

**PLASTIC VERSUS FIXED INFLUENCES ON CORAL RESPONSE TO
FLUCTUATING ENVIRONMENTAL CONDITIONS: SOURCES OF CORAL
RESILIENCE AND STRESS TOLERANCE IN A CHANGING CLIMATE**

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ABSTRACT

The degree to which coral reef ecosystems will be impacted by global climate change depends on regional and local differences in corals' susceptibility and resilience to environmental stressors. This dissertation attempts to investigate the physiological basis behind the unique stress tolerance of a group of back reef corals that experience daily environmental extremes thought to cause bleaching and mortality in most corals. I examined the response of a suite of stress protein biomarkers in the common reef coral *Porites lobata* from multiple back reef sites and a neighboring forereef on Ofu and Olosega Islands, American Samoa, using field reciprocal transplant experiments (RTEs) and controlled laboratory manipulations. Additionally, I examined the genetic diversity of *P. lobata* hosts and their algal symbionts in both back reef and forereef populations to assess the degree of gene-flow between areas and the influence of symbiont genotype on coral response. RTEs performed during the southern summer revealed a combination of both fixed and environmental influence on biomarker response. Fixed influences were strongest for ubiquitin-conjugated proteins with consistently higher levels found in back reef versus forereef source colonies both pre and post transplant; suggesting a greater amount of or capacity to process irreversible protein denaturation in back reef corals. Algal endosymbiont populations were genetically indistinguishable between the forereef and back reef sites based on nuclear ribosomal DNA, while genetic analyses of nuclear microsatellite and mitochondrial loci of the coral host revealed both large scale (<100 km) and small scale (>1 km) population subdivision. Fixed and environmental influences

on biomarker responses were consistent over seasonal and long-term changes, with a significant reduction in ubiquitin-conjugates during winter versus summer seasons when daily extremes in back reef characteristics were lowest. Controlled acclimation experiments demonstrated that coral thermal tolerance increased following exposure to fluctuating versus stable temperatures, although back reef corals still exhibited elevated thermal tolerance compared to forereef corals despite acclimation treatments. These experiments demonstrate that exposure to environmental variability promotes coral stress tolerance at both the level of phenotypic plasticity as well as over longer time-scales through epigenetic changes to or evolutionary adaptation of the coral stress response.

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GENERAL INTRODUCTION

Coral reefs are the most diverse ecosystems in the marine environment (Paulay 1997). They provide key ecosystem goods and services vital to the economic and social stability of tropical maritime nations around the world (Moberg & Folke 1999). They are also areas with high primary productivity (Hatcher 1988), species diversity and sensitivity to disturbance. There is now no doubt that reefs are in a state of ecological decline and will continue to be impacted further by the increasing severity and frequency of environmental disturbance associated with global climate change (Anthony *et al.* 2008; Birkeland 2004; Carpenter *et al.* 2008; Hoegh-Guldberg *et al.* 2007; Wilkinson 2004). Projected increases in ocean temperatures in the 21st century are expected to exacerbate the stressors already affecting many reefs, resulting in additional coral bleaching and mortality on national (Pandolfi *et al.* 2005) and global scales (Hughes *et al.* 2005). Coral reef sensitivity to disturbance has led to estimates of severe damage in 30% of the World's, with up to 60% of reefs predicted to be lost in the next 30 years (Wilkinson 2004)

At the heart of the coral reef ecosystem lie the reef-building scleractinian (hard) corals. Reef corals consist of a symbiotic association between the cnidarian animal host and a group of symbiotic dinoflagellate algae of the genus *Symbiodinium* that are generically known as zooxanthellae (Birkeland 1997; Veron 2000). The cnidarian-algal symbiosis provides nutrients to the host and symbiont that are otherwise rare or unavailable in the surrounding environment (Birkeland 1997). The symbionts receive

shelter and metabolic nitrogenous wastes from the host and, in turn, provide the host with the photosynthetic products necessary for completion of numerous metabolic pathways (Jokiel 2004). "Coral Bleaching" occurs when this symbiosis becomes unfavorable due to changes in numerous environmental parameters acting alone or in combination (Jokiel 2004). The bleaching response consists of degradation and/or expulsion from the host of the zooxanthellae, the resulting loss of photosynthetic pigment leaves the coral tissue with a white or "bleached" appearance (Jokiel 2004). Bleaching is induced by increased sea surface temperatures (Jokiel & Coles 1990), low temperature (Gates *et al.* 1992), infection by bacteria (Kushmaro *et al.* 1996), altered spectral quality in the visible range (Kinzie *et al.* 1984), altered spectral quality in the ultraviolet range (Gleason 1993), low salinity (Coles 1978), increased irradiance (Lesser 1990), decreased irradiance (Franzisket 1970), sedimentation (Meehan 1997), and exposure to low tide, oil contamination, and toxic materials (Guzman 1991; Jones 1997). If bleached corals do not quickly re-establish their symbiotic relationship, they can suffer mortality. In observations of large-scale coral bleaching and mortality during warming events, there is some survival of scattered colonies, localized communities, and whole reef sections (Loya 2001). Bleached and unbleached corals are often found in very close proximity (Hughes *et al.* 2003). The survival of some corals and not others in the face of high temperatures may be attributed to the intrinsic ability of corals and zooxanthellae to acclimatize and adapt to varying conditions and exhibit increased thermotolerance (Coles & Brown 2003), or to extrinsic environmental factors that reduce the severity of the thermal stressors (Jokiel 2004; Smith 2006; Smith & Birkeland 2003, 2007).

The relationship between global climate change, increasing frequency of coral bleaching events, and higher atmospheric concentrations of greenhouse gases is now unquestionable (Glynn 1993; Hoegh-Guldberg 1999). Much of the debate about the future of coral reefs in this changing climate has centered on the differential abilities of corals to acclimatize and adapt to current and future rates of environmental change (Baird & Maynard 2008; Brown 1997; Coles & Brown 2003; Rowan 2004). Baird and Maynard (2008) argue an optimistic perspective that some fast-growing coral species possess the shorter generation times and necessary responses to environmental influence that could favor rapid adaptation to climate change. Hoegh-Guldberg and colleagues (2008) argue that rapidly growing species of corals are exceptions to the general rule and that most corals, which are generally slow growing, likely lack the qualities necessary to combat continued decline over the coming years.

While adaptation, in the evolutionary sense, involves fixed changes in organismal phenotype over multiple generations (Williams 1966), acclimatization refers to plastic, reversible phenotypic changes in response to natural environmental stresses that result in changes to an organisms tolerance levels (West-Eberhard 1989). Acclimatization mechanisms therefore, operate over shorter time-scales and within individual colonies and populations (DeWitt & Scheiner 2004; Miner *et al.* 2005; West-Eberhard 1989). For instance, a history of exposure to sublethal environmental stressors such as temperature (Coles 1978; Griffin *et al.* 2006) and irradiances (Brown *et al.* 2002b) has been shown to correlate with increased upper thermal tolerance thresholds in at least three reef-building coral species, and even within different parts of the same coral colony (Brown *et al.*

2002a). Yet while much attention has been focused on large scale environmental changes and their impacts, an emerging area of research aims to develop a better understanding of how small-scale heterogeneity in abiotic factors shapes physiological responses and tolerance limits (e.g., Anthony & Hoegh-Guldberg 2003; Helmuth & Hofmann 2001; Leichter *et al.* 2006; Smith *et al.* 2007).

Generally, it is thought that most corals exist at, or near, their upper thermal tolerance thresholds, with small increases of 1 to 2 °C above local mean summer maximum temperatures initiating a bleaching response (Coles *et al.* 1976; Jokiel 2004; Jokiel & Coles 1990). However, corals in some environments experience daily and seasonal fluctuations in temperature up to an order of magnitude greater. Corals have been found at temperatures up to 56 °C in the Lakshadweep Islands (Gardiner 1903) and at various other high temperatures in a multitude of other locations (see Coles & Brown 2003 for a detailed review). Craig and colleagues (2001) observed over 50 different coral species surviving repeated daily exposures of up to 34.5 °C in a back reef environment in American Samoa despite a mean summer temperature of 29.3 °C. These particular corals appeared to survive the repeated exposures without exhibiting signs of bleaching (Craig *et al.* 2001). The apparent contrast between the tolerance of some corals of extreme thermal events and the widespread, consistently observed patterns of limited upper thermal stress tolerances across many other locations provoke two particular questions: (i) are the corals found in these extreme thermal environments particularly unique, and (ii) what is the physiological impact of these daily and seasonal temperature exposures?

The back reef environment surveyed in Craig and colleagues (2001) provides an ideal setting in which to explore the relationship between short term environmental variability and coral physiological response. The fringing reefs of Ofu and Olosega islands in American Samoa (14°11'S, 169°40'W; Fig. 0.1) host a diverse community of approximately 85 shallow reef-building coral species, many of which are consistently exposed to seawater temperatures (Craig *et al.* 2001) and irradiances (Smith & Birkeland 2007) much higher than neighboring areas. The extreme conditions on Ofu are caused by a prominent reef crest that blocks circulation between the waters of the back reef and the open ocean, limiting mixing of back reef waters during midday low tides (Smith & Birkeland 2003). This limited mixing coupled with high midday solar irradiance produces large (up to 6°C) daily fluctuations in temperature (Smith & Birkeland 2003, 2007). In addition, frequency of large storm events, corresponding rainfall, and potential groundwater release sporadically cause drops in back reef salinity (up to 5 ppt; Smith *et al.* 2007).

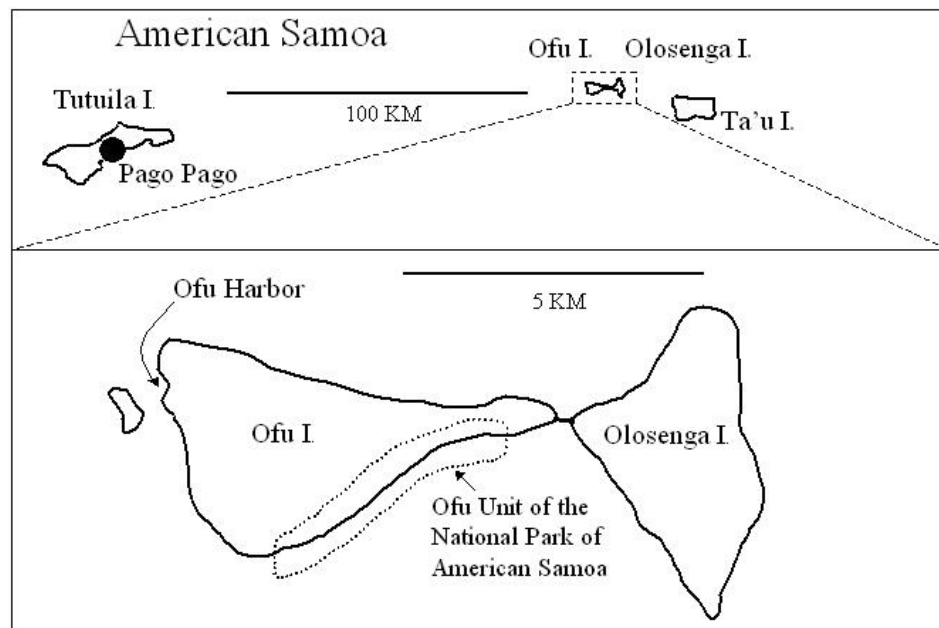


Figure 0.1 Locator map of study area

A series of back reef pools are found along the southern coast of Ofu Island (Fig. 0.2). These pools vary in physical conditions, water quality, and coral communities, thus providing useful study sites for comparative experiments. Two particular pools were used during the following studies: Pool 300 is ~1 m deep, known to be undergo larger daily fluctuations than many other pools in the Ofu back reef, and supports about 80 coral species; while Pool 400 is ~2 m deep, known to undergo more intermediate fluctuations than Pool 300, and supports approximately 105 coral species (Craig *et al.* 2001). In comparison, the shallow (<3m low tide depth) forereef of Olosega island represents a more typical, average reef environment. The lack of a prominent reef crest creates a more

constant flow regime, significantly lower temperature, irradiance, and salinity variability, and extremes of each (Smith *et al.* 2007; Smith & Birkeland 2003, 2007; summarized in Chapter 1, Figs. 1.2, Appendix 1.3-1.8).

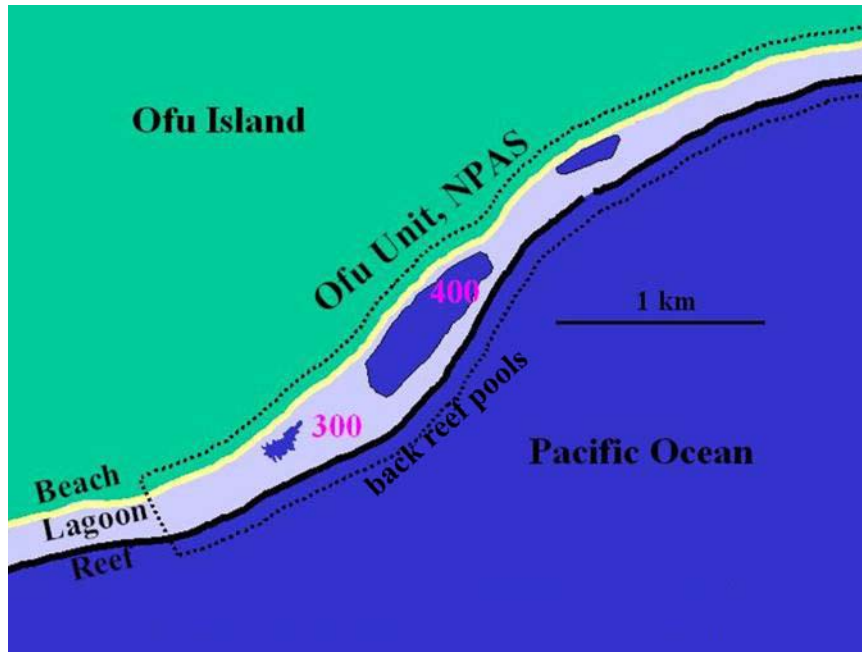


Figure 0.2 Map showing location of the back reef pools of Ofu island.

