant analysis of principal components (DAPC) on variance stabilized gene expression of significant DEGs (padj < 0.01, log fold change >2) to visualize clustering of treatment groups along a discriminant function.

**Functional Enrichment Test**

To characterize the functional discrepancy in DEGs between color morphs and time points, gene ontology enrichment analyses were performed by using adaptive clustering of GO categories and Mann-Whitney U tests based on ranking of signed log p-values (GO_MWU, https://github.com/z0on/GO_MWU). This methodology includes the entire ranked list of genes instead of those chosen by an arbitrary significance cut off, which provides the advantage of determining whether certain GO categories contain significantly more over- or underexpressed genes than expected by chance. Based on clustering results obtained from both PCA and DAPC, we first enriched functional terms for comparisons between DH and BH+AH within each color for both heat stress assays 1 and 2. We then enriched functional terms for comparisons of Red vs Brown for every sampling point of both heat shocks.

# Results

## Transect Survey

Temperature and light regimes were significantly different between depths (Figure 4; ANOVA, $p < 0.001$). Mean temperatures were highest at 2m, followed closely by 4m, 8m, and 6m (Table 1). Mean irradiance was highest at 2m, followed by 4m, 8m, and 6m (Table 1). Levene's test revealed that variance of temperature was significantly different ($p < 0.001$) for comparisons between all depths except for 4m vs. 8m, with 2m having the highest variance (stdev 0.9°C) and 6m having the lowest variance (stdev 0.8°C). The variance of irradiance was significantly different ($p < 0.001$) as well for comparisons between all depths except for 6m vs. 8m, with 2m having the highest variance (stdev 11066 lux), and 6m having the lowest variance (stdev 4788 lux). In summary, temperature and irradiance were generally highest, and most variable, at 2m, while they were lower, and least variable, at 6m.

**Table 1:** Average, maximum, and minimum temperature and irradiance recorded at each depth of the transect over 6 months.

| | **Temperature** | | | **Irradiance** | | |
|---|---|---|---|---|---|---|
| Depth | Average | Maximum | Minimum | Average | Maximum | Minimum |
| 2m | 29.3°C | 32.3°C | 27.8°C | 5966 lux | 126756 lux | 0 lux |
| 4m | 29.3°C | 31.7°C | 27.9°C | 3462 lux | 55112 lux | 0 lux |
| 6m | 29.1°C | 31.4°C | 27.8°C | 2699 lux | 37200 lux | 0 lux |
| 8m | 29.3°C | 31.5°C | 27.8°C | 2868 lux | 49600 lux | 0 lux |

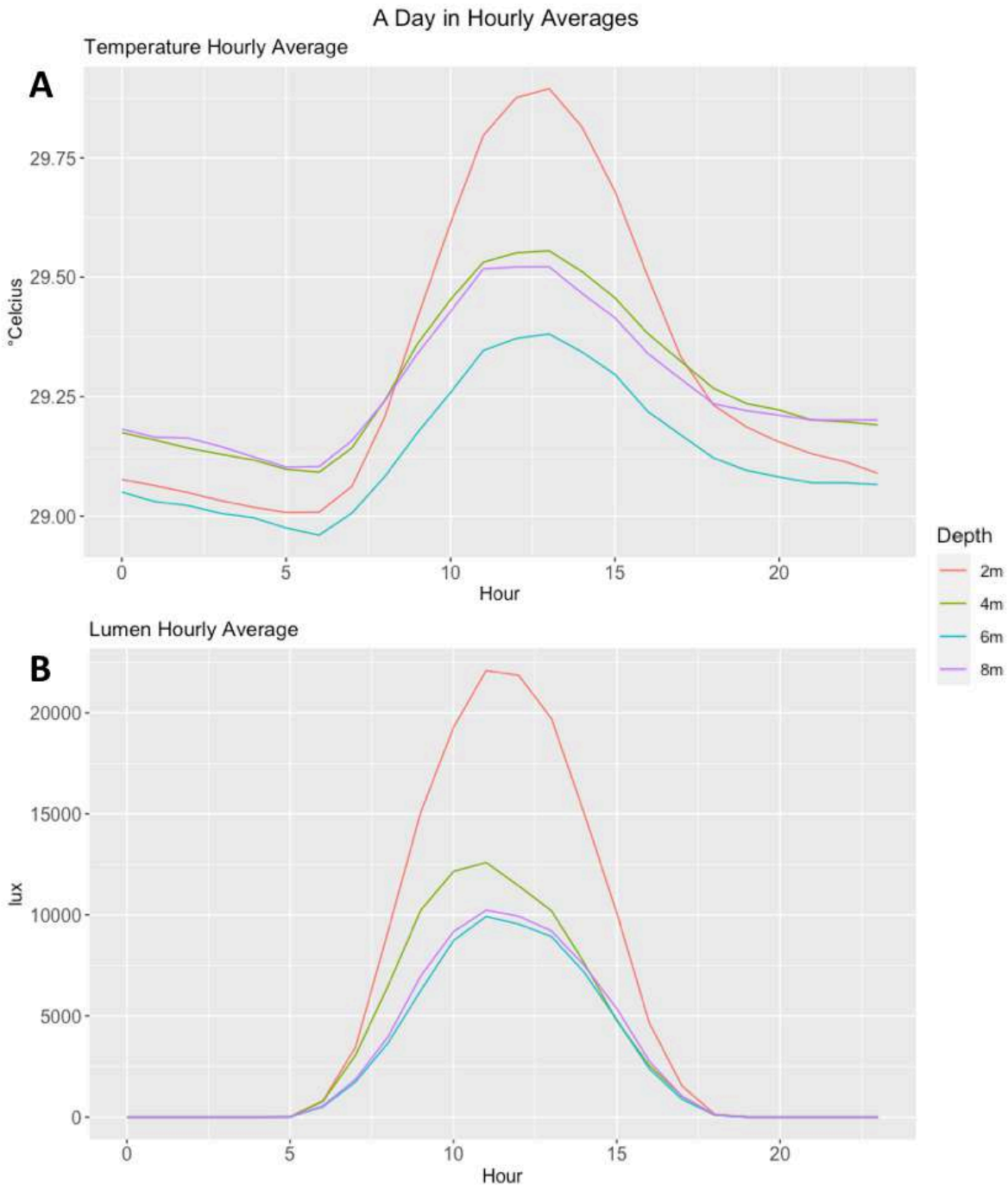


**Figure 4:** Six months of temperature and irradiance data collected on the transect represented as averages by hour for a 24-hour period.

The abundance and distribution of *A. surculosa* color morphs was not evenly distributed across depths for the duration of the survey period. Brown colonies were found in significantly higher numbers at 2m than red and orange, while red colonies were in significantly higher abundance at 6m and 8m than brown and orange (Table S3, S4, Supp. material). Additionally, there were significantly more brown colonies at 2m than at 4m, 6m, and 8m, and significantly more red colonies at 8m than 2m (Table S3, S5, Supp. material). In the majority of colonies, colony color remained consistent over the course of the survey. At each depth, however, at least one colony regularly changed color between red, brown, and orange from month to month, 1 colony at 2m, 1 colony at 4m, 3 colonies at 6m, and 2 colonies at 8m. Change in color did not seem to correlate with time of year or any other observed environmental parameter. Additionally, red colonies died at a higher rate than orange and brown. Sample sizes were too small for statistical testing, however, and the exact cause of death remains unclear. Over the course of the surveys 10 colonies died; 6 were red, 2 were brown, and 2 were orange. Interestingly, 3 of the red colonies that died were recorded as brown in the initial months of the survey period but then switched to red, and 1 of the brown colonies were recorded as switching between red and brown from month to month.

**Heat stress tank experiment: physiological parameters**

Analysis of CoralWatch data revealed that nubbins from one red colony (Colony "A") were significant outliers due to severe paling after collection. Data for these nubbins were subsequently removed from all analyses. CoralWatch color intensity changes were significantly more variable in red than in brown corals ($p < 0.05$; Levene's Test). Despite this, color intensity changes themselves were not significantly different between red and brown over the course of

the experiment (Figure 5A). Photosynthetic efficiency measured by PAM (Figure 5B) revealed that maximum quantum yield of symbiont photosystems in red colonies were not impacted until two days after the second heat stress ($p < 0.05$), when values significantly increased (Table S2, Supp. material). Brown colonies were significantly impacted by the first heat stress, in which Fv/Fm values significantly increased two days after the first heat stress ($p < 0.05$) (Table S2, Supp. material). Between red and brown colonies, Fv/FM values for brown colonies were significantly higher than red before the second heat shock ($p < 0.001$; ANOVA). At all other time points, Fv/Fm values between colors were not significantly different from each other.

**Symbiont density and composition**

Symbiont densities for both color morphs were not significantly affected by either heat treatment, and they were not significantly different between color morphs (Figure 5C). Filtered symbiont transcriptomic data revealed that symbionts in all coral samples were predominantly from the genus *Cladocopium* with ~94.0%, followed by *Breviolum* with ~5.7%, *Symbiodinium* with ~.2%, and *Durusdinium* with ~0% in all colonies. (Figure S1, Supp. material).

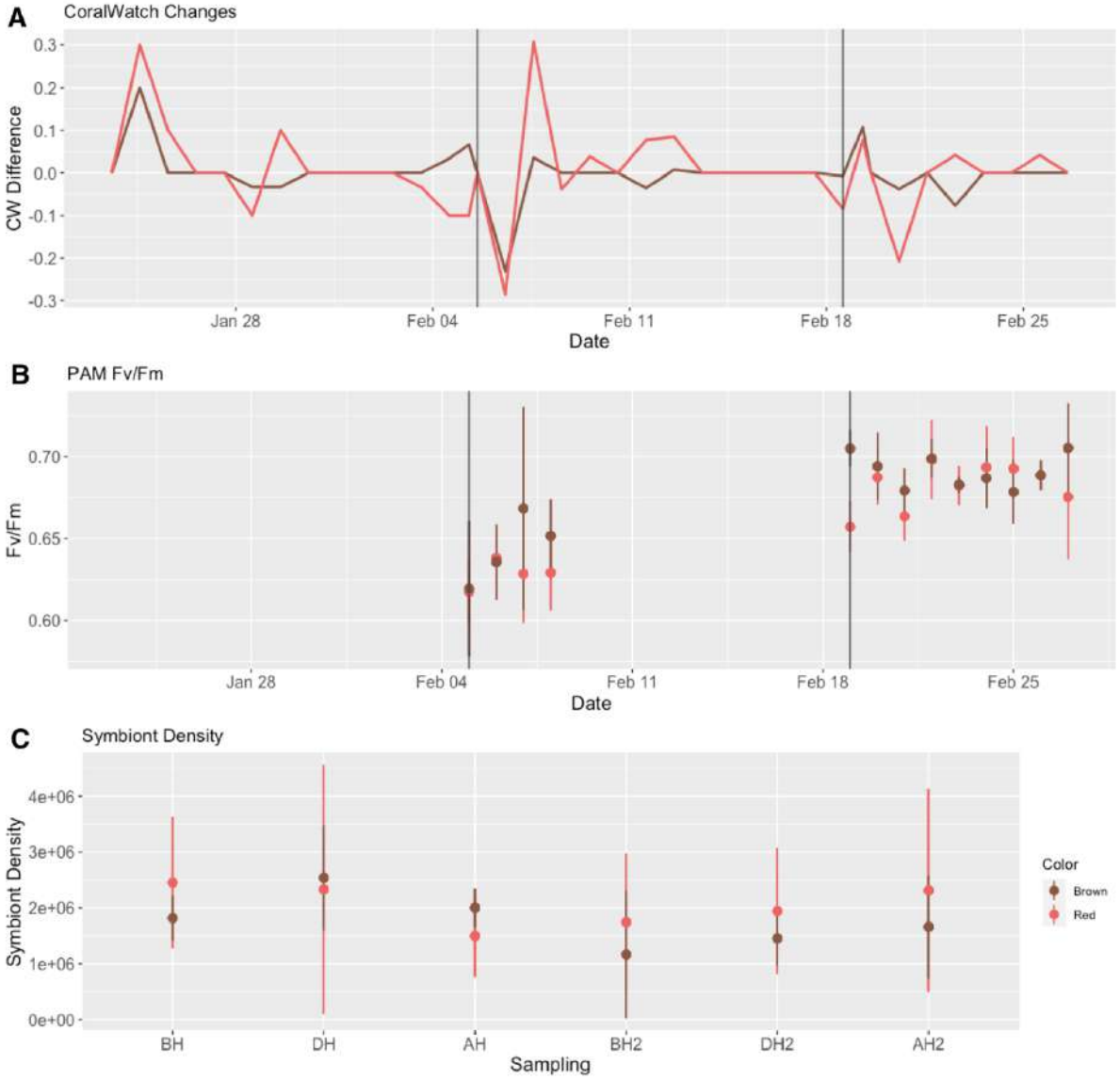


**Figure 5:** CoralWatch Color Chart differences (**A**), Fv/Fm collected using PAM (**B**), and symbiont density (**C**) collected throughout the tank experiment. Symbiont density and Fv/Fm are expressed as averages by color morph +/- standard deviation. Tissue color recorded with a CoralWatch color chart is expressed as average differences from day to day by color morph. In Fv/Fm and ColorWatch plots, black vertical lines denote days when heat stress was applied.

**Sequencing Yield and Transcriptome Assembly Statistics**

Sequencing runs yielded an average of 33 million raw reads per sample for a total of 1.18 billion reads. Approximately 31 million reads per sample remained after quality filtering (adapters, low quality reads, rRNAs and symbiont reads removal, details in supp. material Table S6). The final reference transcriptome had a total of 170,443 transcripts. Downstream analyses were conducted with transcripts that returned an annotation and that were counted at least 10 times in each sample, resulting in 112,664 unique annotated transcripts.

