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AN ABSTRACT OF THE THESIS OF James E. Fifer for the Master of Science in Biology presented August 16, 2018.

Title: Examining Gene Expression of Heat-Stressed Staghorn Coral Under Different Flow Environments

Approved: _____

Laurie J. Raymundo, Chairman, Thesis Committee

Increased water flow provides corals with a variety of health benefits including mitigation of heat stress. Here we take a transcriptomic perspective to the questions of water flow and coral resilience. We examined gene expression of *in situ Acropora* cf. *pulchra* colonies in discrete flow environments during a natural heat stress event and *ex situ A.* cf. *pulchra* nubbins exposed to an artificial severe heat stress. Differential gene expression and GO enrichment analyses reveal flow-driven transcriptomic signatures relating to increased metabolism and growth. We find previously described transcriptomic responses to heat stress, and this response changes based on flow regime. Corals exposed to high flow show "frontloading" of stress genes, likely due to increased oxidative metabolism generated by flow. This "frontloading" effect could explain coral's improved resilience against heat stress under high flow.

The members of the committee approve the thesis of James E. Fifer presented August 16, 2018.

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EXAMINING GENE EXPRESSION OF HEAT-STRESSED STAGHORN CORAL UNDER DIFFERENT FLOW ENVIRONMENTS

BY

JAMES E. FIFER

A thesis defense submitted in partial fulfillment of the requirements for the degree of

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CHAPTER 1

CORAL REEFS ON GUAM

Coral reefs offer resources integral to the success and well-being of millions of people (Cinner et al., 2012). Worldwide, they play major roles in fisheries, tourism, coastal protection, habitat provision for valuable species, and cultural values (Hicks, 2011). On Guam specifically, reefs play a vital role in the provision of income and food for fishers, as well as being a major contributor to the tourism industry which accounts for a third of the island's employment (Guam Visitors Bureau, 2016). The success of Guam's reefs is inseparable from the prosperity of Guam's economy and culture. Managing this resource through examining the nature of coral resilience will help to maintain that mantle.

Increasing concern for avoiding the worst-case scenarios of climate change has led to a call for management actions to restore and maintain the resilience of coral reefs (Obura & Grimsditch, 2009). To manage remaining populations, as well as potentially restore them, there are certain key questions at the local level need to be addressed first. Restoration practices will only be effective if we understand the current state of coral reefs. The success of these practices is dependent on understanding the causes of degradation and nature of resilience (Edwards et al., 2010; Johnson, Holbrook, Schmitt, & Brooks, 2011). Certain habitats can provide refugia against both local and global stressors (Fabricius, De'ath, Humphrey, Zagorskis, & Schaffelke, 2013; Golbuu et al., 2007; Maynard, Anthony, Marshall, & Masiri, 2008). Informed management to protect coral reefs against the threats of climate change thus requires and understanding of local environmental processes that drive resilience or recovery.

The coral genus *Acropora* is the most diverse among the Scleractinia (Veron, 2000; Wallace, 1999). Members of *Acropora* are fast-growing, dominant reef builders (Perry et al., 2012) and staghorn Acroporids disproportionally contribute to reef accretion, marking them as biological pillars for the ecosystem functions delivered by coral reefs (Wild, 2011). Their dominance adds to the structural complexity and rugosity of reefs, providing essential unique habitats for many different marine organisms, evidenced by the higher fish abundance and species richness associated with staghorn patches (Johnson et al., 2011). Staghorns are largely restricted to shallow waters, a habitat frequently exposed to highly variable conditions such as high irradiance, extreme low tides, high sea surface temperatures, sedimentation, eutrophication, pollution, and physical damage (Guilcher, 1988) and as a result, they are particularly susceptible to coral bleaching (Guest et al., 2012; McClanahan, Graham, & Darling, 2014). Staghorn populations have declined 80–90% throughout the Caribbean and western Atlantic since the late 1980s (Bruckner, 2002).

While Guam, and many other Pacific islands, have so far avoided this disturbing scale of decline, recent bleaching events have put the future of their staghorn populations into question. Guam experienced two island-wide bleaching events in the summers of 2013 and 2014 (triggered by high temperatures), as well as high mortality from extreme low tides starting in 2014 and extending through 2015. The total estimated mortality for staghorn populations was 50% after these three years with an estimated total 17.8 ha coral cover lost (Raymundo, Burdick, Lapacek, Miller, & Brown, 2017). However, there was a distinctive spatial pattern of mortality among coral thickets. During the three-year mortality event, large staghorn thickets lost much live growth over the majority of their surface area, with healthier surviving colonies limited to the edges of the thickets. Additionally, colonies closer to the wave-flushed reef crest showed

significantly less mortality (Raymundo et al., 2017). The spatial differences in coral mortality suggest that local processes may have mitigated against these various stressors. Specifically, differential fluid dynamics within and outside staghorn colonies may have influenced survival.

Understanding how hydrodynamics may influence coral survival of environmental stressors could be useful for determining habitats that act as refugia from the threats of climate change. Water flow can lead to a number of physiological changes in corals. Increased flow can explain differences in calcification rates (Dennison & Barnes, 1988; Edmunds & Burgess, 2018), respiration (Bruno & Edmunds, 1998), growth rates (Mass, Brickner, Hendy, & Genin, 2011), particle capture efficiency (Helmuth & Sebens, 1993), photosynthesis (Mass et al., 2011), susceptibility to bleaching (Bayraktarov, Pizarro, Eidens, Wilke, & Wild, 2013; Nakamura & Van Woesik, 2001), increased production of UV-absorbing compounds (Jokiel, Lesser, & Ondrusek, 1997), rates of nutrient uptake (Badgley, Lipschultz, & Sebens, 2006), and oxygen transport across concentration boundaries (Mass et al., 2010). Thus, certain high flow environments might provide mitigation against some of the environmental stressors associated with climate change.

INTRODUCTION TO HYDRODYNAMICS

Benthic Boundary Layers

The Boundary Layer Model (Denny, 1988) describes the creation of the Benthic Boundary Layers (BBL) (Fig. 1) and is vital for understanding how flow impacts benthic organisms in marine systems. The BBL is defined as the fluid between the substrate and the fluid layer where the velocity of the water is no longer significantly affected by the substrate (Denny, 1988). The establishment of the BBL is based on the "no-slip" condition, which states the fluid immediately in contact with the substrate does not move or "slip". This creates a velocity

gradient, where layers of water are slowed according to their distance to the floor (Denny, 1988). Supporting this, in an *ex situ* study of *Dichocoenia stokesii* and *Stephanocoenia michilinismall* in unidirectional flow, flow velocity at the top of the septa of a corallite was roughly half that of flow 4 cm higher in the water column (Gardella & Edmunds, 2001). Within the BBL lies the Momentum Boundary Layer (MBL) which corresponds to the flow around an organism's surface (Finelli, Helmuth, Pentcheff, & Wethey, 2006). Often less than 1.0 mm thick, and within the MBL, is the Diffusion Boundary Layer (DBL) where molecular diffusion governs transport between layers. The DBL is the region closest to the coral surface where molecular diffusion, rather than convective mass transfer, dominates transport (Finelli et al., 2006). It is formed from the concentration gradient that arises as a result of the production and consumption of solutes on the coral surface (Stocking, Rippe, & Reidenbach, 2016). In the case where diffusion of a metabolite through this layer is slower in comparison to the rate of absorption at the organism's surface, the diffusion controls the exchange of that metabolite with the water column (Bilger & Atkinson, 1992).

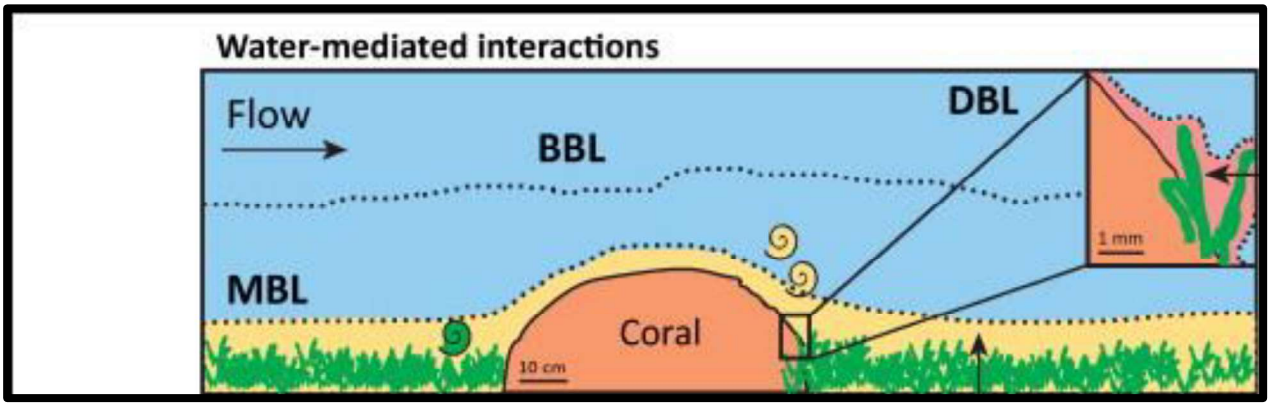

Figure 1 Depiction of the benthic boundary layers, reprinted from Barott and Rohwer (2012).

The size of these boundary layers (relative thickness or thinness) will vary based on the level of shear stress. Shear stress refers to the force exerted by the bottom drag (friction) on the water (Hearn, Atkinson, & Falter, 2001). In laminar conditions (i.e. fluid layers are smooth and do not mix with each other), the size of the boundary layer will grow as it moves across an object, due to increased frictional drag, until it finally separates itself and moves downstream in the form of a wake. As the flow velocity increases, the velocity gradient becomes steeper. However, on coral reefs these benthic boundary layers rarely exhibit laminar flow (Denny, 1988). Instead, the MBL is usually turbulent, (i.e. chaotic changes in pressure and velocity), and the thickness of this layer is characterized by the size, shape, and texture of the organism itself and neighboring solids (Patterson & Sebens, 1989). Turbulence in the MBL will influence vertical mixing, with increased turbulence in the water column decreasing the thickness of the MBL and subsequently the DBL (Hearn et al., 2001). The DBL is influenced by the flow in the MBL and is also controlled by surface roughness, flow speed, and the molecular diffusivities of relevant dissolved materials (e.g., O_2, CO_2, PO_4^{3-}, NH_4, HCO_3) (Stocking et al., 2016).

The thickness of these boundary layers is relevant because it controls the concentration and delivery of solutes between the water column and the surface of a coral. The thickness of the DBL determines the concentration gradient of dissolved materials. For example, this layer has been found to modulate bicarbonate diffusion into aquatic plants and determine the maximum rate of photosynthesis (Patterson & Sebens, 1989). The DBL ultimately controls the rate of mass flux for coral polyps (Jørgensen & Des Marais, 1990). Occasionally, the BBL will be severely depleted of planktonic food because of grazing by the reef community (Yahel, Yahel, & Genin, 2005). Replenishment of these food sources depends upon turbulent mixing near the reef (Genin, Yahel, Reidenbach, Monismith, & Koseff, 2002). When the DBL is thin, concentrations of

important solutes are easily replenished, and transfer into, and out of, the coral tissue is facilitated. On the other hand, thick DBLs might encumber physiological processes reliant on the solutes transferred through them, for example the resupply of essential nutrients or elimination of harmful waste products (Finelli et al., 2006).

The transfer rates of chemical material between fluids and surfaces (e.g. through the BBL) is referred to as "mass-transfer" (Bilger & Atkinson, 1992). Mass transfer occurs bidirectionally between the coral and the water column, for example corals acquire nitrogen, phosphates and bicarbonate from the water column and transfer back oxygen and metabolic by-products (Finelli et al., 2006). When uptake kinetics for a solute on the surface of a coral exceeds the rate of diffusion through the DBL the solute is referred to as "mass-transfer limited" (Hearn et al., 2001). If a solute is mass-transfer limited then the uptake of this solute is controlled by the flow environment (Finelli et al., 2006). Flow influences the concentration gradients within the BBL and increases the availability of the mass-transfer limited solutes (Brown & Carpenter, 2014). The rate and quantity of mass exchange between the coral and the water column is thus controlled by convective motions near the surfaces of the coral (Reidenbach, Koseff, & Monismith, 2007).

Hydrodynamics within the microenvironment of a coral colony

Examining spatial variability of flow inside a coral colony is challenging as outer branches prevent instruments from measuring within. The conventional methods of measuring flow, such as acoustic or optical velocimeters, are not suited for describing flow at a spatial scale such as within a colony. This means much of the *in situ* flow dynamics that are important to mass transfer are inaccessible. It is only through the use of magnetic resonance velocimetry (MRV) that researchers have been able to get a complete map of these areas (Chang, Elkins,

Alley, Eaton, & Monismith, 2009; Elkins, Markl, Pelc, & Eaton, 2003). Exploration of flow variations within a colony is important because of its potential to reveal connections with intra-colony variations in coral physiology. If flow is channeled almost exclusively along a few paths through a colony, then mass transfer will likely occur at higher levels in those areas compared to other locations around the colony (Chang et al., 2009). This will, in turn, influence physiological processes differentially within a colony.

There is evidence suggesting that a stagnant region can develop within a coral colony. *Porites porites* and *Porites furcata* develop stagnant regions within a coral colony, for both high flow morphologies (thicker branches with less spacing between) and low flow morphologies (thinner branches that are spaced apart), even at relatively high velocities (20cm/s) (Chamberlain & Graus, 1975). In high flow morphologies of *Agaricia tenuifolia*, the average core flow velocity will increase with increasing Reynolds number (Re), a dimensionless index of inertial to viscous forces, but stagnant regions still remain (Helmuth, Sebens, & Daniel, 1997). Densely packed branching tends to divert more flow to the exterior, but in *Madracis mirabilis,* for both low-flow and high-flow morphs, stagnant regions developed (Kaandorp et al., 2003). This demonstrates stagnant regions could be present for a variety of different branching morphologies, depending on the flow environment.

Even if stagnant regions are absent, flow through a colony can be variable and create regions of decreased velocity that are not present on the exterior. The coral geometry acts as a bluff body (a structure that separates flow over a substantial part of its surface) that perturbs the flow, resulting in recirculation zones and regions of localized acceleration and deceleration (Chamberlain & Graus, 1975). Compared to solitary branches, mass flux rates have been found to decrease with increasing colony size and the closer branches are to the middle of an

aggregation (Helmuth et al., 1997). Lesser et al. (1994), reported external and internal flow differences for both high flow and low flow morphologies under lab conditions, although the differences were more pronounced in the high flow morphologies. Chang et al. (2009) found that in *Stylophora pistillata*, the flow-facing surfaces blocked by upstream branches had flow comparable to downstream facing surfaces. Increasing velocity could lead to even greater flow variability through a colony. Wakes behind branches and colonies will get longer and more turbulent in higher flow (Hench & Rosman, 2013). With high Re, the distribution of flow velocities around and within the colony form shows a highly complex pattern, especially in the low-flow morphology (Kaandorp et al., 2003). This makes it impossible to accurately characterize the flow within a colony using just one measurement of velocity. When spacing between branches is high, the flow is drag-dominated and velocities inside the colony are much lower than in the free stream (flow on the exterior that is not perturbed by branches or other objects) (Lowe, Koseff, & Monismith, 2005; Lowe, Koseff, Monismith, & Falter, 2005). This agrees with MRV imaging taken throughout a colony, which revealed a complex interaction of wakes created from upstream branches leading to a blocking of flow on downstream branches (Elkins et al., 2003). X-ray and computerised tomography (CT) scans have shown that flow at the edge of the coral retains its high velocity and has little direction change, while flow at the center will slow down and disperse in different directions (Chang et al., 2009). Much of this variability can be attributed to blockage effects which lead to slow regions behind branches and fast regions caused by channeling of the flow between branches (Chang et al., 2009).

Intra colony flow variation could have consequences for the health of the colony. Flow variation could potentially be smoothed out via translocation of photosynthates, nutrients, *etc.* to areas receiving lower flows (Oren, Rinkevich, & Loya, 1997). However, in unidirectional flow

there can be asymmetry in the coral's photosynthetic symbiotic zooxanthellae distribution and skeleton mass between the parts of a colony facing flow and the downstream sides, which suggests a limit to the abilities of translocation across a colony (Mass & Genin, 2008). Flow variations inside the colony may induce specific preferential growth forms (Kaandorp et al., 2005; Kaandorp et al., 2003; Kaandorp & Kübler, 2001; Lesser et al., 1994), or may lead to branch orientation that optimizes nutrient uptake or prey capture (Helmuth & Sebens, 1993). Such spatially and temporally variable flow, in turn, leads to differential local nutrient concentrations and boundary layer thickness throughout a coral colony, which after a long enough time, may induce localized calcification (Chang, Iaccarino, Elkins, Eaton, & Monismith, 2004).

Factors that mitigate intra-colony flow variation

In certain situations, the variation between the interior and exterior of colonies may become negligible. Coral form adjusts *in situ* to changes in the coral's hydrodynamics condition (Mass & Genin, 2008) and thus colony morphologies are likely adapted to their local environment. Intra-colony flow differences can be mitigated by differences in morphologies, for example colonies with branches spaced out (i.e. low flow morphology) will allow for more flow penetration (Reidenbach et al., 2007). In addition, the flow that corals are exposed to in natural environments is much more dynamic than the flow produced in artificial tank experiments, yet most of our knowledge on water flow is derived from the latter. Intra-colony flow differences observed in tank experiments might be greater, weakened or absent *in situ*. For example, the stagnant region observed by Kaandorp et al. (2003) within a high flow morph of *Madracis mirabilis* was interpreted to be a feature exclusive to their particular tank flow regime and unlikely to be observed in the wild. While Mass and Genin (2008) observed flow-attributed

asymmetrical growth of *Pocillopora verrucosa* in their tank study, under natural conditions *P. verrucosa* experiences bidirectional tidal flows and instead grows symmetrical. The discrepancy between lab experiments and *in situ* observations can also be attributed to the presence of various other objects on natural substrate, each with their own roughness and drag, diverting and changing the flow. Hench and Rosman (2013) observed a flow phenomenon that could be positive for corals living downstream of other organisms. Wakes are produced by upstream organisms and corals that exists within those wakes could be subject to more vigorous velocity fluctuations and increased vertical mixing. A recirculation zone on the downstream side of flow could trap particles and draw them back to the coral, increasing feeding on the downstream side of colonies (Hench & Rosman, 2013). Additionally, Chang et al.'s (2009) X-ray and CT scan experiment demonstrated that the tips of the branches, regardless of their position within the colony, experienced the highest velocities, suggesting that these areas are the most important for mass transfer and could mitigate the effect of stagnant regions within the colony via translocation. Morphology and *in situ* flow conditions could, therefore, potentially mitigate within-colony flow variations observed in aquarium experiments.

Wave energy can also alter mass transfer in benthic organisms. Studies have reported benthic macrophytes showing increased rates of nitrogen fixation and ammonium uptake under oscillatory versus steady flow (Thomas & Cornelisen, 2003; Williams & Carpenter, 1988). Atkinson et al. (2001) measured 2-3-fold increases in nutrient transfer when a living reef system was exposed to wave-dominated conditions. Gypsum studies have offered supporting evidence of increased mass transfer under oscillatory flow. Porter et al. (2000) used plaster spheres to demonstrate that oscillatory flow of low to moderate speed had greater mass flux than a flow regime with less oscillatory motion but higher velocity. Several studies have reported that rates

of mass transfer to benthic communities under oscillatory flow are increased by factors ranging from 1.2 to 3, relative to rates measured under steady flow (Carpenter, Hackney, & Adey, 1991; Falter, Atkinson, & Coimbra, 2005; Reidenbach, Koseff, Monismith, Steinbuck, & Genin, 2006). In addition, Reidenbach et al. (2006) found more uniform mass transfer throughout a colony during oscillatory flow compared to unidirectional flow, demonstrating less flow was blocked by upstream branches. These studies suggest oscillating flow can increase mass transfer and reduce intra-colony flow variation.

Oscillating flow alters mass transfer within a colony by changing fluid dynamics around a coral's surface. Reidenbach et al. (2007), using the tightly branched *Porites compressa* as a model, show that wave action increases the flux of particles inside a colony through higher vertical flow, but decreases horizontal flow. A unique feature of oscillatory flow is the appearance of a thin oscillatory wave boundary layer, characterized by sharp increases in velocity due to the fluid acceleration around a coral's branches (Lowe, Koseff, Monismith, et al., 2005). This thin layer causes sharp velocity gradients along the surfaces of the coral which in turn generate periodic large magnitude shear stresses and prevents buildup of a stable BBL (Hearn et al., 2001). The density of *P. compressa*'s branching structure is too great to allow flow to penetrate within the coral colony in low flow environments. However, under oscillatory currents, the combined effects of increased bed shear and pressure-driven flow within the colony allow for these essential exchange processes to occur (Reidenbach et al., 2007). Oscillatory flow prevents the buildup of a stable BBL and increases shear stress, which results in higher mixing between the substrate and the water column.

THE EFFECTS OF FLOW ON CORAL REEF HEALTH AND ITS ROLE AS A HEAT STRESS MITIGATOR

Flow regime can have a variety of positive effects on coral health and can provide relief from thermal stress. Studies have shown that raising flow increases calcification (Smith, Barshis, & Birkeland, 2007), photosynthesis (Osinga, Derksen-Hooijberg, Wijgerde, & Verreth, 2017), respiration (Finelli et al., 2006), nutrient uptake (Atkinson et al., 2001), particle capture (Helmuth & Sebens, 1993), reproduction (Mass et al., 2011), and gas exchange (Mass et al., 2010) in corals, and shifts in coral-associated microbial community compositions (Lee, Davy, Tang, & Kench, 2017). It is thus relatively unsurprising that increased water flow can also mitigate coral bleaching and induce bleaching recovery. Nakamura and Van Woesik (2001) found increased survival of corals in high water flow during elevated sea surface temperatures and irradiance levels that induced bleaching in corals exposed to low flow. Even intermittent flow has been reported to reduce bleaching (Smith & Birkeland, 2007). Additionally, bleached *Stylophora pistillata* recovered chlorophyll and zooxanthellae in high flow regimes, likely via encroachment of zooxanthellae from neighboring tissue that had not been damaged (Nakamura, Yamasaki, & Van Woesik, 2003). In Tayrona National Natural Park, Colombia, sites exposed to faster currents exhibited less bleaching and higher bleaching recovery compared to sheltered sites (Bayraktarov et al., 2013). Water flow decreases bleaching in both small and large *Palythoa caribaeorum* colonies, and upstream sides of large colonies can have reduced bleaching compared to downstream sides (Fujimura & Riegl, 2017). It has even been suggested that wind-induced high flow in the northern Red Sea could explain the observed tolerance of coral reefs in this area to heat stress events (Osman et al., 2017). The recovery from, and mitigation of, bleaching by high flow is likely due to enhanced mass transfer of gases and metabolites (Bayraktarov et al., 2013). Examining the mechanisms in which flow positively influences coral health could provide answers for the role of high flow as a heat stress mitigator.