This natural gradient of environmental variability found in the nearshore environment of Ofu and Olosega provided the opportunity to explore a set of questions investigating coral physiological response to small-scale heterogeneity in environmental factors. *Porites lobata*, a massive, pan-Pacific reef building species, was chosen as the study species for a variety of characteristics. First, *P. lobata* is a dominant member of the back reef community on Ofu, with colonies frequently attaining large sizes (>2 m in diameter; personal observation). Second, *P. lobata* also occurs in the Hawaiian

archipelago (Fenner 2005), which provided easy access to fresh coral material during methodological development. Third, *P. lobata* is a gonochoric, broadcast-spawning species (Richmond & Hunter 1990) with a wide distribution range and is common across the Indo-Pacific (Veron 2000), assuring that the methods developed during this research could be applied in other settings to expand upon any interesting results.

Research Questions and Experimental Approaches

The following set of questions and experiments comprise the individual units of my dissertation research:

- Q₁: Do the different environmental conditions of the forereef and back reef cause intrinsic physiological differences between forereef and back reef populations of *P. lobata*?
- Q₂: If there are physiological differences between corals from the two environments, are they fixed (e.g., due to adaptation) or plastic (e.g., due to acclimatization) in nature?
- Q₃: Do the forereef and back reef populations of *P. lobata* differ in the genetic makeup of their *Symbiodinium* endosymbionts?
- Q₄: How well are the Cnidarian *P. lobata* populations genetically subdivided within American Samoa, are there patterns of genetic subdivision that correlate with habitat designations?

- Q₅: Are there seasonal or temporal differences in coral physiological responses that correlate with temporal and seasonal variability in environmental conditions?
- Q₆: What is the long-term acclimatization potential of forereef and back reef *P. lobata* populations?
- Q₇: Is temperature one of the driving variables behind observed physiological response differences between back reef and forereef populations of *P. lobata*?

To address Q₁, Q₂, and Q₃, a Reciprocal Transplant Experiment (RTE) was performed between one of the back reef pools (Pool 400) and the neighboring Sili forereef during the southern summer season when back reef environmental variability is greatest (Chapter 1). The RTE framework allows the investigation of whether or not the source of phenotypic variability is likely due to environmental influences (e.g., phenotypic plasticity) or possible epigenetic (e.g., developmental plasticity) or genetic (e.g., adaptive) influences (DeWitt & Scheiner 2004; Schluter 2000). A previous study had identified both environmental and epigenetic/genetic influence on the growth physiology of *P. lobata* from these two populations (Smith *et al.* 2007). The RTE in Chapter 1 attempts to determine if these physiological differences are apparent at the molecular level by investigating a suite of stress biomarker proteins within coral tissue samples. The specific roles and functions of these proteins are discussed in detail within Chapter 1.

Many scleractinian corals are relatively flexible in the types of symbionts they can contain (Baker 2001; Rowan & Knowlton 1995; Rowan *et al.* 1997b) although one type is usually dominant in any given species and environment (LaJeunesse *et al.* 2003;

Rowan & Powers 1991). Since symbiont type has been shown to affect the physiological response of the coral holobiont (e.g., Abrego *et al.* 2008; Little *et al.* 2004), genetic characterization of the algal endosymbiont communities of these two coral populations was also performed to address Q_3 . Additionally, to determine the possibility that the coral hosts were adapted to their respective locations, genetic characterization of host tissues was performed for samples from back reef and forereef populations as well.

Based on the results of significant genetic differentiation of coral hosts between forereef and back reef populations (Chapter 1), a wider-scale survey of *P. lobata* populations was performed across multiple forereef and back reef sites on the islands of Ofu, Olosega, and Tutuila in an attempt to address Q_4 . Coral connectivity studies have advanced significantly in recent years with the development of highly polymorphic genetic markers such as microsatellites for a number of different species (Magalon *et al.* 2004; Miller & Howard 2004; Underwood *et al.* 2006). Most coral reef animals disperse via a pelagic larval life stage, a process fundamental to shaping patterns of marine biodiversity, evolution, and gene flow. Historically, eggs and larvae were considered passive drifters with dispersal distances and gene flow assessments based on estimates of maximum pelagic larval duration (PLD) and prevailing oceanographic currents (Scheltema 1986). However, a growing body of evidence using recently developed methodologies suggests that many mechanisms besides larval duration may limit dispersal, thus increasing local settlement and decreasing population connectivity (Mora & Sale 2002; Rocha *et al.* 2007; Taylor & Hellberg 2003). Reef-building corals are no exception, and small-scale population structure at within-island and within-reef spatial

scales has been found for a number of coral species and reproductive strategies (Ayre & Hughes 2000; Magalon *et al.* 2005; Miller & Ayre 2008; Severance & Karl 2006; Sherman *et al.* 2006; Underwood *et al.* 2007, 2009; Whitaker 2006). Selective forces are thought to be among the factors that influence this maintenance of small-scale population subdivision, thus Chapter 2 was designed to assess the degree of population connectivity between *P. lobata* in different locations and habitat types.

To address Q_5 and Q_6 , two additional RTEs were performed, one during the southern winter season when forereef and back reef environmental variability are more similar in nature, and one for an extended (261 day) time period to assess long term acclimatization potential in these coral populations (Chapter 3). An additional temporal profiling of biomarker response was performed on a set of back reef corals in Pool 400 to examine the extent of natural variation in these markers over a 72 hr period of large midday low tides during the southern summer (Chapter 3). Seasonal and temporal variability in stress protein levels has been observed in intertidal marine mussels (Dutton & Hofmann 2008; Hofmann & Somero 1995, 1996b; Roberts *et al.* 1997), yet the degree of variability in corals is unknown. A review by van Oppen and Gates (2006) found a surprising degree of inconsistency in biomarker response between two studies of the same corals performed in different years; raising the question of what degree of resolution these markers provide in field collected corals. Hence, accurate characterization of seasonal and temporal differences in marker response is critical for the successful interpretation of results using these tools.

Lastly, to address Q_7 , coral samples from two back reef sites (Pool 300 and 400) and one forereef site (Sili) were transported to an experimental aquarium system at the Romberg Tiburon Center for Environmental Studies in Tiburon, CA for a set of temperature manipulations and acclimation experiments (Chapter 4). The acclimation treatments were designed in an attempt to recreate the natural daily thermal fluctuations found in the Ofu back reef environment and their effect on coral acclimation potential and upper thermal tolerance limits. Teasing apart the specific influences of the multitude of environmental parameters that differ between the forereef and back reef habitats requires strict evaluation of each variable individually. Acclimation or acclimatization to fluctuating temperatures has been shown to elicit increased growth responses relative to constant temperatures in turtles (Les *et al.* 2009), trout (Spigarelli *et al.* 1982), and a marine alga (Wang *et al.* 2007). Fluctuating temperature exposure also increased thermal tolerance limits in pupfish (Feldmeth *et al.* 1974), freshwater perch (Feminella & Matthews 1984), and black abalone (Hines *et al.* 1980). Significant changes in cellular growth regulation, molecular chaperone transcription, and transcriptome composition have also been observed in the annual killifish (Podrabsky & Somero 2004) when exposed to fluctuating thermal conditions. Yet the only study, to date, examining fluctuating versus constant temperature exposure in corals found significant reductions in photosynthetic yield, symbiont density and respiration in corals exposed to fluctuating versus steady temperature conditions (Putnam & Edmunds 2009). However, Putnam and Edmunds (2009) mimicked fluctuating temperatures by exposing corals to high and low

separate temperature tanks for consecutive 12 hr durations instead of gradual temperature variation within a single tank.

Due to the complicated nature of the RTE design and consecutive conclusions, a brief theoretical framework is outlined here. Transplant groups are designated as either native transplants (taken from their source environment and then placed back in their original source environment) or cross transplants (taken from one source environment and placed in a foreign environment). Comparison between native and cross transplant groups examines variability by transplant destination (back reef native vs. back reef cross, forereef native vs. forereef cross) and by source colony origin (back reef native vs. forereef cross, forereef native vs. back reef cross; Smith *et al.* 2007). Within the RTE framework, variability by transplant destination is indicative of environmental influence on response (i.e., phenotypic plasticity), while variability by source colony origin indicates potential genetic or epigenetic influence on the response variable (DeWitt & Scheiner 2004; Schluter 2000; Smith *et al.* 2007). A reaction norm can be used to illustrate the interplay of influences on physiological responses of native (N) and cross (C) transplants in multiple environments. Five theoretical reaction norms are given in Fig. 0.3 to illustrate potential outcomes of an RTE.

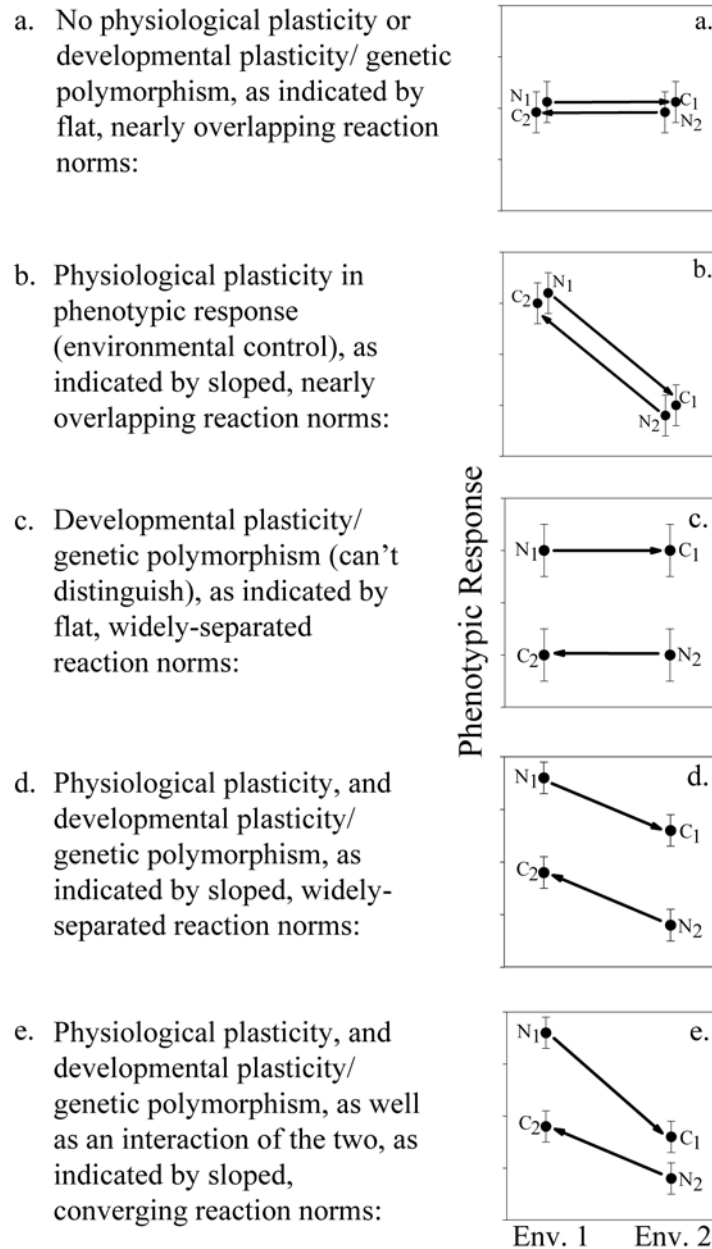


Figure 0.3 Theoretical reaction norms illustrating the potential response patterns of a hypothetical variable due to physiological plasticity, developmental plasticity/genetic polymorphism or a combination thereof. (adapted from (Smith 2008) and used with permission).

A drawback associated with RTE reaction norms is the inability to distinguish between true genetic (adaptive) and epigenetic (developmental plasticity) influences on phenotypic response (DeWitt & Scheiner 2004; Schluter 2000). Developmental plasticity is advantageous when the current environment of an organism is a good predictor of future environmental conditions (Meyers & Bull 2002). On the other hand, adaptive theory suggests that ecological factors predicted to promote local adaptation include limited gene flow between populations and strong selection against genotypes adapted to other habitats with more moderate selection against intermediate genotypes (Kawecki & Ebert 2004). Significant population subdivision therefore, raises the possibility that observed limitations to phenotypic response could also be due to local adaptation as opposed to developmental plasticity (Kawecki & Ebert 2004).

All of the following chapters were written with the intent to submit each one as an individual manuscript; hence the format of each chapter includes all necessary descriptions, sections and components of an individual unit.

CHAPTER ONE:

PROTEIN EXPRESSION AND GENETIC STRUCTURE OF THE CORAL *PORITES* *LOBATA* IN AN ENVIRONMENTALLY EXTREME SAMOAN BACK REEF: GENOTYPIC VS. ENVIRONMENTAL INFLUENCES

Abstract

The degree to which coral reef ecosystems will be impacted by global climate change depends on regional and local differences in corals' susceptibility and resilience to environmental stressors. Here, we present data from a reciprocal transplant experiment (RTE) using the common reef building coral *Porites lobata* between a highly fluctuating back reef environment that reaches daily environmental extremes typically considered stressful, and a more stable, neighboring forereef. Protein biomarker analyses assessing physiological contributions to stress resistance showed evidence for both fixed and environmental influence on biomarker response. Fixed influences were strongest for ubiquitin-conjugated proteins with consistently higher levels found in back reef source colonies both pre and post transplant when compared to their forereef conspecifics. Additionally, genetic comparisons of back reef and forereef populations revealed significant population structure in both the nuclear ribosomal and mitochondrial genomes of the coral host ($F_{ST}=0.146$ $p<0.0001$, $F_{ST}=0.335$ $p<0.0001$ for rDNA and mtDNA respectively), while algal endosymbiont populations were genetically indistinguishable between the two sites. We propose that the genotype of the coral host may drive limitations to the physiological responses of these corals when faced with new environmental conditions. This result is important in understanding genotypic and environmental interactions in the coral algal symbiosis and how coral may respond to future environmental changes.