**Differential Gene Expression Analyses**

The principal component analysis (PCA) conducted with significant DEGs (padj < 0.01 and log fold change > 2) displayed a striking clustering pattern between treatment times and tissue color. Samples cluster by treatments, DH1+2 vs. BH1+2 and AH1+2 on principal component 1 and by color on principal component 2, which explained 46% and 25% of the variation, respectively (Figure 6).

The DAPC conducted with significant DEGs showed red and brown samples clustered on opposite ends of the discriminant function for all time points (Figure 7). In both colors, direction of treatment groups along the discriminant function move the same way, with during heat groups to the left and after heat groups to the right of before heat. Interestingly, in samples collected throughout the first heat stress, red treatment groups are farther apart from each other with notable gaps between each group, while brown treatment groups overlap each other. For samples collected throughout the second heat stress, treatment groups cluster more closely together for both colors than they did for the first heat stress assay.

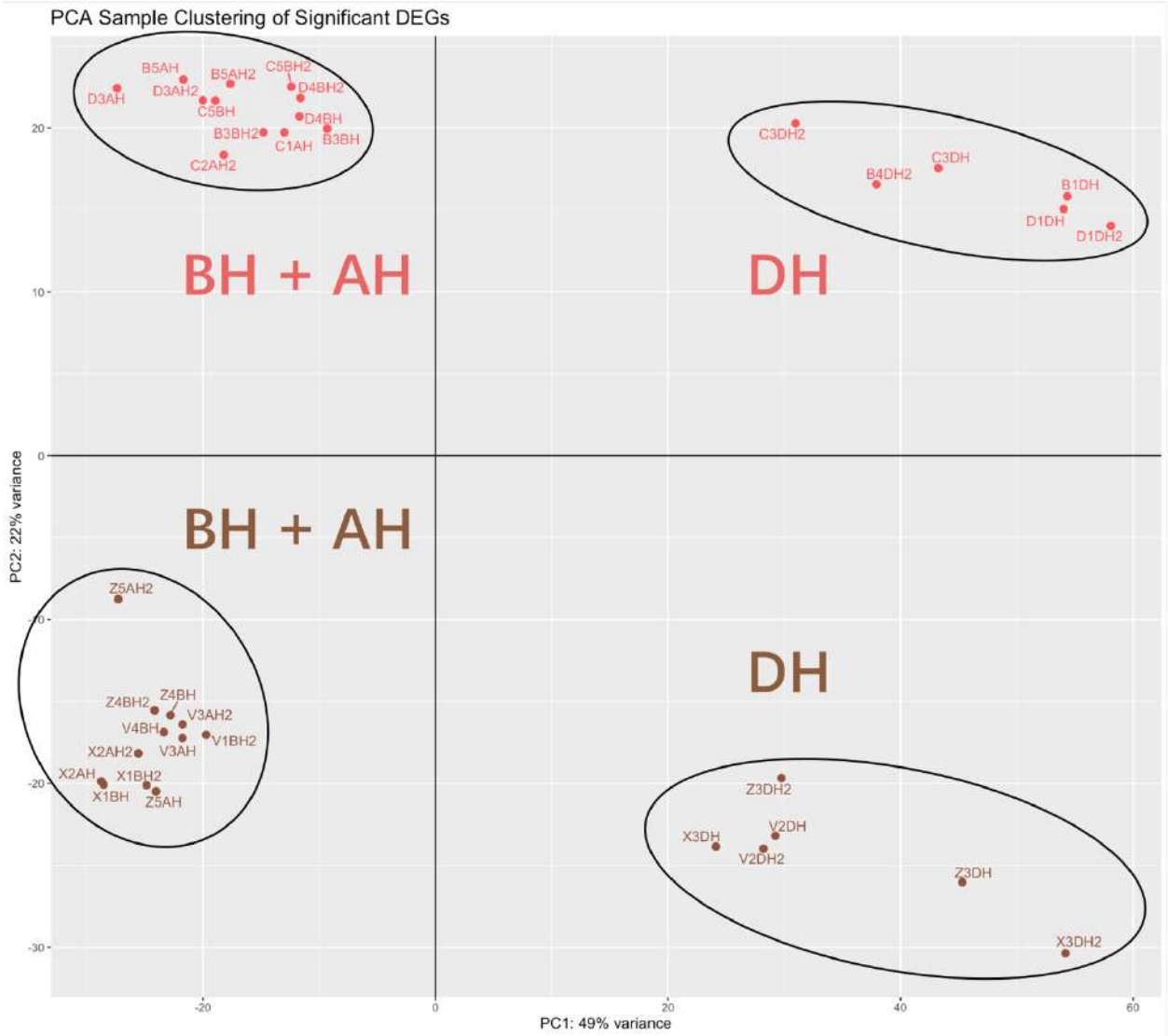


**Figure 6:** Principal component analysis performed on significant differentially expressed genes (padj < 0.01, log fold change > 2). Brown and red samples are labeled in brown and red respectively. The first two characters of each label correspond to sample name, followed by collection time point as follows: BH for before heat, DH for during heat, and AH for after heat. Labels ending with a 2 correspond to samples collected during the second heat stress assay.

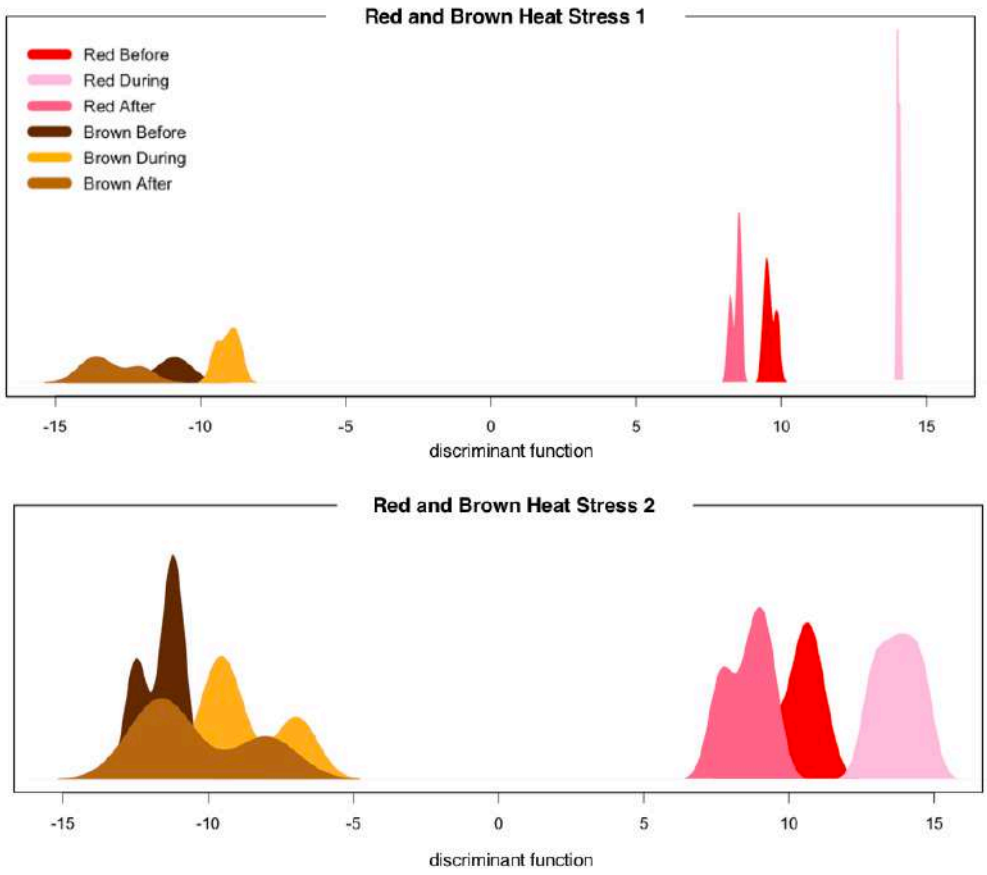


**Figure 7:** Discriminant analysis of principal components of significant DEGS (padj < 0.01, log fold change > 2). Samples from red color morph colonies are represented in various shades of red and samples from brown colonies in various shades of brown. Different shadings correspond to treatment groups from the experiment (ie. During Heat is light pink for red colonies, and yellow for brown colonies).

The number of significant DEGs in the DH1 vs. BH1+AH1 and the DH2 vs. BH2+AH2 comparisons were 1,557 and 768 respectively for red colonies and 589 and 1,128 respectively for brown colonies. The number of significant DEGs at the treatment times BH1, DH1, and AH1 between red and brown phenotypes were 189, 138 and 123 respectively. At treatment times BH2, DH2, and AH2 significant DEGs between brown and red phenotypes were 107, 128 and 146 respectively. For comparisons between red and brown, 38 significant DEGs were shared among all comparisons (Figure 8).

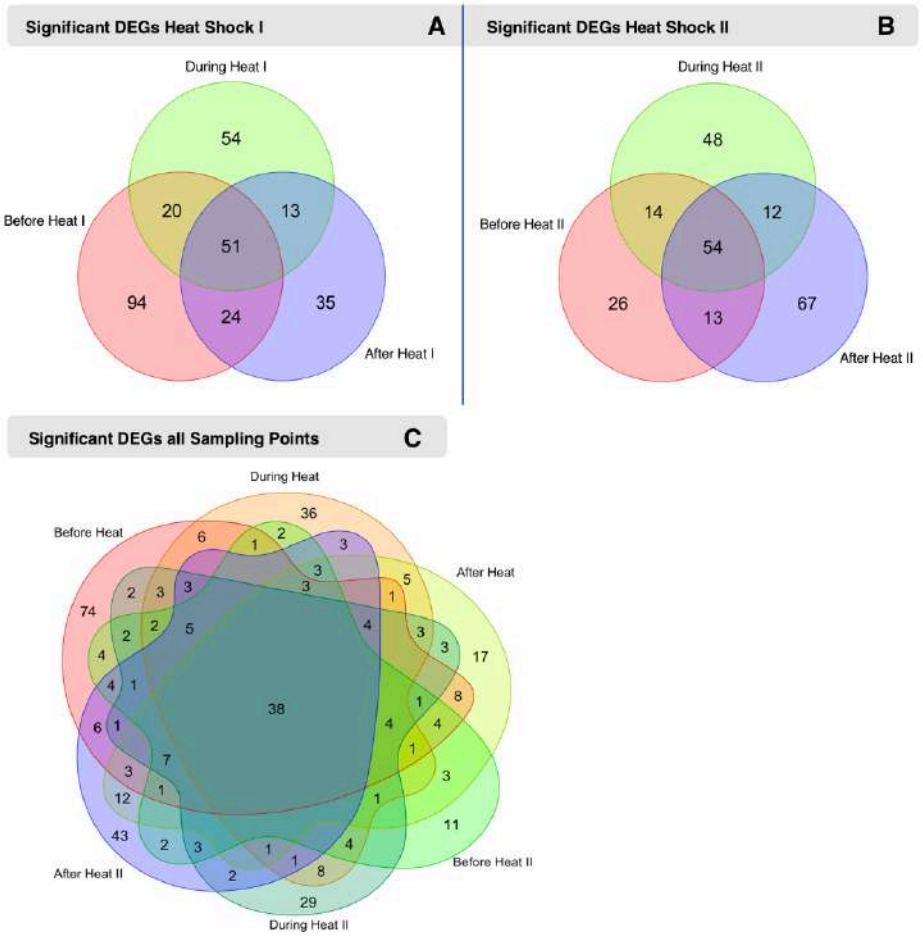


**Figure 8:** Venn diagrams of significant DEGs (padj < 0.01, log fold change >2) comparing red and brown phenotypes at each sampling point. (**A**) represents DEGs shared between heat stress 1 sampling points. (**B**) represents DEGs shared between heat stress 2 sampling points. (**C**) represents DEGs shared between all sampling points.

## Functional Enrichment Analysis

*Heat Stress Experiment 1*

GO enrichment analyses were conducted on all DEGs obtained in the comparisons between DH1 vs. BH1+AH1 within red and brown phenotypes to explore the main functional differences (Figure 9). Within the red phenotype, ADP binding, and cation binding ($p < 0.001$), were among the most significantly enriched molecular functions with overexpressed genes in

DH1 compared to BH1 and AH1, followed by ubiquitin-like protein transferase ($p < 0.01$), and protein tyrosine kinase ($p < 0.01$). Interesting biological processes include signaling, regulation of gene silencing, cytokine-mediated signaling pathway and DNA integration ($p < 0.001$) as the most significantly enriched terms with overexpressed genes in DH1 compared to BH1 and AH1 samples. In red phenotypes, enriched terms with underexpressed genes in DH1 compared to BH1 and AH1 include amino acid binding ($p < 0.01$), ribosome binding ($p < 0.05$), and oxidoreductase ($p < 0.05$). Cellular response to stimulus was among the most significantly enriched biological process with underexpressed genes in DH1, in addition to several biosynthetic processes (including organonitrogen compound, amide, and macromolecule), RNA processing and metabolism, and protein metabolism ($p < 0.001$). DNA replication ($p < 0.01$) and negative regulation of cell death ($p < 0.05$) were also enriched with underexpressed genes in red samples DH1 compared to BH1 and AH1, which is characteristic of stressed corals.

Within the brown phenotype, the most significantly enriched biological processes among overexpressed genes in DH1 compared to BH1 and AH1 are regulation of response to stress, regulation of immune system process, and DNA integration ($p < 0.001$). Cell redox homeostasis ($p < 0.01$), oxidation-reduction process ($p < 0.05$), protein folding ($p < 0.05$), regulation of gene silencing ($p < 0.01$), and positive regulation of defense response ($p < 0.01$) were enriched with overexpressed genes, whereas biosynthetic process ($p < 0.001$), RNA processing and metabolism ($p < 0.01$), and DNA replication ($p < 0.01$) were enriched with underexpressed genes in brown phenotypes in DH1 compared to BH1 and AH1 samples.