Water flow-mediated nutrient uptake

Nutrient uptake plays an important role in many processes that sustain coral health. The coral host obtains its nitrogen (N) and phosphorous (P) via heterotrophic feeding and translocated substances from its zooxanthellae. Dissolved metabolites such as ammonium, nitrates and phosphates are essential for sustaining growth, tissue repair, mucus production, reproduction, photosynthesis and respiration (Badgley et al., 2006; Lowe, Koseff, Monismith, et al., 2005). Zooxanthellae take these nutrients up in their inorganic form (Osinga et al., 2011) and deprivation of one or both of N and P will result in a larger symbiont cell with increased lipid bodies, starch granules and fragmentation of their accumulation bodies, all of which are characteristic of unhealthy cells (Rosset, D'Angelo, & Wiedenmann, 2015). Reducing nutrient uptake will subsequently lead to dysfunction in the coral-algal symbiosis and, in turn, bleaching, reduced growth, and possibly mortality of the coral.

When uptake of dissolved inorganic nitrogen (DIN) is limited, translocated substances become nitrogen-deprived. These low-nitrogen substances are referred to as "junk food" because of their limited ability to provide the host with metabolic energy and the nitrogen-based building blocks required for biosynthesis. The addition of DIN can promote growth and pigment production in zooxanthellae (Osinga et al., 2011), stimulating the overall net photosynthesis rates of the coral holobiont (Dubinsky et al., 1990; Stambler, Cox, & Vago, 1994). Rosset et al. (2017) found that under low nitrogen and high phosphate conditions, zooxanthellae contained classic biomarkers of nutrient limitation, such as elevated levels of lipid bodies and starch granules. However, cell proliferation rates were still high enough to sustain adequate zooxanthellae densities. Rosset et al. (2017) suggest that nitrogen limitation results in slowed growth, but if coupled with high phosphate concentrations, is sufficient to maintain photosynthesis.

Phosphate limitation, caused by either low phosphate concentrations or high phosphate demand mediated by relatively higher nitrogen concentrations, can result in decreased coral health and increased susceptibility to bleaching. Phosphorus is a key compound for many biological molecules, such as DNA, RNA and phospholipids, and has a role in coral growth and photosynthesis (D'Elia, 1977). The influence of phosphate limitation on corals was first revealed through the direct negative effects of eutrophication on zooxanthellae. These effects are caused by the starvation of one of the essential nutrients, in this case phosphate, because of their scarcity relative to the other essential nutrients (nitrogen) (Parkhill, Maillet, & Cullen, 2001). Higher nitrogen will lead to an increased demand by algal populations for phosphorous that is subsequently not met. This starvation for phosphorous, in turn, can lead to a decrease in zooxanthellae's tolerance for heat and light stress and a drop in photosynthetic efficiency (Wiedenmann et al., 2013). The necessity of a balance among essential nutrients is responsible for the markedly similar detrimental effects of low nitrogen/low phosphate and high nitrogen/low phosphate concentrations (Rosset et al., 2017).

Many coral communities are located in nutrient-limited environments and thus the rates of nutrient transfer between the coral and the water column can have important consequences for coral health, and can be mediated by flow. The demand for essential nutrients results in concentration boundary layers where transfer is controlled by the size of these layers (Lowe, Koseff, Monismith, et al., 2005). Nutrient uptake through these layers is governed by their concentration, water velocity, and friction caused by the reef (Baird & Atkinson, 1997). Indeed, phosphate (Atkinson & Bilger, 1992; Falter, Atkinson, & Merrifield, 2004), ammonium (Atkinson, Kotler, & Newton, 1994; Falter et al., 2004; Thomas, 1997) and nitrate (Badgley et al., 2006; Falter et al., 2004) uptake are positively correlated with water velocity. Water flow can

influence nutrient uptake, which subsequently provides the coral with substances essential for a functional zooxanthellae-coral relationship.

Water flow mediated increased heterotrophy

Heterotrophic feeding on particulate organic matter (POM) is important for coral nutrition (Johnson & Sebens, 1993; Sebens, Grace, Helmuth, Maney, & Miles, 1998; Sebens, Vandersall, Savina, & Graham, 1996). The importance of heterotrophic feeding in coral is demonstrated by the rarity of complete autotrophy, the fact that inorganic nutrients alone do not maximize coral growth, and the heterotrophic origin of components of the coral's organic matrix (Osinga et al., 2011). Feeding supplies the coral holobiont with nitrogen, carbon and phosphorous in a balanced ratio, unlike the uptake of DIN and DIP which can lead to the "junk food" photosynthates mentioned earlier. Increased feeding on organic nitrogen and phosphates can promote zooxanthellae growth, pigmentation and photosynthesis in *Stylophora pistillata* (Ferrier-Pagès, Witting, Tambutté, & Sebens, 2003; Houlbrèque, Tambutté, & Ferrier-Pagès, 2003). Heterotrophic feeding increases growth in corals (Houlbreque, 2004), demonstrating its importance in supplying important building blocks for biosynthesis. Some corals also compensate for decreased photosynthesis by increasing heterotrophy, although this response appears to be species-specific (Anthony & Fabricius, 2000). Heterotrophy may ameliorate thermal stress effects; fed corals show less skeletal growth reduction and less of a symbiont density decrease under higher temperatures, as compared to starved corals (Aichelman et al., 2016).

Water flow can increase heterotrophic feeding through higher prey encounter rates. Sebens et al. (1998), showed flow speed has a positive effect on capture rates of cysts, zooplankton and non-copepod plankton in *Madracis mirabilis* and zooplankton for *Montastrea*

cavernosa. Water movement affects the concentration of plankton near the substrate: strong currents will produce greater mixing, transfering plankton from the substratum into the surrounding water (Denny, 1988). Enhancing flow speeds also increases an organism's encounter rate with prey, and encounter rate is correlated with higher prey capture and ingestion (Sebens et al., 1998). Colonies oriented perpendicular to flow direction will show higher capture rates than those with a parallel orientation (Helmuth & Sebens, 1993; Leversee, 1976). Thus, the increased capture rates in high flow are likely due to higher rates of predator-prey encounters, and thus food availability. Supporting this theory, Piniak (2002) demonstrated that the total amount of prey captured for *Oculina arbuscula* increased under high flow, but capture efficiency decreased. Thus, the capacity of water flow to serve as a promoter for heterotrophy could be a mechanism through which flow promotes coral growth and weaken the effects of thermal stress.

Water flow and gas flux

The rate of gas exchange in corals dictates respiration; higher water flow can increase the rate of gas flux. Corals acquire inorganic nutrients (N, P, HCO_3) from the water column, and must transfer O_2 back to the water column whenever the production rate by the zooxanthellae exceeds the uptake rate of the host tissue. The degree of gas flux will determine coral host respiration (Dennison & Barnes, 1988; Patterson & Sebens, 1989; Patterson, Sebens, & Olson, 1991; Shick, Malcolm, & Shick, 1990) and flow speed can have a strong effect on this rate of gas flux in cnidarians (Patterson & Sebens, 1989). High flow results in thinning of the DBL which promotes gas exchange by increasing the concentration gradient. In turn, a thin DBL decreases the time needed to equilibrate changes in gas concentration that occur as a result of the environment or the organism (Finelli et al., 2006). Aggregates of corals with loosely spaced branches maintain a higher rate of respiration in low flow conditions than those with tightly

spaced branches (Bruno & Edmunds, 1998). While there is evidence for elevated light respiration under high flow (Mass et al., 2010) increased dark respiration appears to be a common occurrence under high flow (Osinga et al., 2017; Patterson et al., 1991; Sebens, Helmuth, Carrington, & Agius, 2003). During the day, even at low irradiance, oxygen production by zooxanthellae is much greater than oxygen used for zooxanthellae and coral respiration, and thus oxygen concentrations will often be at a maximum regardless of the flow speed (Edmunds & Davies, 1988; Shick et al., 1990). However, at night there is an almost complete depletion of oxygen near coral surfaces in low flow. High flow increases oxygen availability and consequently, increases dark respiration (Sebens et al., 2003).

Oxygen efflux can potentially increase photosynthesis in corals by enhancing CO_2 binding. Increasing water velocity was observed to instantaneously increase photosynthesis in *Galaxea fascicularis* (Miriam Schutter, Kranenbarg, Wijffels, Verreth, & Osinga, 2011). Mass et al. (2010) found the onset of flow leading to enhanced photosynthesis and reduced oxygen concentration in the tissues of three different corals. Water flow's influence on oxygen transport initiates more efficient use of irradiance and leads to higher rates of photosynthesis (Sebens et al., 2003). Maintaining a low concentration of oxygen inside the coral can enhance CO_2 binding by Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) (Finelli et al., 2006). In low flow environments, an accumulation of photosynthetically produced oxygen within the tissue reduces photosynthesis and channels carbon to the photorespiration pathway (Kremien, Shavit, Mass, & Genin, 2013). The switch from photosynthesis to photorespiration is metabolically very costly and potentially detrimental to coral health (Lesser, 1997). Photorespiration may be especially problematic for corals because zooxanthellae possess Rubisco Form II, which has a higher affinity for oxygen than for CO_2 (Rowan, Whitney, Fowler, & Yellowlees, 1996). While

high flow has a propensity to prevent a shift to photorespiration and has short-term benefits of flow-mediated oxygen efflux, it is the avoidance of photoinhibition and harmful ROS buildup that is likely to be the source of heat stress mitigation by water flow.

Reduction of chronic photoinhibition in corals could be the primary means by which increasing water flow mitigates the heat stress response in corals. Photoinhibition is the reduction of photosynthetic activity due to excess light and it has been suggested that water flow reduces photoinhibition in the zooxanthellae of *Acropora digitifera* (Nakamura, Van Woesik, & Yamasaki, 2005), *Porites lobata* and *Porites cylindrica* (Smith & Birkeland, 2007). Dynamic photoinhibition is the relatively harmless form of photoinhibition and refers to the reversible inactivation of reaction centers in Photosystem II (PS II) in response to excess light energy. Chronic photoinhibition, on the other hand, can be dangerous to the photosystem. Chronic photoinhibition occurs during the Mehler reaction; the electron transfer to O_2 in Photosystem I (PS I) generates superoxide radicals (O_2^-). O_2^- can be reduced by superoxide dismutase to form a less reactive, highly permeable ROS, H_2O_2 (R. Armoza-Zvuloni & Shaked, 2014). However, if H_2O_2 is not scavenged rapidly it may produce hydroxyl radicals (·OH) which are the most reactive and harmful ROS (Yamasaki, 2000). Energetically costly (Finelli et al., 2006) antioxidant pathways are subsequently overwhelmed and irreversible damage to PS II can occur (Jones and Hoegh-Guldberg, 2001; Lesser and Farrell, 2004). Maximum quantum yield of PS II can be used to indicate the presence of photoinhibition. Nakamura et al. (2005) and Smith and Birkeland (2007) both found rates of down-regulation in PS II activity increased in low flow during acute irradiance stress. In addition, Smith and Birkeland (2007) found that in low flow conditions, maximum quantum yield of PS II did not return to previous levels at night, indicating chronic damage. Flow-mediated oxygen efflux could provide thermal stress relief via the

removal of highly permeable H_2O_2 (Nakamura, 2010), which shows higher efflux under increased flow (Armoza-Zvuloni & Shaked, 2014), however the effect of flow on photoinhibition appears species-specific (Nakamura & Yamasaki, 2013).

It is still unclear whether the higher influx of carbon dioxide (Dennison & Barnes, 1988; Lesser et al., 1994) or a higher efflux of oxygen (Finelli et al., 2006; Mass et al., 2010; Nakamura et al., 2005; Schutter et al., 2011) is responsible for the observed increase of photosynthesis under high flow. Dissolved inorganic carbon (DIC) is an essential metabolite for both calcification and photosynthesis (Dennison & Barnes, 1988) and CO_2, specifically, accounts for 60% of the carbon needed by zooxanthellae during high rates of photosynthesis (Muscatine, Falkowski, Dubinsky, Cook, & Mccloskey, 1989). Mass et al. (2010), while submitting to the theory that flow-dependent influx of DIC over the long-term can positively influence coral growth, demonstrated that the immediate influence on photosynthesis in high flow is due to the efflux of O_2 from the coral, rather than the influx of DIC. However, Osinga et al. (2017) challenged the assertions made by Mass et al. (2010) when they found that photosynthesis was not affected by elevated oxygen levels in *Galaxea fascicularis*. Osinga et al. (2017) theorized that the effect of a higher efflux of oxygen on coral photosynthesis is species-specific and could depend on the efficiency of the carbon concentrating mechanism (CCM). Efficient CCMs result in decreased susceptibility to photorespiration for zooxanthellae and it seems that *G. facicularis* has a highly efficient CCM. Additionally, in *G. fascicularis,* elevating CO_2 levels stimulated net photosynthesis, and this effect only occurred under high flow. Thus DIC was mass-transfer limited for photosynthesis, which contrasts studies that did not show this limitation (Marubini, Ferrier-Pagès, Furla, & Allemand, 2008; Reynaud et al., 2003; Schneider & Erez, 2006; Tansik, Fitt, & Hopkinson, 2015). However these studies involved different species and did not use as

high irradiance or flow, suggesting they might not have reached thresholds required for mass transfer of CO_2 (Osinga et al., 2017). The theory that flow exerts its positive effects on photosynthesis via an efflux of oxygen is likely a species-specific phenomenon and does not fully address the mechanism of flow benefits. However, regardless of the mechanism, it is clear that mass transfer processes can enhance the coral-zooxanthellae relationship and flow could mediate effects of stressors, such as increased temperatures, that disrupt the coral-zooxanthellae symbiosis.

In summary, water flow impacts corals by thinning the DBL, which facilitates mass transfer of essential chemicals and can ultimately provide resilience against heat stress. Heat stress mitigation under high flow is likely due to the ability of water flow to increase heterotrophy, nutrient uptake and gas flux. Intra-colony variation in flow could thus potentially result in spatially distinct rates of mass transfer (Chang et al., 2009), which in turn could explain observed patterns on differential response to bleaching within colonies (Rowan, Knowlton, Baker, & Jara, 1997). Examining spatial variability of the health states in different positions within a colony could help shed light on the importance of flow in this microenvironment.

TAKING A TRANSCRIPTOMIC PERSPECTIVE ON FLOW-MEDIATED HEAT STRESS MITIGATION IN CORALS

Examining the transcriptomes of corals in different flow and temperature conditions can elucidate genes involved in flow-associated health benefits for corals. Analyzing the expression of thousands of genes from the transcriptome (i.e., the sum total of all transcribed messenger RNAs) using RNA-seq allows for studying associations of phenotypic traits to gene expression changes (De Wit, Pespeni, & Palumbi, 2015). In an ecological context, RNA-seq has enabled

examination of gene expression differences underlying inter-individual or inter-population variation in important traits, including thermal tolerance (Bellantuono, Granados-Cifuentes, Miller, Hoegh-Guldberg, & Rodriguez-Lanetty, 2012), CO_2 response (Moya et al., 2012), the process of bleaching (Pinzón et al., 2015) and disease stress (Anderson, Walz, Weil, Tonellato, & Smith, 2016). Using gene expression to determine a coral's response to heat stress, as opposed to more traditional biomarkers, provides the advantage of identifying less obvious responses (Goff & Dubinsky, 2016) which becomes particularly important when looking at small changes in the environment (Pavey, Bernatchez, Aubin-Horth, & Landry, 2012), such as flow differences around or within a coral colony. Because there have been no studies on the effects of water flow on coral gene expression, research on thermal stress-prompted expression is used here as a proxy to identify genes that might show differential gene expression (DGE) under different flow regimes and elevated temperatures. In this study, the groups of genes identified by Louis et al. (2017) as biomarkers for heat stress were used for this purpose. While all available coral genes were examined, the genes explored in the next section are candidate genes that have been previously studied.

Heat shock proteins

Genes encoding heat shock proteins (HSPs) are the most common candidate genes for heat stress biomarkers (Louis et al., 2017) (Table 1). During heat stress, protein misfolding can occur. HSPs serve as chaperones to regulate protein structure and functions, and promote cellular repair processes (Li & Srivastava, 2004). Their rapid initiation is the main source of protein stability in the face of heat stress (Oakley et al., 2017) and their up-regulation occurs when the transcription factor HSF1 is activated or suppressed in the face of a stressor (Louis et al., 2017). While most genes have a plateaued response to heat stress, genes encoding for HSPs can show a

linear up-regulation with increased stress, making them the gene family that often shows the greatest fold change in expression after a heat stress event (Kenkel et al., 2014).

The Hsp90 gene shows potential as a biomarker for an early response to heat stress. This gene has shown up-regulation in the span of just 3 hr in both *Acropora millepora* (Rodriguez-Lanetty, Harii, & Hoegh-Guldberg, 2009) and *Porites astreoides* (Kenkel et al., 2011) and during natural tidal heat pulses in *Acropora hyacinthus* (Ruiz-jones & Palumbi, 2017). Other studies have shown a response of Hsp90 in *Acropora tenuis* (Yuyama et al., 2012) and the sea anemone *Aiptasia* sp. (Oakley et al., 2017) after 27 hr and 24 hr heat stress events, respectively. Hsp90 can exhibit a linear increase as temperature is raised in *Acropora aspera* (Leggat, Yellowlees, & Medina, 2011), and maintaining up-regulation for days after temperature returns to below-bleaching levels, as demonstrated in an 11-day experiment with *Orbicella faveolata* (Desalvo et al., 2008). HSP90 also exhibited a 1.5- fold up-regulation between 29 °C and 31 °C and a 2.4-fold up-regulation between 31 °C and 33 °C (Kenkel et al., 2011). Interestingly, Rosic et al. (2014) found differential gene expression (DGE) in Hsp90 after 1 day but not 3 for *A. aspera*, demonstrating an acclimation response for this gene during longer durations of heat stress. Additionally, while *P. astreoides* showed an up-regulation after 4 hr (Kenkel et al., 2011), down-regulation of Hsp90 occurred when heat stress was extended to 6 weeks (Kenkel, Meyer, & Matz, 2013). After 6 weeks, colonies that paled exhibited a 2.4-fold decrease in Hsp90 expression, while colonies that bleached showed a 1.6-fold down-regulation. This dampening in down-regulation for bleached corals could have been caused by extreme cellular damage limiting the response (Louis et al., 2017).

Hsp16 is a small HSP and, like other small HSPs, it assists other chaperones in the maintenance/refolding of protein structures (Veinger, Diamant, Buchner, & Goloubinoff, 1998). Hsp16 is involved in cytoprotection (Kamradt et al., 2005), preventing protein aggregation and highly sensitive to temperature-induced structural changes (Datta, 1999). Hsp16 is one of the most responsive genes to heat stress reported to date in corals (Louis et al., 2017). It was identified as one of four genes that were particularly well-suited for detecting heat stress in *Acropora cervicornis*, out of a suite of potential biomarkers (Parkinson et al., 2017) and one of the top stress response genes in bleached *Acropora hyacinthus* (Thomas & Palumbi, 2017). Up-regulation of Hsp16 was observed in two different acute stress experiments that heat-stressed *Porites astreoides* (Kenkel et al., 2011), while a third follow-up study showed 10 times less up-regulation of Hsp16 after a more mild thermal stress (Kenkel et al., 2014). *A. hyacinthus* shows reduced up-regulation of Hsp16 after 72 hr of heat stress in previously acclimated corals compared to those that are thermally sensitive (Barshis et al., 2013). Hsp16 also shows rapid down-regulation after the heat stress event is over (Kenkel et al., 2011), making it a good candidate as a biomarker for early heat stress.

Hsp60 is a chaperonin found in mitochondria, cytosol, vesicles, extracellular space, and cell membranes (Cappello, Conway De Macario, Marasà, Zummo, & Macario, 2008). This protein mediates cell stress signaling, activates the innate immune response, and induces nitric oxide production (Colaco, Bailey, Walker, & Keeble, 2013). Up-regulation of this Hsp has been detected after 24 hr heat stress in *Orbicella faveolata* (Desalvo et al., 2008) and *Aiptasia* sp. (Oakley et al., 2017). Up-regulation was observed in *Porites astreoides* with a 1.3-fold increase from 29°C to 31°C and a subsequent 2.2 fold increase from 31°C to 33°C (Kenkel et al., 2011), suggesting that, like other HSPs, Hsp60's response is progressive (Kenkel et al., 2014). Hsp60

showed comparable up-regulation in three different species, *Seriatopora hystrix*, *Montipora monasteriata* and *Acropora echinata*, that were heat shocked at 34 °C. For all three, an initial increase in Hsp60 protein expression was noted but sustained stress brought about a down-regulation of the protein expression as a result of severe breakdown of cellular functions (Seveso et al., 2014). Another Hsp60 family member, T-complex polypeptide (Tcp-1), exhibited a delayed response to heat stress after other HSPs responded in *Acropora millepora*; no up-regulation of this gene was detected in the first 10 hr of heat stress (Rodriguez-Lanetty et al., 2009). This is in contrast to the Hsp60 gene examined in the Kenkel et al. (2014) study, which showed up-regulation after just 3 hr of heat stress. Unlike other HSPs, Tcp-1 is a potential biomarker for long-term effects of heat stress (Desalvo et al., 2008), but not as an immediate responder.