Introduction

Although climate warming scenarios (IPCC 2007) predict the most dramatic warming at high latitudes, even a small amount of warming at low latitudes may have large impacts on marine organisms as many tropical marine organisms presently experience temperatures at or near their heat tolerance thresholds (Carpenter *et al.* 2008; Stillman 2003; Tomanek 2008). Climate anomalies (such as the El Niño Southern Oscillation) increasing in severity and frequency due to global climate change (IPCC 2007), in combination with rising levels of ocean acidification are further compromising coral reef ecosystems (Hoegh-Guldberg *et al.* 2007). While coral reefs are distributed throughout the warm tropical and sub-tropical oceans and seas, most corals are found within 1°C to 2°C of their upper limits of temperature tolerance during the summer months (Jokiel 2004). Consequently, conspecific corals from different geographic regions can have very different heat tolerance limits and temperatures at which bleaching (the functional loss of their endosymbiotic algae) occurs. These regional differences in bleaching susceptibility are generally attributed to host and symbiont adaptation to local thermal environments (Hughes *et al.* 2003).

During mass coral bleaching events induced by extreme environmental temperatures and irradiances, there is generally survival of scattered colonies, specific communities, or whole reef sections (Sotka & Thacker 2005; West & Salm 2003). The mechanisms underlying continued survival of some corals and not others have been the focus of considerable research over the past few decades (Brown *et al.* 2002a; Grottoli *et al.* 2006; Lesser 1997; Rowan *et al.* 1997a). In many cases, results have been conflicting

and questions remain as to what degree host or symbiont-specific mechanisms contribute to increased resilience of the entire holobiont (defined here as the collective unit of the coral animal, endosymbiotic dinoflagellates of the genus *Symbiodinium*, and associated community of microorganisms).

Many of the proposed mechanisms for differences in holobiont stress tolerance and resilience can be grouped into three broad categories. First, it has been shown that different genotypes of the algal endosymbiont, *Symbiodinium*, significantly affect coral thermal susceptibility (Baker 2001; Little *et al.* 2004; Rowan *et al.* 1997a); with thermally tolerant symbiont types found in corals living in higher temperature and post-disturbance environments (Baker *et al.* 2004; Rowan 2004; van Oppen *et al.* 2005). Second, host genetic background has been shown to alter such physiological responses as upper thermal tolerance limits across taxonomic and latitudinal gradients (Smith-Keune & van Oppen 2006; Ulstrup *et al.* 2006), symbiont type effect on holobiont physiology (Abrego *et al.* 2008), and capacity for heterotrophic feeding during bleaching and recovery (Grottoli *et al.* 2006). Third, it is thought that the history of the thermal conditions a given coral has experienced may drive acclimatization responses in the form of increased capacity for cellular stress defense and thermotolerance (Brown *et al.* 2002a; Brown *et al.* 2000, 2002b). In this study, we use an integrative approach to address the contributions of these three factors (symbiont and host genotypes, and thermal experience) to the stress resistance of corals from habitats with different thermal conditions that are extreme compared to those of most extant reefs (Coles 1997), but

which could represent “normal” thermal conditions of future reefs under projected climate warming scenarios (IPCC 2007).

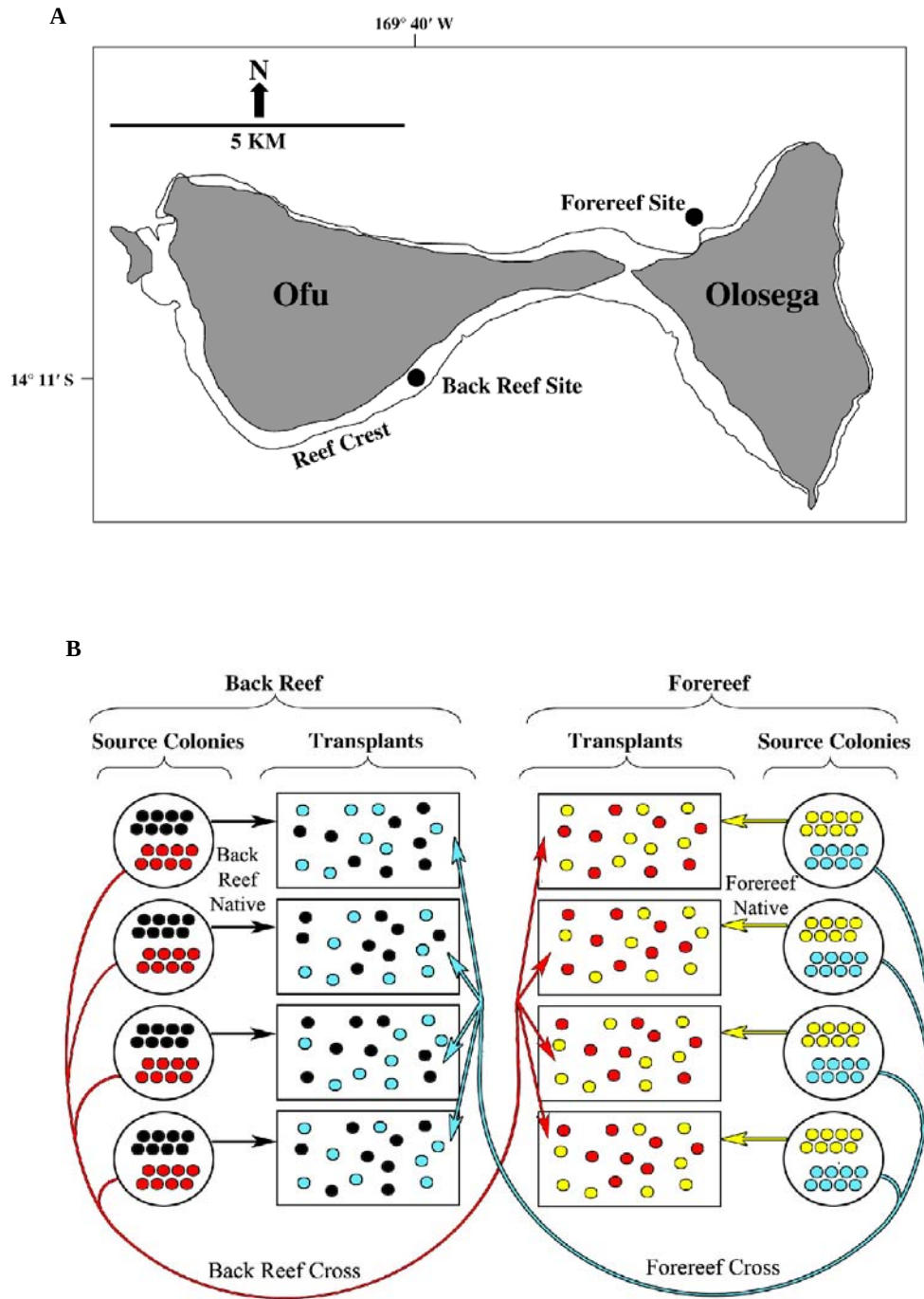


Fig. 1.1 (A) Map of Ofu and Olosega islands, American Samoa, showing source colony collection and transplant sites. **(B)** RTE transplant schematic illustrating experimental design. The RTE consisted of four replicate source colonies in each site, back reef and forereef, designated by black circles. Eight replicate cores from each source colony were designated for cross (red and blue for back reef and forereef respectively) transplantation to the opposite environment, and native (black and yellow for back reef and forereef respectively) transplantation to the same environment. Two cores per source colony per environment were sampled at time 0 and once daily at 13:45 for four consecutive days following a 24 hour acclimation period.

Our study was conducted on Ofu and Olosega islands in American Samoa (14°11'S, 169°40'W; Fig 1.1A). These islands host diverse communities of ~85 shallow reef-building coral species, many of which are consistently exposed to seawater temperatures (Craig *et al.* 2001) and irradiances (Smith & Birkeland 2007) much higher than neighboring areas. The extreme conditions on Ofu are caused by a prominent reef crest that blocks circulation between the waters of the back reef and the open ocean, limiting mixing of back reef waters during midday low tides (Smith & Birkeland 2003). This limited mixing coupled with high midday solar irradiance produces large (up to 6 °C) daily fluctuations in temperature (Fig. 1.2, Appendix 1.3; Smith & Birkeland 2003, 2007). In addition, frequency of large storm events, corresponding rainfall, and potential groundwater release sporadically cause drops in back reef salinity (up to 5 ppt; Appendix 1.4; Smith *et al.* 2007). In comparison, the shallow (<3m low tide depth) forereef of

Olosega island represents a more typical, average reef environment with a more constant flow regime, significantly lower temperature, irradiance, and salinity variability, and extremes of each (Fig. 1.1, Appendix 1.3-1.8; Smith *et al.* 2007; Smith & Birkeland 2003, 2007).

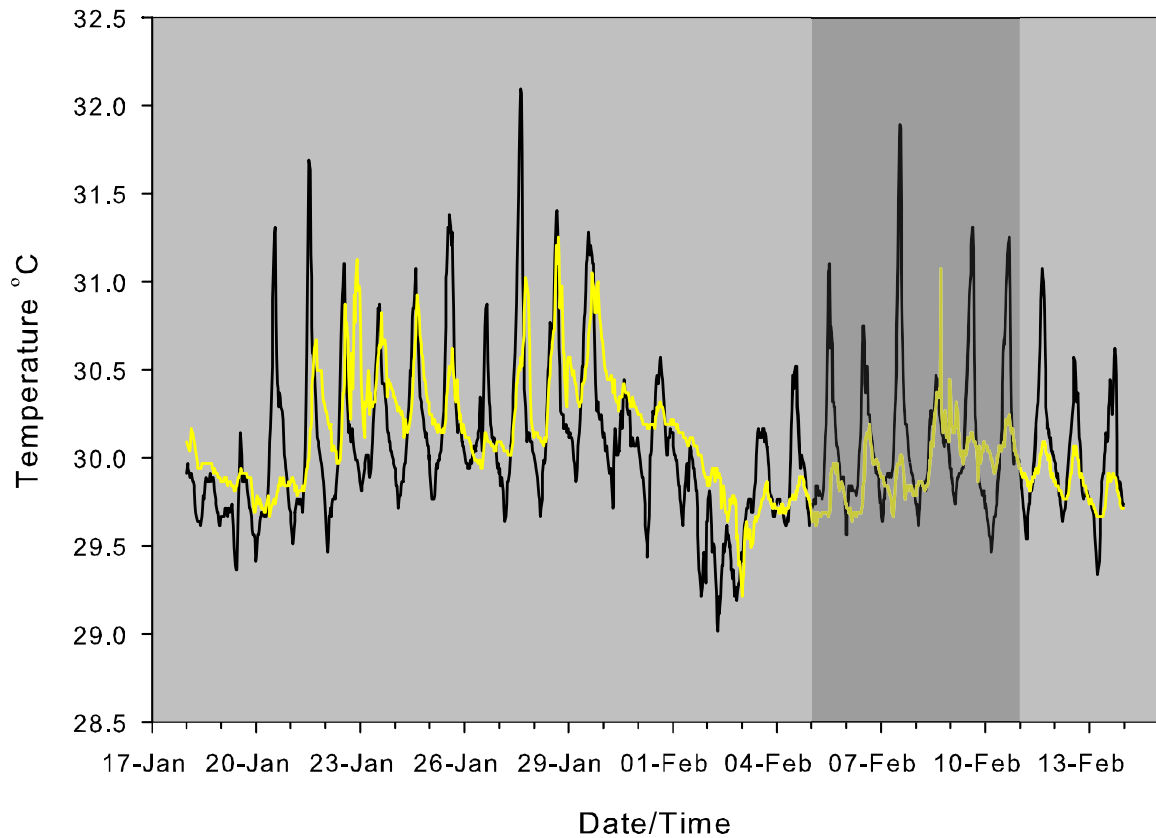


Fig. 1.2 Temperatures (°C) in the back reef (black line) and forereef (yellow line) recorded every 30 minutes continuously for 27 days surrounding the RTE using HOBO Water Temp Pro[®] temperature loggers (Onset Computer Corp.)

Using the common reef-building coral *Porites lobata*, we investigated (i) coral holobiont (cnidarian host and *Symbiodinium*) protein expression, (ii) *Symbiodinium* genetic identity (referred to herein as symbiont), and (iii) cnidarian host population

structure in a reciprocal transplant experiment (RTE) between a thermally extreme back reef on Ofu island and the more stable, neighboring forereef on Olosega. Our study revealed evidence for genotypic influence on all four of the biomarkers under investigation which correlated with differences in coral host population genetic structure. Genetic differentiation of symbionts in corals from the two study locations was not observed, thus these results suggest a host genetic influence on response differences between corals from contrasting thermal environments.

Materials and Methods

Source Colony Selection and Environmental Monitoring

Individual *Porites lobata* source colonies were initially identified based on surface morphology and skeletal characteristics (Veron 2000). Additionally, all sequences obtained from the host genetic study were integrated into a comprehensive *Porites* phylogeny (Forsman *et al.* 2009) and all shown to be contained within Clade I, a monophyletic clade containing known *P. lobata* genotypes from other sites across the Pacific.

Temperatures at 1 m low tide depth in the Ofu back reef have been continuously recorded every 30 min since 1999 with Tidbit[®] (1999-2003) or Water Temp Pro[®] (2004-present) temperature loggers (Onset Computer Corp., Pocasset, MA). Forereef temperatures were recorded every 30 min with Water Temp Pro loggers during the experimental period (18-Jan-05 to 13-Feb-05), and continuously since April, 2006 for long-term comparisons. Water quality data loggers, Sonde 6600 (YSI inc, Yellow

Springs, OH), measuring salinity, conductivity, and dissolved oxygen were deployed in both fore-reef and back-reef sites during 12 days in March 2006. A 48hr profile of nutrient dynamics in the water column was also performed during March 2006. Additional water quality measurements were taken in the back-reef during a previous study (Smith & Birkeland 2003).

Sample Collection and Reciprocal Transplant Experiment (RTE)

A pneumatic drill and hole saw attachment was used to remove 18 cores, 19 mm diameter, from the upward facing surfaces of each four source colonies in the back reef and fore reef sites; two for immediate freezing (day 0) and eight each for cross (new location) or back (original location) transplantation (Fig. 1.1B). Holes were filled and transplants affixed to the substrate with Z-Spar, Splash Zone marine epoxy (Carboline Company, St Louis, MO).