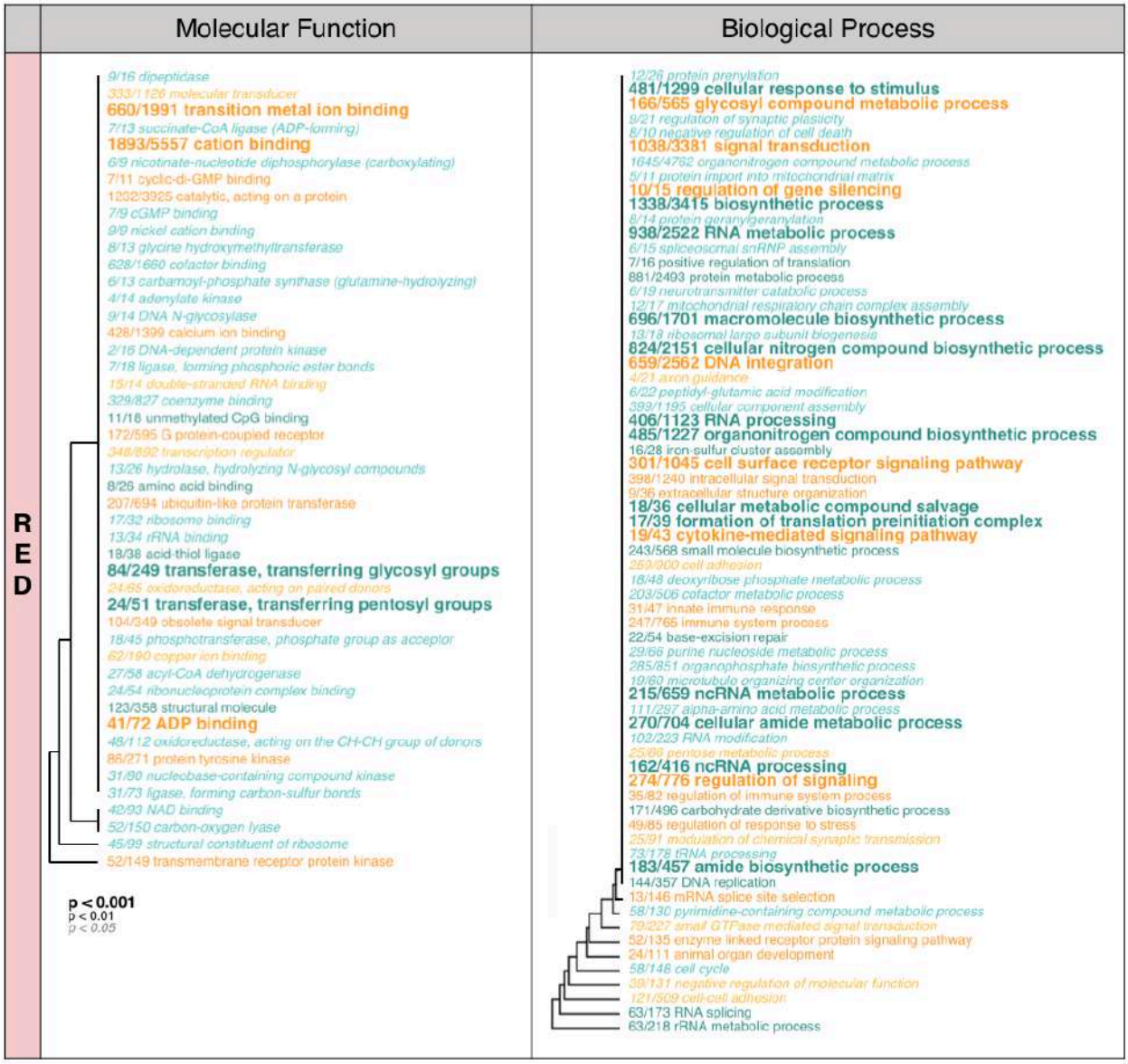


**Figure 9:** See legend below

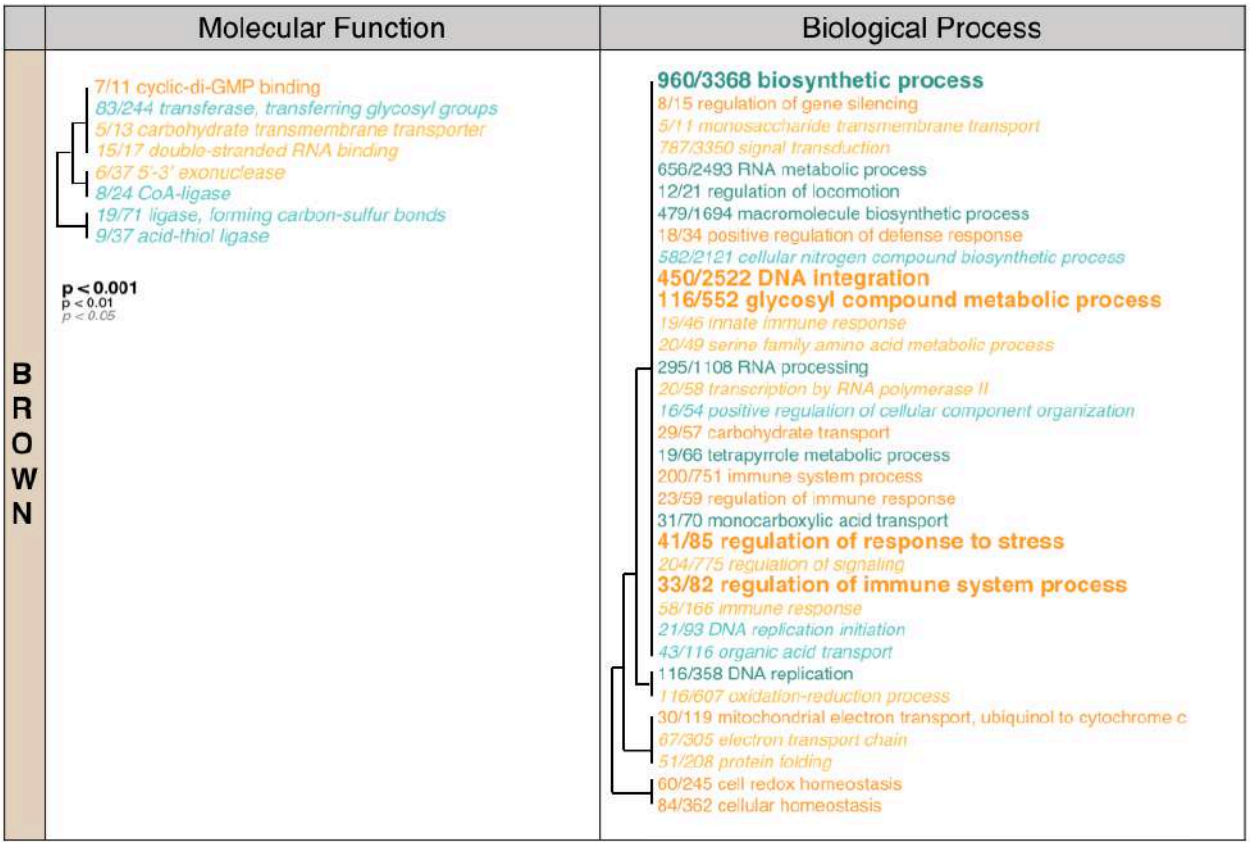


**Figure 9 (continued):** GO categories significantly enriched with up/down-regulated genes from comparisons between DH1 vs BH1+AH1. Orange text indicates terms overexpressed in DH1 compared to BH1 and AH1; turquoise text indicates terms underexpressed in DH1 compared to BH1 and AH1. Large bold font is equivalent to a p-value less than 0.001, medium font is equivalent to a p-value less than 0.01, and the small italicized font is equivalent to a p-value less than 0.05. Dendrograms show hierarchical clustering of GO categories based on shared genes within the dataset.

GO enrichment analyses were also conducted on all DEGs obtained in comparisons between red and brown phenotypes at each treatment time point (Figure 10). In BH1 samples, no biological processes were enriched. The molecular function calcium ion binding ($p < 0.001$) was enriched with overexpressed genes in brown phenotypes, whereas ATPase and carbamoyl-phosphate synthase ($p < 0.01$) were enriched with overexpressed genes in red phenotypes. In DH1 samples, stress related molecular functions and biological processes including ADP binding ($p < 0.001$), ubiquitin-like protein transferase ($p < 0.01$), protein tyrosine kinase ($p < 0.01$),

signal transduction ($p < 0.001$), and DNA integration ($p < 0.001$), were most significantly enriched with overexpressed genes in red compared to brown phenotypes. Molecular functions enriched with underexpressed genes in red compared to brown phenotypes in DH1 samples included ribosomal large subunit biogenesis ($p < 0.05$), ribosome binding ($p < 0.05$), and oxidoreductase activity ($p < 0.01$). Biosynthetic process ($p < 0.001$) was also strongly enriched with underexpressed genes in red DH1 samples, followed by several specific biosynthetic processes including organonitrogen compound ($p < 0.001$), amide ($p < 0.001$), phospholipid ($p < 0.05$), macromolecule ($p < 0.05$), and organophosphate ($p < 0.05$). Finally, in AH1 samples, biological processes including organonitrogen and cellular nitrogen compound biosynthetic processes ($p < 0.01$ and $p < 0.05$), organic substance transport ($p < 0.01$), cellular and macromolecule localization ($p < 0.01$) were enriched with overexpressed genes in red phenotypes compared to brown. Molecular functions calcium ion binding ($p < 0.001$), metallopeptidase ($p < 0.01$), cation binding ($p < 0.01$), and biological processes signal transduction ($p < 0.01$), and DNA integration ($p < 0.05$) were enriched with overexpressed genes in brown phenotypes.

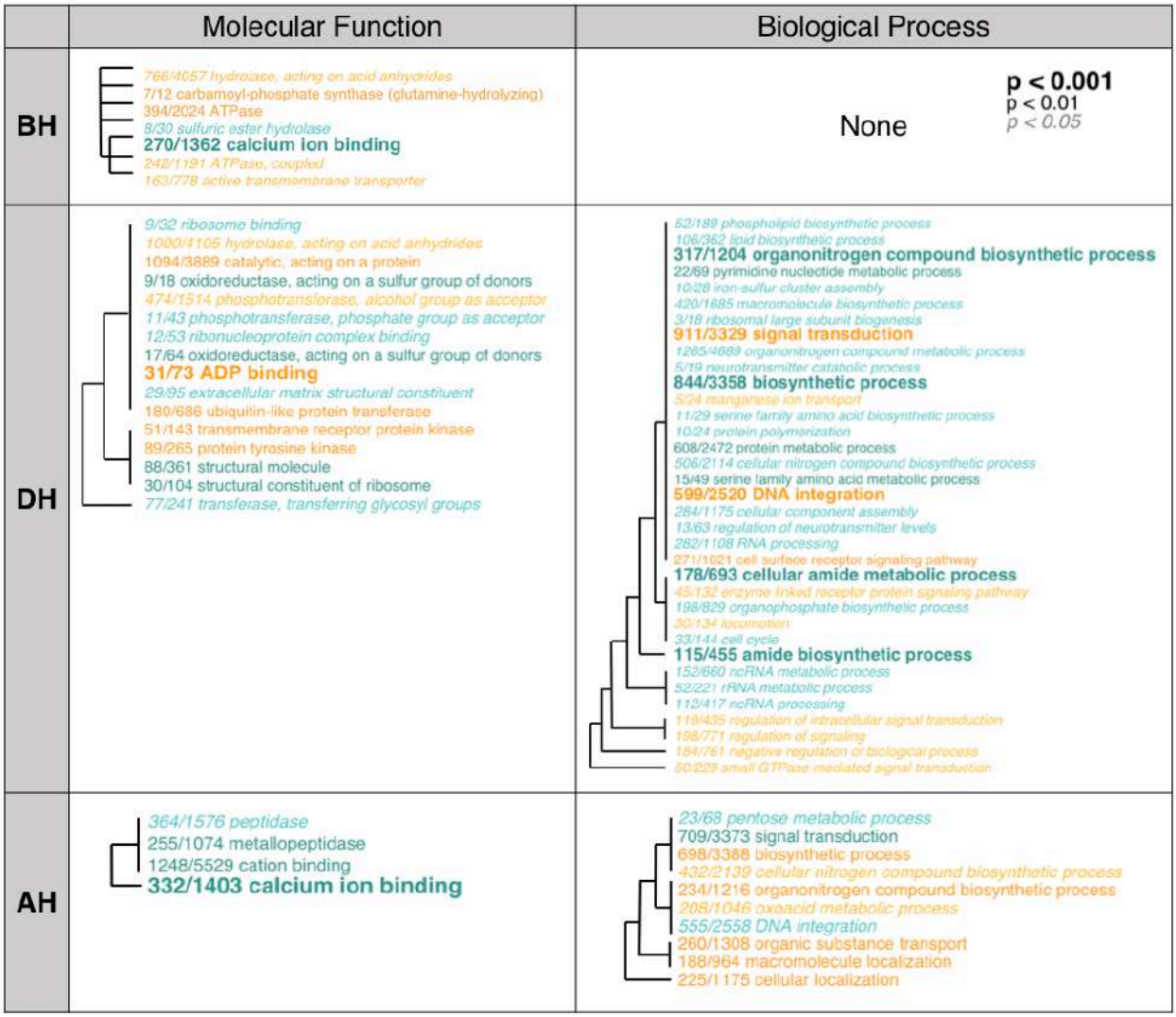


**Figure 10:** GO categories significantly enriched with up/down-regulated genes from comparisons between red and brown phenotypes for each time point during Heat Stress 1. Orange text indicates terms overexpressed in red phenotypes; turquoise text indicates terms underexpressed in red phenotypes. Large bold font is equivalent to a p-value less than 0.001, medium font is equivalent to a p-value less than 0.01, and the small italicized font is equivalent to a p-value less than 0.05. Dendrograms show hierarchical clustering of GO categories based on shared genes within the dataset.