Hsp70s are an important factor in protein folding and repair of stress-induced protein damage (Tavaria, Gabriele, Kola, & Anderson, 1996) and is well-documented during all types of coral stress (Louis et al., 2017). Hsp70s up-regulation moderates ROS damage, increases cell survival, and links the oxidative states of the mitochondria and endoplasmic reticulum (ER) (Oakley et al., 2017). Given their key function as molecular chaperones regulating and preserving protein structure and functionality, Hsp70s are amongst those molecular mediators which show up-regulation in response to heat stress (Barshis et al., 2013; Bellantuono et al., 2012; Carpenter, Patterson, & Bromage, 2010; Crowder, Meyer, Fan, & Weis, 2017; Desalvo et al., 2008; Leggat et al., 2011; Levy et al., 2016; Maor-Landaw et al., 2014; Maor-Landaw & Levy, 2016; Mayfield et al., 2011; Oakley et al., 2017; Poli, Fabbri, Goffredo, Airi, & Franzellitti, 2017; Portune, Voolstra, Medina, & Szmant, 2010; Putnam, Mayfield, Fan, Chen, & Gates, 2013; Rodriguez-Lanetty et al., 2009; Ruiz-jones & Palumbi, 2017; Sharp, Brown, &

Miller, 1997; Voolstra et al., 2009). Since Hsp70s are regulated on a diel cycle (Levy et al., 2011), expression patterns may be affected by internal processes related to the regulation of the metabolic machinery (Oakley et al., 2017). Hsp70s have also been directly linked to water flow, with differential expression between upstream and downstream sections of heat stressed *Montastrea annularis* (Carpenter et al., 2010).

Hsp40, also known as DNAJ, plays an important role in the unfolded protein response (UPR) during ER stress and serves as a co-chaperone to Hsp70 (Cyr, Langer, & Douglas, 1994). Genes involved in the UPR, including DNAJ, have been shown to be up-regulated in response to brief thermal stress as well as in corals that are bleaching (i.e. longer term thermal stress) (Ruiz-jones & Palumbi, 2017; van de Water et al., 2017). The UPR is a set of signaling pathways that are activated after a buildup of unfolded proteins in the ER. When the stress is non-lethal, the UPR appears to serve as an adaptive response, restoring homeostasis, and when the stress is more severe, the UPR has a terminal response that leads to apoptosis (Oakley et al., 2017). Thus, genes related to the UPR, like Hsp40, are potential biomarkers for both immediate and chronic heat stress.

Hsp40 is reported to be differentially expressed (DE) in heat-stressed Acroporids (Desalvo, Sunagawa, Voolstra, & Medina, 2010; Yuyama et al., 2012) and *Stylophora pistillata* (Maor-Landaw et al., 2014). In a heat stress experiment, out of seven previously identified heat stress biomarkers, Hsp40 was the only common temperature-related expression feature in three different coral species, and the two branching species in the experiment showed expression before bleaching occurred (Maor-Landaw & Levy, 2016).

Table 1 Heat shock proteins differentially expressed in cnidarian heat stress experiments.

Specific gene	Host organism	Max Temperature	Duration of exposure	Up/ Down	Reference
Hsp90	*A. millepora* (larvae)	31	3hr	Up	(Rodriguez-Lanetty *et al*., 2009)
	A. millepora (larvae)	31	10hr	Down/ None	(Rodriguez-Lanetty *et al*., 2009)
	P. astreoides	30.9	3hr	Up	(Kenkel *et al*., 2011)
	M. faveolata	31.5 ± 1.1	10d	Up	(DeSalvo *et al.,* 2010)
	A.millepora (larvae)	31.4	4hr	Up	(Meyer *et al*., 2011)
	A. tenuis	32	27hr	Up	(Yuyama *et al*., 2012)
	Aiptasia sp.	33, 30-33	24h, 12d	Up	(Oakley *et al*., 2017)
	A. aspera	32	5,7,8d	Up	(Leggat *et al*., 2011)
	O. faveolata	32	10d	Up	(Desalvo *et al*., 2008)
	P. astreoides	31	3h	Up	(Kenkel *et al*., 2014)
	P. astreoides	33	3h	Up	(Kenkel *et al*., 2014)
	P. astreoides	31	6 wks	Down	(Kenkel *et al.,* 2013)
	A. aspera	36 ± 1	1d	Up	(Rosic *et al*., 2014)
	A. aspera	36 ± 1	3d	None	(Rosic *et al*., 2014)
	O. faveolata	31.5	12h	None	(Voolstra *et al*., 2009)
	A. hyacinthus	30.5	(In-situ)	Up	(Ruiz-Jones & Palumbi, 2017)
	M. aequituberculata	29.5, 32	22d	Up	(van de Water *et al.,* 2017)
Hsp16	*A. cervicornis*	35	1hr	Up	(Parkinson *et al*., 2017)
	P. astreoides	35-36	48hr	Up	(Kenkel *et al., 2011)*
	P. astreoides	31,33	3-4hr	Up	(Kenkel *et al., 2014*)
	A. hyacinthus	N/A	2, 10, 12 months after bleaching	Up	(Thomas & Palumbi, 2017)
	A. hyacinthus	37.6	72hr	Up	(Barshis *et al.,* 2013)
	P. astreoides	30.8	48hr	None	(Olsen *et al.,* 2013)
Hsp60	*Aiptasia* sp.	33, 30-33	1d, 12d	Up	(Oakley *et al.,* 2017)
	P. astreoides	30.9	3h	Up	(Kenkel *et al.,* 2011)
	P. astreoides	31, 33	3h	Up	(Kenkel *et al.,* 2014)
	A. echinata	34	6h, 12h, 18h, 24h	Up	(Seveso *et al.,* 2014)
	A. echinata	34	30h	Down	(Seveso *et al.,* 2014)
	S. hystrix	34	6h, 12h	Up	(Seveso *et al.,* 2014)
	S. hystrix	34	18h	Down	(Seveso *et al.,* 2014)
	M. monasteriata	34	6h, 12h, 18h	Up	(Seveso *et al.,* 2014)
	M. monasteriata	34	24h	Down	(Seveso *et al.,* 2014)
	P. astreoides	31	6 weeks	Up	(Kenkel *et al*., 2013)
	S. pistillata	500µmoles photons m^{-2} s^{-1}	15d	Up	(Levy *et al.,* 2016)
	S. caliendrum	33	6h, 12h	Up	(Seveso *et al.,* 2016)
	S. caliendrum	33	24hr	Down	(Seveso *et al.,* 2016)
Tcp-1	*A. millepora*	32	10hr	None	(Rodriguez- Lanetty *et al*., 2009)
	O. faveolata	32.72	1d, 2-4d,9d	Up	(Desalvo *et al.,* 2008)

Specific gene	Host organism	Max Temperature	Duration of exposure	Up/ Down	Reference
Hsp70	*O. faveolata*	32.72	24hr	None	(Desalvo *et al.,* 2008
	A. millepora	32	9d	Up	(Császár *et al.*, 2009)
	O. faveolata	31.5	12hr, 48hr	None	(Voolstra *et al.,* 2009
	A. millepora	31	3hr	Up	(Rodriguez-Lannety *et al.,* 2009)
	M. annularis	^2	1-6d	Up	(Carpenter *et al.*, 2010)
	M. annularis	^2	7d	Down	(Carpenter *et al.*, 2010)
	A. palmata	30	24.5	Up	(Portune *et al.,* 2010)
	A. aspera	32	7d, 8d	Up	(Leggat *et al., 2011)*
	S. hystrix	30	48hr	None	(Mayfield *et al.,* 2011)
	A. hyacinthus	27–37.6	72hr	Up	(Barshis *et al.,* 2013)
	A. millepora	31	2d, 4d, 8d	None	(Bellantuono *et al.,* 2012)
	P. damicornis	29	9d	Up	(Putnam *et al.,* 2013)
	S. pistillata	32, 34	13d	Up	(Maor-Landaw *et al.,* 2014)
	S. pistillata	32, 34	10d, 13d	Up	(Maor-Landaw & Levy, 2016)
	A. eurystoma	34	13d	None	(Maor-Landaw & Levy, 2016)
	A. eurystoma	32	10d	Up	(Maor-Landaw & Levy, 2016)
	Porites sp.	32	10d	None	(Maor-Landaw & Levy, 2016)
	Porites sp.	34	13d	Up	(Maor-Landaw & Levy, 2016)
	S. pistillata	500µmoles photons m^{-2} s^{-1}	15d	Up	(Levy *et al.,* 2016)
	A. hyacinthus	30.5	In-situ	Up	(Ruiz-Jones & Palumbi, 2017)
	P. verrucosa	31	7d	Up	(Poli *et al.,* 2017)
	Aiptasia sp	33.5	24hr	Up	(Oakley *et al.,* 2017)
	P. damicornis	28	7d, 14d, 21d	Up	(Crowder *et al.,* 2017)
	M. aequituberculata	29.5, 32	22d	Up	(van de Water *et al.,* 2017)
Hsp40/ DNAJ	*A. tenuis*	32	27hr	Up	(Yuyuma *et al.,* 2012)
	S. pistillata	32, 34	10d, 13d	Up	(Maor-Landaw *et al.,* 2014)
	S. pistillata	32	10d	Up	(Maor-Landaw & Levy, 2016)
	A. eurystoma	32	10d	Up	(Maor-Landaw & Levy, 2016)
	Porites sp.	32, 34	10d, 13d	Up	(Maor-Landaw & Levy, 2016)
	A. hyacinthus	30.5	In-situ	Up	(Ruiz-Jones & Palumbi, 2017)
	S. pistillata	500µmoles photons m^{-2} s^{-1}	15d	Up	(Levy *et al.,* 2016)
	M. aequituberculata	29.5, 32	22d	Up	(van de Water *et al.,* 2017)

Calcium

Calcium ions play a role in calcification, the zooxanthellae-coral host symbiosis (Rosic, Ling, et al., 2014), and as an important secondary messenger ion, inducing respiratory, TCA cycle activity, and apoptosis (Rainbolt, Saunders, & Wiseman, 2014). Calcium signaling pathways (Rosic, Kaniewska, et al., 2014), genes related to calcium ion homeostasis (Barshis et al., 2013; Crowder et al., 2017; Maor-Landaw et al., 2014; Portune et al., 2010; Vidal-Dupiol et al., 2014), and calcium transporter genes (Kenkel et al., 2013; Meyer, Aglyamova, & Matz, 2011) are differentially expressed during heat stress in corals. Ruiz-Jones and Palumbi (Ruiz-jones & Palumbi, 2017) identified several genes involved in calcium ion binding and calcium homeostasis with temperatures above 30.5°C. Louis et al. (2017), noted that while two studies have observed down-regulation in calmodulin (CaM), a calcium binding protein, in response to 24 hr (Desalvo et al., 2008) and 10 days of mild heat stress (Desalvo et al., 2010), aposymbiotic larvae of *Acropora millepora* showed stable expression of CaM in response to thermal stress, suggesting a role for calmodulin in the host-zooxanthellae relationship (Rodriguez-Lanetty et al., 2009). Calumenin, another calcium binding protein, also shows differential expression in response to thermal stress. Calumenin is localized to the ER and plays a chaperone-like role in alleviating ER stress and subsequent apoptosis (Dunn, Pernice, Green, Hoegh-Guldberg, & Dove, 2012). Up-regulation of calumenin in the symbiotic sea anemone *Anemonia viridis* (Ganot et al., 2011), suggests a role in the zooxanthellae-coral host symbiosis. Calumenin is down regulated in bleached *Acropora millepora* after heat stress except for samples previously acclimated to heat stress which, instead, showed up-regulation and did not bleach (Bellantuono et al., 2012). This finding was later confirmed in the field (Ruiz-jones & Palumbi, 2017).

Calumenin was also found to be greatly up-regulated in heat-shocked *Aiptasia* sp., further demonstrating calcium's role in the control of cnidarian thermal stress (Oakley et al., 2017).

Table 2 Calcium genes differentially expressed in cnidarian heat stress experiments.

Specific gene	Host organism	Max Temperature	Duration of exposure	Up/ down	Reference
Calcium signaling pathway	*A. aspera*	36 ± 1	1d	Up	(Rosic *et al.,* 2014)
Calcium ion binding gene	*A. hyacinthus*	2, 6 months after bleaching	In-situ	Up	(Thomas & Palumbi, 2017)
Cacna1s	*A. millepora* (larvae)	31.4	4hr	Up	(Meyer *et al.,* 2011)
Cacna1s	*P. astreoides*	30.9	6 wk	Down	(Kenkel *et al.,* 2013)
Calcium ion binding and calcium homeostasis	*A. hyacinthus*	30.5	In-situ		(Ruiz-jones & Palumbi, 2017)
Calcium-transporting ATPase	*S. pistillata*	34	13d	Up	(Maor-Landaw *et al.,* 2014)
Calcium-transporting ATPase	*S. pistillata*	32	10d	None	(Maor-Landaw *et al.,* 2014)
Calcium homeostasis	*P. damicornis*	28	7d, 14d, 21d	Up	(Crowder *et al.,* 2017)
Calcium homeostasis	*P. damicornis*	32.5	12d	DGE	(Vidal-Dupiol *et al.,* 2014)
Calcium homeostasis	*A. hyacinthus*	27–37.6	72hr	DGE	(Barshis *et al.,* 2013)
Calcium homeostasis	*A. palmata*	30, 31.5	12-131h	DGE	(Portune *et al.,* 2010)
Calmodulin	*O. faveolata*	32.72	24hr	Down	(DeSalvo *et al.,*2008)
Calmodulin	*M. faveolata*	31.5 ± 1.1	10d	Down	(DeSalvo *et al.,* 2010)
Calmodulin	*A. millepora*	32	3hr	Up	(Rodriguez- Lanetty *et al.,* 2009)
Calmodulin	*P. damicornis*	32	15d	Up	(Vidal Dupiol *et al.,* 2009)
Calmodulin	*O. faveolata*	Bleached	In-situ	DGE	(Pinzon *et al.,* 2015)
Calumenin	*A. millepora*	31	2d, 4d, 8d	Up	(Bellantuono *et al.,* 2012)
Calumenin	*Aiptasia* sp.	33.5	24hr	Up	(Oakley *et al.,* 2017)
Calumenin	*Aiptasia* sp.	33.5	24hr	Up	(Oakley *et al.,* 2017)

Oxidative stress genes

Genes related to oxidative stress, an imbalance between the production of ROS and antioxidant defenses (Betteridge, 2000), show differential regulation when exposed to changing temperature. Corals have an adaptive response to an alteration of ROS levels that involves an

increased or decreased expression of antioxidant genes (Dalton, Shertzer, & Puga, 1999). Antioxidants play an important role in improving photosynthetic activity and mitigating bleaching by scavenging ROS (Lesser, 1997). In response to ROS production, genes encoding for superoxide dismutase and the iron-binding protein ferritin are up-regulated to allow for the binding and neutralization of ROS (Császár, Seneca, & van Oppen, 2009). Enzymes such as superoxide dismutase (SoD), peroxidasin, and thioredoxin convert ROS to hydrogen peroxide (H_2O_2) and water. Catalase activity then regulates the increasing amount of H_2O_2 in host cells (Merle, Sabourault, Richier, Allemand, & Furla, 2007) and glutathione-S-transferase (GST) detoxifies some of the secondary ROS that are produced when they reacts with cells (Veal, Mark Toone, Jones, & Morgan, 2002). Additionally, oxidative stress stimulates a signaling pathway that integrates with other stress signals such as HSPs (Oakley et al., 2017). Up-regulation of these genes is expected following heat stress, as a direct consequence of increased oxidative stress. However some genes have such a broad response to a variety of stressors that they might not be the best biomarkers for heat stress (Louis et al., 2017).

Regardless of the non-specificity of oxidative genes to thermal stress, their usefulness as general stress detectors could help parse apart the effects of flow as a stress mitigator. GST shows both up-regulation (Desalvo et al., 2008; Oakley et al., 2017; Rosic, Kaniewska, et al., 2014) and down-regulation (DeSalvo et al., 2010; Desalvo et al., 2008; Polato et al., 2010) in heat stressed corals. *Acropora millepora* has shown up-regulation of a catalase homolog in bleached samples (Seneca et al., 2010) and after 10 hr heat stress (Portune et al., 2010; Vidal-Dupiol et al., 2014), as has *Orbicella faveolata* subjected to a 48 hr heat stress event (Polato et al., 2010; Voolstra et al., 2009) and partially bleached samples (Desalvo et al., 2008). Catalase genes were up-regulated after 1 day of heat stress, but not 3 days in *Acropora aspera* (Rosic,

Kaniewska, et al., 2014), suggesting a reduced role of catalase in chronic heat stress. Ferritin genes show up-regulation in *A. millepora* after both an 8 (Bellantuono et al., 2012) and a 9 day increase in temperature (Császár et al., 2009) and naturally bleached samples (Seneca et al., 2010). In addition, up-regulation was observed after a 48 hr temperature increase in *O. faveolata* (Polato et al., 2010; Voolstra et al., 2009) and a 24 hr heat stress in *Acropora elegantissima* (Richier, Rodriguez-Lanetty, Schnitzler, & Weis, 2008). However, studies have also shown down-regulation of ferritin after heat stress (Desalvo et al., 2008; Portune et al., 2010). Thioredoxin has been reported to be up-regulated in corals following thermal stress (DeSalvo et al., 2010; Desalvo et al., 2008; Maor-Landaw et al., 2014; Maor-Landaw & Levy, 2016; Rosic, Kaniewska, et al., 2014) and high irradiance (Levy et al., 2016; Starcevic et al., 2010). However, thioredoxin seems to be a part of a more general environmental stress response, with expression shown at a variety of different reefs that had other environmental stressors (Morgan, Edge, & Snell, 2005), and is induced by different oxidative stress conditions (Das & White, 2002; Das & Das, 2000). Peroxiredoxin elevation has been documented after heat stress in *Stylophora pistillata* (Maor-Landaw et al., 2014) *A*cropora *eurystoma* and *Porites* sp. (Maor-Landaw & Levy, 2016). Out of several up-regulated oxidative stress genes, a peroxidasin-like protein showed the highest response to a 48 hr heat stress in *O. faveolata* embryos (Voolstra et al., 2009). Peroxidasin seems to show upregulation consistently after heat stress (Desalvo et al., 2008; Polato et al., 2010; Rosic, et al., 2014; Souter et al., 2011; Vidal-Dupiol et al., 2014; Voolstra et al., 2009). Souter et al. (2011) observed significant and consistent up-regulation of MnSoD and SoD by 1-fold in adult *A. millepora* after 9 days of exposure to 32 °C. Several forms of SoD were enriched after 1 day, but not 3, in *A. aspera* (Rosic, Kaniewska, et al., 2014). Barshis et al. (2013) also noticed reduced expression of MnSoD in thermo-tolerant corals

compared to thermo-sensitive genotypes of *Acropora hyacinthus*. Oxidative stress genes in corals are expected to show differential expression in response to heat stress, likely as a consequence of ROS buildup.

Table 3 Genes involved in oxidative stress response differentially expressed in cnidarian heat stress experiments.