To minimize confounding factors associated with clonal structure and microhabitat differences, original source colonies were selected >5 m apart and similar in size to reduce the likelihood of selecting clones, and resulting fore-reef and back-reef cores were grouped randomly and transplants were affixed in random orientation >5 m apart and near (<1 m) original source colony locations to include microhabitat variation. The transplant experiment was initiated on February 5th, 2005 (day 0) and concluded on February 10th, 2005 (day 5). After an initial 24 hr recovery period (day 1), all samples were destructively sampled for four consecutive days (days 2, 3, 4 and 5) between 13:45 and 14:15. At each sampling day, 2 cores from each parent colony in each transplant

group were placed on pre-incubated -80 °C ice packs and stored at -80 °C until used for protein or DNA extractions.

Coral Protein Extraction

Coral and algal symbiont protein was extracted using a protocol adapted from a previous study (Brown *et al.* 2002a). Each core was flash frozen in liquid nitrogen before the tissue layer (up to 1 cm below surface) was removed with bone cutting pliers and placed in a pre-frozen, 50 ml stainless steel mixing jar (Glennmills, Clifton, NJ). The tissue and skeleton was crushed using a TissueLyser[®] (Qiagen, Valencia, CA) at 25 rpm for 5 s. Resulting sample was transferred to individual 2.5 ml cryovials and stored at -80 °C until further analyses.

Between 280-380 mg of crushed tissue was added to a prechilled 2 ml microcentrifuge tube to which 3.5 µl/mg of protein extraction buffer was added for a total volume of 1-1.5 mls. The extraction buffer was made fresh daily and consisted of 50 mM Tris-HCl (pH 6.8), 2 % (w/v) SDS, 25 mM dithiothreitol (DTT), 10 mM EDTA, 4 % (w/v) polyvinylpolypyrrolidone (PVPP), and 1 % (v/v) DMSO. 1 µL of Halt Protease Inhibitor Cocktail (Pierce Biotechnologies, Rockford, IL) was added per 100 µl of extraction buffer. Tubes were vortexed for 20 s then incubated at 90 °C for three minutes, vortexed again for 20 s, and incubated for an additional three minutes at 90 °C. The tubes were cooled at room temperature for five minutes then centrifuged at 13,000 *g* for 15 minutes. After the centrifugation, approximately 300 – 400 µL was aspirated from the middle phase of the tube, being careful not to collect any supernatant that had been

contaminated with the whitish matrix that was the top-most phase. Total protein concentration was determined using the Bio-Rad RC/DC protein concentration assay (Bio-Rad, Hercules, CA).

SDS-PAGE and Western Blotting

Standard curve dilutions of 0, 2, 6, 10, 14 and 18 μg total protein for Ubiquitin-Conjugate assays and 0, 1, 3, 5, 7 and 9 μg total protein for all other assays of a single, standard coral sample were added to all gels for reference. Note that an increased range of standard was needed for Ubiquitin-Conjugate assays in order to accurately capture the larger range of the experimental samples. 2 μg total protein of each experimental sample was added to 4 μl of E-PAGE Sample Loading Buffer (Invitrogen, Carlsbad, CA), and water to a final volume of 18 μl . All samples and standards were run in triplicate, boiled at 95 °C for 5 min before loading onto E-PAGE 96-well 6% gels (Invitrogen) and run for 14 min with the provided E-PAGE power supply according to manufacturers instructions.

Gels were removed, incubated for 30 minutes in a western transfer buffer consisting of 25 mM Tris, 192 mM Glycine, 20% v/v Methanol, 0.025% w/v Sodium Dodecyl Sulfate (SDS) pH 8.3 before transfer using a Criterion blotting system (Bio-Rad) according to manufacturers instructions with PVDF (Millipore, Bedford, MA) membrane. Transfers were conducted for 30-40 min at 100 V. Membranes were blocked overnight in 50 mls 1x TBST (50 mM Tris, 150 mM NaCl pH to 7.4, 0.1% (v/v) Tween-20) and 1% (w/v) Bovine Serum Albumin (BSA) at 4 °C. After blocking, membranes were incubated with primary antibody in TBST/BSA blocking buffer for 2 hr at room temperature,

washed three times for 10 min each with fresh changes of TBST, incubated with secondary antibody in blocking buffer for 1 hour, washed three times for 5 min each in 1x TBS with 0.3% (v/v), and three times for 5 minutes each in 1x TBST before detection with SuperSignal West Dura Extended Duration Substrate (Pierce). All images were recorded using a ChemiDoc XRS molecular imager (Bio-Rad) and analyzed with National Institute of Health Image J software.

Antibodies against heat shock protein 70 (hsp70; Cat. #SPA-822), Ubiquitin (Cat. #SPA-200), Manganese Superoxide Dismutase (MnSOD; Cat.#SOD-110), anti-rabbit- and anti-mouse-conjugated (Cat. #SAB-300, SAB-100) horseradish peroxidase antibodies were obtained from Stressgen Biotechnologies (Victoria, British Columbia, Canada). Anti-4-Hydroxynonenal (4HNE; Cat. #AB5605) and anti-goat-conjugated horseradish peroxidase (Cat. #AP180P) antibodies were obtained from Chemicon International (Temecula, CA). Antibody specificity was verified by molecular weight comparisons with known standards (Fig. 1.3). For the MnSOD and hsp70 antibodies, a single band of the predicted molecular weight was visualized, while for the ubiquitin and 4HNE antibodies, a variety of bands were observed due to the conjugation of these markers to a wide range of proteins (Fig. 1.3). Antibody reactivity against *Symbiodinium* protein was verified using protein extracts from 3 different *Symbiodinium* cultures (KB8, AipB, and *Montipora Capitata*) graciously provided by the lab of Dr. Robert Kinzie III at the Hawai'i Institute of Marine Biology (Fig. 1.3). These cultures are believed to represent *Symbiodinium* types from taxonomic clades A, B, and D respectively. Antibody recognition of Cnidarian host protein is assumed based on manufacturers

recommendations and previous study (Downs *et al.* 2000). Hence, the data presented herein are from holobiont homogenates comprised of both symbiont and host proteins.

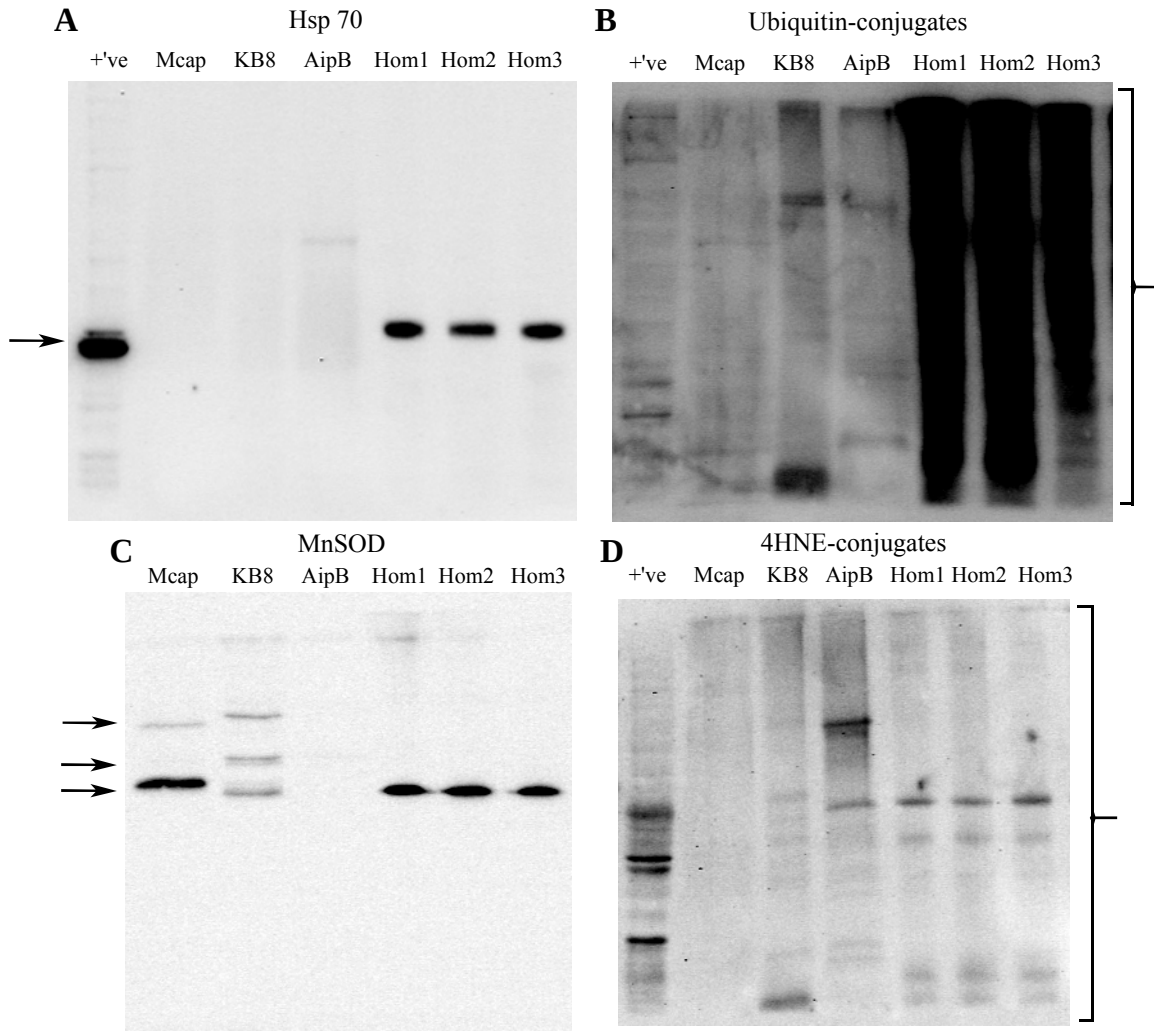


Fig. 1.3 Western blots used for validation of (A) Hsp70 (8% SDS-PAGE), (B) Ubiquitin-conjugate (10% SDS-PAGE), (C) MnSOD (15% SDS-PAGE), and (D) 4HNE-conjugate (10% SDS-PAGE) antibody specificities against protein extractions from *Symbiodinium* cultures and holobiont (host and symbiont) tissue homegenates. Positive control lanes (+ve) consisted of 10 μ g of Heat Shocked HeLa Cell Lysate (A, D) and Rat Brain Tissue Extract (B) (Stressgen LYC-HL101F and LYT-RB100F respectively). Lanes Mcap,

KB8, and AipB consisted of 15 µg of total protein from extracts of cultured *Symbiodinium* while lanes Hom1, Hom2, and Hom3 consisted of 5 µg of total protein from three separate host/symbiont homogenate extractions prepared from a heat shocked *P. lobata* colony collected at the Hawai'i Institute of Marine Biology. Gels were run at 200 V for 45min with a 100 V, 50min wet transfer. Note, no +ve control was available when running gel C, however, the prominent band pictured is ~25 kDa, the expected size of MnSOD and similar size was observed using rat brain tissue extract previously (data not shown).

Protein biomarkers were chosen based on their functional roles in stress defense and stress-caused damage: heat shock protein 70 (hsp70) is a molecular chaperone that helps maintain protein structure and function under heat related denaturation, functionally serving as a cellular defense against temperature related stress (Feder & Hofmann 1999), Manganese Superoxide Dismutase (MnSOD) deactivates the reactive superoxide anion that is formed under oxidative stress and symbiont photosynthesis before it can damage other proteins involved in cellular processes (Halliwell & Gutteridge 1999), 4-Hydroxynonenal (4HNE) is a reactive lipid peroxide that causes oxidative protein damage (Halliwell & Gutteridge 1999), and ubiquitin is a cellular protein tag that is bound to damaged proteins, marking them for degradation and reuse (Hawkins 1991). Increases in ubiquitinated proteins are generally thought to be indicative of elevated levels of stress induced protein damage, although non-damage related functions of

ubiquitination related to protein turnover, trafficking, and maintenance of cellular protein homeostasis could also produce similar increases (Hawkins 1991; Welchman *et al.* 2005).

Protein Statistical Analysis

Experimental protein concentration values were calculated relative to a standard coral sample dilution curve for each blot image based on integrated optical density using National Institute of Health software Image J. Statistical analyses were performed using Minitab[®] 15 (Mintab Inc., State College, PA) and JMP Ver. 8 (SAS Institute Inc., Cary, NC). A repeated measures analyses of variance (ANOVA) was performed with marker concentration as the response variable for each transplant day compared against source colony origin, transplant destination, and source colony number nested within origin. To investigate detailed responses within each day, five separate ANOVA's were conducted as above using marker concentrations split by individual days. All data were natural log transformed prior to the repeated measures analysis and a weighted least squares correction was used on untransformed data for the within day tests to correct for unequal variances.

DNA Extraction, Amplification and Sequencing

DNA was extracted following a previous study (Concepcion *et al.* 2006) for subsequent use as template in all polymerase chain reactions (PCRs). Optimal annealing temperatures for each primer set were determined empirically by temperature gradient PCR on a MyCycler Thermal Cycler (Bio-Rad). An approximately 650 base pair (bp)

sequence of coral host nuclear ribosomal DNA was amplified using primers “ITSZ1” (5’-TAAAAGTCGTAACAAGGTTTCCGTA-3’) and “ITSZ2” (5’-CCTCCGCTTATTGATATGCTTAAAT-3’; Forsman *et al.* 2009). This fragment consisted of partial sequences of the 18S and 28S ribosomal RNA genes, and complete sequences of the internal transcribed spacer regions 1 and 2 (ITS1 and ITS2), and the 5.8S ribosomal RNA gene. ITS hot-start PCR amplification was performed as follows: 95 °C for 7 min (1 cycle), followed by 35 cycles consisting of 94 °C for 30 sec, 50 °C for 30 sec, and 70 °C for 2 min, and a final extension at 72 °C for 1 min.