*Heat Stress Experiment 2*

GO enrichment analysis of comparisons between DH2 vs. BH2 and AH2 showed fewer terms for red phenotypes in both molecular function and biological process than in the first heat

stress assay (Figure 11). Transcription factor ($p < 0.001$) was the most enriched molecular function with overexpressed genes in red phenotypes DH2. Biological processes such as biosynthetic process, RNA processing, cellular response to stimulus, and DNA replication ($p < 0.001$) were the most enriched in underexpressed genes in red DH2 samples. Additional molecular functions that were found significantly enriched with underexpressed genes in the red phenotypes at DH2 included cell-matrix adhesion ($p < 0.01$), as well as functions involved in DNA repair such as nucleotide-excision repair ($p < 0.05$) and double-strand break repair via nonhomologous end joining ($p < 0.05$). Finally, the biological processes regulation of response to stress ($p < 0.01$), signal transduction ($p < 0.01$), toll-signaling pathway ($p < 0.05$), and cytokine-mediated signaling pathway ($p < 0.05$) were enriched with overexpressed genes in red phenotypes at DH2.

Conversely, in brown phenotypes, we enriched more terms for the second heat stress assay compared to the first. Among the molecular functions, ligand-gated channel, and cyclic-di-GMP binding ($p < 0.001$) were the most significantly enriched terms associated with overexpressed genes. The molecular functions cation binding ($p < 0.01$), ADP binding ($p < 0.01$), protein tyrosine kinase ($p < 0.05$), iron ion and ferric ion binding ($p < 0.05$), and ubiquitin-like protein transferase ($p < 0.05$) were enriched with overexpressed genes in brown DH2. Molecular functions enriched with underexpressed genes included DNA binding ($p < 0.05$), amino acid binding ($p < 0.05$), oxidoreductase ($p < 0.05$), and chromatin binding ($p < 0.05$). Several biological processes enriched with overexpressed genes in brown phenotypes at DH2 were similar to red phenotypes at DH2, including regulation of defense response ($p < 0.001$), signal transduction ($p < 0.001$), cytokine-mediated signaling pathway ($p < 0.05$), and regulation of gene silencing ($p < 0.05$), showing that brown and red phenotypes implemented similar gene

pathways during the second heat shock. Brown phenotypes overexpressed more genes with terms enriched for the immune system at DH2 than red ones, including positive regulation of immune system process signal transduction ($p < 0.05$), innate immune response ($p < 0.01$), positive regulation of immune system process ($p < 0.01$), and positive regulation of innate immune response ($p < 0.001$). Cellular response to stimulus, DNA replication, and a few biosynthetic processes including cellular nitrogen compound, amide, organonitrogen, and macromolecule biosynthetic processes ($p < 0.001$) were enriched with underexpressed genes in brown phenotypes DH2.

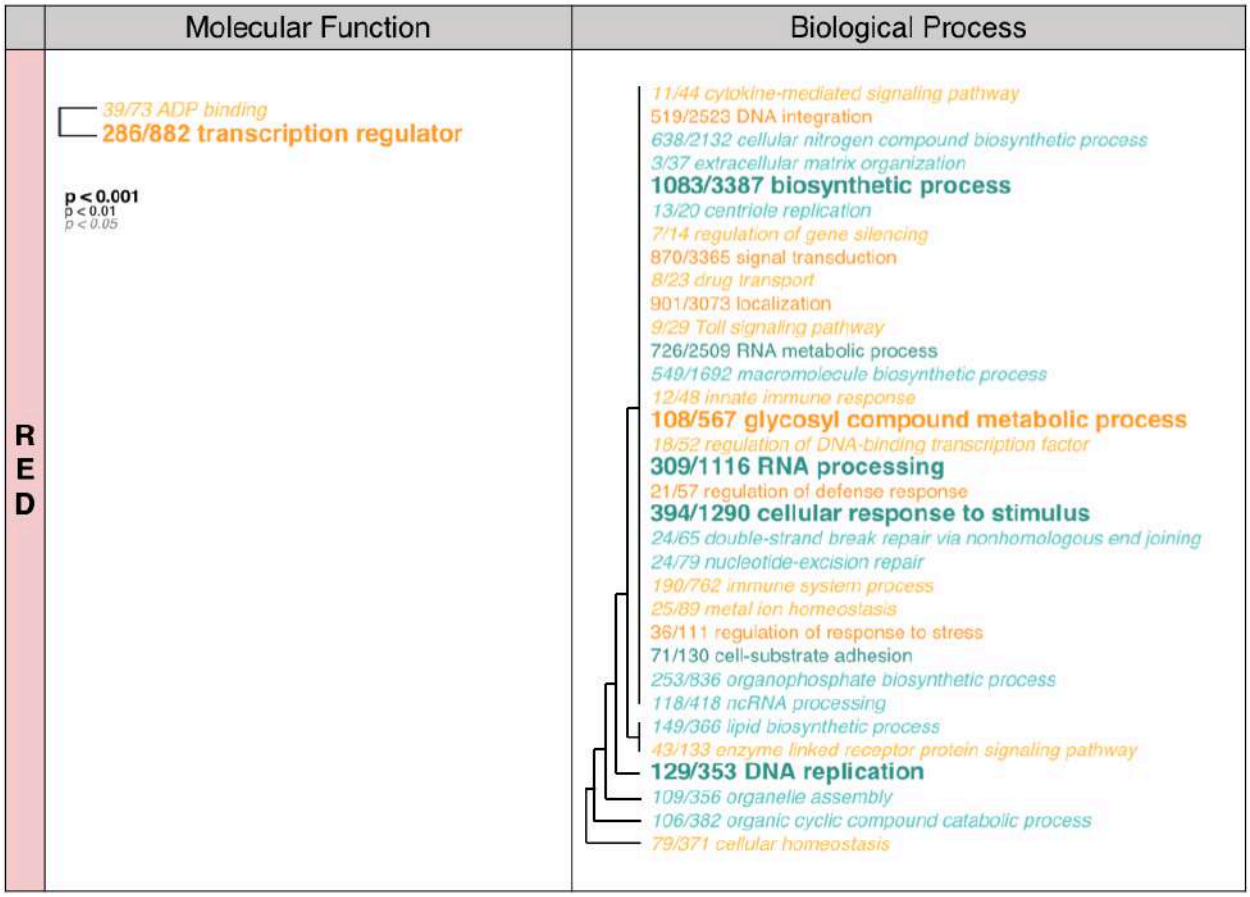


**Figure 11:** see legend below

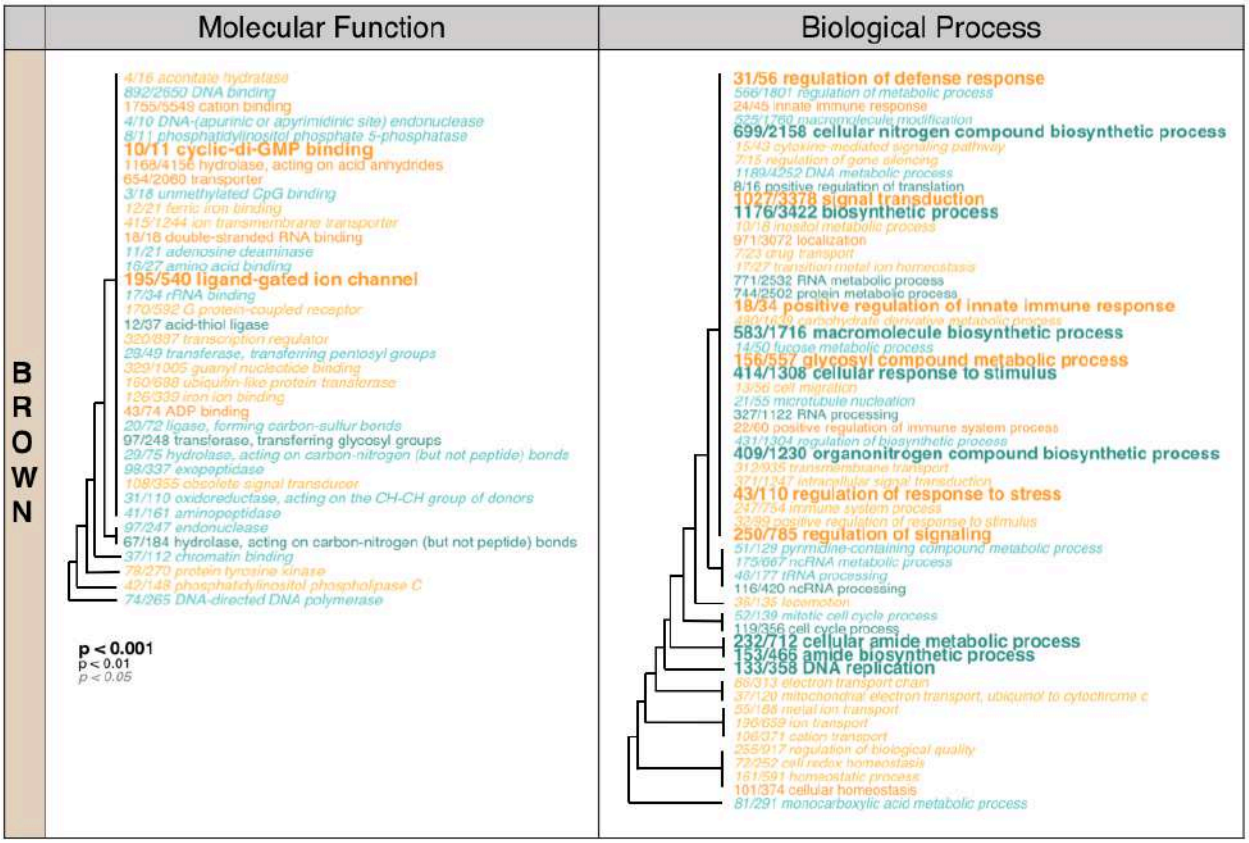


**Figure 11 (continued):** GO categories significantly enriched with up/down-regulated genes from comparisons between DH2 vs BH2+AH2. Orange text indicates terms overexpressed; turquoise text indicates terms underexpressed. Large bold font is equivalent to a p-value less than 0.001, medium font is equivalent to a p-value less than 0.01, and the small italicized font is equivalent to a p-value less than 0.05. Dendrograms show hierarchical clustering of GO categories based on shared genes within the dataset.

GO enrichment analysis for comparisons between red and brown phenotypes at each time point throughout the second heat shock revealed that both phenotypes responded similarly to the second heat stress (Figure 12). No biological processes could be enriched for BH2 samples, however enrichment of molecular functions showed that transporter ($p < 0.05$), ligand-gated channel ($p < 0.01$), and ion transmembrane transporter ($p < 0.01$) were associated with overexpressed genes in red phenotypes compared to brown at BH2. We did not enrich any molecular functions between red and brown phenotypes for DH2 samples, and only found two enriched biological processes, including glycosyl compound metabolic process ($p < 0.001$) and

protein modification by small protein conjugation ($p < 0.05$), enriched with underexpressed genes in red phenotypes.

The AH2 time point showed the greatest disparity between color phenotypes. Molecular functions such as DNA binding, DNA polymerase, alcohol binding, and protein kinase inhibitor ($p < 0.05$) were enriched with underexpressed genes in red phenotypes, while dipeptidase ($p < 0.05$) was enriched with underexpressed genes in brown phenotypes AH2 samples. DNA integration and DNA metabolic process ($p < 0.001$) were among the most significant enriched biological process terms with overexpressed genes in brown phenotypes, in addition to JAK-STAT signaling pathways ($p < 0.05$), modulation of chemical synaptic transmission ($p < 0.05$), and mRNA splice site selection ($p < 0.01$). Microtubule-based process ($p < 0.001$) was the most strongly enriched biological process with overexpressed genes in red phenotypes.

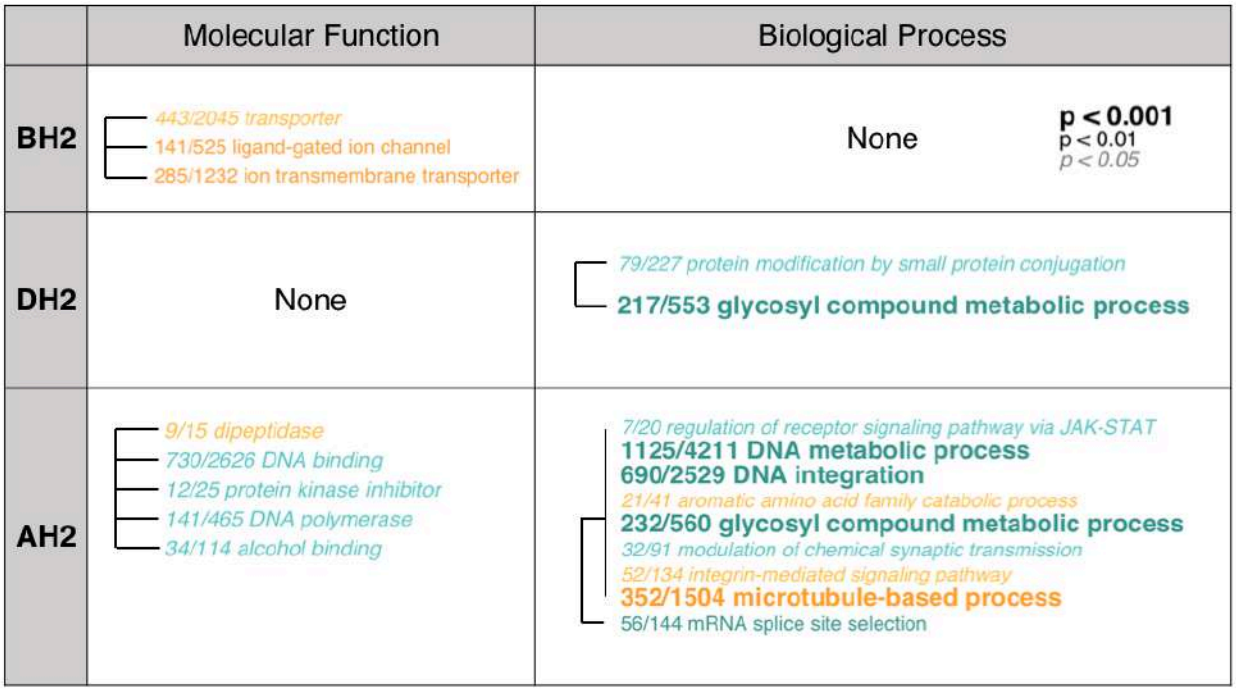


**Figure 12:** GO categories significantly enriched with up/down-regulated genes from comparisons between red and brown phenotypes for each time point during Heat Stress 2. Orange text indicates terms overexpressed in red phenotypes; turquoise text indicates terms

underexpressed in red phenotypes. Large bold font is equivalent to a p-value less than 0.001, medium font is equivalent to a p-value less than 0.01, and the small italicized font is equivalent to a p-value less than 0.05. Dendrograms show hierarchical clustering of GO categories based on shared genes within the dataset.