Specific gene	Host organism	Max Temperature °C/ stressor	Duration of exposure	Up/down	Reference
Catalase	*A. millepora*	Naturally bleached	In situ	Up	(Seneca *et al.,* 2010)
Catalase	*M. faveolata*	29, 31.5	48h	Up	(Voolstra *et al.,* 2009)
Catalase	*A. aspera*	36 ± 1	1d	Up	(Rosic *et al.,* 2014)
Catalase	*A. aspera*	36 ± 1	3d	Down	(Rosic *et al.,* 2014)
Catalase	*M. faveolata*	32.72	Partial bleaching	Up	(Desalvo *et al.,* 2008)
Catalase	*P. damicornis*	32.5+	12d	Up	(Vidal-Dupiol *et al.,* 2014)
Catalase	*A. millepora*	31	10h	Up*	(Rodriguez-Lannety *et al.,* 2009)
Catalase	*M. faveolata*	31.5 ± 1 Bleached	20d	Down	(DeSalvo *et al.,* 2010)
Catalase	*A. millepora* (larvae)	31	10h	None	(Portune *et al.,* 2010)
Catalase	*A. millepora*	32	9d	None	(Souter *et al.,* 2011)
Catalase	*M. faveolata*	30-31.5	24h, 48h	Up	(Polato *et al.,* 2010)
Ferritin	*A. millepora*	32	6d	Up	(Császár et al., 2009)
Ferritin	*M. faveolata*	29, 31.5	48h	Up	(Voolstra *et al*., 2009)
Ferritin	*A. millepora*	Naturally bleached	In situ	Up	(Seneca *et al.,* 2010)
Ferritin	*A. millepora*	31	10h	Up*	(Rodriguez-Lannety *et al.,* 2009)
Ferritin	*A. millepora*	31	8d	Up	(Bellantuono *et al.,* 2012)
Ferritin	*M. faveolata*	31.23	Partial bleaching	Down	(DeSalvo *et al.,* 2008)
Ferritin	*A. palmata* (larvae)	30, 31.5	24.5h	Down	(Portune *et al.,* 2010)
Ferritin	*A. millepora*	32	9d	None	(Souter *et al.,* 2011)
Soma ferritin	*M. faveolata*	30-31.5	24h, 48h	Up	(Polato *et al.,* 2010)
Ferritin	*A. elegantissima*	20	24h	Up	(Richier *et al.,* 2008)
GST	*M. faveolata*	30-31.5	24h, 48h	Down	(Polato *et al.,* 2010)
GST	*A. aspera*	36 ± 1	1d	Up	(Rosic *et al.,* 2014)
GST	*A. millepora*	31	10h	Up*	(Rodriguez-Lannety *et al.,* 2009)
GST sig	*M. faveolata*	32	10d	Down	(DeSalvo *et al.,* 2008)
GST mu	*M. faveolata*	32	10d	Up	(DeSalvo *et al.,* 2008)
GST Mu 5	*M. faveolata*	31.5 ± 1 Bleached	20d	Down	(DeSalvo *et al.,* 2010)
GST	*Aiptasia* sp.	33.5	2h	Up	(Oakley *et al.,* 2017)
Peroxidasin	*M. faveolata*	30-31.5	24h, 48h	Up	(Polato *et al.,* 2010)
Peroxidasin	*M. faveolata*	30-31.5	24h, 48h	Up	(DeSalvo *et al.,* 2008)
Peroxidasin	*M. faveolata*	29, 31.5	48h	Up	(Voolstra *et al.,* 2009)
Peroxidasin precurser	*A. millepora*	32	9d	None	(Souter *et al.,* 2011
Unspecified peroxidase	*P. damicornis*	32.5+	12d	Up	(Vidal-Dupiol *et al.,* 2014)
Unspecified peroxidase	*A. aspera*	36 ± 1	1d, 3d	Up	(Rosic *et al.,* 2014)
Thioredoxin	*M. faveolata*	32.72	Partial bleaching	Up	(DeSalvo *et al.,* 2008)
Thioredoxin	*M. faveolata*	31.5 ± 1.1	20d	Up	(DeSalvo *et al.,* 2010)
Thioredoxin	*S. pistillata*	32, 34	10d, 13d	Up	(Maor-Landaw *et al.,* 2014)
Thioredoxin	*S. pistillata*	32	10d	Up	(Maor-Landaw & Levy, 2016)
Thioredoxin	*A. eurystoma*	28, 32	5d, 10d	Up	(Maor-Landaw & Levy, 2016)
Thioredoxin	*Porites* sp.	34	13d	Up	(Maor-Landaw & Levy, 2016)
Thioredoxin	*A. microphthalma*	50–1950 PAR	36h	Up	(Starcevic *et al.,* 2010)
Thioredoxin	*A. aspera*	36 ± 1	3d	Down	(Rosic *et al.,* 2014)
Thioredoxin	*A. aspera*	36 ± 1	1d	Up	(Rosic *et al.,* 2014)

Thioredoxin	*S. pistillata*	500µmoles photons $m^{-2}s^{-1}$	15d	Up	(Levy *et al.,* 2016)
Thioredoxin	*Aiptasia* sp.	33.5	24h	Up	(Oakley *et al.,* 2017)
Thioredoxin	*A. millepora*	32	9d	None	(Souter *et al.,* 2011
Thioredoxin peroxidase	*A. millepora*	31	10h	Up*	(Rodriguez-Lannety *et al.,* 2009)
SOD	*P. damicornis*	32.5+	12d	Up	(Vidal-Dupiol *et al.,* 2014)
SOD	*A. aspera*	36 ± 1	1d	Up	(Rosic *et al.,* 2014)
SOD	*S. pistillata*	500µmoles photons $m^{-2}s^{-1}$	15d	Up	(Levy *et al.,* 2016)
SOD	*Aiptasia* sp.	33, 30-33	24h, 12d	None	(Oakley *et al.,* 2017)
MnSOD, SOD	*A. millepora*	32	9d	Up	(Souter *et al.,* 2011)
MnSOD	*A. millepora*	31	10h	Up*	(Rodriguez-Lannety *et al.,* 2009)
MnSOD	*A. millepora*	32	6d	Up	(Császár *et al.,* 2009)
MnSOD	*A. millepora* (larvae)	31	3h, 10h	None	(Rodriguez-Lannety *et al.,* 2009)
Cu/ZnSOD	*A. millepora* (larvae)	31	10h	None	(Portune *et al.,* 2010)
Cu/ZnSOD	*A. palmata*	32.72 ± 0.32	1d, 2d	Up*	(DeSalvo *et al., 2010a)*
Cu/ZnSOD	*M. aequituberculata*	32	22d	Down	(van de Water *et al.,* 2017)
MnSOD	*A. millepora* (larvae)	31	3h, 10h	None	(Rodriguez-Lannety *et al.,* 2009)
Peroxiredoxin-6	*S. pistillata*	28	5d	Up	(Maor-Landaw & Levy, 2016)
Peroxiredoxin-6	*A. eurystoma*	28	5d	Up	(Maor-Landaw & Levy, 2016)
Peroxiredoxin-6	*Porites* sp.	34	13d	Up	(Maor-Landaw & Levy, 2016)
Peroxiredoxin-6	*S. pistillata*	32, 34	10d, 13d	Up	(Maor-Landaw *et al.,* 2014)
Peroxiredoxin	*A. aspera*	36 ± 1	1d	Up	(Rosic *et al.,* 2014)
Peroxiredoxin	*A. aspera*	36 ± 1	3d	Down	(Rosic *et al.,* 2014)
Peroxiredoxin	*S. pistillata*	500µmoles photons $m^{-2}s^{-1}$	15d	Up	(Levy *et al.,* 2016)
Peroxiredoxin-6	*A. millepora*	31	2d	Down	(Bellantuono *et al.,* 2012)
Peroxiredoxin-5	*A. millepora*	31.4	5d	Up	(Meyer *et al.,* 2011)
Peroxiredoxin-5	*A. millepora*	31.4	4h	None	(Meyer *et al.,* 2011)
Peroxiredoxin	*M. aequituberculata*	29.5, 32	22d	Up	(van de Water *et al.,* 2017)

Immune system genes

Several studies highlighted the correlation between bleaching events and subsequent disease outbreaks (Muller, Rogers, Spitzack, & Van Woesik, 2008; Palmer, Bythell, & Willis, 2010; Toller, Rowan, & Knowlton, 2001). Heat stressed corals experiencing dysfunction of the coral-*Symbiodinium* symbiosis can result in increased susceptibility to coral pathogens (Maynard et al., 2015), which could potentially lead to an increase in gene expression of immunity related pathways (Kaniewska et al., 2015). Thus, coral immunity has a likely role in how corals are able, or unable, to adapt to increased temperatures (Hayes, Eytan, & Hellberg, 2010). A component of the immune system is the complement system, which selects foreign molecules to be cleared from the organism (Carroll, 1998) and could be a critical pathway in the bleaching response

(Traylor-Knowles & Connelly, 2017). Complement component 3 (C3) is a major component of the complement system (Carroll, 1998), and *Orbicella faveolata* demonstrated suppression of the complement system during both bleaching, and non-bleaching (Pinzón et al., 2015). In *Porites* sp., complement C3 also had down-regulation in response to long-term heat stress (Kenkel et al., 2011) but not acute heat stress (Kenkel et al., 2014).

Another immune pathway affected during heat stress is nuclear factor (Nf)-kβ signaling, which regulates physiological processes involved in innate immune response, cell death and inflammation (Kaniewska et al., 2015; Perkins, 2007; Zhou, Wu, et al., 2017). MnSoD and ferritin genes are also under the transcriptional control of Nf-kβ (Shen, 2006). Nf-kβ expression increases in bleached *Exaiptasia pallida*, and the authors suggest Nf-kβ could control an immune pathway that needs to be suppressed for *Symbiodinium* uptake (Mansfield et al., 2017). Other studies have also observed up-regulation of Nf-kβ after heat stress (Anderson et al., 2016; Desalvo et al., 2010; Souter et al., 2011). One study observed down-regulation of Nf-kβ in *A. aculeus* after 12 hr heat stress (Zhou et al., 2017), however this could be explained by the relative short heat stress event in their experiment; increased expression of Nf-kβ might only occur after a significant loss of symbionts (Traylor-Knowles & Connelly, 2017).

The Nf-kβ pathway is activated by a variety of different upstream receptors including tumor necrosis factor receptors (TNFRs) and TNF receptor-associated factors (TRAFs) (Shen, 2006), regulators of the apoptosis cascade (Quistad et al., 2014). Corals possess more TNF family members than any organism sequenced so far (Traylor-Knowles, Rose, & Palumbi, 2017). TRAF1, TRAF3 and TRAFD1 can inhibit Nf-kβ activity (Carpentier & Beyaert, 1999; Shi et al., 2006; Yamamoto, Kishimoto, & Minamoto, 1998), however many studies have shown up-regulation of these genes in response to heat stress (Barshis et al., 2013; Desalvo et al., 2010;

Palumbi, Barshis, Traylor-Knowles, & Bay, 2014; Thomas & Palumbi, 2017; Zhou, et al., 2017) and only one showed down-regulation (Parkinson et al., 2017). Similarly, studies examining TRAF6, which activates the Nf-kβ pathway (Wong et al., 1998), shows down-regulation after heat stress (Pinzón et al., 2015; van de Water et al., 2015). The counterintuitive nature of the expression of TRAF as a regulator of the Nf-kβ pathway could be explained by the influence of the other existing receptors, pathways, and effector mechanisms on this particular signaling pathway. The study by Desalvo et al. (2010), which observed up-regulation of both NF-KB and TRAF3 in the same heat stressed *A. palmata* colonies, corroborates this theory.

The DGE of TNFRs after heat stress could be from dysfunctional *Symbiodinium* cells identified as foreign bodies which, in turn, triggered the TNFR signaling pathway leading to apoptosis, an immune response, or cell survival (Traylor-Knowles & Connelly, 2017; Traylor-Knowles & Palumbi, 2014). A variety of TNFRs have shown DGE after acute (Barshis et al., 2013; Palumbi et al., 2014; Traylor-Knowles et al., 2017; Zhou et al., 2017) or long term (Crowder et al., 2017; van de Water et al., 2017) heat stress. However, TNFR expression is not exclusive to heat stress (Wecker et al., 2018; Yuan et al., 2017) and expression of many TNFR genes are likely not involved in the bleaching mechanism, but part of the generalized stress response. This is supported by the finding that differentially expressed TNFR genes during a heat stress experiment were found in the coral's epidermis and oral gastrodermis instead of in the symbiotic cells (Traylor-Knowles et al., 2017).

Table 4 Genes involved in the immune response differentially expressed in cnidarian heat stress experiments.

Specific gene	Host organism	Max Temperature °C/ stressor	Duration of exposure	Up/ Down	Reference
Complement C3	*P. astreoides*	30.9	3h	Down	(Kenkel *et al.,* 2011)
	O. faveolata	Bleached vs non-bleached	In situ	None	(Kenkel *et al.,* 2013)
	P. astreoides	31, 33	3h	None	(Kenkel *et al.,* 2014)

	P. astreoides	Bleached vs non-bleached 30+	In situ	None	(Kenkel *et al.,* 2014)
	A. millepora	Summer months	In situ	Up	(van de Water *et al.,* 2015)
MASP1	*O. faveolata*	Recovery from bleaching	In situ	Down	(Pinzón *et al*., 2015)
	O. faveolata	Unbleached corals during bleaching event		Up	(Pinzón *et al*., 2015)
	A. aspera	32-33.5	8d	Up	(van de Water *et al.,* 2015)
	P. damicornis	32.5+	12d	Down	(Vidal-Dupiol *et al.,* 2014)
Nf-kβ pathway	*P. damicornis*	32.5+	12d	Up	(Vidal-Dupiol *et al.,* 2014)
Nf-kβ pathway	*O. faveolata*	Bleached		Up	(Anderson *et al.,* 2016)
Nf-kβ1, Nf-kβ2	*A. millepora*	32	9d	Up	(Souter *et al.,* 2011)
Nf-kβ	*A. palmata*	32	1d, 2d	Up	(DeSalvo *et al.,* 2010b)
Nf-kβ	*A. millepora*	N/A	Winter	Down	(van de Water *et al.,* 2015)
Nf-kβ	*E. pallida*	35 (Bleached)	6d	Up	(Mansfield *et al.,* 2017)
Nf-kβ	*E. pallida*	Inoculated with *Symbiodinium*	4-5d	Down	(Mansfield *et al.,* 2017)
Nf-kβ inhibitor (IkBa)	*A. millepora*	31	8d	Down	(Bellantuono *et al.,* 2012)
Nf-kβ	*A. aculeus*	32	12h	Down	(Zhou *et al.,* 2017a)
Nf-kβ & TRAF6	*A. aspera*	33.5	8d	Down	(van de Water *et al.,* 2015)
TNFRS	*M. aequituberculata*	29.5, 32	22d	Up	(van de Water *et al.,* 2017)
TNFR	*P. damicornis*	32	12h	Up	(Zhou *et al.,* 2017b)
TNFRS	*A. hyacinthus*	35	5hr	Up	(Traylor-Knowles *et al.,* 2017)
TNFR	*P. damicornis*	28	7d	Up	(Crowder *et al.,* 2017)
TRAFD1	*A. hyacinthus*	30-35	HV vs MV	Up	(Palumbi *et al.,* 2014)
TRAF3	*A. cervicornis*	35	1hr	Down	(Parkinson *et al.,* 2017)
TRAF3	*A. cervicornis*	Post summer thermal maximum	In-situ	Up	(Parkinson *et al.,* 2017
TRAF3	*A. hyacinthus*	2,10,12 months after bleaching	In situ	Up	(Thomas & Palumbi, 2017)
TRAF1	*O. faveolata*	Bleached	In situ	Up	(Pinzon *et al.,* 2015)
TRAF3	*A. palmata*	32	1d, 2d	Up	(DeSalvo *et al.,* 2010b)
TRAF3	*A. hyacinthus*	35	5hr	DGE	(Seneca & Palumbi, 2015)
TRAF3, TNFR	*A. hyacinthus*	27–37.6 °C	72hr	Up	(Barshis *et al.,* 2013)
TRAF1 TRAF2 TRAF3	*P. damicornis*	32	12h	Up	(Zhou *et al.,* 2017b)

Structural genes

Some structural genes, although previously used in gene expression studies as control genes (Louis et al., 2017), show differential expression under heat stress. Actin is the most abundant protein in the eukaryotic cytoskeleton and the principle component of the microfilament network (Oakley et al., 2017). It is also a muscle fiber constituent and sensitive to

oxidative damage; the buildup of ROS results in actin reorganization. While not immediate responders like HSPs, actin genes are still highly responsive to acute thermal stress, as demonstrated by observed differential expression in *Porites* sp. (Kenkel et al., 2013; Kenkel et al., 2011) *A. palmata* (Desalvo et al., 2010) and *A. aspera* (Rosic, Kaniewska, et al., 2014), and to long-term stress, as observed in *O. faveolata* (Desalvo et al., 2008) and *Stylophora pistillata* (Maor-Landaw et al., 2014). Seneca and Palumbi found that extracellular matrix proteins and genes involved in the regulation of actin were differentially regulated 20 hr after peak heat stress, but not 1 hr after (Seneca & Palumbi, 2015). A "*Porites* stress index" was developed based on the magnitude of differential expression between the up-regulation of Hsp 16 and down-regulation of actin in *Porites astreoides* (Kenkel et al., 2011), and was a useful biomarker in a later study (Kenkel et al., 2013). *A. millepora* showed down-regulation of actin-related proteins after 8 days of heat stress, but only in corals that had been preconditioned to thermal stress (Bellantuono et al., 2012). In addition these genes showed no change in expression when *S. pistillata* was exposed to a pollutant suggesting that they might be specific to heat stress (Maor-Landaw et al., 2014).

Table 5 Structural genes differentially expressed in cnidarian heat stress experiments.

Specific gene	Host organism	Max Temperature °C/ stressor	Duration of exposure	Up/ Down	Reference
Actin	*P. astreoides*	30.9	3h	Down	(Kenkel *et al.,* 2011)
	P. astreoides	Bleached inshore	In situ	Up	(Kenkel *et al.,* 2013)
	P. astreoides	33	3h	Down	(Kenkel *et al.,* 2014)
	M. faveolata	32.23 ± 0.48	10d 17h	Down	(DeSalvo *et al.,* 2008)
	A. palmata	32.72 ± 0.32	1d	Down	(DeSalvo *et al.,* 2010b)
	A. hyacinthus	35	1h after peak stress	None	(Seneca & Palumbi, 2015)
	A. hyacinthus	35	20h after peak stress	Down	(Seneca & Palumbi, 2015)
	A. aspera	36 ± 1	1d, 3d	Up	(Rosic *et al.,* 2014)
	A. millepora	31	8d	Down	(Bellantuono *et al.,* 2012)
	A. palmata	30, 31.5	24.5	Down	(Portune *et al.,* 2010)
	S. pistillata	32, 34	10d, 13d	Down	(Maor-Landaw *et al.,* 2014)
	A. elegantissima	20	24h	Down	(Richier *et al.,* 2008)

Differential gene expression is dependent on length of heat stress

Corals have distinct short-term versus long-term thermal stress responses. The most prominent responses to acute thermal stress in adult corals include immediate up-regulation of HSPs and antioxidant enzymes (Császár et al., 2009; Desalvo et al., 2008; Desalvo et al., 2010; Kenkel et al., 2011; Poli et al., 2017; Seneca et al., 2010; Seneca & Palumbi, 2015). Longer-term (several days or more) thermal stress causes broad-scale down-regulation of basic metabolic processes (Vidal-Dupiol et al., 2014) and heat shock proteins (Poli et al., 2017). *Aiptasia pallida*, during early exposure to heat stress, suppressed expression of rheostat enzymes, important for cell survival, proliferation and mediating cell death, while Hsp90 increased. With extended exposure, rheostat enzymes responsible for cell survival increased, suggesting that these enzymes function only in response to prolonged heat stress (Kitchen & Weis, 2017). Kenkel et al. (2014) separates the coral response into two groups based on their timing: the immediate *cellular stress response* and the subsequent *cellular homeostasis response*. The *cellular homeostasis response* reestablishes homeostasis at the new norm if the stressor persists or if there is lasting change in the environment. Biomarkers of this response to thermal stress include down-regulation of Ca^{2+} and ribosomal proteins, and changes to cytoskeleton and extracellular matrix proteins (Kenkel et al., 2014). The observed differences in expression, based on exposure time to heat stress, underscores the importance of examining water flow impacts with both long-term and short-term heat stress experiments.

Water flow could potentially dampen gene expression responses to heat stress as has been observed with other environmental or physical factors that mitigate the effect of heat stress on corals. Prior exposure to heat reduces the expression of stress-related genes in two different ways. Corals that have undergone long-term exposure to heat stress will show a dampened

response to an acute heat stress (Barshis et al., 2013; Palumbi et al., 2014) because the genes are already up-regulated. In comparison, the dampened response after short-term acclimation is caused by a lower reaction of those genes to acute stress (Bay & Palumbi, 2015). Environmental memory has been shown to exist for more than 10 years in corals (Brown, Dunne, Edwards, Sweet, & Phongsuwan, 2015), suggesting acclimation to heat stress can be long-lived. Water flow could potentially dampen gene expression through both means of acclimation, either by allowing corals to survive even as they elevate gene expression in response to heat stress (as per the long-term acclimation) or by dampening the reaction in the first place. As mentioned before, water flow can increase photosynthesis by raising the influx of CO_2. Increasing CO_2 levels has shown to lead to an up-regulation in calcium pathways and metabolic genes in *Pocillopora damicornis* (Vidal-Dupiol et al., 2013), and in *Siderastrea sidereal*, increasing CO_2 will still result in an up-regulation of metabolic processes even when combined with increased temperatures (Davies, Marchetti, Ries, & Castillo, 2016). Thus, increased flow could mitigate the effects of heat stress on the DGE of calcium and metabolic related genes. Water flow could alter gene expression after heat stress through increased nutrient uptake. A study examining heat stressed *P. damicornis* under elevated ammonium conditions found that while genes encoding for the TNF signaling pathway were upregulated in both treatments, genes relating to apoptosis and cell death was exclusive to the heat stress without increased ammonium. The authors suggest the TNF signaling pathway under elevated ammonium is triggered to regulate the immune response (Zhou et al., 2017). Finally, the demonstrated benefit of flow for heterotrophy could dampen the thermal stress response. Levy et al. (2016) found that when comparing fed and starving corals under light stress, the starved corals showed the decreased expression of genes related to energy metabolism and calcium pathways was due to the inability to meet the energetic

demand of cellular processes such as protein degradation, refolding and DNA repair. Fed corals, on the other hand, presented fewer differentially expressed genes, suggesting that high flow's propensity to increase heterotrophic feeding via increased food delivery could result in similar expression dampening under heat stress. Thus, flow-mediated increases in oxygen flux, nutrient uptake, and heterotrophy could result in dampened expression responses to heat stress as observed by other factors that increase coral resilience to higher temperatures.

OBJECTIVES OF THIS STUDY

The objectives of the study are as follows:

1) To determine the impact of colony-level flow differences on corals during natural mild heat stress;

2) To determine the impact of reef-level flow differences on corals during natural mild heat stress.

3) To determine how the impact of colony-level flow differences on corals during mild heat stress changes in oscillatory and unidirectional flow environments;

4) To determine the impact of reef level flow differences on corals during a bleaching event;

5) To determine the impact of flow on acutely heat-stressed corals; and

6) To determine the impact of flow on corals at ambient temperature.

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CHAPTER 2

GOING WITH THE FLOW: CORAL COLONIES IN HIGH FLOW ENVIRONMENTS CAN BEAT THE HEAT

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Abstract

Coral reefs on a global scale are suffering from previously unprecedented declines in cover and overall health state. That said, varying responses of colonies from the same species (separated by either small or large spatial scales) have been observed in reaction to these global stressors. Such variation can be accredited to the specific micro-environment, high versus low flow exposure for example. Increased water flow provides corals with a variety of health benefits including mitigation of heat stress. In this study we aimed to take a transcriptomic perspective to the questions of water flow and coral resilience. Gene expression of *Acropora* cf. *pulchra* colonies in discrete flow environments were measured *in situ* during a natural heat stress event. These were then compared to *A.* cf. *pulchra* nubbins exposed to different flow regimes and an artificial severe heat stress *ex situ*. We observed distinct flow-driven transcriptomic signatures related to metabolism and growth. We found previously described transcriptomic responses to heat stress, and this response changed based on flow regime. For example, corals that were exposed to high flow levels showed "frontloading" of heat stress genes. A result likely due to increased oxidative metabolism generated by higher water movement. This "frontloading" effect could (at least in part) explain why colonies found in higher flow environments exhibit greater

resilience in the face of increased sea surface temperatures compared to those exposed to lower flow.