A 498 bp sequence of host coral mitochondrial DNA from the *NAD5* intron region was amplified using the primers “NAD_700F” (5’-TGCCGGATGCYATGGAG-3’; Concepcion *et al.* 2006) and “NAD_157R” (5’-VCCATCYGCAAAAGGCTG-3’; Concepcion *et al.* 2006) under the following conditions: 95 °C for 10 min (1 cycle), followed by 35 cycles consisting of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 90 sec, and a final extension at 72 °C for 1 min. A second mitochondrial host coral fragment from the putative control region was amplified with primers “CRf” (5’-GCTTAGACAGGTTGGTTGATTGCCC-3’; Vollmer & Palumbi 2002) and “CO3r” (5’-CTCCCAAATACATAATTTGAACTAA-3’; Vollmer & Palumbi 2002) under the following conditions: 95 °C for 7 min (1 cycle), followed by 35 cycles consisting of 94 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 2 min, and a final extension at 72 °C for 5 min.

For symbiont genetic analysis a ~280 bp fragment of the ITS2 region of the symbiont ribosomal DNA array was either amplified with primers “Sdino” (5’-

CGCTCCTACCGATTGAGTGA-3'; Pochon *et al.* 2001) and "L0" (5'-GCTATCCTGAGRGAACTTCG-3'; Pochon *et al.* 2001), or primers "ITSintfor2" (5'-GAATTGCAGAACTCCGTG-3'; LaJeunesse & Trench 2000) and "ITS-Reverse" (5'GGGATCCATATGCTTAAGTTCAGCGGGT-3'; Coleman 1994) under the following conditions: 95°C for 7 min (1 cycle), followed by 35 cycles consisting of 94 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 2 min, and a final extension at 72 °C for 5 min.

All mitochondrial PCR amplifications (25 µL) consisted of 2 µL of DNA template, 2.5 µL of 10x ImmoBuffer, 0.1 µL IMMOLASE DNA polymerase (Bioline Inc., London, United Kingdom), 1.5 µL of 50 mM MgCl₂, 0.5 µL of 10 mM total dNTPmix (Bioline), 0.325 µL of each 10 µM primer, and deionized H₂O to volume. All ITS host and symbiont PCR amplifications (50 uL) consisted of 2 µL of DNA template, 5 µL of 10x ImmoBuffer, 0.2 µL IMMOLASE DNA polymerase (Bioline), 2 µL of 50 mM MgCl₂, 1 µL of 10 mM total dNTPmix (Bioline), 1 µL of each 10 µM primer, and deionized H₂O to volume. All PCR products were visualized using 1.0% agarose gels (1×TAE) stained with 0.5% v/v Gelstar[®]. PCR products were treated with 2 U of exonuclease I and 2 U of shrimp alkaline phosphatase (Exo:SAP) at 37 °C for 60 min, followed by an incubation at 80 °C for 10 min. Cleaned mitochondrial PCR products were then cycle-sequenced using the ABI Prism Big Dye Terminator Reaction Kit (Perkin Elmer-Applied Biosystems, Foster City, CA) and run on an ABI-3100 automated sequencer. Clone libraries were constructed for each host and symbiont ITS PCR product using the pGEM[®]-T easy vector system (Promega Corp., Madison, WA). Positive inserts

were verified by PCR using M13 primers and plasmids (8-10 per library) were sequenced as above.

Sequence Analysis

Resulting sequences were inspected using Sequencher version 4.5 (Gene Codes Corp., Ann Arbor, MI) and aligned using Bio-edit (Hall 2001) and by eye. Population genetic structure was estimated using an analysis of molecular variance (AMOVA) in Arlequin 2.0 (Schneider 2000). Molecular phylogenetic networks were constructed using the median-joining algorithm and maximum parsimony post-processing calculation in NETWORK ver 4.5.0.0 (Fluxus Technology Ltd.; Polzin & Daneschmand 2003). Host mtDNA fragments from the *NAD5* and putative mitochondrial control regions were concatenated into a single sequence for alignment and analysis.

It is known that a range of copy number and intragenomic variability exists within the Scleractinian ITS region (Coleman & van Oppen 2008; Vollmer & Palumbi 2004; Wei *et al.* 2006). Thus, multiple approaches were used in analyses of the Host ITS sequences in this study. Results presented herein are from calculation of population differentiation performed on the entire data set including all sequence variants from each individual. However, as this approach treats each cloned sequence as an individual, host ITS intragenomic variants were also combined into single consensus sequences for each individual using Sequencher for additional alignment and analysis. No difference in statistical outcome for host ITS analysis was observed between sequences built by consensus by inclusivity versus plurality, hence data presented are from analysis of

consensus sequences generated by inclusivity. By incorporating all nucleotide polymorphisms detected within the clone libraries from individuals using International Union of Pure and Applied Chemistry (IUPAC) ambiguity codes, the entire diversity of an individual's ITS region is represented by a single inclusivity consensus sequence. This approach tests the level of genetic differentiation between the entire suite of ITS diversity contained within individuals among the sampling populations. Network constructions were made using both the complete and consensus-based data sets; however, for purposes of clarity, only the consensus-based network is presented in the main text.

There is a similar occurrence of intragenomic variation in *Symbiodinium* rDNA (Thornhill *et al.* 2007; van Oppen & Gates 2006), which dictates a level of caution when handling cloned symbiont sequences. Hence, all symbiont ITS2 sequences were screened for potential PCR error following a previous study (Manning & Gates 2008). Unique sequence types were only included in the analysis if they were found from multiple clone libraries or if they differed from published sequence types by three or more base pair (bp) substitutions. A cluster-based network analysis was used to examine divergence between cloned sequences and assess identity of the dominant symbiont type following published approaches (Correa & Baker 2009; Sampayo *et al.* 2009).

Results

Protein Biomarker Investigation

Antibody recognition of proteins extracted from cultured *Symbiodinium* was observed for MnSOD, Ubiquitin-conjugated proteins, and 4HNE-conjugated proteins (Fig. 1.3). Antibody recognition of *Symbiodinium* hsp70 protein was not observed (Fig. 1.3), possibly due to the specificity of the antibody for animal hsp70 or to limited expression or non-recognition of hsp70 in these specific cultured *Symbiodinium* strains. Additionally, multiple SOD bands were observed in extracts from *Symbiodinium* cultures Mcap and KB8 (Fig. 1.3C), a phenomenon which has been found previously in coral symbionts (Richier *et al.* 2003) due to the increased number of SOD isoforms found within plant tissues. However, multiple bands were not observed for any holobiont homogenate extracts, suggesting undetectable levels of these isoforms in the results presented herein.

All four protein biomarker concentrations were evaluated from the RTE consisting of four source colonies of *Porites lobata* in each location selected for cross (new location) or native (original location) reciprocal transplantation (Fig. 1.1B). Comparison between native and cross transplant groups examines variability by transplant destination (back reef native vs. back reef cross, forereef native vs. forereef cross) and by source colony origin (back reef native vs. forereef cross, forereef native vs. back reef cross; Smith *et al.* 2007). Within the RTE framework, variability by transplant destination is indicative of environmental influence on response (i.e., phenotypic

plasticity), while variability by source colony origin indicates potential genetic influence on the response variable (DeWitt & Scheiner 2004; Schluter 2000; Smith *et al.* 2007).

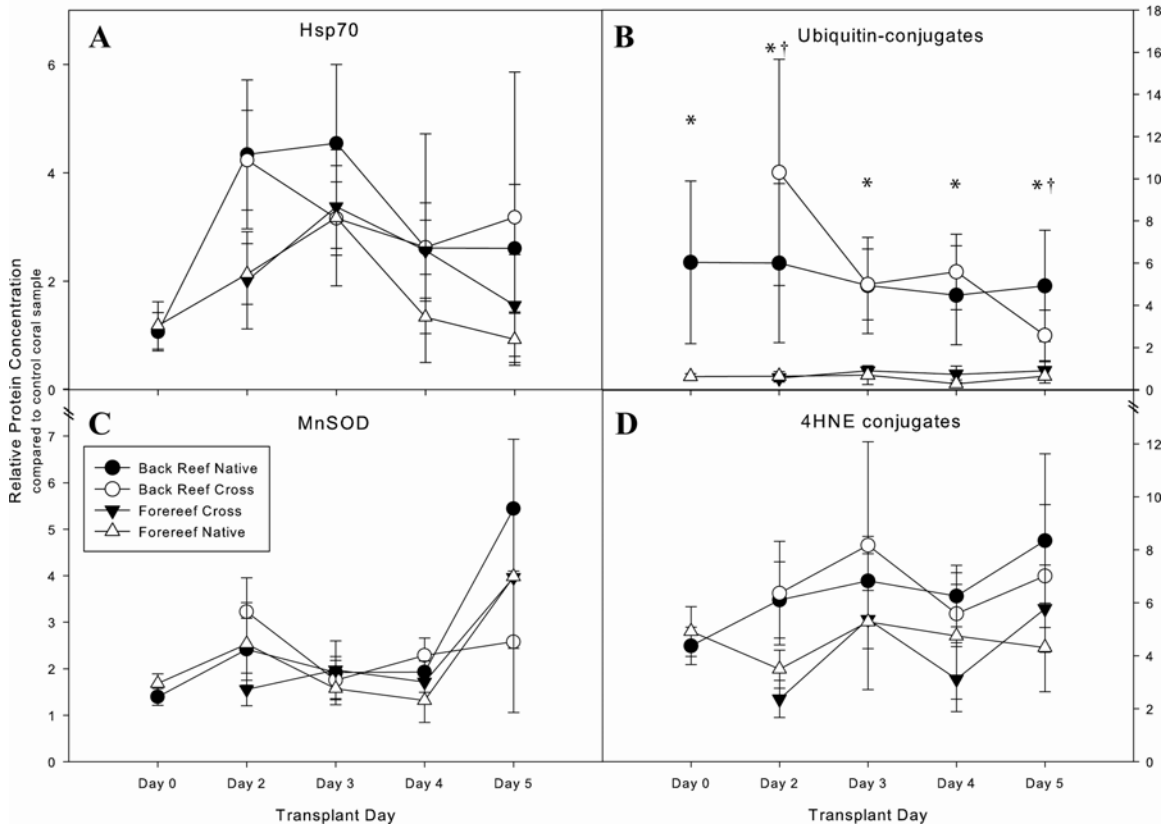


Fig. 1.4 Molecular marker concentrations (A) Hsp70, (B) Ubiquitin-conjugates, (C) MnSOD, and (D) 4HNE-conjugates calculated from western blots of host and symbiont homeogenates relative to a standard coral sample (optical density of marker concentration per 2 μ g total soluble protein) for pre (day 0) and subsequent days of the RTE. Means $\pm$ 1 SD. Black and white triangles and black and white circles represent back reef native and cross transplants and forereef native and cross transplants respectively. A * or † above symbol values indicates significant differences for source colony origin and

transplant destination respectively for that particular day ($p < 0.05$; note these tests were only carried out for ubiquitin-conjugates).

Figure 1.4 shows the relative concentrations of each biomarker protein observed throughout the time course of the experiment. The results for hsp70, MnSOD, and 4HNE indicated similar trends as the ubiquitin-conjugates although with much greater response variability on both temporal and treatment scales. Significant source colony origin and individual effects were observed for all three markers in addition to an effect of transplant destination (MnSOD, and 4HNE) and an source colony origin by transplant destination interaction (MnSOD only; Table 1.1A).

Table 1.1 (A) Three-way repeated measures ANOVA (Source colony origin, transplant destination, and source colony individual) for protein biomarker concentrations (ubiquitin conjugates, hsp70, MnSOD, and 4HNE) repeated over the five day course of the experiment **(B)** Three-way ANOVA for ubiquitin conjugate concentrations analyzed for each day separately

(A)		MS				F				p			
Factor	df	ubiq	hsp70	MnSOD	4HNE	ubiq	hsp70	MnSOD	4HNE	ubiq	hsp70	MnSOD	4HNE
Day		0.796	6.518	3.121	0.681	5.52	40.04	134.10	15.53	0.0010	< 0.0001	< 0.0001	< 0.0001
Source colony origin	4	1.473	0.901	0.404	0.719	10.21	5.53	17.36	16.41	< 0.0001	0.0010	< 0.0001	< 0.0001
Transplant destination	4	0.924	0.249	0.616	0.308	6.41	1.53	26.47	7.02	< 0.0001	0.2050	< 0.0001	< 0.0001
Source colony indiv	24	0.508	0.306	0.078	0.225	3.52	1.88	3.37	5.13	< 0.0001	0.0250	< 0.0001	< 0.0001
Origin x destination	4	0.856	0.447	0.421	0.097	5.94	2.75	18.08	2.22	< 0.0001	0.0360	< 0.0001	0.0800

(B)		Mean Square					F					p				
	df	Day0	Day2	Day3	Day4	Day5	Day0	Day2	Day3	Day4	Day5	Day0	Day2	Day3	Day4	Day5
Source colony origin	1	15.74	42.40	67.75	73.62	32.22	30.31	120.40	91.71	101.56	32.12	0.001	<0.001	<0.001	<0.001	<0.001
Transplant destination	1	-	3.55	0.026	0.393	5.88	-	10.09	0.040	0.540	5.86	-	0.004	0.853	0.469	0.025
Source colony indiv	6	1.64	3.38	1.96	2.01	0.990	3.16	9.58	2.65	2.77	0.990	0.068	<0.001	0.043	0.037	0.459
Origin x destination	1	-	3.29	0.069	2.18	3.71	-	9.35	0.090	3.00	3.70	-	0.006	0.763	0.097	0.068
Error	8	0.519	0.35	0.739	0.725	1.00										

The consistent response differences observed in the ubiquitin-conjugate response allowed for a more detailed analytical approach which will be presented in detail for this marker only. Ubiquitin-conjugate levels were consistently higher in back reef when compared to forereef source colonies for both transplant treatments and across all days. There were significant effects of both source colony origin and individual on Ubiquitin response (Table 1.1A), suggesting a genotypic influence at both the population and individual level. Tests within each day revealed that source colony origin was significant pre-transplant and for all four days post transplant while source colony individual was significant only on transplant days two, three and four (Table 1.1B). Significant effects of

transplant destination, and a transplant destination by source colony origin interaction were also observed (Table 1.1A). However these effects were only significant for within-day tests on days two (destination and interaction) and five (destination only), were opposite in nature, and appear to be driven by the changing response of back reef cross transplants for those given days (Fig. 1.4B, Table 1.1B). Additionally, all four markers showed a significant effect of transplant day (Table 1.1), likely indicating a response to handling stress and/or some component of daily variability between measurements.