## Discussion

### Red phenotypes are most sensitive to environmental variation

The non-random distribution across depths of *A. surculosa* color phenotypes in Pago Bay indicates that different coral color morphs are better adapted to different habitats. We recorded significantly more brown than red colonies at 2m, where temperature and light regimes are more variable compared to 6m and 8m depths. This result suggests that brown colonies may have higher abundances of fluorescent proteins than red or orange colonies, which allow them to better tolerate the high variation in light and temperature at 2m. Higher abundance of fluorescent proteins is often found in corals from shallow, high-light habitats (Gittins et al., 2015; Smith et al., 2013). In particular, high relative proportions of cyan fluorescent protein in visibly green *A. millepora* corals has been linked to higher thermal tolerance (Paley et al., 2012). Paley et al. (2014), noted higher abundances of red colonies overall despite the higher rate of bleaching and mortality on the Great Barrier Reef. Our study had a similar finding in which we noted higher abundances overall and higher mortality rates of red colonies during the length of our survey. Moreover, several *A. surculosa* colonies switched from brown to red before dying of unidentified causes. Our sample size was too small to statistically test our observation, but it aligns with other studies that significantly correlated visible coral color and mortality (Paley et al., 2012; Paley,

2014; Satoh et al., 2020). Whether this change in color from brown to red (or vice-versa) is directly related to coral health in our study organisms remains unclear.

In our tank experiments, red phenotypes displayed signs of stress, such as significant underexpression of genes enriched for calcium ion binding compared to brown phenotypes (Figure 10), even before the beginning of the actual heat stress assay. This suggests that disruption in cell-signaling pathways (Kaniewska et al., 2015) and skeleton calcification (Moya et al., 2015) was likely occurring in red phenotypes before heat had ever been applied. Downregulation of calcium ions has been consistently observed in corals exposed to various stressors including *p*CO2 (Kaniewska et al., 2015), increased UV radiation (Aranda et al., 2011), thermal stress, and bleaching (Davies et al., 2016; Desalvo et al., 2008). Other signs of pre-heat stress in red phenotypes include overexpression of carbamoyl-phosphate synthase (Figure 10), which is linked to increased nitrogen cycling and coral hosts suppressing symbiont productivity (Hemond et al., 2015). Increased productivity of symbionts can lead to symbiont parasitism within the holobiont (D. M. Baker et al., 2018). This in turn may trigger coral hosts to expel symbionts, thus suppressing overproduction of symbionts is likely a useful tool to avoid the shift to a parasitic relationship. Similar results were found in Paley et al. (2014), who found that red colonies of *A. millepora* began to bleach before heat stress was applied in their tank experiments, while yellow and green colonies did not. In our study, it is possible that slight variations in light and ambient temperature between our tanks and the corals' original environment were enough to raise background levels of stress only in red phenotypes, while brown phenotypes were not affected. This would explain why, despite random distribution of colonies in all tanks, only red phenotypes responded to changes in their environment.

In summary, we found that red colonies are more common in stable environments, suffered mortality in greater numbers, and reacted more profoundly to environmental changes between the transect and tanks than brown colonies. In contrast, brown phenotypes were more common in highly variable environments, typically turned red before suffering mortality, and showed no response to differences between transect and tank environments.

**Red phenotypes show elevated physiological and molecular responses to acute heat stress**

We found that red phenotypes were overall more responsive to acute heat stress. Physiological changes can be challenging to detect, especially on such a short time scale. While higher variation in CoralWatch changes of red phenotypes suggests erratic changes in either fluorescent/chromoprotein type, concentration, or symbiont density, we could not confirm the latter with our symbiont density measurements (Figure 5C). Both color morphs hosted the same genus of symbionts, which remained consistent for each sampling point (Figure S1; supp. materials). Damage or destabilizing of photosystems (evident by a decrease in Fv/Fm) is a sign of symbiont stress, and though to be a significant driver of bleaching (Nielsen et al., 2018; Weis, 2008). However, the general lack of significant differences in Fv/Fm measurements over the course of the experiment for both color morphs, aside from the significant increases after heat stresses, indicate that symbiont photosystems were not damaged or otherwise altered by either heat stress assay. As symbiont type and photosynthetic efficiency did not change throughout the course of the experiment, observed physiological and molecular differences in corals can be associated solely with the color phenotype of the coral host.

Molecular responses to stress in corals are highly conserved (Dixon et al., 2020). This is evident in our study by the segregation of samples collected during the actual heat shocks and all other time points, in both red and brown phenotypes (Figure 6, 7). However, the clustering of samples by color morph implies that the response to heat shock was significantly different between the two color morphs, as was their gene expression before and after heat stress. Analyses of significant DEGs between DH1 vs. BH1+AH1 shows that red phenotypes differentially regulated nearly three times more genes than brown phenotypes during the first heat shock (1557 and 589, respectively). This reflects an elevated response to the first heat shock in red compared to brown phenotypes. Functional analyses of all DEGs between DH1 vs. BH1 + AH1 for each color phenotype (Fig. 9), as well as between the color phenotypes at DH1 (Figure 10), reveals that red phenotypes appeared to be more negatively impacted by the first heat shock than brown ones. The overexpression of signaling, cytokine pathways, and ubiquitin protein transferases in red phenotypes during the first heat shock demonstrates the negative impact of thermal stress in red phenotypes. Signaling is a main component of the immune system response (Rosic et al., 2014; Vidal-Dupiol et al., 2014) and the environmental stress response (Dixon et al., 2020). We found more terms related to signaling, and more genes within these terms, in red phenotypes than in brown ones during the first heat shock, highlighting the increased reaction to stress in red phenotypes. Cytokines are a broad group of signaling proteins linked to DNA damage (Martindale et al., 2002), pro-inflammatory responses (van de Water et al., 2018), and immune system pathways (Weis, 2019), which we found to be overexpressed exclusively in red phenotypes during the first heat stress (Figure 9). Overexpression of ubiquitin protein transferases in red phenotypes during the first heat shock conveys increased degradation/denaturing of proteins as a result of thermal stress (Downs et al., 2000; Kenkel et al.,

2014). In contrast, terms for protein folding overexpressed in brown phenotype, implies activation of pathways to facilitate protein folding of degraded proteins, which is associated stress resistance (Sørensen et al., 2003). This suggests that red phenotypes experienced protein degradation and apoptosis as a result of the heat shock, whereas brown phenotypes were not. Furthermore, red phenotypes also strongly underexpressed cellular response to stimulus (Figure 9), a pattern thought to predict coral bleaching after thermal stress (Rose et al., 2016).

Altogether, red phenotypes mounted a greater molecular response in response to heat shock, appeared to be less effective in mitigating stress, and showed more signs of cell damage than brown phenotypes during the first heat shock. Brown phenotypes showed less reactivity to acute heat stress, and activated molecular pathways to mitigate and manage stress, compared to red phenotypes.

**Red phenotypes recover less between heat stresses**

Red phenotypes did not appear to recover much during the 2 weeks between the first and second heat stress. Comparisons of red and brown phenotypes after the first heat shock continued to exhibit underexpression of calcium ion binding and additionally, metallopeptidase, implying disruption of the cytoskeleton (Figure 10). Downregulation of metallopeptidase is regularly associated with corals experiencing stress (Dixon et al., 2020). This result could either indicate a signal of quick recovery by brown phenotypes that is lacking in red phenotypes, or that brown phenotypes simply dedicate more energy towards growth and calcification than red phenotypes. For comparisons DH2 vs BH2 + AH2, we found less significant DEGS and enriched fewer GO terms for red phenotypes than in the same comparison for the first heat shock. This suggests gene

expression may not have changed much from the first to the second heat stress in red phenotypes, implying that they remained stressed and did not recovery between the two heat stress assays. On the other hand, we found a higher number of significant DEGs and enriched more GO terms for brown phenotypes comparing DH2 vs BH2 + AH2 than during the first heat stress. This implies that brown phenotypes had a greater reaction to the second heat shock, thus it is unclear whether recovery occurred between heat stress assays or if this was simply the first signs of stress in brown phenotypes.

In red phenotypes, the presence of new functional terms during the second heat shock in red phenotypes indicates a continued decrease in their overall health. We found underexpression of genes enriched for DNA repair in red phenotypes during the second heat shock (Figure 11), which is somewhat contrary to other studies that have found upregulation of DNA repair pathways in *A. millepora* (Kaniewska et al., 2015) and *Stylophora pistillata* (Maor-Landaw et al., 2014) exposed to heat stress. However, our heat shock was shorter than the heat shocks in these other studies, suggesting that DNA repair pathways may take more time to be activated than other immunity pathways when corals are under acute thermal stress. Another possible explanation for this observation is that the second heat shock was disruptive to DNA repair pathways in red phenotypes, potentially due to red phenotypes prioritizing other processes, as DNA repair is energetically expensive (Kaniewska et al., 2015). During the second heat shock red phenotypes overexpressed genes enriched for the toll signaling pathway (Figure 11). This is in contrast to other studies that have examined thermal stress in combination with bacterial infection (Vidal-Dupiol et al., 2014), or physical injury (van de Water et al., 2015), in which toll-like receptors were downregulated under thermal stress. Toll-like receptors activate downstream innate-immunity pathways and pro-inflammatory proteins (Traylor-Knowles et al., 2017).

Overexpression in red phenotypes here may therefore signal irritation of coral tissue as a result of thermal stress.

In short, red phenotypes showed limited recovery between heat stress events. Red phenotypes continued expressing genes linked with coral stress after the first heat stress compared to brown, and gene expression did not appear to change much between the heat stress assays. This ultimately implies recovery in red phenotypes was unlikely.

**Red phenotypes allocate more energy to heat stress management**

During both heat stress assays, red phenotypes were under higher strain to mitigate the heat shocks than brown phenotypes were. Overexpression of ADP binding was found exclusively in red phenotypes during heat shock 1 (Figure 9, 10), which implies increased energy harvesting in red phenotypes. This is often associated with thermal stress as corals' energy demands increase to mitigate the stress (Maor-Landaw et al., 2014). In addition, we found many more biosynthetic processes underexpressed in red than in brown phenotypes during the first heat shock (Figure 10), indicating that red phenotypes reduced the biosynthesis of non-essential compounds, compared to brown ones. Cell cycle and ribosome biogenesis, typical indicators of growth (Vrede et al., 2004), were also underexpressed in red during the first heat shock (Figure 9). Together, this suggests that red phenotypes downregulated general cell functions (ie cell cycle, ribosome biogenesis, biosynthetic processes, and growth) to satisfy increasing energy demands for managing thermal stress. This pattern of increased energy harvesting and decreased general cellular processes has also been found in other coral thermal stress studies (Barfield et al., 2018; Rocker et al., 2019).

Brown phenotypes also exhibited underexpression of some biosynthetic process terms during the first heat shock, which indicates that brown corals also reduced the biosynthesis of non-essential proteins. However, this reduction was much more pronounced in red phenotypes, as outlined before. During the second heat shock, brown phenotypes had a stronger response that mimicked the reaction of red phenotypes to the first heat shock. For example, a larger number of biosynthetic processes and cell cycle terms were underexpressed, while terms associated with protein degradation/denaturing and cytokine signaling pathways were overexpressed in brown phenotypes during the second heat shock (Figure 11). These terms were previously only found to be enriched in red phenotypes during the first heat shock (see previous section). This pattern indicates that the brown phenotype's response to the second heat stress was more similar to the response of red phenotypes to the first heat stress implying that brown phenotypes recovered almost completely between heat stress assays. We did not find overexpression of terms related to increased energy in brown phenotypes. This shows that similar expression of stress terms for red and brown phenotypes results in higher energy allocation for red phenotypes than brown ones.

To summarize, red phenotypes consistently downregulated more biosynthetic pathways, genes associated with the cell cycle, and genes associated with growth, than brown phenotypes in both heat stress assays. Concurrently, red phenotypes also upregulated genes associated with higher energy demands, which was not found in brown phenotypes during the first heat shock. This shows that red phenotypes allocated more energy towards managing heat stress than brown phenotypes.

**Brown phenotypes utilize more oxidative stress and immune system management pathways**

Our analysis found consistent evidence that brown phenotypes activated more oxidative stress management and immune system pathways than red phenotypes in response to both heat stress assays. During the first heat shock, we found more functional terms relating to immune system responses in brown phenotypes. This observation could be the result of the pathways utilized by brown phenotypes to mitigate stress in contrast to the evidence of protein and cell damage found in red phenotypes. The effect of heat stress on coral immune systems is complex. It appears that corals suppress their immune systems in response to thermal stress (Traylor-Knowles et al., 2017), typically to reduce apoptosis as a way of preventing bleaching (Dunn et al., 2007; Pinzón et al., 2015). However, there are components of the immune system that are activated in response to, and play a critical role in, mitigating thermal stress (Louis et al., 2017; Traylor-Knowles et al., 2017). One of the immune system components critical to survival during thermal stress are antioxidant pathways (Lesser, 2006; Traylor-Knowles et al., 2017) which we also found to be overexpressed more in brown than red phenotypes during both heat stress assays. Oxidative injury can result in disruption of cytoskeletal organization and calcification, damage to proteins, lipids, and DNA, and cell death (Lesser, 2006; DeSalvo et al., 2008), which we did not find in brown phenotypes during the first heat shock. This suggests that overexpression of oxidative stress terms in brown phenotypes during the first heat stress can be linked to mitigation.