Introduction

The genus *Acropora* is the most diverse among the reef-building stony corals of Scleractinia (Veron, 2000; Wallace, 1999). They are all characterized as fast-growing, dominant reef builders (Perry et al., 2012) and staghorn *acroporids* disproportionally contribute to reef accretion, making them biological pillars for the ecosystem functions delivered by coral reefs (Wild, 2011). Their dominance adds to the structural complexity of reefs, providing essential habitats for many marine organisms, evidenced by the higher fish abundance and species richness associated with staghorn corals (Johnson et al., 2011). Staghorns are largely restricted to shallow reefs, a habitat frequently exposed to variable levels of irradiance, extreme tidal flux, sea surface temperatures, sedimentation and eutrophication (Guilcher, 1988). Furthermore, due to their location near the shoreline, these same environments are under increasing threat from anthropogenic stressors such as pollution and physical damage (Hughes, 1994; Thiel et al., 2013). Such a variable habitat coupled with the staghorns life history strategy (fast growth but highly susceptible to stress) (Loya et al., 2001), has resulted in an overall increase in their bleaching records (Guest et al., 2012; McClanahan et al., 2014). Indeed many studies have highlighted the dramatic decline of staghorns across much of their former range (upwards of 80–90% in the Caribbean and western Atlantic for example since the late 1980s (Bruckner, 2002)).

Until recently, Guam, and many other Pacific islands, have avoided this scale of decline (Burdick et al., 2008; Paulay, 1999). However, recent bleaching events, and their projected increase in frequency (Logan, Dunne, Eakin, & Donner, 2014) questions the future of staghorn

populations in the region. Guam experienced two temperature-induced bleaching events in 2013 and 2014 and additional mortality from extreme low tides in 2015. Total mortality for staghorn populations was estimated at 52% after these events with a total 17.8 ha coral cover lost (Raymundo et al., 2017). However, staghorn coral loss was not uniform across all habitats and a distinctive spatial pattern of resistance to bleaching conditions was observed. During the three-year bleaching event, healthier surviving staghorn coral branches were limited to the edges of the colonies, with extensive mortality in colony centers. Additionally, colonies closer to the wave-flushed reef crests showed significantly less bleaching mortality (Raymundo et al., 2017). These spatial differences suggest that local processes (such as flow) may mitigate the effect of certain stressors such as increased sea surface temperatures (Bayraktarov, Pizarro, Eidens, Wilke, & Wild, 2013; Nakamura & Van Woesik, 2001).

Corals in high flow environments have indeed been shown to have higher survival rates and are in general, less likely to experience bleaching during elevated sea surface temperatures and irradiance levels than those exposed to low flow (Bayraktarov, Pizarro, Eidens, Wilke, & Wild, 2013; Nakamura & Van Woesik, 2001). Bleached corals also recover faster in high flow environments (Nakamura, Yamasaki, & van Woesik, 2003). Even intermittent flow reduces bleaching (Smith & Birkeland, 2007). This effect can also cause differential responses within colonies; with upstream sides of large colonies showing less bleaching than downstream sides (Fujimura & Riegl, 2017). Flow has impacts on a macro scale as well. For example, wind-induced high flow could explain the heat-stress tolerance of coral reefs in the northern Red Sea (Osman et al., 2017).

Such observations can in part be explained by the number of flow-mediated physiological changes in various coral species. Higher flow causes a thinning of the diffusive

boundary layer (DBL), allowing for efficient transport of solutes between coral tissue and the surrounding water (Bayraktarov et al., 2013). This means increased nutrient uptake (Atkinson & Bilger, 1992; Badgley, Lipschultz, & Sebens, 2006; Falter, Atkinson, & Merrifield, 2004), which allows for greater availability of essential nutrients (Rosset et al., 2015, 2017), and also increased gas exchange rates, thus impacting respiration (Osinga et al., 2017). Greater oxygen efflux under higher flow can potentially increase photosynthesis in corals by enhancing CO_2 binding (Finelli et al., 2006) and reducing photorespiration in the coral photo-symbiont (Kremien et al., 2013; Tali Mass et al., 2010). Additionally, flow-mediated increases in the influx of dissolved inorganic carbon can promote greater calcification and photosynthesis (Dennison & Barnes, 1988). However, not all of flow's impact can be explained by the thinning of the DBL. Water flow can also increase heterotrophic feeding through higher prey encounter rates (Sebens et al., 1998) and decrease the water temperature at the coral tissue level (Fabricius, 2006).

This study aimed to assess, via a transcriptomic perspective, the "health benefits" attributed to corals under increased water flow and its role as a heat stress mitigator through *in situ* observations during a natural bleaching event and an *ex situ* controlled acute heat stress experiment.

Materials and Methods

Study Site

The site for *in situ* work (Fig. 1) is located on the reef flat of West Hagåtña Bay, Guam. At this site, the major currents are determined, primarily, by the wind direction, which ~80% of the year are easterly (NOAA National Centers for Environmental information, 2017). As a result, during this time a strong westerly current is present across the reef flat. During high tide, farshore colonies experience oscillatory motion from waves while nearshore colonies only

experience these waves to a minimal degree or not at all (Fifer pers. obs; see results section). Two sites were therefore selected, hereafter denoted as “high flow” and “low flow”. The high flow site exhibits both oscillatory flow and the primarily westerly wind-driven current with little buffering. The low flow site receives little oscillatory flow and weaker wind driven flow due to buffering from staghorn coral patches farshore and east of it. Both sites share similar-sized staghorn patches (~6m^2), which allows for a standardized comparison of the effects of flow rates on individual colonies (Edmunds & Burgess, 2018). Finally, the two areas have distinct differences in scale of mortality as a result of the 2013-2015 bleaching events, with an estimated > 80% and < 20% mortality for low and high flow sites respectively (Raymundo et al., 2017).

Water flow, temperature, irradiance, and phosphate and nitrate concentrations were measured to characterize the environment of the *in situ* study sites. Temperature and irradiance were measured with HOBO light and temperature loggers (Onset, Bourne, MA), and PO_4^{3-} and NH_4 concentrations were analyzed by the Water and Environmental Research Institute of the Western Pacific (WERI) (Mangilao, GU). These measurements were taken from the outside and the inside of all colonies that were sampled for sequencing. Temperature and irradiance data were taken for 6 months at thirty-minute intervals, and flow and nutrient data were collected for 6 hr on three different days during the summer where winds were reflective of yearly norms (both speed and direction). The temperature data from the days experiencing the highest fluctuation were extrapolated (i.e. maximum and variation) to create the acute heat stress conditions used in the *ex situ* experiment.

Clod cards were deployed to estimate short term mass transfer rates. Clods were deployed on the outside and inside of all colonies that were sampled for sequencing. Mean dissolution of clod cards over a 6 hr period on three different days was calculated using the protocol from

Reidenbach et al. (2006). Mean dissolution measurements for each day were standardized (mean = 0, sd =1) and averaged. A linear mixed effects model was fit using the lme4 package (Bates, Machler, Bolker M., & Walker C., 2015) in R (R Development Core Team, 2018) to determine differences in dissolution between sites and outside versus inside of the colonies. Our model had one fixed factor (colony position) and two random factors (colony number, branch number) nested within. There was less than a 4% variance in the dissolution rate of the still control (see Reidenbach et al. (2006)) between sites, indicating that water temperature or chemistry were not causing differences in dissolution at the low-flow versus high-flow site. Residuals were tested for normality using the Shapiro-Wilk test.

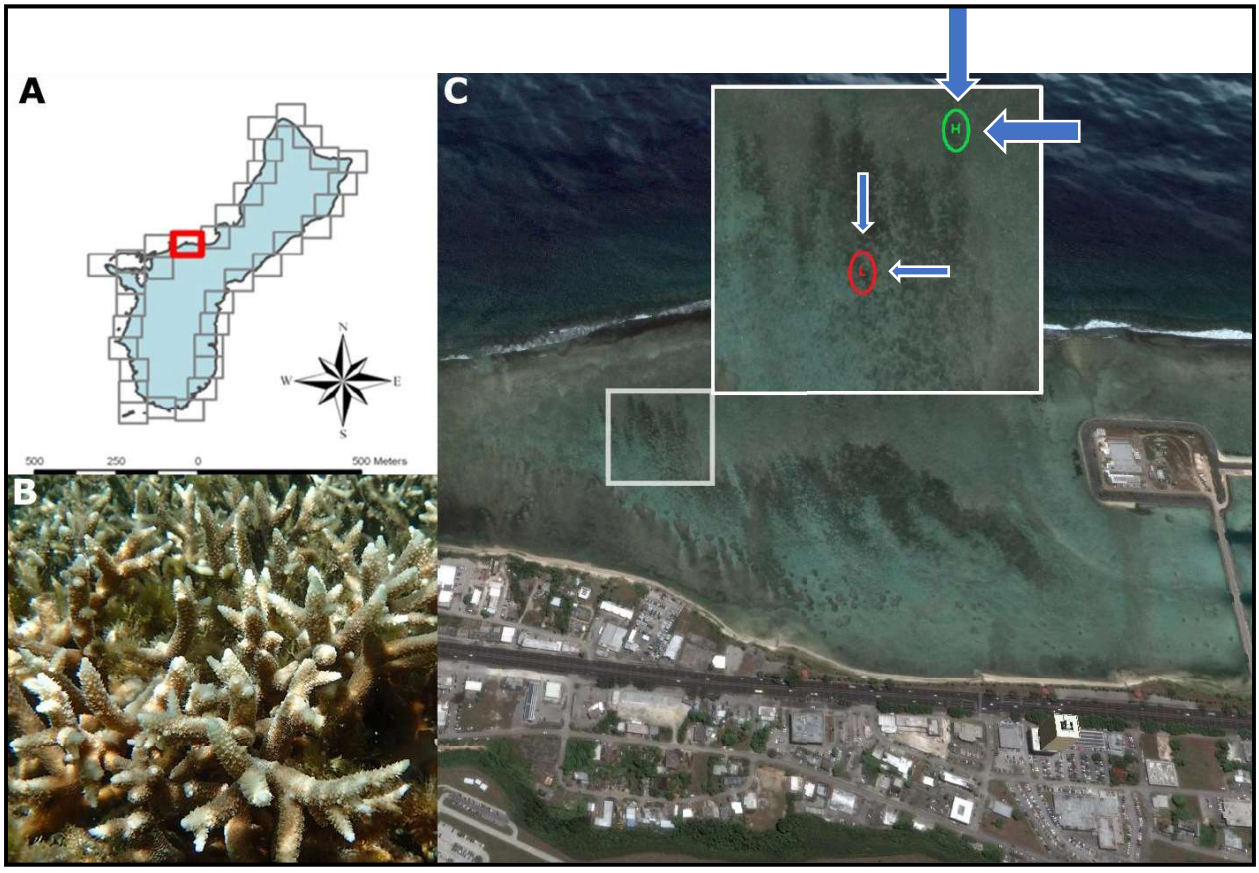

Figure 1 A) Location of West Hagåtña Bay (red rectangle), Guam. B) Typical morphology of staghorn colonies found in the study area. C) Study Area (white square), size of blue arrows

depict the relative velocity and direction of water flow. Abbreviations in white squared insert: high flow (H) and low flow (L).

Assessing gene expression of *Acropora* cf. *pulchra* colonies in different flow environments *in situ*

Six *Acropora* cf. *pulchra* colonies (3 in each of the high flow and low flow sites) were tagged. Tissue samples for transcriptomics and *Symbiodinium* counts were taken from these colonies during elevated summer temperatures in August, 2017 (1) and again after signs of bleaching in September, 2017 (2). These samples were compared against environmental parameters collected at both sites to assess for similarities and differences in gene expression between the colonies. Lastly, *Symbiodinium* densities were determined in coral tissue (3).

1) Tissue sampling for transcriptomics during a summer heat stress event

Here we examined differential gene expression in corals growing in different flow environments under heat stress, as experienced during Guam summer temperatures in August 2017 (Island-wide NOAA Bleaching Alert level 1, NOAA Coral Reef Watch (2013)) (Supplemental Fig. 1) before bleaching was apparent (i.e. obvious paling). Branches from the edge (referred throughout as outside) (n=18) and center (referred throughout as inside) (n=18) of staghorn colonies (n=3 colonies per site) were sampled for transcriptomics to determine the influence of colony scale water flow differences on gene expression. Samples were taken from both the high flow (n=18) and low flow (n=18) sites to compare gene expression across sites (i.e. high flow and low flow) and the impact of oscillatory flow at the colony level (i.e. outside versus inside).

2) Coral sampling for transcriptomics during a bleaching event

The second sampling period was at the end of summer in September 2017 (Supplemental Fig. 1) when paling was evident (Island-wide NOAA Bleaching Alert level 2, NOAA Coral Reef

Watch (2013)). This allowed us to examine differential gene expression between different flow environments during a bleaching event. Only branches from the outside of the colonies (n=18) were sampled for transcriptomics due to scarcity of live coral tissue on the inside of the low flow site colonies. The same 6 colonies from the previous collection event (1) were sampled.

3) *Symbiodinium* counts

All branches from both heat stress and bleaching sampling periods collected for gene expression (n=54) were also analyzed for *Symbiodinium* counts and used as a proxy for coral health. The coral tissue remaining after extractions was airbrushed with filtered seawater (0.45µm) and *Symbiodinium* were counted using a hemocytometer. Counts were scaled to the surface area of the branch airbrushed which was calculated using the protocol from Marsh (1970) (For full protocol on *Symbiodinium* counts see supplemental methods). A linear mixed effects model was fit using the lme4 package (Bates et al., 2015) in R (R Development Core Team, 2018) to determine differences in *Symbiodinium* density. Our model had two fixed factors (site and colony position) and two random factors (colony number, branch number) nested within.

Assessing gene expression of heat stressed *Acropora* cf. *pulchra* nubbins under high and low flow regimes

Here we examined how water flow regime influences gene expression in acutely heat stressed corals under controlled conditions *ex situ*. For this, small unbranched (~4 cm) nubbins from the outside of the West Hagåtña high flow colonies (the same colonies sampled during August *in situ*) were acclimated for two weeks in a raceway at the University of Guam Marine Lab with free-flowing water at ambient temperature (29-29.5 °C) under shade cloth. After acclimation, 16 nubbins were then placed in four uni-directional flow tanks (built using guidelines from Denny, 1988) for 11 days at the same ambient temperature. Each tank was split

into two different flow regimes (low flow and high flow) with two nubbins assigned to each flow type. The low flow condition was set to 4 cm^2/s and the high flow condition was set to 16 cm^2/s. Flow velocity was determined using the FlowTracker Acoustic Doppler Velocimeter (SonTek, San Diego, CA). On day 11 after acclimation, temperature was increased to 35°C over 3 hr, maintained at 35°C (Mimicking the most extreme observed temperature increase during the bleaching season at the study site, Supplemental Fig. 1) for 45 minutes and then lowered back to 29.5°C over a 2 hr period (Fig. 2). Temperature was regulated with a Tank TK-3000 Aquarium chiller (TECO, Ravenna, Italy) and HC-810M water temperature controller (Finnex, Countryside, IL). The other two tanks were maintained at ambient temperature for the same period. A ~2 cm sample from each nubbin in each tank (n=16) was collected immediately after the heat stress event for RNA extraction and sequencing.

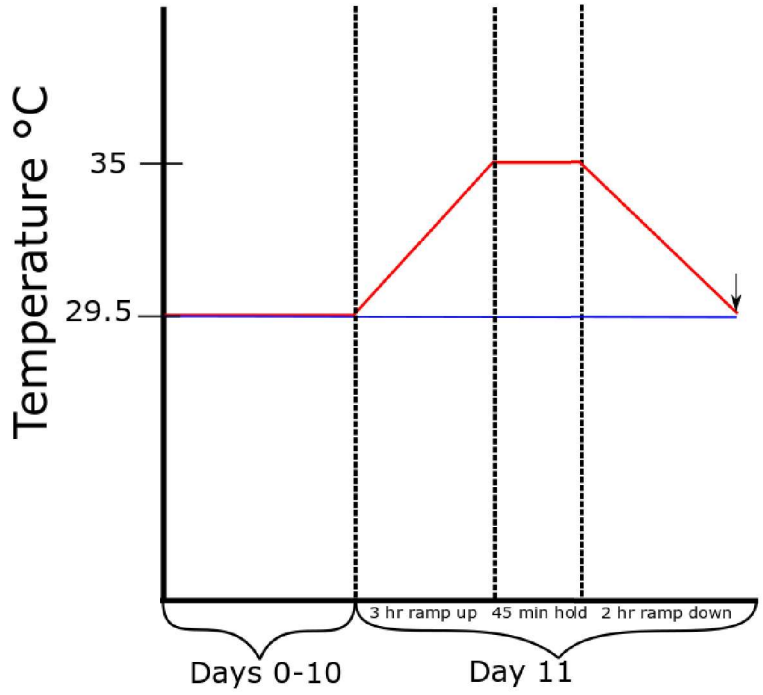

Figure 2 Temperature profile for *ex situ* experiment. Arrow denotes sampling time. Red line depicts heated tanks (n=2) with 8 individual corals exposed to low flow (n=4) or high flow

(n=4). Blue line depicts ambient temperature tanks (n=2) with 7 individual corals exposed to low flow (n=3) or high flow (n=4).

Tissue sampling, library preparation and sequencing

All samples for both *in situ* and *ex situ* experiments (n=70) were fragmented to approximately 2 cm, avoiding the branch tips, and immediately placed in RNAlater (Sigma-Aldrich, St. Louis, MO). They were stored frozen at -80°C until extraction. Total RNA was extracted from half the fragment (~1cm) using an RNeasy kit (Qiagen, Hildenheim, Germany) on a QIAcube DNA/RNA extraction robot (Qiagen, Hildenheim, Germany). RNA was quantified fluorometrically using a Qubit (Life Technologies, Carlsbad, CA) and RNA integrity was determined using a Bioanalyzer (Agilent Technologies, Santa Clara, CA). The samples with the highest quality and quantity of RNA were used to prepare cDNA libraries (n=33) for sequencing using the Illumina NeoPrep system (Illumina, San Diego, CA. The libraries were normalized, pooled and sequenced in 2 multiplexed runs on the NextSeq500 sequencer (150 cycles) in house, according to the manufacturer's instructions.

Reference transcriptome filtering and annotation

Sequences were trimmed using TRIMMOMATIC (ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:35:-phred33) (Bolger, Lohse, & Usadel, 2014), which removes low quality nucleotides with bp ≤35 and sequencing adapters, per default settings. All quality-filtered paired reads were aligned against the publicly available *A. digitifera* genome (Shinzato et al., 2011) using the splice-junction mapper TopHat2 (Kim et al., 2013). Using the resulting BAM files, a reference transcriptome for *A.* cf. *pulchra* was assembled via the genome-guided version of the Trinity assembler (Haas et al., 2013), with the *A. digitifera* genome (Shinzato et al., 2011) as a guide.

BLASTN searches were performed against NCBI's nt database (evalue 1e-5), retaining only scleractinian matches; contigs shorter than 300bp were removed. Five sequences showed significant similarity (sN, e-value ≤ 1×10−8) to the SILVA LSU and SSU rRNA databases (http://www.arb-silva.de/) and were removed from further analysis.

BLASTX searches with an evalue cutoff of 1e-5 were performed using the assembled coral reference transcriptome against the cnidarian sequences contained in the uniprot database (www.uniprot.org) to annotate the filtered reference transcriptome and provide genes with gene ontology (GO) terms (Ashburner et al., 2000).

Differential gene expression analysis

Sequences for each sample were mapped against the reference host transcriptome and RNA expression was estimated using the expectation maximization algorithm implemented in RSEM (Li & Dewey, 2011). Differentially expressed genes (DEG) between treatments were identified using the R (R Development Core Team, 2018) package edgeR (Robinson, McCarthy, & Smyth, 2010) with a false discovery rate (FDR) adjusted p-value (adj-p) < 0.05 and log fold change (logFC)s ≥ 2, and package Deseq2 with the same parameters (Love, Huber, & Anders, 2014). Genes were classified as differentially expressed if they were significant using both statistical packages. To identify significant functional differences among the up and downregulated genes we conducted a GO analysis using a Fisher's exact test and the GO_MWU rank-based methodology with adaptive clustering of GO terms (Wright, Aglyamova, Meyer, & Matz, 2015), downloaded at https://github.com/z0on/GO_MWU. Blast2GO analysis (BioBam, Valencia, Spain) was also performed on all differentially expressed genes between treatments, and gene ontologies were mapped to transcripts using the uniprot GO annotations. Top Blast2GO

results for biological processes, molecular functions and cellular components were reported in supplemental materials.

For the *ex situ* samples, principal coordinate analysis of differentially expressed genes was performed to determine if there was clustering among heat stress and flow treatments or among source colonies. For the *in situ* samples, principal coordinate analysis of differentially expressed genes was performed to determine clustering among different flow conditions and sampling periods. We implemented the "vegan" package (Oksanen et al., 2017) in R using Manhattan distances between samples, corresponding to the sum of log-fold differences across all genes.

For both the *ex situ* and *in situ* high flow/low flow comparisons we examined a list of "heat stress candidate genes". Heat stress candidate genes were identified through the following steps: 1) we compiled a list of genes commonly shown to be differentially expressed in previous cnidarian heat stress experiments, 2) these genes were filtered to include only genes that were also identified in this *ex situ* heat stress experiment, 3) only genes that were also present in the *ex situ* ambient high flow/low flow comparison or the *in situ* high flow versus low flow site were included.

Symbiodinium **community**

To determine cladistics of sampled *Symbiodinium,* sequences for each sample were mapped against a reference *Symbiodinium* ITS2 database (Franklin, Stat, Pochon, Putnam, & Gates, 2012). Methods for determining cladistics were adopted from Davies et al. (2018). All possible alignments were identified and used in the analysis if all alignments for that read mapped to a single clade.

To determine possible differences in clades of *Symbiodinium* between treatments that might account for differences in host differential gene expression analyses, we mapped RNAseq libraries against the *Symbiodinium* COI BOLD database (Ratnasingham & Hebert, 2007) and publicly available COI sequences from NCBI, using bowtie2 (Langmead & Salzberg, 2013) with the --no-unal flag. Reads that were mapped successfully were then assembled against the consensus sequence of our *Symbiodinium* reference alignment, using the reference-guided assembly algorithm implemented in Geneious (version 10.0.2; Biomatters, Auckland, NZ). All *Symbiodinium* sequences and a *Protodinium* plus *Polarella glacialis* outgroup were aligned using MUSCLE (Edgar, 2004) with the default options implemented in Geneious, followed by removing sites of ambiguous alignment using Gblocks (Castresana, 2000) with the default settings implemented in the alignment viewer Seaview version 4 (Gouy, Guindon, & Gascuel, 2010). Phylogenetic relationships were inferred using PhyML version 3.2 (Guindon et al., 2010) under the GTR+I+G model of nucleotide substitution. Robustness of the phylogeny was evaluated with 100 non-parametric bootstrap replicates.