Symbiodinium Community Analysis

An additional 11 and 12 source colonies of *P. lobata* were sampled from the forereef and back reef respectively for the *Symbiodinium* genetic analysis and were included with the original four source colonies from each location used in the RTE to ensure robust comparisons. A total of 153 back reef and 145 forereef *Symbiodinium* cloned sequences from the Internal Transcribed Spacer region 2 (ITS2) of the nuclear ribosomal tandem array were collected, comprising three previously published sequence types and 72 novel sequence identities (Appendix 1.2). Molecular phylogenetic network examinations of the back reef and forereef *Symbiodinium* populations (Fig. 1.5) showed no systematic differences in symbiont identity between back reef and forereef corals with most genotypes identical to or clustering closest to ITS2 type C15 (Appendix 1.2, average genetic distance $p=0.0119$), a genotype known to associate with Pacific *Porites* sp. (LaJeunesse *et al.* 2003). All colonies sampled contained sequences identical to type

C15, with additional sequences exhibiting no discernable pattern based on source location.

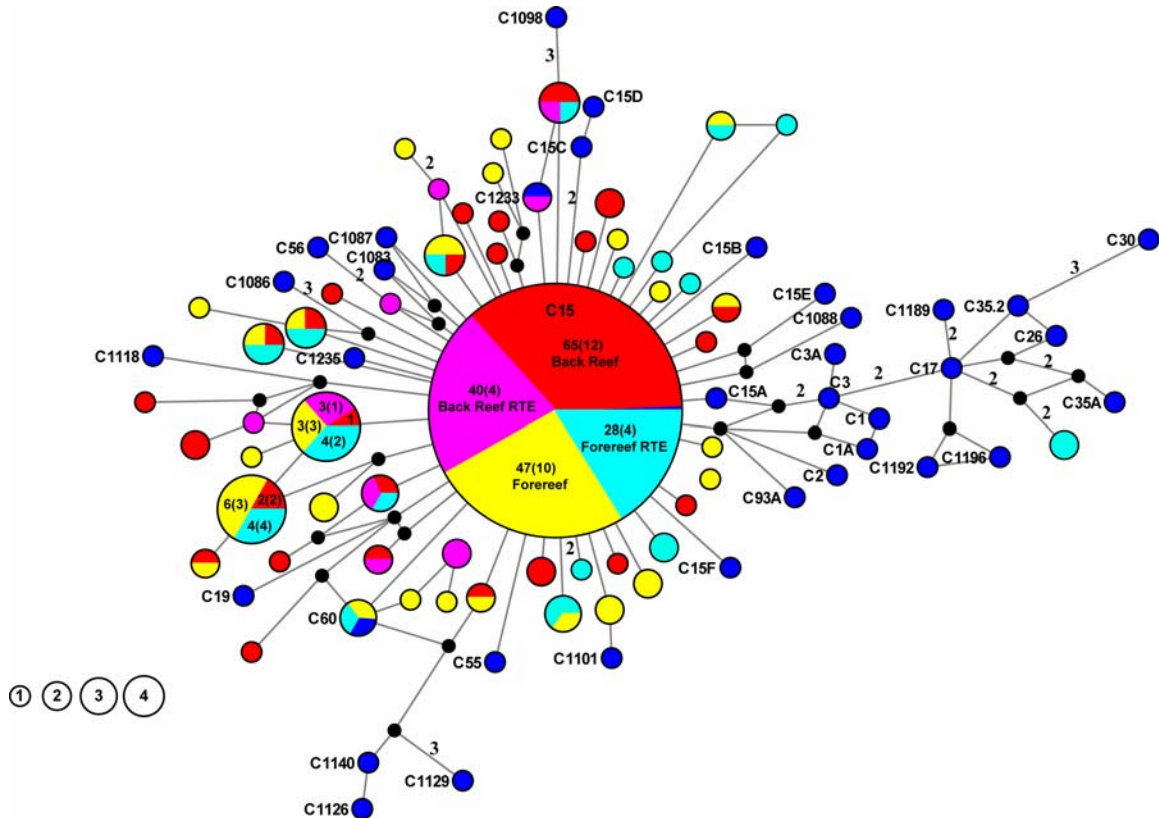


Fig. 1.5 Maximum parsimony phylogenetic network reconstruction of *Symbiodinium sp.* ITS2 isolates from symbiont genetic survey compared to previously published sequences. Lines between nodes represent single substitutions, gaps and/or indels; multiple substitutions are denoted using single black digits located next to each line. Gaps and/or indels were weighted as single changes regardless of length. Diameter of circle at each node is proportional to the number of clones with identical sequences. Nodes with greater than 4 sequences have the corresponding number of sequences marked within the circle followed by the number of individuals from which those sequences were obtained in parentheses. Sequences from back reef individuals are shown in red, those from colonies

used in the reciprocal transplant experiment shown in light purple, forereef individuals shown in yellow and forereef RTE colonies shown in light blue, and previously published sequence types shown in dark blue. Black circles represent hypothetical intermediate sequence types that were not sampled. Sequence accession numbers can be found in Appendix 1.2 and 1.3.

Porites lobata Genetic Population Structure

Multilocus sequence data from the Internal Transcribed Spacer region (ITS) of the nuclear ribosomal tandem array (rDNA) and two mitochondrial (mtDNA) regions (the “putative” control region, and the NAD5 intron) were obtained from 27 and 28 back reef and 22 and 26 forereef *P. lobata* source colonies (mtDNA and rDNA respectively). The two mtDNA fragments were concatenated to produce six unique haplotypes, two shared between forereef and back reef and four unique to the forereef. Cloning of ITS intragenomic variants resulted in 96 and 126 unique sequence types for the back reef and forereef populations respectively, with three sequence types shared between them. All individual coral samples were found to contain at least one unique sequence type not found in any other individual, hence no individual samples are thought to be clonal pseudo-replicates.

Host genetic comparisons using both ITS and mtDNA revealed significant genetic differentiation between these back reef and forereef corals ($F_{ST}=0.146$ $p<0.0001$, $F_{ST}=0.335$ $p<0.0001$ for ITS and mtDNA respectively). Molecular phylogenetic network examinations showed separation of four of the forereef mtDNA haplotypes, while 10

forereef individuals shared the same two intermediate haplotypes with the back reef samples (Fig. 1.6). Additionally, the back reef population had lower haplotype diversity with only two of the six total mtDNA haplotypes occurring in the back reef samples while all six occurred in the forereef samples. The ITS network showed similar grouping of both forereef and back reef sequences types, with additional intermediate sequence types (Fig. 1.7, Appendix 1.8). There was also much greater overall sequence diversity and distribution among the two populations in the ITS marker when compared to the mtDNA. No difference in statistical outcome was observed between the individual sequence and consensus methods with the greater F_{ST} of the consensus method ($F_{ST}=0.221$) likely due to the reduced number of sequence types within that analysis.

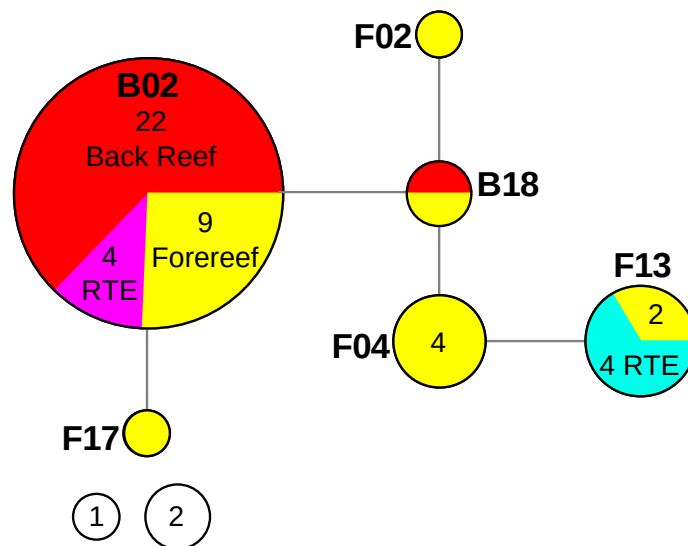


Fig. 1.6 Maximum parsimony network of mtDNA haplotypes. Color scheme is the same as in Fig. 1.5 excluding dark blue. Individual sequence names are shown in black with a B or F for back reef and forereef individuals respectively followed by colony number, X's denote the source colonies used in the RTE.

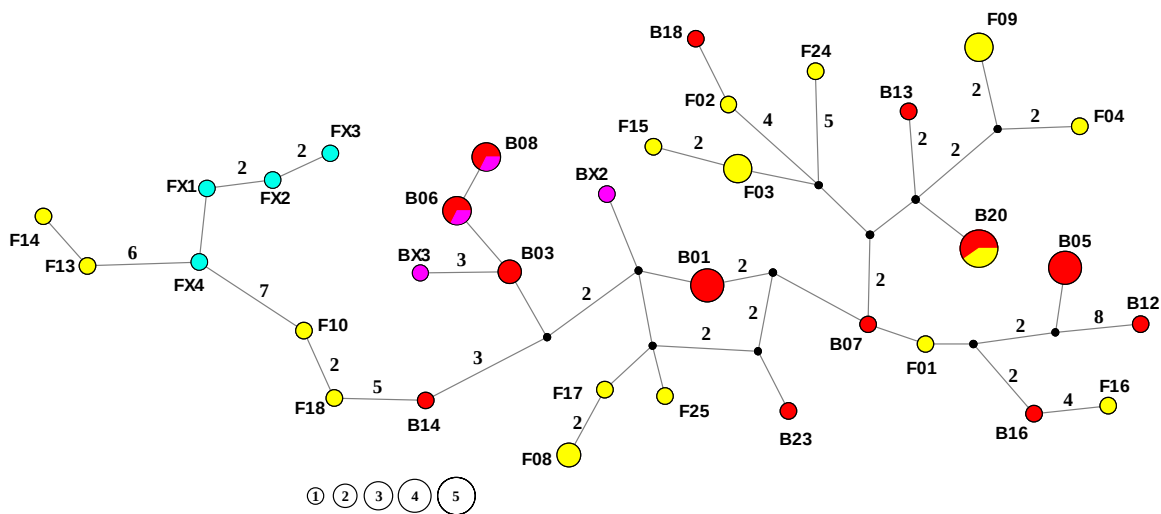


Fig. 1.7 Maximum parsimony network of ITS consensus sequences. Back Reef individuals are shown in red, Forereef individuals are shown in yellow. Individual sequence names are shown in black with a B or F for back reef and forereef individuals respectively followed by colony number, X's denote the source colonies used in the RTE. Lines between nodes represent single substitutions, gaps and/or indels; multiple substitutions are denoted using single black digits located next to each line. Gaps and/or indels were weighted as single changes regardless of length. Diameter of circle at each node is proportional to the number of clones with identical sequences.

Discussion

Back Reef and Forereef P. lobata Exhibit Variability in Protein Response

The significant source colony and transplant effects for all biomarkers under investigation demonstrate that coral response is both a function of genotypic and environmental influences. Similar trends were observed for all four of the biomarkers under investigation, although three of the four showed substantial variability in response.

A high degree of variability in these markers has been observed previously for field-collected corals (van Oppen & Gates 2006), and in this particular case, likely indicates strong influence on biomarker response of the rapid time-scale of fluctuations in many characteristics of the fore and back reef environments (e.g., temperature, salinity, light, flow; Fig 2, Appendix 1.3-1.8). Back reef source colonies generally showed increased levels of hsp70, MnSOD, and 4HNE-conjugates when compared to forereef source colonies, as indicated by the significant source colony origin effects (Table 1A, Fig. 1.4). This may be evidence of increased stress loading in the back reef environment, however the variable nature of the responses, as well as the lack of consistent differences between cross and native transplant groups, make it difficult to draw any substantial conclusions from these three markers.

In contrast to the hsp70, MnSOD, and 4HNE trends illustrated above, more consistent differences were observed between the levels of ubiquitin-conjugated proteins in back reef and forereef source colonies and the remainder of the discussion will focus on this marker in particular. The significant influences of both source colony origin and transplant destination suggest a combination of genotypic and environmental influence on ubiquitin-conjugate response. Information regarding the relative influence of each of these factors may be gained by looking at the consistency of significant effects for each factor. A significant effect of source colony origin was found for all within-day tests while an effect transplant destination was only significant for two of the five days tested with an opposing direction of difference on day two than day five (Table 1). Additionally, the magnitude of average differences between ubiquitin-conjugate levels

based on source colony origin was much greater than that based on transplant destination (~790% average difference between origins vs ~41% between destinations).

Taken as a whole, the influence of source colony origin on the ubiquitin response appears to be stronger than that of transplant destination. Additionally, the lack of dramatic upregulation of protein responses in forereef cross transplants suggests limitations to the capacity for phenotypic change when faced with new environmental conditions. The significance of the genetic effect on ubiquitin-conjugates, in addition to the more variable markers, indicates potential limits to the plastic response either due to long-term acclimatization or fixed genetic constraints on the phenotypic response.

An important factor to consider is that the five day duration of the RTE may not have been long enough to adequately allow full acclimatization of protein responses to new environmental conditions. During the experiment, a 30% increase and 57% decrease over pre RTE (day 0) ubiquitin-conjugate levels was observed in forereef and back reef cross transplants respectively on day five (Fig. 1.4B). While we cannot draw any substantial conclusions from this trend, it raises the possibility that a longer experimental duration could elicit continued changes in cross transplant responses, eventually approaching those of the native transplants in each location.

A concurrent, 6-month transplant study using the same *Porites lobata* source colonies and RTE design observed a similar combination of genotypic and environmental influences on growth responses over a longer experimental duration (Smith *et al.* 2007). Smith and colleagues found that back reef source corals had higher survivorship and elevated growth when compared to forereef source corals (genotypic influences) while

corals from both origins showed elevated growth in the back reef versus the forereef transplant destinations (environmental influence; Smith *et al.* 2007). The findings of Smith and colleagues (2007) agree with those of the current study in that both demonstrate a response in the physiology of these corals to changes in environmental conditions (e.g., cross transplantation) that is limited by source colony origin; a finding that has been found previously for the congeneric *P. attenuata* (Raymundo 2001). Furthermore, the concordant results of the two studies serve to illustrate the benefits of combining the more variable molecular approaches with classical measures of fitness such as growth and survival.