During the second heat shock, oxidative stress management continued to be more prominent in brown phenotypes response to stress than red phenotypes. For example, we found overexpression of genes enriched for ferric iron and iron ion binding in brown phenotypes. Increased iron and ferric iron binding are associated with iron sequestration by corals to

successfully combat oxidative stress (Berlett & Stadtman, 1997; Császár et al., 2009). After the second heat stress we found overexpression of genes enriched for "receptor signaling pathway via JAK-STAT" in brown compared to red phenotypes. The JAK/STAT pathway is a molecular signaling mechanism linked to regulation of Hsp70, and ultimately, enhanced cell survival or proliferation (Martindale et al., 2002).

One reason for the higher use of oxidative stress pathways in brown phenotypes may be varying concentrations of different fluorescent protein types present in each phenotype. As previously mentioned, distinct visible color in corals is due to the abundance of fluorescent proteins in coral host tissue (Dove et al., 2001; Smith et al., 2013). Measurements of relative fluorescent protein concentrations of different color morphs of *Acropora millepora* indicated that heat-sensitive red morphs had the highest relative concentration of chromoproteins (CPs), but lowest relative concentration of cyan and green fluorescent proteins (Paley, 2014). Thus, it is possible that the red phenotypes in our study have higher concentrations of CPs than the brown phenotypes. As previously mentioned, Palmer et al., (2009) found that CPs and red fluorescent protein scavenged ROS more efficiently in vivo, accounting for lower abundance of those proteins in host tissue. Lower concentrations of red fluorescent proteins or CPs in brown coral tissue would explain why they activated more immune and oxidative stress management pathways to mitigate thermal stress than red corals. Red phenotypes in our study likely have higher concentrations of red fluorescent and chromoproteins, however, unexpectedly, we found more signs of oxidative injury in red than brown phenotypes. Ultimately, the relationship between FP/CP type, abundance, and ROS scavenging remains unclear. Measuring FP/CP abundance concurrent to measuring differential gene expression of different coral color morphs in response to thermal stress would help clarify this relationship.

In summary, we found that brown phenotypes consistently upregulated more genes associated with immune system and oxidative stress management than red phenotypes. This could be due to differences in variant antioxidant fluorescent protein type abundances, however this relationship still remains unclear.

## Conclusions

In this study we found that red colonies are more common in stable environments, suffered mortality in greater numbers, and reacted more profoundly to environmental changes between the transect and tanks than brown colonies. In contrast, brown phenotypes were more common in highly variable environments, typically turned red before suffering mortality, and showed no response to differences between transect and tank environments. In our tank experiment, red phenotypes mounted a greater molecular response in response to heat shock, appeared to be less effective in mitigating stress, and showed more signs of cell damage than brown phenotypes during the first heat shock. Brown phenotypes showed less reactivity to acute heat stress, and activated molecular pathways to mitigate and manage stress, compared to red phenotypes. Between heat stress assays, red phenotypes showed limited recovery, while brown phenotypes either fully recovered or were unaffected by the first heat stress. Red phenotypes continued expressing genes linked with coral stress after the first heat stress compared to brown, and gene expression did not appear to change much between the heat stress assays, ultimately implying that recovery in red phenotypes was unlikely. Additionally, red phenotypes consistently downregulated more biosynthetic pathways, genes associated with the cell cycle, and genes associated with growth, than brown phenotypes in both heat stress assays. At the same time, red

phenotypes also upregulated genes associated with higher energy demands, which was not found in brown phenotypes. This shows that red phenotypes allocated more energy towards managing heat stress than brown phenotypes. Finally, we found that brown phenotypes consistently expressed more immune system and oxidative stress management pathways than red phenotypes, likely due to differences in variant fluorescent protein type.

This study provides a new perspective for coral reef monitoring and the development of management practices. Our observations about color distributions across a depth gradient in conjunction with different bleaching susceptibilities amongst color polymorphisms in corals has implications for restoration efforts that plan to use deep water corals as refuge populations. As coral color is easy to identify, utilizing color data from established databases such as CoralWatch can help to further characterize distributions of color morphs in a wide range of different environments and aid predictions about future coral compositions.

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# Supplementary Materials

**Table S1**: Kruskal-Wallace p-values for comparisons of CoralWatch Color Chart differences at different time points during the heat stress assays. BH: Before heat 1, AH: After heat 1, BH2: Before heat 2, AH2: After heat 2. Significance is as follows: * for P<0.05, ** for P<0.01, *** for P<0.001

| **Red ColorWatch Kruskal Wallace Results** | | | | | |
|---|---|---|---|---|---|
| | BH | DH | AH | BH2 | DH2 |
| BH | - | - | - | - | - |
| DH | 1.000 | - | - | - | - |
| AH | 1.000 | **0.039 *** | - | - | - |
| BH2 | 0.086 | 1.000 | **0.001 ***** | - | - |
| DH2 | 1.000 | 1.000 | 1.000 | 0.505 | - |
| AH2 | 1.000 | 0.186 | 1.000 | **0.008 **** | 1.000 |
| **Brown ColorWatch Kruskal Wallace Results** | | | | | |
| | BH | DH | AH | BH2 | DH2 |
| BH | - | - | - | - | - |
| DH | 1.000 | - | - | - | - |
| AH | 1.000 | 1.000 | - | - | - |
| BH2 | 1.000 | 1.000 | 1.000 | - | - |
| DH2 | 1.000 | 1.000 | 1.000 | 1.000 | - |
| AH2 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| **Red vs Brown Kruskal Wallace Results** | | | | | |
| BH | 1.000 | | | | |
| DH | 1.000 | | | | |
| AH | 1.000 | | | | |
| BH2 | 1.000 | | | | |
| DH2 | 1.000 | | | | |
| AH2 | 1.000 | | | | |

**Table S2:** ANOVA P-values for comparisons of Fv/Fm at different time points during the heat stress assays, using PAM. BH: Before heat 1, AH: After heat 1, BH2: Before heat 2, AH2: After heat 2. Significance is as follows: * for P<0.05, ** for P<0.01, *** for P<0.001

| **Red Fv/FM ANOVA Results** | | | | **Brown Fv/FM ANOVA Results** | | | | **Red vs Brown** | |
|---|---|---|---|---|---|---|---|---|---|
| **Heat Stress 1** | | | | **Heat Stress 1** | | | | **Heat Stress 1** | |
| | BH | AH | 36hr AH | | BH | AH | 36hr AH | BH | 0.4 |
| BH | - | - | - | BH | - | - | - | AH | 0.93 |
| AH | 0.277 | - | - | AH | 1 | - | - | 36hr AH | 0.063 |
| 36hr AH | 1 | 1 | - | 36hr AH | 0.013 | 0.233 | - | 60hr AH | **0.021 *** |
| 60hr AH | 1 | 1 | 1 | 60hr AH | 0.35 | >0.001 | 1 | | |
| **Heat Stress 2** | | | | **Heat Stress 2** | | | | **Heat Stress 2** | |
| | BH2 | AH2 | 36hr AH2 | | BH2 | AH2 | 36 hr AH2 | BH2 | **<0.001 *** ** |
| BH2 | - | - | - | BH2 | - | - | - | AH2 | 0.56 |
| AH2 | 0.243 | - | - | AH2 | 1 | - | - | 36hr AH2 | 0.077 |
| 36hr AH2 | **0.018 *** | 0.243 | - | 36hr AH2 | 0.358 | 1 | - | 60hr AH2 | 0.99 |
| 60hr AH2 | **0.018 *** | 1 | **0.018 *** | 60hr AH2 | 1 | 1 | 0.616 | | |

**Table S3:** Mean abundance and proportions of colors at each depth. Means are reported due to color changing of colonies during survey period.

| | | Abundance | | | Proportion | | |
|---|---|---|---|---|---|---|---|
| | **Total Colonies** | **Red** | **Orange** | **Brown** | **Red** | **Orange** | **Brown** |
| **2m** | 9 | 1.8 | 0.4 | 3.5 | 0.32 | 0.08 | 0.60 |
| **4m** | 11 | 4.1 | 2.0 | 2.7 | 0.43 | 0.27 | 0.30 |
| **6m** | 8 | 3.6 | 1.5 | 1.7 | 0.53 | 0.22 | 0.25 |
| **8m** | 10 | 4.3 | 1.3 | 1.1 | 0.62 | 0.21 | 0.17 |

**Table S4:** Kruskal Wallace p-values for comparisons of color distributions between depths for each color. Proportion of color at each depth Significance is as follows: * for P<0.05, ** for P<0.01, *** for P<0.001

| **Red** | | | |
|---|---|---|---|
| | **2m** | **4m** | **6m** |
| **4m** | 0.311 | - | - |
| **6m** | **0.017 *** | 0.334 | - |
| **8m** | **<0.001 ***** | 0.070 | 0.334 |
| **Orange** | | | |
| | **2m** | **4m** | **6m** |
| **4m** | **0.027 *** | - | - |
| **6m** | **0.049 *** | 1.000 | - |
| **8m** | 0.084 | 1.000 | 1.000 |
| **Brown** | | | |
| | **2m** | **4m** | **6m** |
| **4m** | **0.002 **** | - | - |
| **6m** | **<0.001 ***** | 0.561 | - |
| **8m** | **<0.001 ***** | 0.155 | 0.561 |

**Table S5:** Kruskal Wallace p-values for comparisons of color proportions between colors for each depth. Significance is as follows: * for P<0.05, ** for P<0.01, *** for P<0.001

| **2m** | | |
|---|---|---|
| | **Brown** | **Orange** |
| **Orange** | **<0.001 ***** | - |
| **Red** | **<0.001 ***** | **<0.001 ***** |
| **4m** | | |
| | **Brown** | **Orange** |
| **Orange** | 0.640 | - |
| **Red** | 0.220 | 0.120 |
| **6m** | | |
| | **Brown** | **Orange** |
| **Orange** | 0.762 | - |
| **Red** | **0.001 ***** | **0.001 ***** |
| **8m** | | |
| | **Brown** | **Orange** |
| **Orange** | 0.57 | - |
| **Red** | **<0.001 ***** | **<0.001 ***** |

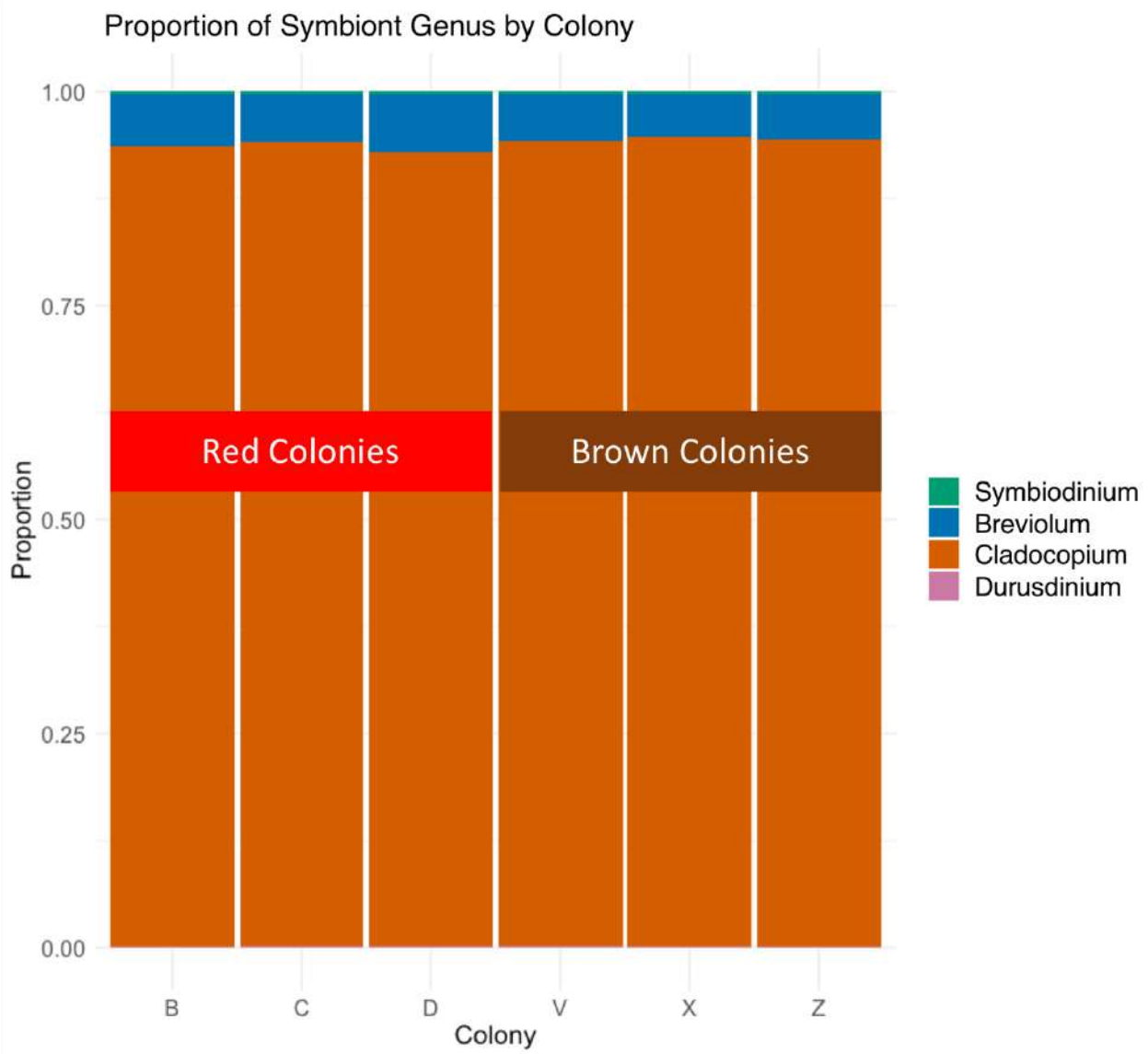



**Figure S1**: Proportion of symbiont clade by colony. Proportion reflects the relative proportion of highly unique reads (mapping quality > 40) matching each clade. Colors in the barplot correspond to different symbiont clades as in the figure legend.