Results

Acropora cf. *pulchra* reference transcriptome

After adapter trimming and quality filtering, library size ranged from ~2.3 to ~59 million paired-end reads. The first draft assembly comprised 150,719 contigs, 34,231 of which were determined as being of sceleractinian origin with an average length of 779bp and an N50 of 923. Annotations were obtained for 21,265 genes.

In situ clod cards and environmental data

Flow differences between the outside and inside of colonies at the high flow site were not significantly different ($p > 0.05$) according to our linear mixed effects model. Flow was

significantly higher at the high flow site compared to the low flow site and, for the low flow site only, on the outside of colonies compared to the inside ($p < 0.05$) (Fig. 3). Temperature data is reported in supplemental materials (Supplemental Fig. 1). Nutrient analysis revealed low concentrations of nitrate and phosphate in the water column (< 0.01 mg/L) and trace amounts of ammonia (0.10- 0.013 mg/L) at both sites.

Symbiodinium **data**

Symbiodinium counts did not reflect the aforementioned flow differences and there were no significant differences ($p > 0.05$) between sites or between the outside and inside of colonies within each site according to our linear mixed effects model (Supplemental Fig. 2). However, there does seem to be a trend where *Symbiodinium* counts were higher at the high flow site compared to the low flow site ($p <0.1$), during both the heat stress and bleaching events (Supplemental Fig. 2).

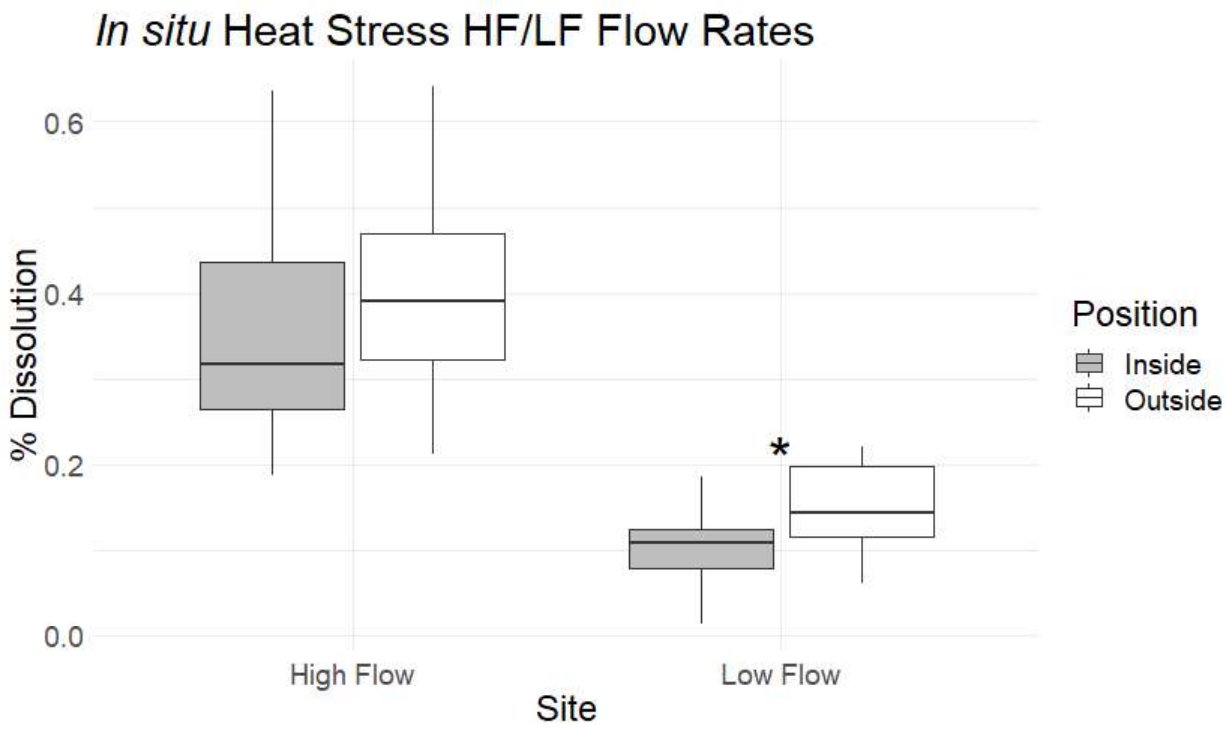

Figure 3 Average percent dissolution of gypsum per time measured on the inside and outside of colonies for both high flow and low flow site conditions. Box plot shows distribution of % clod card dissolution for individuals (n = 88) by treatment. The box represents the inter-quartile range (IQR) between the upper and lower quartile. The whiskers maximally extend 1 times beyond the IQR. Effect significances are represented by (*) = $p < 0.05$.

***Symbiodinium* community**

Symbiodinium were determined to be clade C dominant. COI phylogenetic analysis revealed no clustering according to flow environment (Supplemental Fig. 3).

Differential gene expression

Our differential gene expression analysis produced no differentially expressed genes in three comparisons (*ex situ* heat stress high flow versus low flow, *in situ* heat stress low flow site outside versus inside and heat stress high flow site outside versus inside) and very few differentially expressed genes (n=69) in a fourth comparison (*in situ* bleaching high flow versus

low flow site). The highest number of differentially expressed genes (2211 DEG, 1892 annotated) was found in the only comparison that was not heat stressed (i.e. *ex situ* ambient temperature high flow versus low flow). This was followed by the *in situ* heat stress high flow versus low flow site comparison (922 DEG, 441 annotated), and the two ambient temperature versus heat stress comparisons (798 DEG, 569 annotated and 608 DEG, 396 annotated) (Table 1). Principal coordination analysis for the *ex situ* experiment revealed clustering according to treatment with source colony playing no apparent role in the explained variation (Fig. 4).

Table 1 Number of genes differentially expressed between comparisons (intersection between DESeq2 and edgeR analysis; FDR $p < 0.05$, logFC ≥ 2. Rows in grey denote comparisons that had few differentially expressed genes. "Pooled Heat Stress" combined samples from high flow heat stressed and low flow heat stressed conditions in the *ex situ* experiment, because of the degree of similarity between conditions (0 DEG). Abbreviations: HF = High Flow, LF = Low Flow.

Comparison	Differentially Expressed Genes	Upregulated	Downregulated
Ex situ Ambient HF/LF	2211	2162	49
Ex situ Ambient HF/Pooled Heat Stress	798	458	340
Ex situ Ambient LF/Pooled Heat Stress	608	139	469
Ex situ Heat Stress HF/LF	0	0	0
In situ Heat Stress LF Outside/Inside	0	0	0
In situ Heat Stress HF Outside/Inside	0	0	0
In situ Heat Stress HF/LF	922	303	619
In situ Bleaching HF/LF	69	25	44

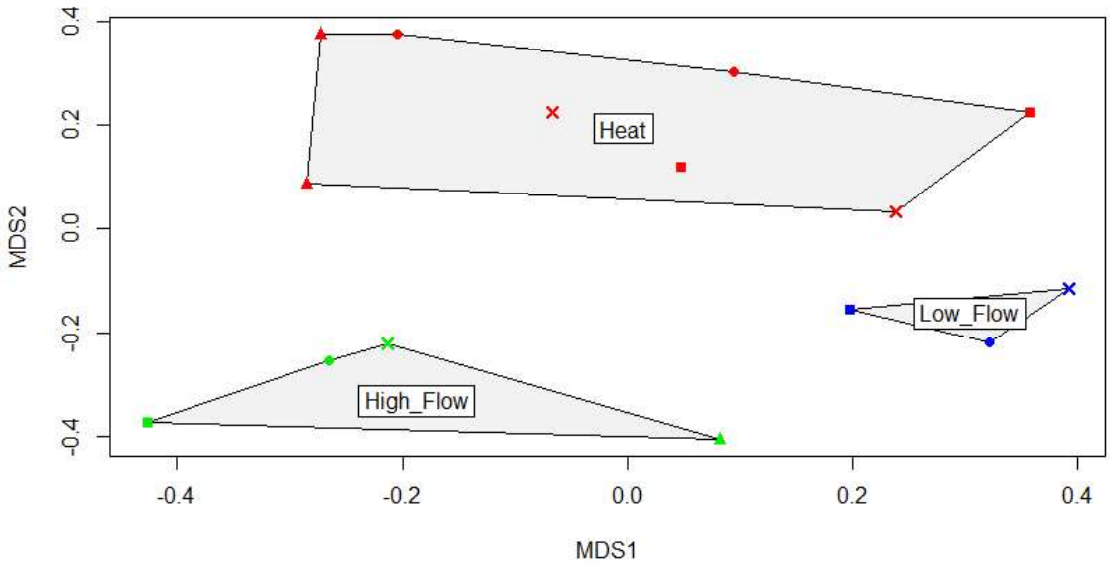

Figure 4 Principal coordinate analysis of *ex situ* gene expression values. Shapes correspond to the four different colonies at the *in situ* high flow site which samples were sourced from.

DEG and GO enrichment analysis for *ex situ* ambient flow/heat stress comparison

Regulation of apoptosis was significantly enriched ($p < 0.01$) with upregulated genes in the heat stress condition compared to both flow conditions at ambient temperature (Table 2). We also found upregulation of specific genes relating to oxidative stress and immune response in the

heat stress condition compared to both ambient flow conditions (Fig. 5). Metabolic and cell adhesion processes were also significantly downregulated under heat stress ($p < 0.01$), but only when compared to the ambient high flow condition (Table 2).

DEG and GO analysis for *ex situ* ambient high flow/low flow comparison

The high flow condition showed significant upregulation for functional terms relating to energy metabolism, ribosome, RNA modification, and calcification (Table 2). There were no significantly enriched terms upregulated in the low flow condition (Table 2).

We also looked for specific genes relating to energy metabolism. In the high flow condition, we found upregulation of genes related to glycolysis including Ketohexokinase, Fructose-bisphosphate aldolase (EC 4.1.2.13), 4-hydroxy-4-methyl-2-oxoglutarate aldolase (HMG aldolase) (EC 4.1.1.3) (EC 4.1.3.17) (Oxaloacetate decarboxylase), 4-hydroxy-4-methyl-2-oxoglutarate aldolase (HMG aldolase) (EC 4.1.1.3) (EC 4.1.3.17) (Oxaloacetate decarboxylase), Phosphoglycerate kinase (EC 2.7.2.3), Glucose-6-phosphate isomerase (EC 5.3.1.9). We also found genes related to oxidative phosphorylation including NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial. NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 3, NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial and Krebs cycle genes, 2-oxoglutarate dehydrogenase, mitochondrial, succinate dehydrogenase (quinone) (EC 1.3.5.1) (Fragment), Isocitrate dehydrogenase [NAD] subunit, mitochondrial, undescribed protein. Growth-related genes were found, which included Alpha carbonic anhydrase 7 (EC 4.2.1.1), Bone morphogenetic protein 7, and Bone morphogenetic protein I-like protein (Predicted protein) (Fragment).

Several GO terms related to stress were upregulated in the high flow condition, but were not significant. This included biological processes relating to cell death and oxidative stress

(Supplemental Fig. 4). We also found several genes relating to oxidative stress and immune response upregulated in the high flow condition (Fig. 5).

Blast2GO and genes of interest for *in situ* heat stress high flow/low flow comparison

Although not significantly enriched, we also found similar functional groups of genes upregulated in the *in situ* high flow site that were also abundant in the *ex situ* high flow condition. These included metabolic processes, cell matrix adhesion and calcium ion binding. Stress related GO terms were also abundant in the high flow condition: cellular protein metabolic process, cellular response to DNA damage stimulus, apoptotic process, and regulation of programmed cell death (Supplemental Fig. 5). These terms were not apparent in the bleaching high flow versus low flow site comparison.

We also looked for upregulation of genes relating to key metabolic processes and found upregulation of genes associated with the Krebs cycle: Isocitrate dehydrogenase [NAD] subunit, mitochondrial, Succinate dehydrogenase (quinone) (EC 1.3.5.1) (Fragment), 2-oxoglutarate dehydrogenase; skeletal organic matrix proteins: Collagen alpha-1(VII) chain, Collagen alpha-2(I) chain extracellular matrix structural constituent, Collagen-like protein 6; protein phosphorylation: Tyrosine kinase receptor Cad96Ca, Tyrosine-protein kinase (EC 2.7.10.2), non-membrane spanning protein tyrosine kinase activity; HCO3 conversion: Alpha carbonic anhydrase 7 (EC 4.2.1.1); and organic matrix: Bone morphogenetic protein 7, Bone morphogenetic protein I-like protein (Predicted protein) (Fragment).

Table 2 Biological processes (BP) and molecular functions (MF) implicated in heat and flow responses by GO enrichment analysis of DEGs. Adjusted P-values obtained from Fisher's exact test comparing the proportion of DEG associated with each process relative to their proportion in the reference transcriptome (FDR controlled at 10%).

Process	Category	Level	Genes	P-value
***Ex situ* ambient high flow/pooled heat stress comparison: Upregulated in heat stress**				
regulation of apoptotic process GO:0042981; GO:0043067; GO:0010941	BP	2	7	0.0005914
***Ex situ* ambient low flow/pooled heat stress comparison: Upregulated in heat stress**				
regulation of apoptotic process GO:0042981; GO:0043067; GO:0010941	BP	4	13	0.0000004
***Ex situ* ambient high flow/pooled heat stress comparison: Upregulated in high flow**				
cellular amino acid metabolic process GO:0006520	BP	2	9	0.0545168
fatty acid metabolic process GO:0006631	BP	2	4	0.0770426
nitrogen compound metabolic process GO:0006807	BP	2	53	0.0302465
cell adhesion GO:0007155; GO:0022610	BP	2	12	0.0793690
cell-matrix adhesion GO:0007160; GO:0031589	BP	4	7	0.0005073
oxoacid metabolic process GO:0019752; GO:0043436; GO:0006082	BP	4	14	0.0034884
carboxylic acid biosynthetic process GO:0046394; GO:0016053	BP	3	8	0.0043001
organonitrogen compound catabolic process GO:1901565	BP	2	5	0.0418247
cellular amino acid biosynthetic process GO:1901607; GO:0008652	BP	6	5	0.0556927
death domain binding GO:0070513	MF	4	4	0.0090267
***Ex situ* ambient high flow/low flow comparison: Upregulated in high flow**				
maturation of 5.8S rRNA GO:0000460	BP	2	5	0.0176854
rRNA metabolic process GO:0006364; GO:0016072	BP	7	12	0.0408308
mRNA splice site selection GO:0006376	BP	6	12	0.0500599
intracellular protein transport GO:0006886	BP	2	26	0.0099190
cellular amino acid catabolic process GO:0009063	BP	4	32	0.0068471

lipid catabolic process GO:0016042	BP	3	9	0.0575547
oxoacid metabolic process GO:0019752; GO:0043436; GO:0006082	BP	3	39	0.0002473
dicarboxylic acid metabolic process GO:0043648	BP	6	5	0.0176854
carboxylic acid biosynthetic process GO:0046394; GO:0016053	BP	2	14	0.0571403
folic acid-containing compound metabolic process GO:0046653; GO:0006760	BP	4	4	0.0541253
organonitrogen compound metabolic process GO:1901564	BP	3	51	0.0541253
alpha-amino acid metabolic process GO:1901605	BP	3	17	0.0068471
cellular amino acid biosynthetic process GO:1901607; GO:0008652	BP	2	11	0.0477510
actin binding GO:0003779	MF	2	36	0.0212948
protein binding GO:0005515	MF	2	82	0.0110194
cytoskeletal protein binding GO:0008092	MF	3	42	0.0285738
ion binding GO:0043167	MF	2	357	0.0057252
***In situ* heat stress high flow/low flow comparison: Upregulated in high flow**				
GTPase activity GO:0003924	MF	2	14	0.0001269
***In situ* bleaching high flow/low flow comparison: Upregulated in high flow**				
GTPase activity GO:0003924	MF	2	9	0.0526552

Ex situ and *in situ* heat stress candidate genes

All heat stress candidate genes were upregulated in the *ex situ* ambient high flow condition as compared to the ambient low flow condition (Fig. 5).

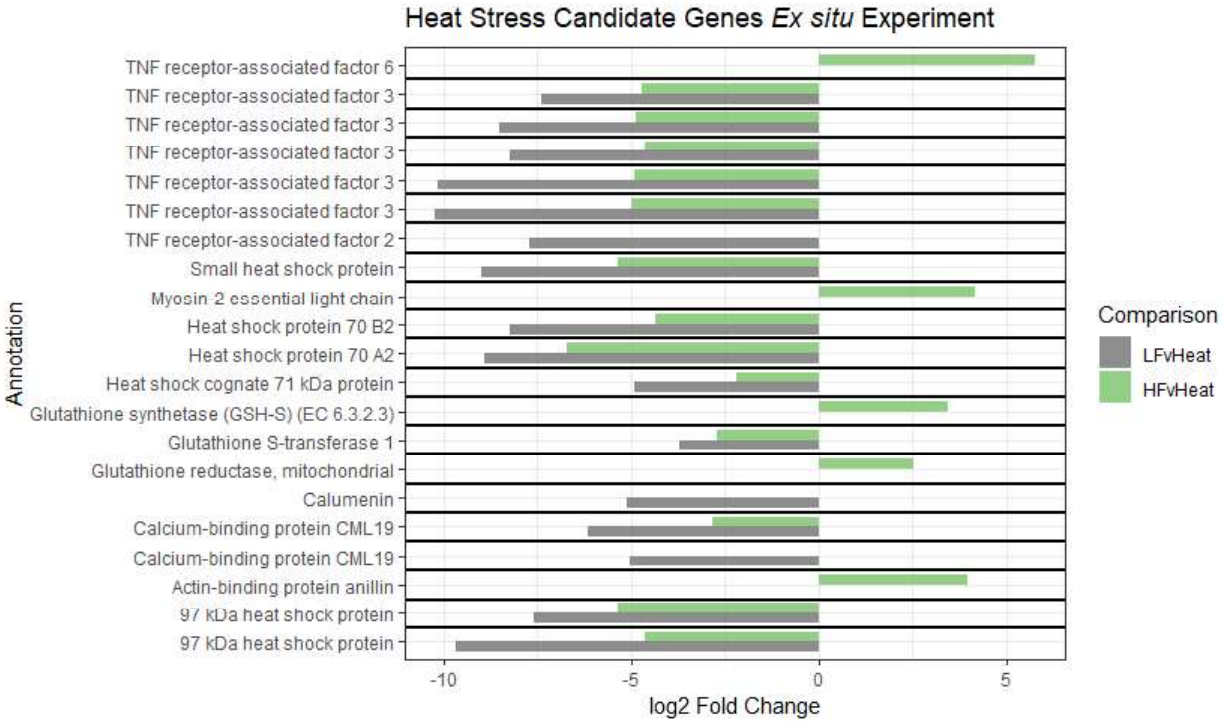

Figure 5 Comparisons examining fold change between each flow condition at ambient temperature and the pooled heat condition. Genes that have no bars were not significantly different for that flow condition at ambient temperature, as compared to the pooled heat condition. Abbreviations: LF= Low Flow, HF= High Flow.

In the *in situ* conditions, one heat shock associated transcript, sacsin, a DNAJ/Hsp40 protein that acts as an Hsp70 co-chaperone, was identified as upregulated in the high flow condition. Two transcripts were associated with the oxidative stress response, peroxidasin-like protein and glutathione peroxidase. One heat stress candidate was associated with calcium homeostasis, calmodulin-dependent protein kinase type II delta chain (Fig. 6).

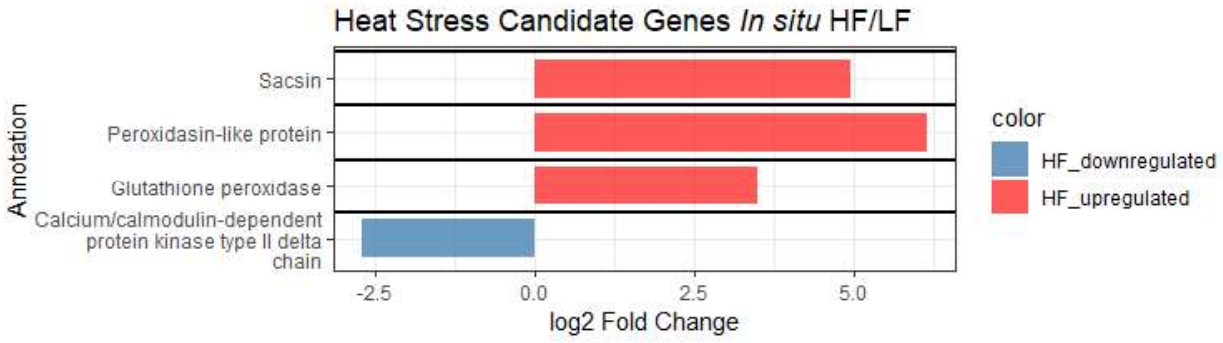

Figure 6 Comparison examining fold change between the high flow and low flow sites during the heat stress event.

Comparison of genes differentially expressed in both *ex situ* and *in situ* experiments

Genes that were differentially expressed in both the *ex situ* ambient high flow/low flow comparison and the *in situ* heat stress high flow/low flow comparison showed similar expression trends. Thirty-nine of these 50 genes generated annotations and the majority of these shared genes (95%) had the same expression pattern in their respective flow condition across experiments (Supplementary Fig. 6). These included genes involved in the immune system: hemicentin-1, NACHT, LRR and PYD domains-containing protein 3, and NLR family CARD domain-containing protein 4; the Krebs cycle: 2-oxoglutarate dehydrogenase, ATP-binding, ATP-dependent DNA helicase PIF1, vesicle/ion/macromolecule transport, BTB/POZ domain-containing protein KCTD7; and the heat shock-associated transcript, sacsin. In addition, an uncharacterized protein with the GO term regulation of apoptotic process [GO:0042981] was also upregulated in both high flow conditions.