It is necessary, however, to discuss the potential contradiction between the greater levels of ubiquitin conjugates in back reef source colonies observed in this study and the increased growth and survival of back reef source colonies observed by Smith *et al.* (2007). Increases in ubiquitin-conjugated proteins have been shown to correlate with increased exposure to toxic pollutants (Downs & Downs 2007; Yum 2006) and high temperatures in corals (Brown *et al.* 2002b), and both warm and cold thermal stresses in other marine organisms (Dutton & Hofmann 2008; Hofmann & Somero 1995, 1996b; Place *et al.* 2004; Teranishi & Stillman 2007). It would follow then, that the elevated concentrations of ubiquitin conjugates observed in back reef source corals indicate higher stress levels caused by the dynamic fluctuations in environmental conditions of the back reef site. In contrast, the increased growth rates of back reef source colonies and transplants observed in the previous study (Smith *et al.* 2007) indicate that the conditions of the back reef environment are also conducive to growth and survival.

Beneficial aspects of exposure to fluctuating temperatures such as increases in growth (Les *et al.* 2009; Spigarelli *et al.* 1982; Wang *et al.* 2007) and thermal tolerance limits (Feldmeth *et al.* 1974; Feminella & Matthews 1984; Hines *et al.* 1980) have been observed for a variety of aquatic organisms. Furthermore, non-lethal stresses have also been shown to elicit large ubiquitin responses both seasonally and over tidal cycles in naturally occurring populations of inter and subtidal marine mussels (Hofmann & Somero 1995, 1996a, b) with contradictory responses mirroring opposing range distributions (Dutton & Hofmann 2008). Similarly, the increased ubiquitin response of back reef source corals may be temporally variable, thus illustrating their capacity to survive the daily extremes of the back reef without a corresponding reduction in other fitness characteristics such as growth and survival. Future work will concentrate on determining the upper limits of ubiquitin response in forereef corals in hopes of elucidating the extent of source population influence and the potential of the ubiquitin-response to limit range distributions.

Back Reef and Forereef Symbiodinium Populations Were Genetically Indistinguishable

Research over the past few decades has revealed that certain genotypic combinations of coral host and algal endosymbiont can exhibit different physiological characteristics such as differential growth (Little *et al.* 2004), thermal tolerance and bleaching resistance (Baker *et al.* 2004; Jones *et al.* 2008; Rowan 2004; Rowan *et al.* 1997a), and susceptibility to disease (Stat *et al.* 2008b). Recently, it has also been shown that the same type of *Symbiodinium* can elicit both increased and decreased thermal

tolerance depending on the host genetic background (Abrego *et al.* 2008). Even the degree of mutualism within the cnidarian-dinoflagellate symbiosis has been called into question, with an association bordering on parasitism found in a particular Hawaiian coral (Stat *et al.* 2008b). To further add to this complexity is an inherent difficulty in characterizing the diversity of the genus *Symbiodinium*. Genetic diversity within this genus can be as great as order-level differences seen in other dinoflagellate groups (Rowan & Powers 1992). Additionally, much of the molecular characterization of *Symbiodinium* diversity is based on nuclear ribosomal gene sequences which, due to their multicopy nature, can exhibit large levels of intragenomic variation (Hillis & Dixon 1991).

The data generated during this study give us the unique opportunity to examine the question of *Symbiodinium* intragenomic variability within a sampling strategy that spans two thermally distinct regions within one host species over a relatively short distance (<5km). The resulting comparison showed that all individuals sampled had at least one sequence type identical to ITS2 type C15, a type found in *Porites* species across the Pacific (LaJeunesse *et al.* 2003). Furthermore, of the 75 unique sequence types found in this study, only three matched previously published sequences, with 68 of the 72 novel types found less than 4 times (Appendix 1.2). The uncorrected average genetic distance between sequence types ($p=0.0119$) was almost half that of what has been found to exist within a single clonal C-type strain (Thornhill *et al.* 2007). These results, in combination with the lack of any distinguishable pattern between back reef and forereef sequence types (Fig. 1.5) suggest that the back reef and forereef *Symbiodinium* populations are

genetically the same and may be identically comprised of the single ITS2 type C15 and intragenomic variants of C15. *Porites lobata* is among the corals found to transmit their symbionts directly from parent to offspring (vertical/maternal transmission) rather than acquire them anew each generation from the surrounding environment (horizontal transmission; Richmond & Hunter 1990). Coral species with a vertical transmission strategy have been found to often associate with a single distinct type or genus-specific lineage of *Symbiodinium* (Stat et al. 2008a). While limiting the flexibility to associate with a variety of symbionts, this strategy can prove advantageous in conserving effective host-symbiont associations over multiple generations (Douglas 1998).

Coral Host Populations Show Significant Differentiation

The pattern of genetic subdivision between back reef and forereef populations of *P. lobata* suggest the presence of a physical or environmental barrier to gene flow between these two populations. There is a growing body of evidence suggesting that small-scale heterogeneity in coral population structure is not uncommon. Some studies have found only regional isolation of coral populations with panmictic patterns at within-region spatial scales (<100km; Baums *et al.* 2005; Ridgway *et al.* 2001; Ridgway *et al.* 2008), while many others have discovered population subdivision at smaller spatial scales of cross-shelf (<35km; Mackenzie *et al.* 2004; Nishikawa 2008), within-island (<10km; Magalon *et al.* 2005) and within-reef (<5km; Ayre & Hughes 2000; Miller & Ayre 2008; Underwood *et al.* 2007, 2009) distances. Subdivision between within-island habitat types has also been shown previously by Sherman *et al.* between lagunal and reef slope

populations of *Pocillopora damicornis*, with 83% of genetic variation attributable to habitat type (Sherman *et al.* 2006). The persistence of subdivision of forereef and back reef populations of *P. lobata* despite their close proximity (<5km) found in our study, demonstrate maintenance of some type of reduction in geneflow between the two environments either via physical or environmental barriers.

A possible explanation for maintenance of this differentiation could be that forereef and back reef genotypes constitute different cryptic species. While massive *Porites* can be difficult to identify based on skeletal and morphological characteristics, every attempt was made to accurately identify the samples prior to inclusion. Also, each mtDNA and ITS sequence used within this study was compared against a recent, comprehensive molecular phylogeny of *Porites* (Forsman *et al.* 2009) and all were found to occur within the monophyletic Clade I, a clade containing all other *P. lobata* samples used in the phylogeny. *P. lutea*, the most closely related species morphologically that is thought to occur in the region, is readily distinguishable genetically from *P. lobata* and found within an entirely different clade from all samples used in this study (Forsman *et al.* 2009). Additionally, the presence of intermediate and shared genotypes in both the mtDNA and ITS markers (Fig. 1.6, 1.7) as well as a lack of a discernable pattern towards reciprocal monophyly in the network constructions, suggest either a high degree of incomplete lineage sorting or maintenance of low levels of connectivity between the two populations. Review of the recent literature suggests that small-scale population structure in reef-building corals is more common than previously thought (Miller & Ayre 2008; Severance & Karl 2006; Underwood *et al.* 2007, 2009; Whitaker 2006), and while the

driving factors behind maintenance of this differentiation remain largely unknown, the implications for reef resilience and conservation certainly warrant continued investigation (Miller & Ayre 2008; Underwood *et al.* 2009).

Conclusions

While the specific mechanisms of increased performance of back reef versus forereef corals remain elusive, our findings and those of previous work (Smith *et al.* 2007) demonstrate physiological response differences between these back reef and forereef *P. lobata* populations. The RTE of these corals showed a combination of environmental and genetic influences on physiological response, suggesting that the thermal history of these corals plays a part in their reaction to novel environmental stresses. The significance of source colony origin on protein response across all four biomarkers in combination with the persistence of this influence on growth characteristics over longer time-scales (Smith *et al.* 2007) demonstrate, however, that previous acclimatization during past thermal experience is subject to strong limitations. The lack of differentiation in symbiont community structure and fine-scale genetic subdivision of the cnidarian host indicates a host-driven influence on the limitations to holobiont phenotypic plasticity. This result is important in furthering our understanding of the interactions between the heterogeneous genescape of reef-building coral populations and the environmentally variable seascape of the coral reef ecosystem.

Appendix 1.1 Summary of unique sequences generated for use in this study for each gene fragment. Sequence labels indicate site (B for back reef, F for forereef), an X indicates colonies that were used in the RTE, colony number, followed by clone number.

Host/Symbiodinium		Back Reef (B)		Forereef (F)		total # seq
Gene Region	Sequence Identity	# of seq	# of indiv	# of seq	# of indiv	
<i>Symbiodinium</i> sp. ITS2	C15	105	16	75	14	180
	C15 like novel seq					
	B02-05	4	2	7	5	11
	B04-05	1	1	9	6	10
	B08-01	1	1	3	2	4
	B19-06	1	1	3	3	4
	68 novel found <4 times	40	13	46	13	86
	C60	-	-	2	2	2
	C1233	1	1	-	-	1
	totals	153	16†	145	15†	298
Host NAD5 mt Intron	B01	28	28	12	12	40
	F04	-	-	10	10	10
	totals	28	28	22	22	50
Host mt control region	B02	26	26	10	10	36
	B18	1	1	6	6	7
	FX1	-	-	6	6	6
	F02	-	-	1	1	1
	F17	-	-	1	1	1
	totals	27	27	24	24	51
Host ITS region	B01	2	2	-	-	2

Appendix 1.1 (continued) Summary of unique sequences generated for use in this study for each gene fragment. Sequence labels indicate site (B for back reef, F for forereef), an X indicates colonies that were used in the RTE, colony number, followed by clone number.

Host ITS region contd.	B02	1	1	-	-	1
	B03	1	1	-	-	1
	B04	1	1	-	-	1
	B05	1	1	-	-	1
	B06	1	1	-	-	1
	B07	1	1	-	-	1
	B08	4	4	-	-	4
	B12	1	1	-	-	1
	B13	1	1	-	-	1
	B15	1	1	-	-	1
	B16	1	1	-	-	1
	B18	1	1	-	-	1
	B19	1	1	-	-	1
	B20	4	4	3	3	7
	B21	1	1	-	-	1
	B22	1	1	-	-	1
	B23	1	1	-	-	1
	BX1	1	1	-	-	1
	BX2	1	1	-	-	1
	BX4	1	1	-	-	1
	F01	-	-	1	1	1
	F02	-	-	1	1	1
	F03	-	-	2	2	2
	F04	-	-	1	1	1
	F08	-	-	1	1	1
	F09	-	-	1	1	1

Appendix 1.1 (continued) Summary of unique sequences generated for use in this study for each gene fragment. Sequence labels indicate site (B for back reef, F for forereef), an X indicates colonies that were used in the RTE, colony number, followed by clone number.

Host ITS region contd.	F10	-	-	1	1	1
	F13	-	-	1	1	1
	F14	-	-	1	1	1
	F16	-	-	1	1	1
	F17	-	-	1	1	1
	F18	-	-	1	1	1
	F19	-	-	1	1	1
	F20	-	-	1	1	1
	F21	-	-	1	1	1
	F23	-	-	1	1	1
	F24	-	-	1	1	1
	F25	-	-	1	1	1
	FX1	-	-	1	1	1
	FX2	-	-	1	1	1
	FX3	-	-	1	1	1
	FX4	-	-	1	1	1
	totals	28	28	26	26	54

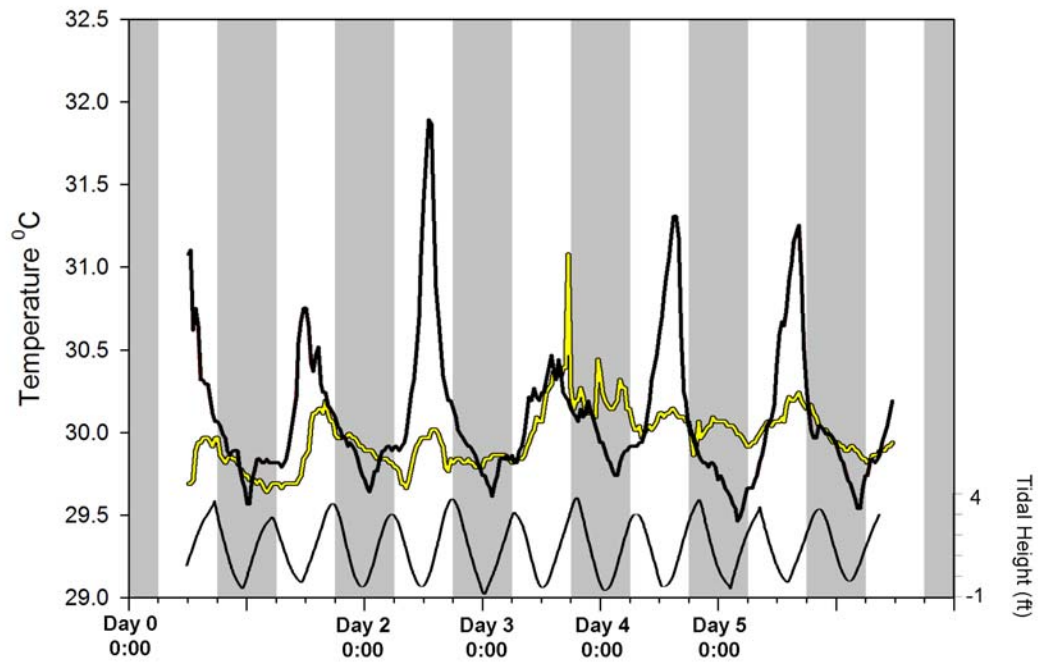
† Note: Multiple sequence types were found within individuals, totals represent total number of individuals within each group

Appendix 1.2 Summary of all previously published *Symbiodinium* sp. Sequences used in Figure 1.4 and their corresponding Genbank Accession numbers.