**Table S6**: Number of combined forward and reverse reads retained for samples after each step of filtering for reference transcriptome construction and generation of gene counts.

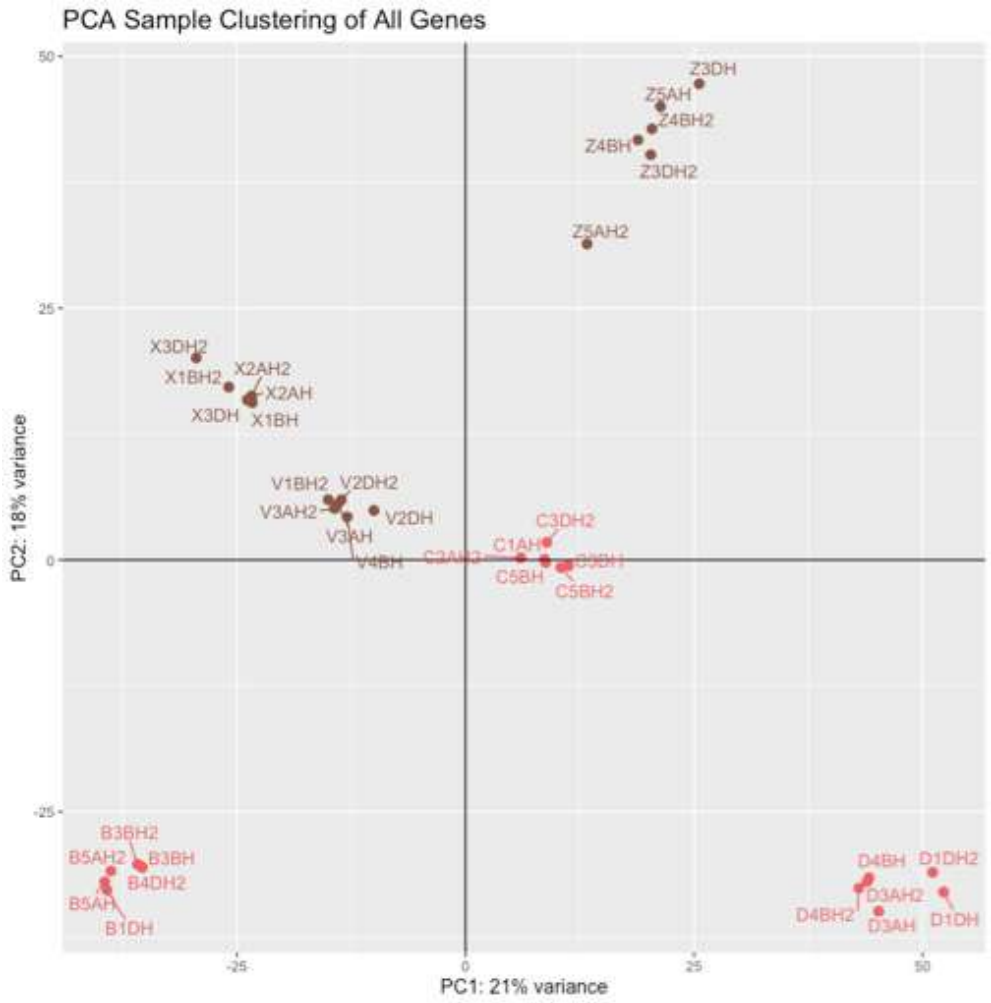
| sample | Raw reads | Reads after trimgalore | Reads after rRNA filtering | Reads after zoox trim |
|---|---|---|---|---|
| **B1DH** | 113,657,106 | 108,412,636 | 108,393,407 | 107,723,426 |
| **B3BH** | 45,523,964 | 43,529,522 | 43,509,570 | 43,213,862 |
| **B5AH** | 196,562,150 | 188,134,592 | 188,076,663 | 186,380,490 |
| **C1AH** | 60,356,614 | 58,098,362 | 58,074,556 | 57,554,792 |
| **C3DH** | 64,360,430 | 61,155,048 | 61,116,634 | 60,881,474 |
| **C5BH** | 44,698,978 | 42,592,954 | 42,550,221 | 42,446,108 |
| **D1DH** | 45,816,164 | 43,731,504 | 43,691,192 | 43,397,142 |
| **D3AH** | 138,571,508 | 131,921,078 | 131,850,435 | 130,610,376 |
| **D4BH** | 35,521,614 | 33,838,652 | 33,779,588 | 33,542,258 |
| **V2DH** | 48,540,992 | 44,658,284 | 44,647,232 | 44,276,572 |
| **V3AH** | 58,890,618 | 56,398,432 | 56,296,804 | 55,956,858 |
| **V4BH** | 36,332,876 | 34,223,946 | 34,115,826 | 34,035,890 |
| **X1BH** | 29,078,522 | 27,683,568 | 27,663,601 | 27,590,748 |
| **X2AH** | 39,196,278 | 36,457,706 | 36,441,248 | 36,205,566 |
| **X3DH** | 26,349,562 | 24,919,470 | 24,898,812 | 24,857,598 |
| **Z3DH** | 85,521,632 | 81,440,372 | 81,421,640 | 80,687,482 |
| **Z4BH** | 36,296,208 | 34,737,016 | 34,679,994 | 34,523,306 |
| **Z5AH** | 88,639,472 | 85,303,164 | 85,268,945 | 84,717,674 |
| **B3BH2** | 43,064,408 | 39,988,164 | 39,979,005 | 39,631,688 |
| **B4DH2** | 35,832,556 | 33,765,598 | 33,762,214 | 33,515,728 |
| **B5AH2** | 121,164,920 | 115,338,958 | 115,325,978 | 114,672,586 |
| **C2AH2** | 38,862,434 | 37,052,712 | 37,045,862 | 36,776,642 |
| **C3DH2** | 63,068,778 | 59,276,624 | 59,268,046 | 58,871,132 |
| **C5BH2** | 46,391,554 | 43,471,528 | 43,458,047 | 42,863,652 |
| **D1DH2** | 28,473,368 | 26,869,088 | 26,863,608 | 26,565,600 |
| **D3AH2** | 64,199,068 | 60,754,598 | 60,745,047 | 60,097,822 |
| **D4BH2** | 35,305,050 | 33,000,582 | 32,991,417 | 32,675,402 |

| | | | | |
|---|---|---|---|---|
| **V1BH2** | 88,236,238 | 83,873,370 | 83,865,018 | 82,774,032 |
| **V2DH2** | 96,653,980 | 91,495,946 | 91,480,253 | 91,031,910 |
| **V3AH2** | 45,409,192 | 43,061,984 | 43,054,629 | 42,855,990 |
| **X1BH2** | 68,183,752 | 64,787,248 | 64,777,881 | 64,225,394 |
| **X2AH2** | 33,478,564 | 31,893,492 | 31,888,795 | 31,641,004 |
| **X3DH2** | 150,782,520 | 142,639,010 | 142,614,095 | 140,876,298 |
| **Z3DH2** | 52,641,852 | 47,951,648 | 47,945,985 | 47,590,458 |
| **Z4BH2** | 38,804,138 | 36,475,264 | 36,469,261 | 36,073,730 |
| **Z5AH2** | 69,577,050 | 66,689,454 | 66,256,226 | 38,236,700 |
| **Total** | 2,314,044,110 | 2,195,621,574 | 2,194,267,735 | 2,149,577,390 |

**Chapter 3**

## Additional Transcriptomic Analyses

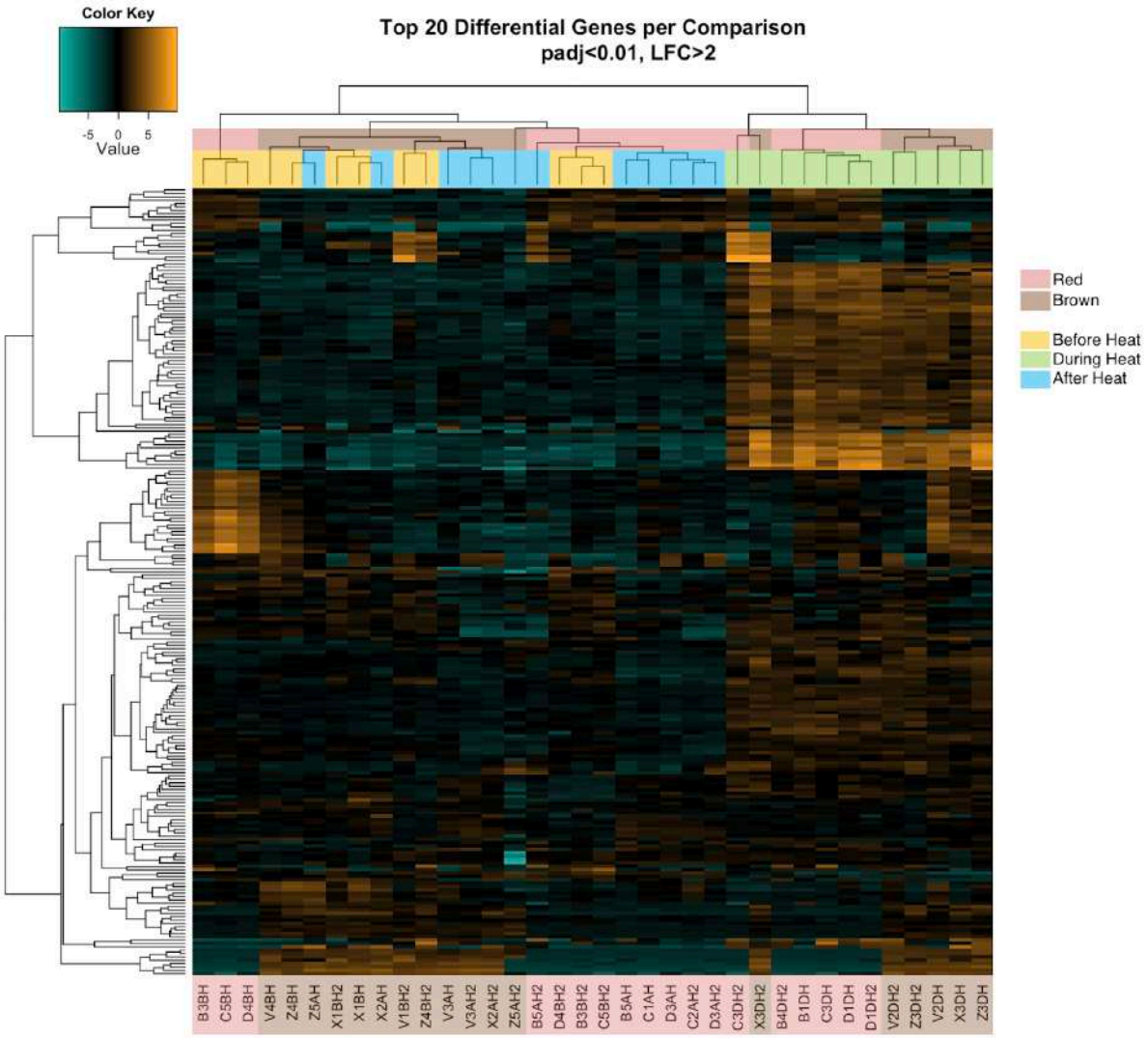
Principal component analysis (PCA) of variance stabilized gene expression was performed using the *plotPCA* function from the DESeq2 package in R to visualize clustering of data for all genes in the dataset. The resulting PCA clustered samples primarily by their colony association (Figure S1). Brown colonies clustered completely above principal component 2, while red colonies clustered mostly below principal component 2, which accounts for 18% of the variance.




**Supplemental Figure 1:** Principal component analysis performed on all expressed genes. Brown and red samples are labeled in brown and red respectively. The first two characters of each label correspond to

sample name, followed by collection time point as follows: BH for before heat, DH for during heat and AH for after heat. Labels ending with a 2 correspond to samples collected during the second heat stress experiment.