Discussion

Water flow differences found *in situ* between sites and at the colony level at low flow site

At the low flow site, we found significant differences in water flow experienced by the coral colony branches, with flow being higher on the outside than the inside of coral colonies

(Fig. 3). High overall water flow close to the reef flat erodes this flow difference at the colony level (Fig. 3). Stagnant regions of low water flow can develop within a variety of different branching coral species (Chamberlain & Graus, 1975; Helmuth, Sebens, & Daniel, 1997; Kaandorp et al., 2003) from the blockage of flow produced by surrounding branches (Chang et al., 2009). We provide evidence for the development of stagnation within staghorn coral colonies. The disappearance of stagnant regions at the high flow site is likely due to wave-driven, oscillating flow closer to the reef margins. In general, oscillating flow can provide increased mass transfer over a coral colony (Falter, Atkinson, & Coimbra, 2005; Reidenbach et al., 2006) by changing fluid dynamics across a coral surface (Reidenbach, Koseff, & Monismith, 2007).

***Symbiodinium* diversity and abundance**

The lack of obvious clustering of *Symbiodinium* COI genes according to flow environment (Supplemental Fig. 3), demonstrates that the dominant *Symbiodinium* clade does not appear to differ in these discrete flow environments. This makes it unlikely that *Symbiodinium* clades played a role in the observed discrete host transcriptomic responses under different flow regimes discussed below.

We further found no significant difference in *Symbiodinium* counts based on colony position, which is counter intuitive given the flow differences we observed on the outside versus the inside of the low flow site colonies. In unidirectional flow there can be asymmetry in *Symbiodinium* distribution and skeleton mass between flow-facing sides of a colony and its downstream sides (Mass & Genin, 2008) induced by asymmetric nutrient concentrations and boundary layer thicknesses (Chang, Iaccarino, Elkins, Eaton, & Monismith, 2004). However, these findings could be exclusive to unidirectional flow (Mass & Genin, 2008), which differs

from the flow experienced by corals *in situ*. Additionally, we observed a lot of variation in *Symbiodinium* counts between colonies (Supplemental Fig. 7), which weakened our ability to detect potentially significant differences.

The slightly increased *Symbiodinium* abundance at the high flow site may reflect the presence of oscillatory flow-driven benefits. Wave energy can alter mass transfer in benthic organisms (Atkinson, Falter, & Hearn, 2001; Carpenter, Hackney, & Adey, 1991; Falter et al., 2005; Reidenbach et al., 2006) and increase rates of nitrogen fixation and ammonium uptake under oscillatory versus steady flow (Thomas & Cornelisen, 2003; Williams & Carpenter, 1988). This is also consistent with the *Acropora* cf. *pulchra* health surveys in 2015, which found higher survivorship at the sites closer to the reef crest (Raymundo et al., 2017)

Heat stress overwhelms transcriptomic response

Interestingly, we found that despite the various flow-regimes there was none or relatively little variation in the expression of genes in four of our comparisons. We found several flow-related comparisons contained none or a relatively small number of differentially expressed genes. Although considerable heat stress is sometimes necessary to detect differences in the transcriptome response in corals (Bellantuono et al., 2012), we speculate that the heat stress in our *ex situ* experiment was so severe it overwhelmed the transcriptomic response, drowning out any flow-driven differences that might have been observed (Table 1). It is important to note that the comparisons that showed no, or few, differentially expressed genes were those with the most severe heat stress (*ex situ* high flow/low flow comparison), the smallest flow differences (*in situ* outside/inside comparison), and where corals were the most stressed (*in situ* bleaching high flow site/ low flow site comparison). This suggests that to increase the signal driven by flow differences it would be necessary to either increase flow or decrease the stressor. Alternatively, a

flow-driven transcriptomic response might also be identified with a larger sample size. Our comparisons were made with a relatively low number of samples per condition (n=3), and the high variance in expression levels which was found in our study is commonly observed among wild coral colonies (Alvarez, Schrey, & Richards, 2015). The absence of differentially expressed genes could also have been due to low coverage or the relatively small fragments that were sequenced (75bp). Finally, we performed a stricter cut off for measuring differentially expressed genes compared to most expression studies. However, reanalysis with a more standard cutoff also resulted in a low number (relative to the other comparisons with significant differential gene expression) of differentially expressed genes (< 30) for these conditions, suggesting our strict cutoff was not the principal cause.

Heat stress induces apoptotic processes and expression of heat stress candidate genes

Consistent with prior work on the coral heat stress response (Kitchen & Weis, 2017), we found enrichment of apoptosis processes upregulated in the heat stress condition compared to both ambient flow conditions. The presence of this term could be a consequence of increased oxidative stress (Linley, Denyer, McDonnell, Simons, & Maillard, 2012) for which we found related genes also upregulated in the heat stress condition. Oxidative stress can lead to cell death and is a theorized major player in bleaching (Lesser, 1997).

The downregulation of general metabolism and growth terms that we observed in the heat stress condition is consistent with other transcriptomic studies on coral heat stress response (Barshis et al., 2013; Bay & Palumbi, 2015; Desalvo et al., 2008; Dixon et al., 2015; Kenkel, Meyer, & Matz, 2013; Meyer, Aglyamova, & Matz, 2011; Portune, Voolstra, Medina, & Szmant, 2010). In the ambient high flow versus heat stress comparison we found downregulation of metabolism and biosynthesis for amino acids, fatty acids, carboxylic acids, and nitrogen

compounds. Reduced metabolism in heat stressed samples could provide energy savings necessary for efficient stress responses (Gust et al., 2014). We also observed downregulation of cell adhesion and cell-matrix adhesion functional terms, also common in coral heat stress responses (Barshis et al., 2013) and suggestive of a disruption in Ca^{2+} homeostasis due to oxidative stress (Desalvo et al., 2008). Interestingly, greater downregulation in both cell adhesion and the reported metabolism terms were found between the heat stress condition and the ambient high flow regime than between the heat stress condition and the ambient low flow regime. This suggests metabolism was already suppressed in the low flow condition, perhaps due to decreased availability of metabolites.

We found upregulation of TRAF2 and TRAF3 genes and downregulation of TRAF6 in our heat stressed condition (Fig. 5). The Nf-kβ pathway is activated by a variety of different upstream receptors including tumor necrosis factor receptors (TNFRs) and TNF receptor-associated factors (TRAFs) (Shen, 2006), regulators of the apoptosis cascade (Quistad et al., 2014). Nf-kβ expression increases in bleached *Exaiptasia pallida*, and Mansfield et al. (2017) suggest Nf-kβ could control an immune pathway that needs to be suppressed for *Symbiodinium* uptake. TRAF2 and TRAF3 impact Nf-kβ activity similarly (Williams et al., 2018) and could possibly be inhibitors of the pathway (Carpentier & Beyaert, 1999; Shi et al., 2006; Yamamoto et al., 1998). However, many studies have shown a contrasting up-regulation of these genes in response to heat stress as well (Barshis et al., 2013; Desalvo et al., 2010; Palumbi, Barshis, Traylor-Knowles, & Bay, 2014; Thomas & Palumbi, 2017; Zhou et al., 2017). Similarly, studies examining TRAF6, which likely activates the Nf-kβ pathway (Wong et al., 1998), have found down-regulation after heat stress (Pinzón et al., 2015; van de Water, Lamb, van Oppen, Willis, & Bourne, 2015). The counterintuitive nature of the expression of TRAF as a regulator of the Nf-kβ

pathway could be explained by the influence of the other existing receptors, pathways, and effector mechanisms on this particular signaling pathway. Desalvo et al. (2010), observed up-regulation of both Nf-kβ and TRAF3 in the same heat stressed *A. palmata* colonies, which corroborates this theory.

Genes related to oxidative stress, an imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses (Betteridge, 2000), showed differential regulation in our *ex situ* heat stress study. Corals have an adaptive response to an alteration of ROS levels that involves an increased or decreased expression of antioxidant genes (Dalton et al., 1999). Antioxidants play an important role in improving photosynthetic activity and mitigating bleaching by scavenging ROS (Lesser, 1997). Glutathione-S-transferase (GST), which was differentially expressed in our study, detoxifies some of the secondary ROS that are produced when ROS reacts with cells (Veal et al., 2002). Upregulation of GST in our study could be stimulating a signaling pathway that integrates with other stress signals and might explain our observed upregulation in heat shock proteins (HSPs) (Oakley et al., 2017).

We found several Hsp70s that were differentially expressed (Fig. 5) in our heat stress experiment. Hsp70s are an important factor in protein folding and repair of stress-induced protein damage (Tavaria et al., 1996) and their presence is well-documented during all types of coral stress (Louis et al., 2017). Hsp70s' up-regulation moderates ROS damage, increases cell survival, and links the oxidative states of the mitochondria and endoplasmic reticulum (ER) (Oakley et al., 2017). Given their key function as molecular chaperones regulating and preserving protein structure and functionality, Hsp70s are amongst those molecular mediators which show up-regulation in response to heat stress (Louis et al., 2017). Since Hsp70s are regulated on a diel cycle (Levy et al., 2011), expression patterns may be affected by internal

processes related to the regulation of the metabolic machinery (Oakley et al., 2017). This could also explain the presence of Hsp70s in the *ex situ* ambient temperature high flow versus low flow comparison.

Some structural genes show differential expression under heat stress. Indeed, in our heat stress experiment we found differential expression of coral structure genes such as actin related genes and Myosin-2 essential light chain. Actin is sensitive to oxidative damage; the differential expression of structural genes in our heat stress experiments could be related to a buildup of ROS resulting in actin reorganization (Dalle-Donne, Rossi, Milzani, Di Simplicio, & Colombo, 2001). Actin genes are highly responsive to both acute (Desalvo et al., 2010; Rosic, Kaniewska, et al., 2014) and to long-term thermal stress (Desalvo et al., 2008; Maor-Landaw et al., 2014). Myosin-2 essential light chain was also downregulated by 1- to 2-fold after 1 day of gradual heat stress of ~32.7 °C (Desalvo et al., 2010).

Differential expression of calcium-homeostasis genes, including calumenin, in our heat stress experiment (Fig. 5) suggests disruption in calcium homeostasis. Calcium ions play a role in calcification, the *Symbiodinium*-coral host symbiosis (Rosic, Ling, et al., 2014), and as an important secondary messenger ion, inducing respiratory, the Krebs cycle activity, and host apoptosis (Rainbolt et al., 2014) which is one proposed mechanism of bleaching (Weis, 2008). Calcium signaling pathways (Rosic, Kaniewska, et al., 2014), genes related to calcium ion homeostasis (Barshis et al., 2013; Crowder et al., 2017; Maor-Landaw et al., 2014; Portune et al., 2010; Vidal-Dupiol et al., 2014), and calcium transporter genes (Kenkel et al., 2013; Meyer et al., 2011) are differentially expressed under heat stress in corals. Calumenin, a calcium binding protein which plays a chaperone-like role in alleviating endoplasmic reticulum stress and

subsequent apoptosis (Dunn et al., 2012), also shows differential expression in response to thermal stress (Ganot et al., 2011; Oakley et al., 2017).

Flow driven transcriptomic responses characterized by increased metabolism and growth GO terms and genes

Higher flow can lead to elevated energy expenditure for metabolic processes (Bruno & Edmunds, 1998; Dennison & Barnes, 1988; Patterson et al., 1991; Schutter et al., 2010). A higher flow regime can result in increased photosynthesis (Osinga et al., 2017), respiration (Finelli et al., 2006; Osinga et al., 2017; Schutter et al., 2010) growth rates (Jokiel, 1978; Schutter et al., 2010) and calcification (Dennisson & Barnes, 1988; Goldenheim & Edmunds, 2011; Smith, Barshis, & Birkeland, 2007). These health benefits are likely mediated by the thinner diffusive boundary layers under high flow which promotes the exchange of dissolved gasses and metabolites in coral (Mass et al., 2010).

For our *ex situ* high flow condition, we found enrichment of GO terms that would demonstrate elevated energy expenditure. Flow's impact on improved coral health could be functioning through the multitude of previously mentioned mechanisms, but broadly speaking we expected to see an upregulation in pathways that reflect increased energy availability. Amino acid, lipid and carboxylic acid biosynthesis were enriched for the genes upregulated under the ambient high flow condition compared to ambient low flow, suggesting a modification of metabolic rate. Downregulation in metabolism and biosynthesis of these compounds involved in translocation (Venn, Loram, & Douglas, 2008) under low flow suggests an unhealthy symbiotic relationship. Our finding that these and related terms were also downregulated under heat stress in our experiment, as well as other coral heat stress studies (Dixon et al., 2015; Thomas & Palumbi, 2017) would appear to corroborate this. We also found several groups of genes

involved in energy metabolism that were differentially expressed between the flow environments including genes involved in glycolysis, Krebs cycle, and oxidative phosphorylation. The expression of these relevant genes in the *in situ* high flow condition as well as in our *ex situ* experiment, suggests this metabolic upregulation is sustainable and present over the long term.

We see evidence for calcium homeostasis disruption and lower growth under low flow conditions for both *ex situ* and *in situ* flow comparisons, however this occurrence was more striking in the *ex situ* experiment. We found the subcategory calcium ion binding to be highly abundant in both high flow conditions (Supplementary Fig. 4-5), and this subcategory seems to be largely responsible for the enrichment of the parent GO term ion binding with the upregulated genes in the *ex situ* high flow condition. A disruption in calcium homeostasis, along with decreased metabolism under low flow, could be responsible for the observed enrichment of GO terms for cytoskeletal rearrangement, cell adhesion, ribosome biogenesis and metabolism (Desalvo et al., 2008) that we find downregulated under low flow compared to high flow conditions. Upregulation of ribosome biosynthesis GO terms: maturation of 5.8S rRNA and rRNA metabolic process, is an indicator of higher growth in the ambient high flow condition (Elser et al., 2003; Vrede, Dobberfuhl, Kooijman, & Elser, 2004). Additionally, in both high flow *ex situ* and *in situ* flow comparisons, we found upregulation of specific genes encoding for skeletal organic matrix proteins: the bone morphogenic protein superfamily and galaxin superfamily that are likely responsible for the upregulation of the extracellular matrix related GO terms.

It is possible that the increase of these growth related terms is a direct manifestation of the flow mediated building blocks for coral growth such as inorganic nutrients, organic food and calcium and carbonate ions (Osinga et al., 2011). Calcification can be diffusion limited by supply

of calcium ions which might have greater influx into coral tissues under high flow via the thinning of the DBL (Goreau, 1961), possibly caused by increased demand from elevated HCO_3^- (Herfort, Thake, & Taubner, 2008). Alternatively, increased calcification under high flow has often been attributed specifically to the increase of dark calcification, which is mediated by higher oxygen influx via thinning of the DBL under normally hypoxic conditions (Finelli et al., 2006). Dark calcification enhanced by oxygen may involve increased ATP production, subsequently promoting Ca^{2+} activity (Wijgerde, Silva, Scherders, van Bleijswijk, & Osinga, 2014), a necessary component of calcification (Tambutté et al., 2011).

Upregulation of the enriched functional term mRNA splice site selection in the *ex situ* high flow condition represents increased RNA processing and modification. RNA splicing function is associated with post transcriptional modification of RNA (Mata, Marguerat, & Bähler, 2005). MicroRNAs that can produce this modification are potentially involved in the heat stress response in corals (Gajigan & Conaco, 2016), changing symbiont types (Barfield, Aglyamova, Galina, Bay, & Matz, 2018), and maintaining cnidarian host-symbiont relationships (Baumgarten et al., 2017). MicroRNAs are critical for the maintenance of cellular homeostasis under stressful conditions (Ebert & Sharp, 2012). If flow is providing this increased post transcriptional response, it could be increasing the ability of corals in the high flow site to maintain normal functioning during the heat stress event and might explain the better health outcome of these corals in comparison to the low flow site.

High flow induces upregulation of stress GO terms and genes

Unexpectedly, we found upregulation of stress related GO terms and heat stress candidate genes under high flow compared to low flow conditions (Fig. 5-6 and Supplemental Fig. 4-5). The upregulation of heat stress candidate genes under high flow could be a function of increased

oxidative metabolism. As mentioned previously, increased water flow can have a variety of positive impacts on photosynthesis and respiration. The upregulation of growth and metabolism related terms and genes, as well as the apparently healthier fragments in the high flow site compared to the low flow site, suggest the corals experiencing high flow in our study are similarly impacted. Higher oxidative metabolism under our high flow conditions could be causing the upregulation of oxidative stress terms present in our analysis; the production of reactive oxygen species can occur in healthy cells under non-stressful conditions. H_2O_2 can be generated during photosynthesis and respiration (Apel & Hirt, 2004; Halliwell, Clement, & Long, 2000; Pamatmat, 1997), and has been observed to increase under high flow (Armoza-Zvuloni & Shaked, 2014) and increased heterotrophy (Rachel Armoza-Zvuloni, Schneider, Sher, & Shaked, 2016) in *Stylophora pistillata*. Additionally, reactive oxygen species production can occur in corals as a response to a bacterial community shift (Rachel Armoza-Zvuloni, Schneider, & Shaked, 2016), which is a biproduct of changing flow regimes (Lee et al., 2017). The upregulation of antioxidant related genes in this experiment does not seem to be negatively impacting the health of the coral, as high flow was associated with gross and increased *Symbiodinium* counts *in situ*. This is consistent with the finding that more thermally tolerant coral species can have higher reactive oxygen species concentrations compared to less resilient species during a bleaching event (Diaz et al., 2016). Additionally, the activation of these anti-oxidant related genes might also explain the upregulation of cell death terms in the high flow conditions as oxidative stress functions in modulating cell death pathways (Martindale & Holbrook, 2002).

Carpenter et al. (2010) found a similarly counter-intuitive response of flow on Hsp70. They found greater upregulation of Hsp70 and decreased photosynthesis on the upstream sides of

heat stressed *Montastrea annularis* compared to the downstream sides. They theorized the differences in stress protein expression might be caused by a detrimental fluid-mechanical pressure effect on the coral tissue induced by the unidirectional flow regime. However, the upregulation of several stress related genes and apoptotic processes also present in our *in situ* high flow condition suggests this was not just an artifact of our unidirectional flow experimental setup.

Reyes-Bermudez et al. (2009) found a similar trend to our study- upregulation of heat stress and oxidative stress genes, accompanied by enrichment of cytoskeleton restructuring functional terms without the presence of heat stress when they examined gene expression of coral planula. They attributed the presence of these terms to processes involved in morphogenesis. As coral morphology can alter according to the flow environment (Mass & Genin, 2008), it is possible that the upregulation of these terms is reflective of hydraulic stress inducing morphogenesis under high flow.

Front loading of stress genes under high flow

A number of our heat stress candidate genes were upregulated at elevated temperatures. The high flow ambient condition showed expression values more similar to the heat stress condition compared to the low flow ambient condition. This effect is known as "frontloading", or the upregulation of stress related genes at baseline (Barshis et al., 2013). Corals that demonstrate frontloading can be more resilient to heat stress events (Barshis et al., 2013; Hochochka & Somero, 2002). Both our study and Barshis et al. (2013) showed frontloading of heat stress proteins, antioxidant enzymes, genes involved in apoptosis regulation, innate immunity and cell adhesion. We also found that Hemicentin-1, a gene related to the buildup of extracellular matrix (Muscatine, Tambutte, & Allemand, 1997) that shows frontloading in resilient corals (Barshis et

al., 2013; Wright et al., 2017), was upregulated in both our *in situ* and *ex situ* high flow conditions. Frontloading theoretically promotes rapid rates of protein translation, and could dictate how well a coral acclimates to stressors (Gates & Edmunds, 1999) by allowing it to respond faster. The increase of post transcription related terms in the high flow condition might be representative of this faster protein turnover response, which is linked to acclimation under stressful conditions (Gates & Edmunds, 1999). We suggest, therefore, that the resilience against bleaching provided by higher water flow is in part due to this frontloading effect on genes implicated in the heat stress response.

Conclusion

We highlight flow-driven differences in the transcriptomic response of *A.* cf. *pulchra,* both in an experimental setting and under a natural heat stress event. We find evidence for molecular mechanisms that are consistent with current understanding of the beneficial impacts of water movement on coral health. Our data suggest these flow-driven responses can be drowned out by severe heat stress, but under natural heat stress conditions there still appears to be a flow-mediated transcriptomic response. We also find a frontloading effect of flow on stress genes, possibly demonstrating that higher flow mediates the ability of corals to respond to heat stress quickly, providing increased resilience.