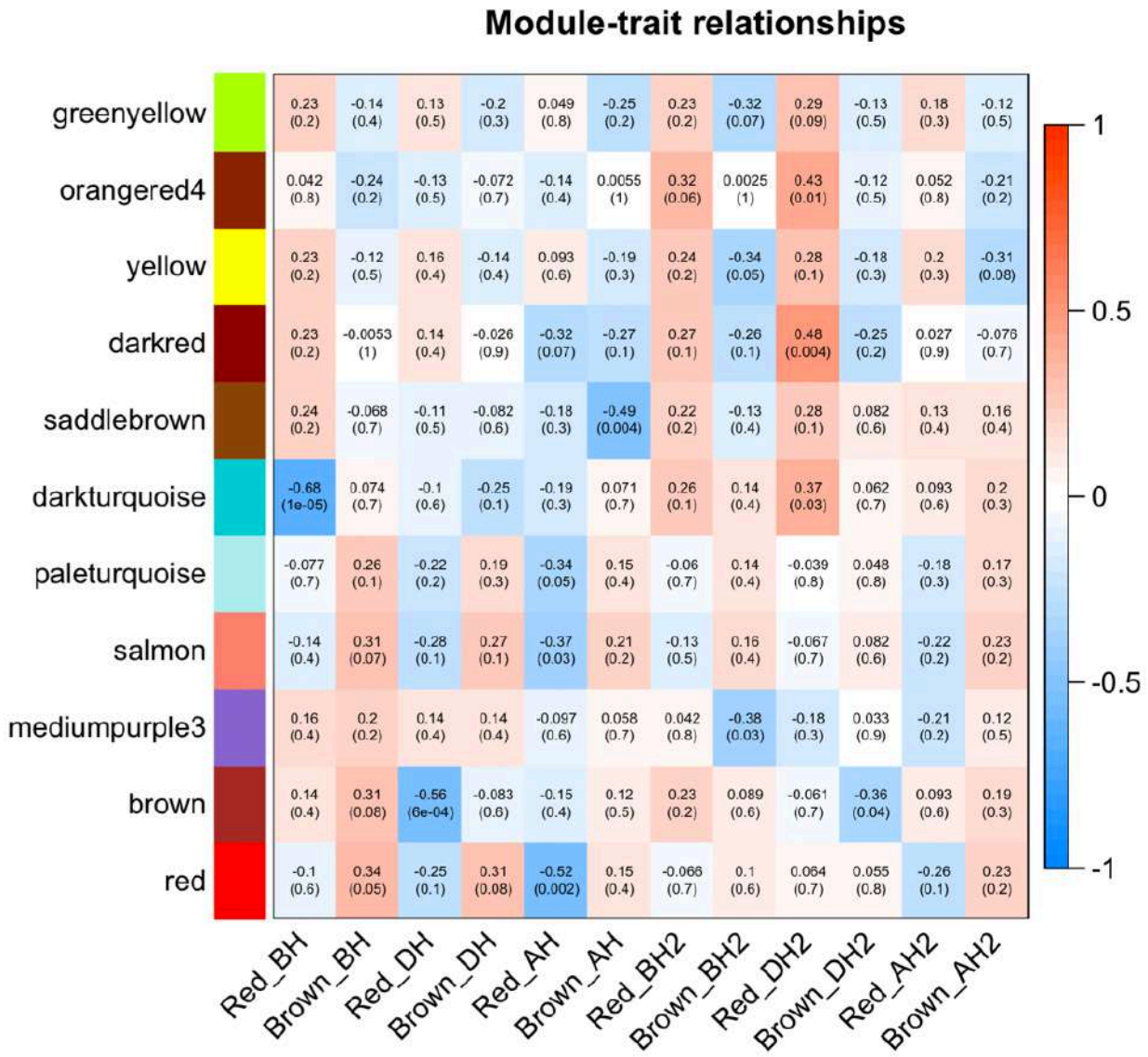
A heatmap of the top 20 significant DEGs per pairwise comparison, generated by the analyze_diff_expr.pl script in Trinity, was created using the *heatmap.3* function of the base R package. The resulting heatmap revealed that samples clustered first by treatment (DH vs BH+AH) and second by tissue color, with a few exceptions (Figure S2). There are clear and opposing contrasts in the expression of the genes between the 2 main clades (DH vs. BH+AH), especially in the first top tier section of the heatmap. This suggests that both color phenotypes had a response to our acute heat stress. Furthermore, it indicates that responses to stress differed between color phenotypes during heat stress, in addition to differences in expression before and after heat stress.



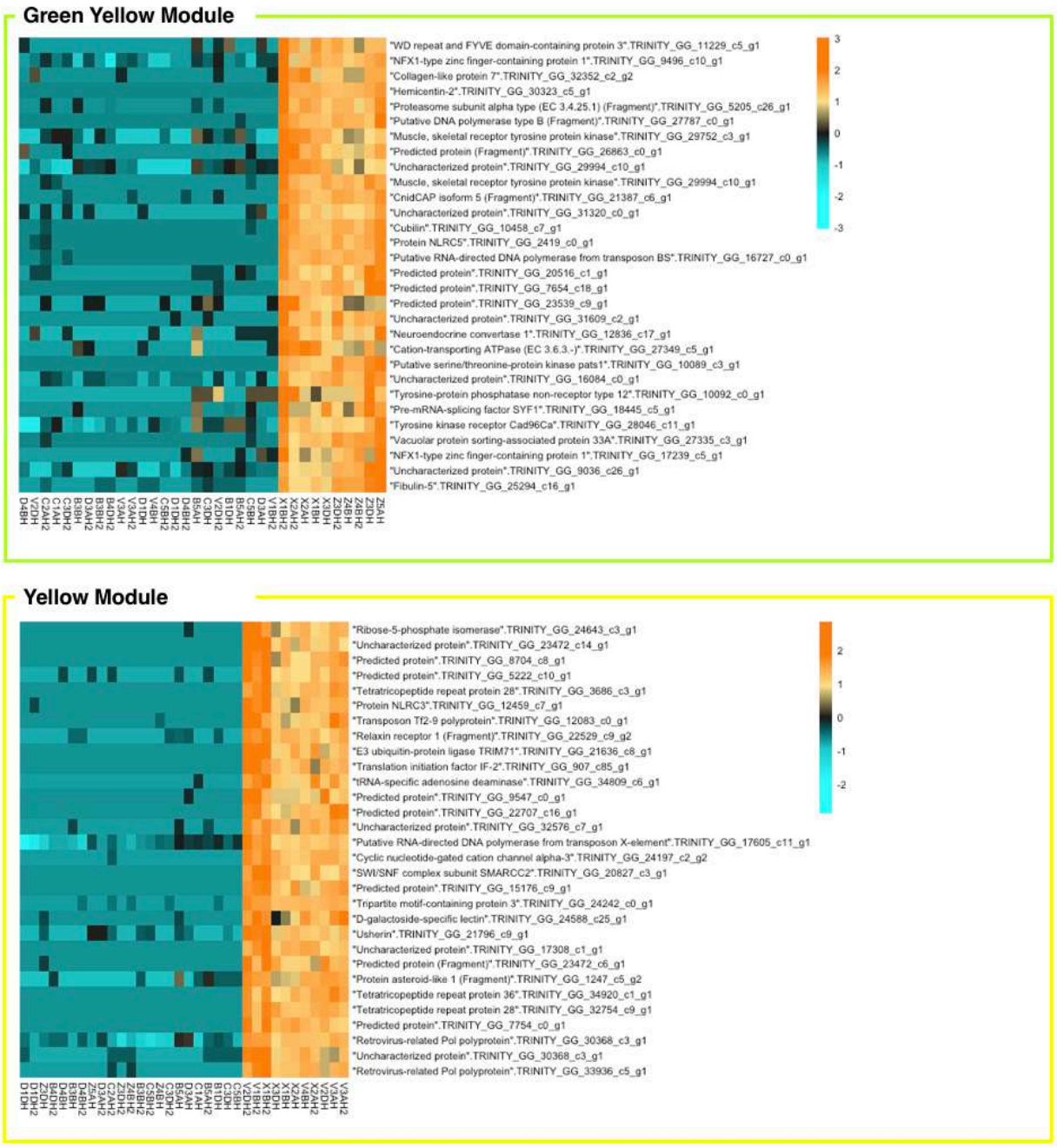

**Supplemental Figure 2:** Heatmap of the top 20 significant DEGs (padj < 0.01, log fold change > 2) per pairwise comparison. Rows are genes, and the vertical dendrogram is a hierarchical clustering of genes using Pearson's correlation. Columns are samples, and the horizontal dendrogram is a hierarchical clustering of samples using Pearson's correlation. Color scale represents log2 fold change of genes. Colors in the sample dendrogram and labels distinguish color morphs and collection time points as indicated in the color key. Sample labeling is the same as in Figure S1.

To examine groups of genes that red and brown phenotypes regulate differently in response to acute heat stress, we performed a weighted gene co-expression network analysis (WGCNA) (Langfelder et al., 2008). Signed co-expression networks were examined using

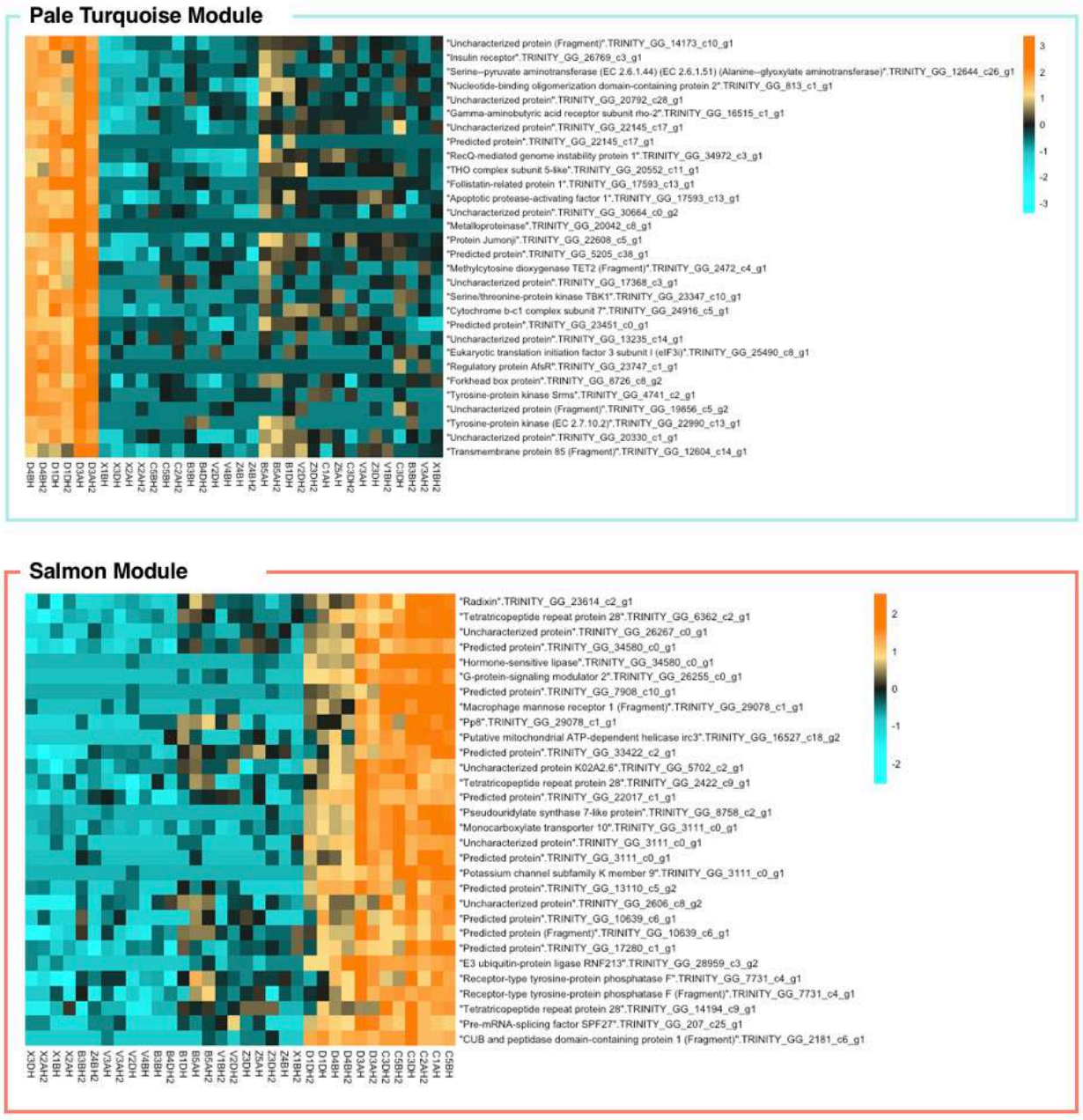
Pearson correlations of all genes across all treatments in an unsupervised manner, without considering any experimental conditions. Genes were clustered into eleven co-expression modules (designated by colors) with a module merging of 0.35, which effectively merged modules with highly correlated genes (Figure S3). Four of the modules were consistently expressed divergently between red and brown phenotypes. Green yellow (563 genes) and yellow (585 genes) modules were consistently upregulated in red phenotypes and downregulated in brown ones. Pale turquoise (112 genes) and salmon (492 genes) modules were consistently downregulated in red phenotypes and upregulated in brown ones. Heatmaps of the top 30 significant genes in each module were generated with the pheatmap package in R to examine expression patterns of the samples for green yellow, yellow, pale turquoise, and salmon color modules (Figure S4, S5). Gene ontology enrichment was performed on the same color modules; however, we could only detect molecular functions for the green yellow module (Figure S6).




**Supplemental Figure 3:** Correlations between modules (rows) and particular treatment groups (columns), in which treatment groups are labeled "Red" or "Brown", followed by their associated sampling point, labeled as follows: BH for before heat, DH for during heat and AH for after heat. Labels ending with a 2 correspond to samples collected during the second heat stress experiment. The two numbers in each cell are Pearson's correlation coefficients and p-values of the correlation test. Coefficients closer to 1 signify that the genes in the module trend towards overexpression for the corresponding treatment group, whereas coefficients closer to -1 signify that genes trend towards underexpression for the corresponding treatment group.




**Supplemental Figure 4:** Gene expression heatmaps of differentially expressed genes in the green yellow and yellow modules that displayed divergent expression between red and brown corals. Sample labeling is the same as in Figure S1. Color scales represents log2 fold change of genes.




**Supplemental Figure 5:** Gene expression heatmaps of differentially expressed genes in the pale turquoise and salmon modules that displayed divergent expression between red and brown corals. Sample labeling is the same as in Figure S1. Color scales represents log2 fold change of genes.


**Supplemental Figure 6:** GO categories significantly enriched with differentially expressed genes from the green yellow module. Large bold font is equivalent to a p-value less than 0.001, small italicized font is equivalent to a p-value less than 0.05. Dendrogram shoes hierarchical clustering of GO categories based on shared genes within the module.

## References

Langfelder, P., Horvath, S. (2008). WGCNA: An R package for weighted correlation network analysis. *BMC Bioinformatics* **9,** 559 https://doi.org/10.1186/1471-2105-9-559