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Author contributions

J.F., L. J. R, B.B., S.L., and A.G.F conceived of this project. J.F. performed field experiments. J.F. and S.L. performed laboratory experiments. J.F. and B.B. analyzed the data. J.F. created the figures. J.F. wrote the manuscript. All authors contributed editorially to the final manuscript

Data accessibility

All scripts and data for the study are available at https://github.com/bastodian/StaghornFlow

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CHAPTER 3

Supplemental Information for:

GOING WITH THE FLOW: CORAL COLONIES IN HIGH FLOW ENVIRONMENTS CAN BEAT THE HEAT

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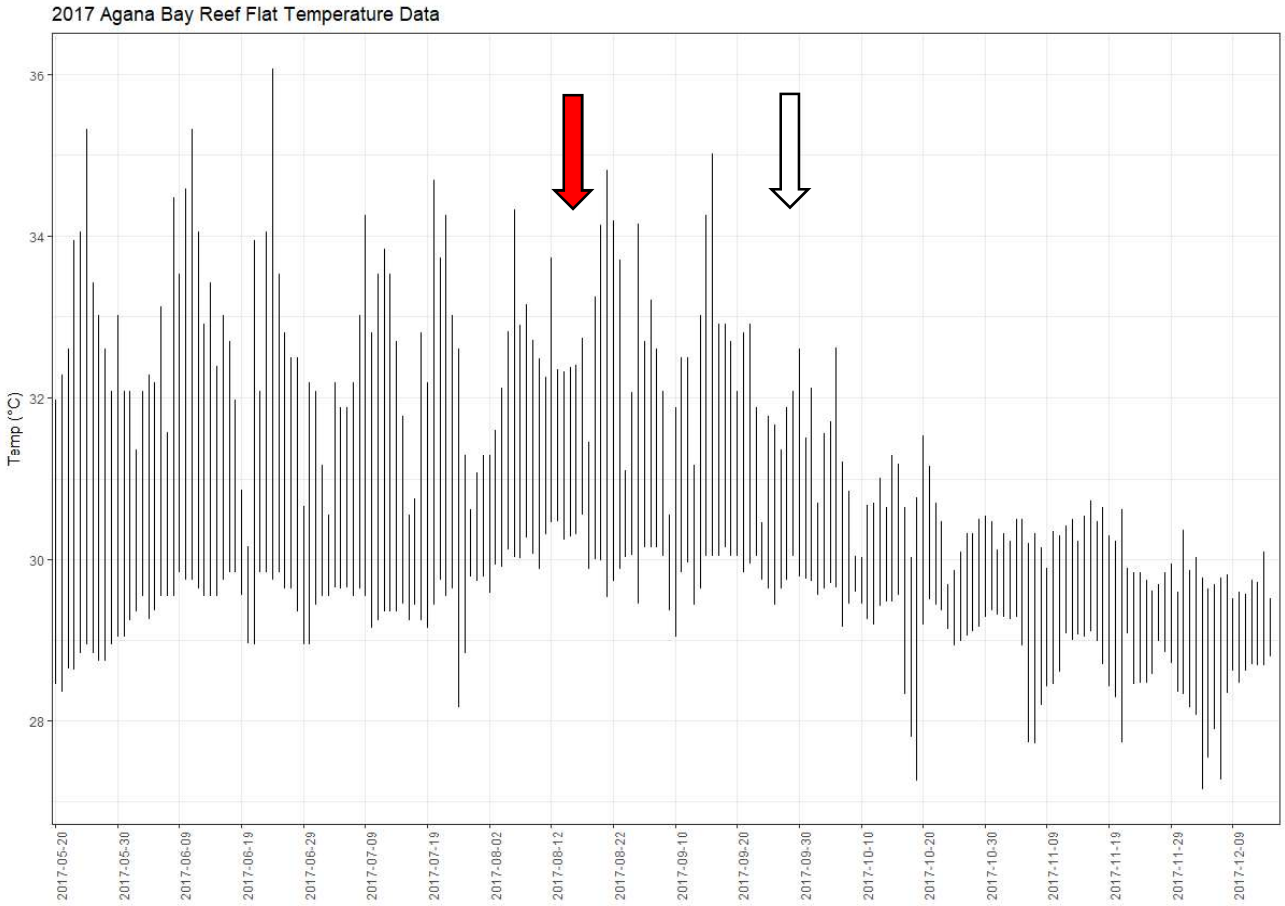
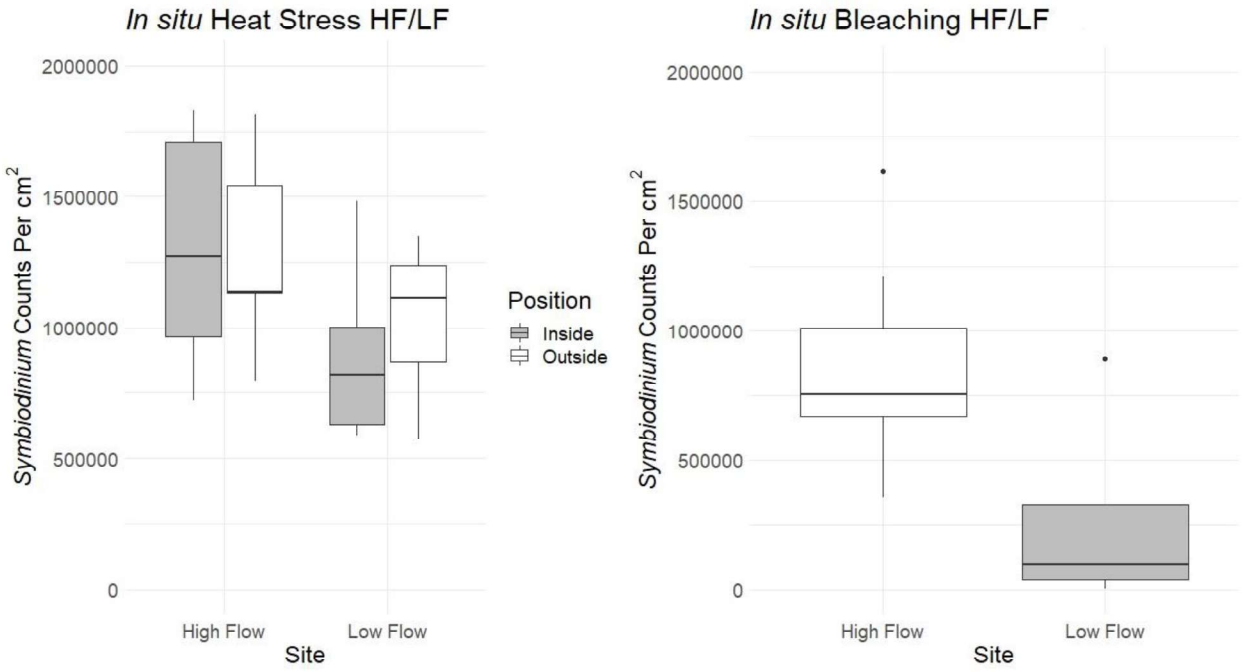
Supplemental Figures

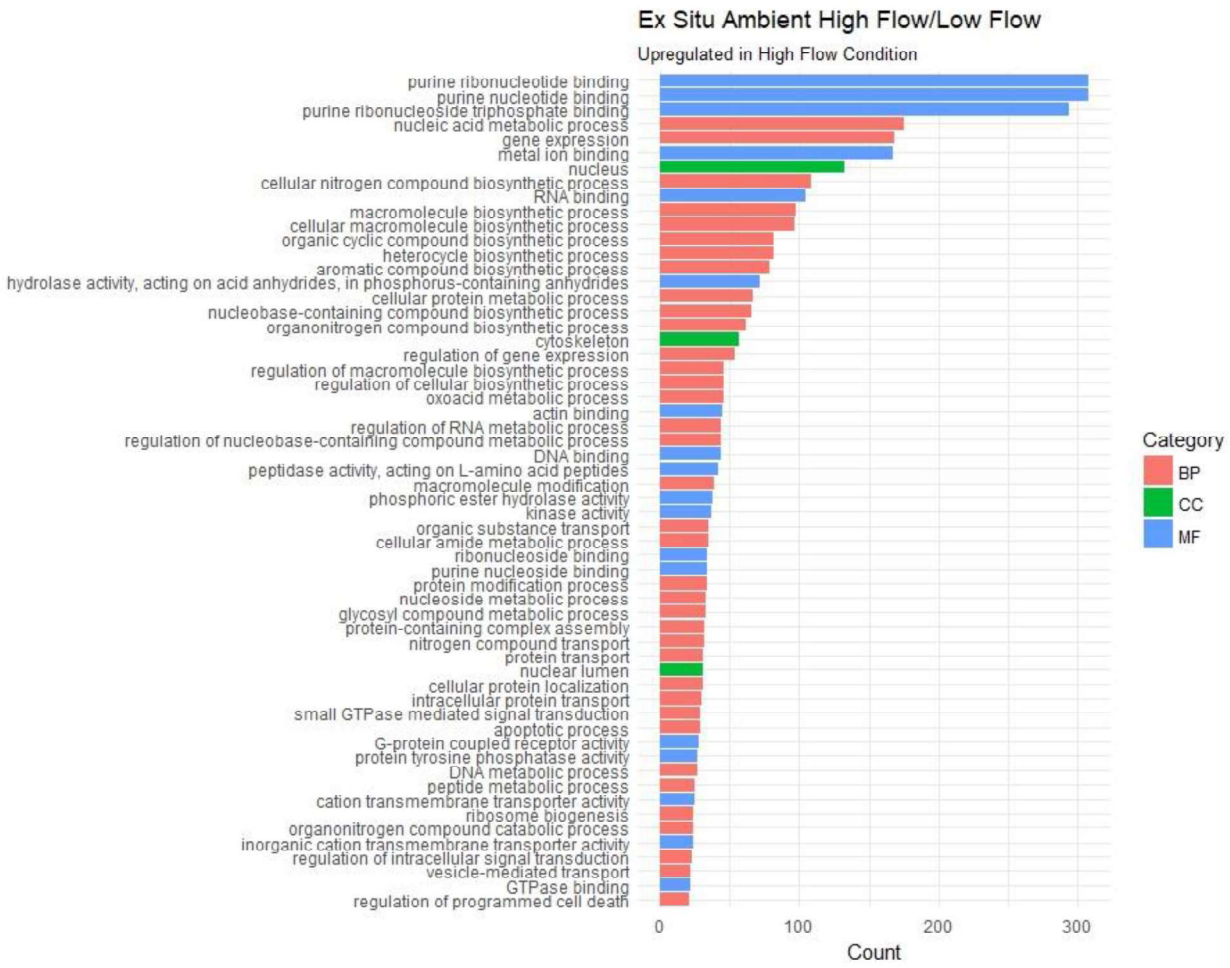


Supplemental Figure 1 Temperature data collected over 7 months at West Hagåtña Bay reef flat. Red arrow denotes initial sampling period at the tail end of the 2017 heat stress event before obvious bleaching occurred. White arrow denotes sampling period when colonies were showing a bleaching response.

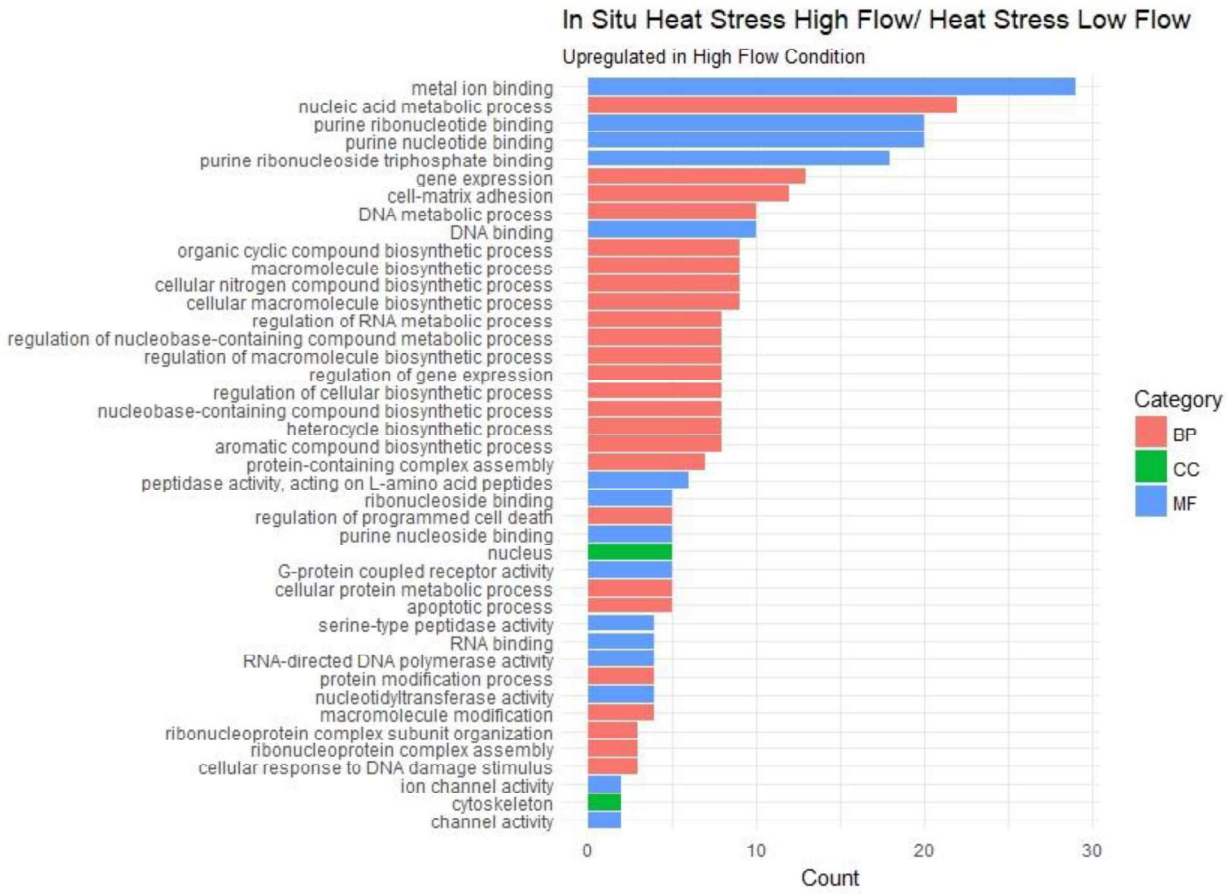


Supplemental Figure 2 A) Comparison of *Symbiodinium* counts per cm^2 in discrete flow environments during heat stress event for each branch (n=33). B) Comparison of *Symbiodinium* counts per cm^2 in discrete flow environments during heat stress event for each branch (n=12) in discrete flow environments during the bleaching event. Branches were collected only on the outside of colonies for this comparison. Average percent dissolution of gypsum per time measured on the inside and outside of colonies for both high flow and low flow site conditions. The box represents the inter-quartile range (IQR) between the upper and lower quartile. The whiskers maximally extend 1 times beyond the IQR.

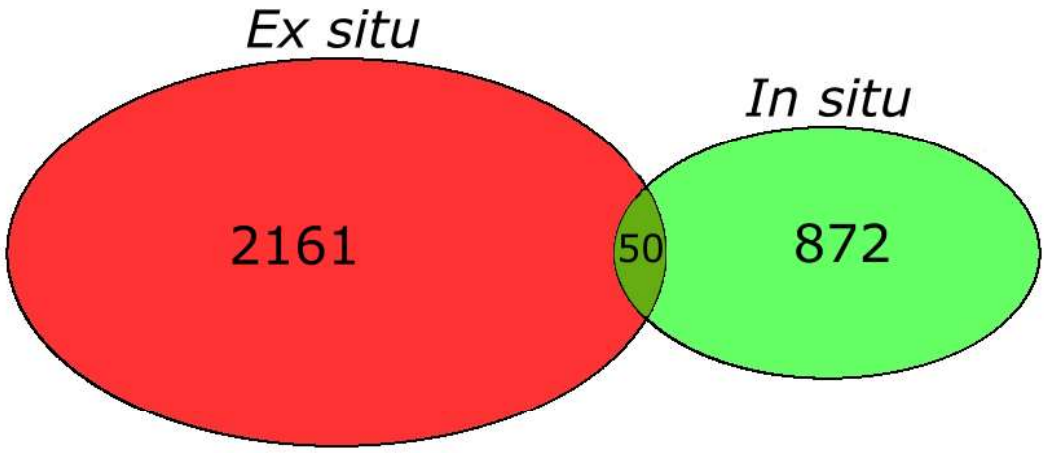
Supplemental Figure 3 Phylogenetic relationship of *Symbiodinium* communities.



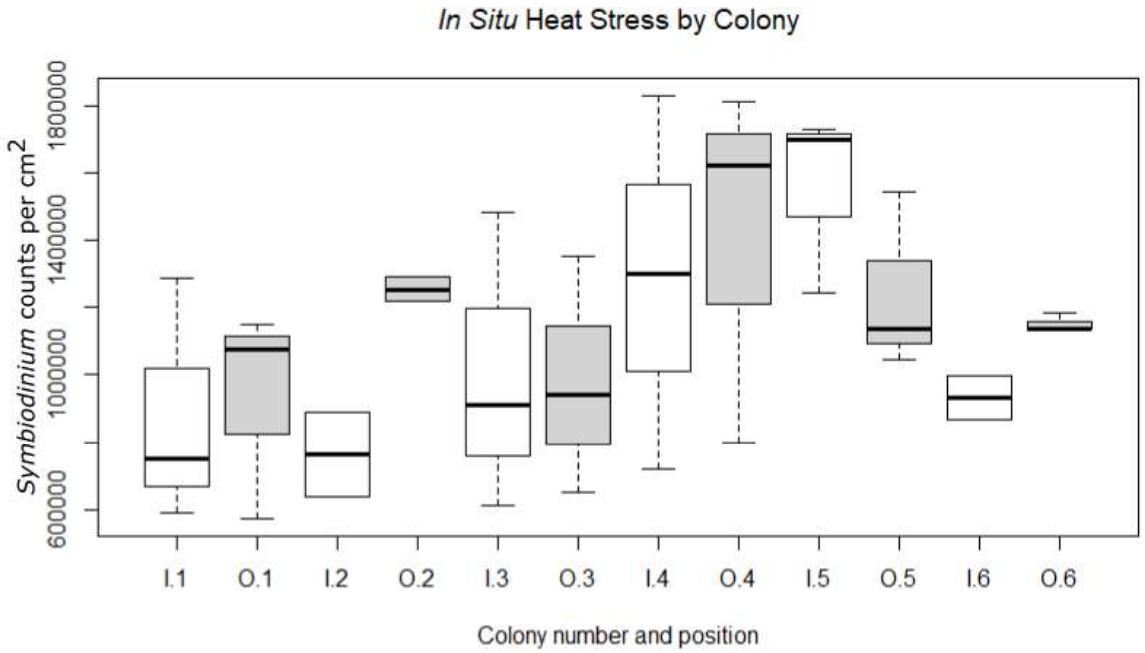
Supplemental Figure 4 Relative counts for the most abundant GO terms for biological processes (BP), molecular functions (MF) and cellular components (CC) for the significantly differentially expressed genes upregulated under high flow in the *ex situ* ambient high flow/low flow comparison. Plot based on Blast2GO analysis level 5 only.



Supplemental Figure 5 Relative counts for the most abundant GO terms for biological processes (BP), molecular functions (MF) and cellular components (CC) for the significantly differentially expressed genes upregulated under high flow in the *in situ* heat stress high flow/ heat stress low flow comparison.



Supplemental Figure 6 Venn-diagram showing overlap of genes that were differentially expressed in both the *ex situ* ambient high flow/low flow comparison (red) and the *in situ* heat stress high flow/low flow comparison (green). Figure was created using the R package "VennDiagram".



Supplemental Figure 7 Colony numbers 1-3 refer to low flow site, colony numbers 4-6 refer to high flow site. Outside of colony = O, inside of colony = I.

Supplemental Methods

Protocol for *Symbiodinium* count preparation

1. Remove the tissue using Air Brush with filtered sea water (FSW) or PB to 50ml falcon tube.

2. Dry the skeleton and save it for surface area measure (see protocol)

3. Measure the total volume of the sample.

4. Homogenize the tissue by vortexing for 30 seconds and breaking up chunks of tissue with 200µl pipette (or using electric homogenizer). *Make sure tissue is sufficiently broken up before moving to next step

5. Remove 1.5ml from falcon tube to a 2ml Eppendorf tube

6. Centrifuge for 5 minutes at 5000rpm at 4°C

7. Remove the supernatant (Host tissue) work with pellet (zoox)

8. Resuspend the zoox with 2ml FSW and homogenize (electric homogenizer or vortex for 30 seconds).

9. Centrifuge for 5 minutes at 5000rpm at 4°C

10. Remove the supernatant

11. Resuspend the zoox with 1ml FSW and homogenize (homogenize well! Vortex for 30 seconds or more if tissue is not broken up, use 200µl pipette as needed)

12. Centrifuge for 5 minutes at 5000rpm at 4°C

13. Remove the supernatant **thoroughly**

14. Resuspend the zoox with 1ml FSW and homogenize

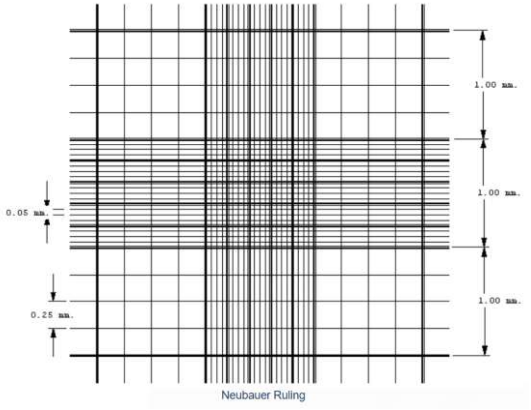
***Symbiodinium* count protocol**

1. **Vortex or shake the sample well so the sample is homogenous throughout. (At least 30 seconds, use 200µl pipette to break up bigger chunks). *Do this before injecting liquid under coverslip EVERY TIME.**

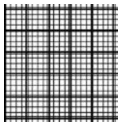
2. Cover the haemocytometer with a glass cover slip.

3. Pipette up 50 µl to inject under the coverslip onto the haemocytometer (Val can help you), enough liquid should be introduced so that the mirrored surface is just covered but do NOT flood the chamber/well in between the two surfaces.
4. Inject under the coverslip from both sides without flooding the chamber (There should be some liquid left in your pipette).

5. Place under the microscope and you will see the gridlines.



6. You are interested in the center most box



7. Count all zoox within this box (including zoox that are on the lines).
8. Switch to the mirror side of the hemocytometer and count the center most box as well.
9. Clean hemocytometer with 70% ethanol or RO water using **KIMWIPES**, being careful not to scratch the surface of the hemocytometer. Clean cover slip with alconox and warm water, use KIMWIPES to dry (**USE BLOTTING TECHNIQUE WHEN DRYING THEY BREAK EASILY).**
10. Repeat this 3 times for each sample (you should get a total of 6 numbers for each sample). ***Remember vortex between replicates.**
11. The number of zoox per branch is calculated as follows:
    a. Example: you count 23 zoox in center box, this is the amount per .1 cubic mm
    b. To get amount per mL multiply # of zoox in center most box (23) by 10^4
    c. To get zooxanthellae counts per branch:

$$\frac{(23 \times 10^4) \times Volume\ of\ dilution}{Surface\ Area\ of\ fragment}$$

(Volume of dilution is the amount of FSW used in step 1 when airbrushing fragment)

Special Remarks:

It's always good to leave spare of the sample in case anything goes wrong.
Protocol adapted from Mote Marine Laboratory SOP.