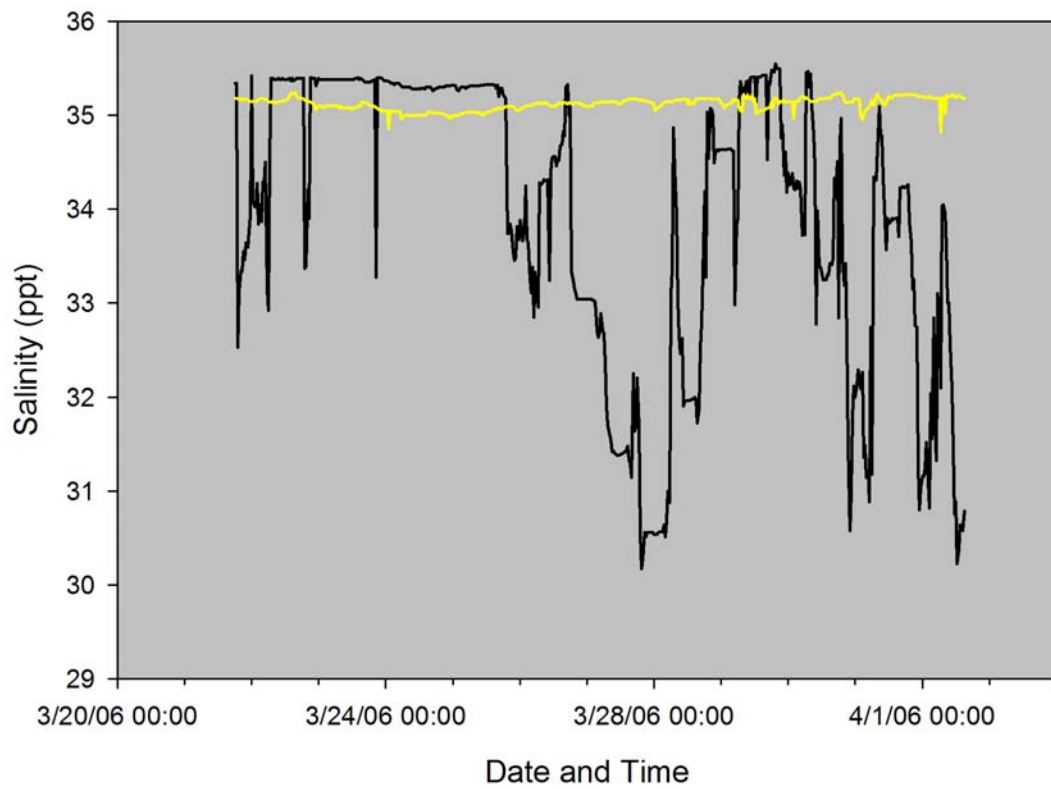
Sequence Identity	GenBank Accession #
C1	AF333515
C1a	AF499788
C2	AF333518
C3	AF499789
C3a	AF499790
C15	AY239369
C15a	AY258476
C15b	AY258491
C15c	AY258492
C15d	AY258493
C15e	AY589742
C15f	AY589743
C17	AY239370
C19	AY258479
C26	AY239378
C30	AY589745
C35.2	AY258500
C35a	AY258501
C55	AY589759
C56	AY589760
C60	AY589764
C93a	AM748563
C1083	AY237300
C1086	DQ182656
C1087	DQ182655
C1088	DQ182640
C1098	DQ182641
C1101	AB253787
C1118	AF180127
C1126	DQ182653
C1129	DQ182651
C1140	DQ182638
C1189	EU099831

Appendix 1.2 (Continued) Summary of all previously published *Symbiodinium* sp. Sequences used in Figure 4 and their corresponding Genbank Accession numbers.

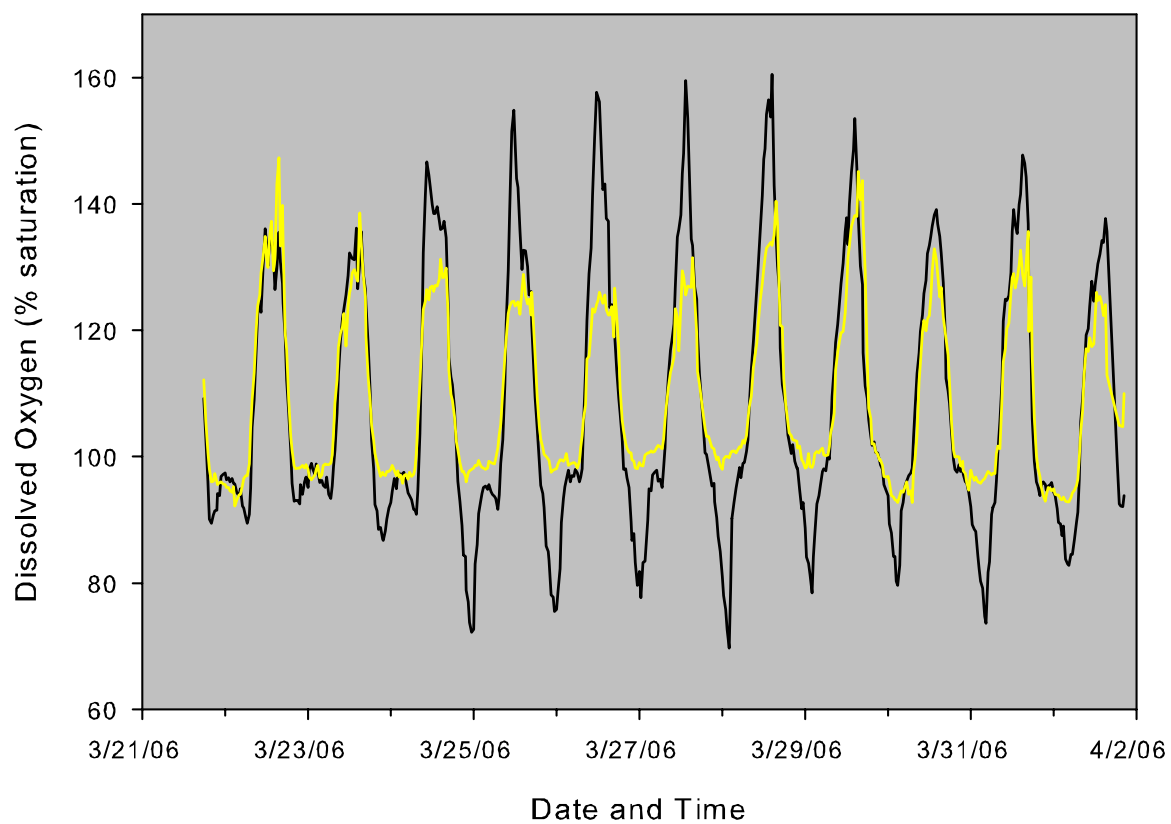
C1192	EU099820
C1196	EU099819
C1233	EU118166
C1235	EU118167



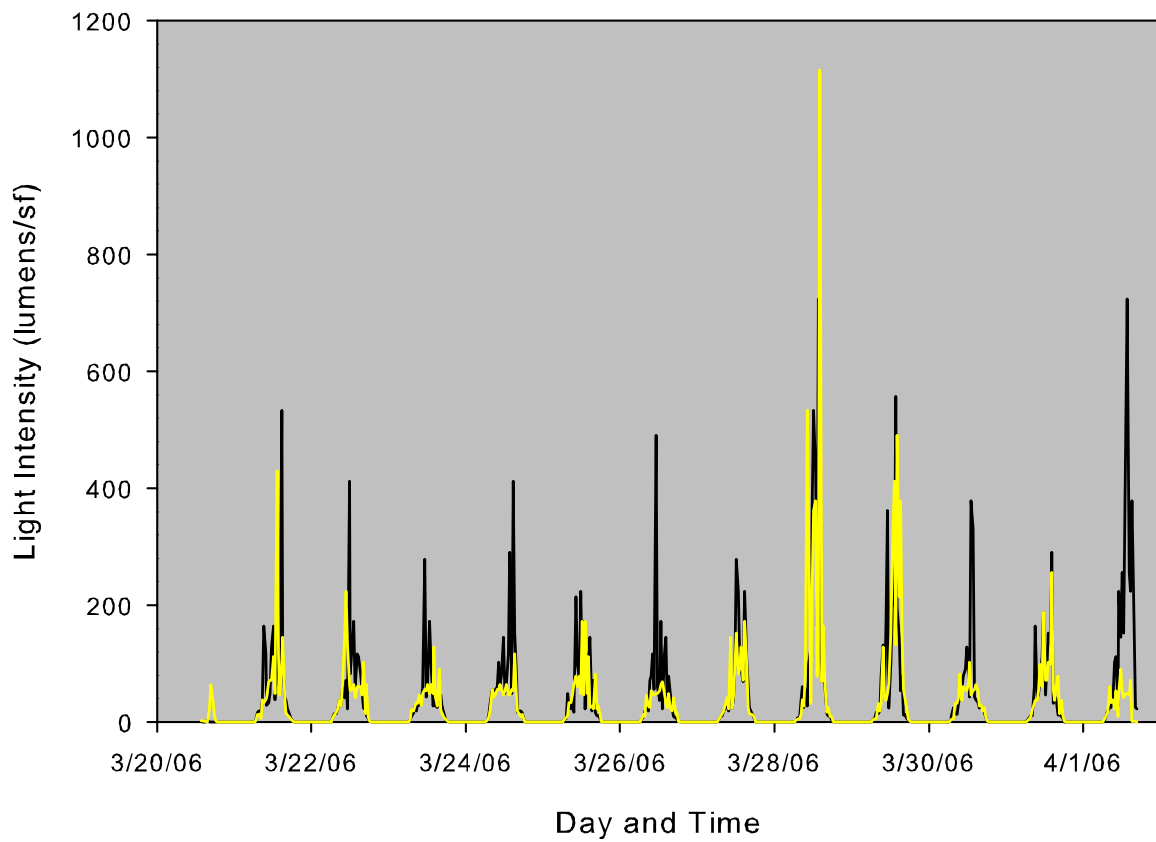
Appendix 1.3 Temperatures (°C) in the back reef (black line) and forereef (yellow line) recorded every 30 minutes during the 5 day RTE.



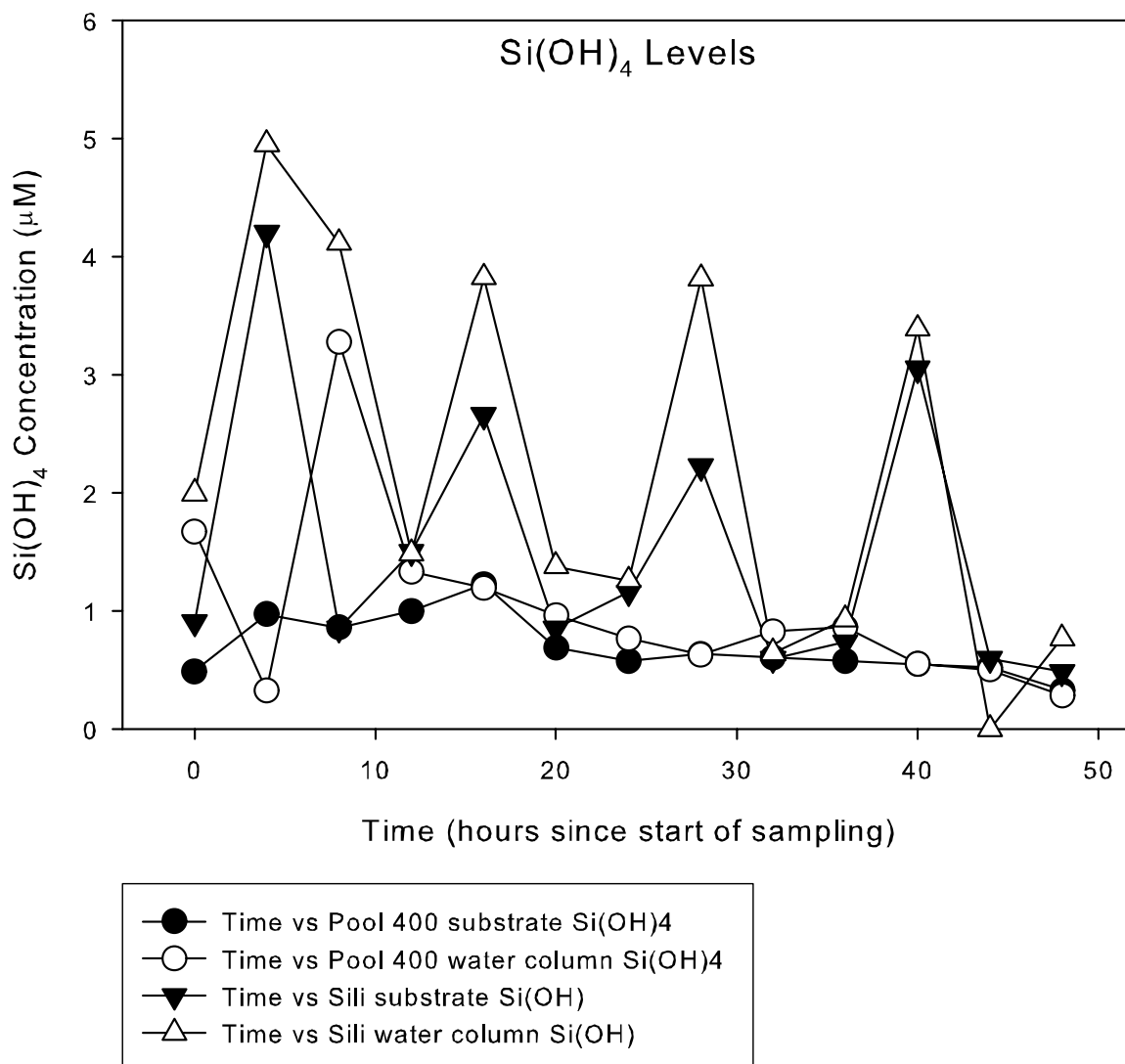
Appendix 1.4 Salinity measurements taken every 30 minutes in the back reef (black line) and forereef (yellow line) from 21-March-2006 to 1-April-2006



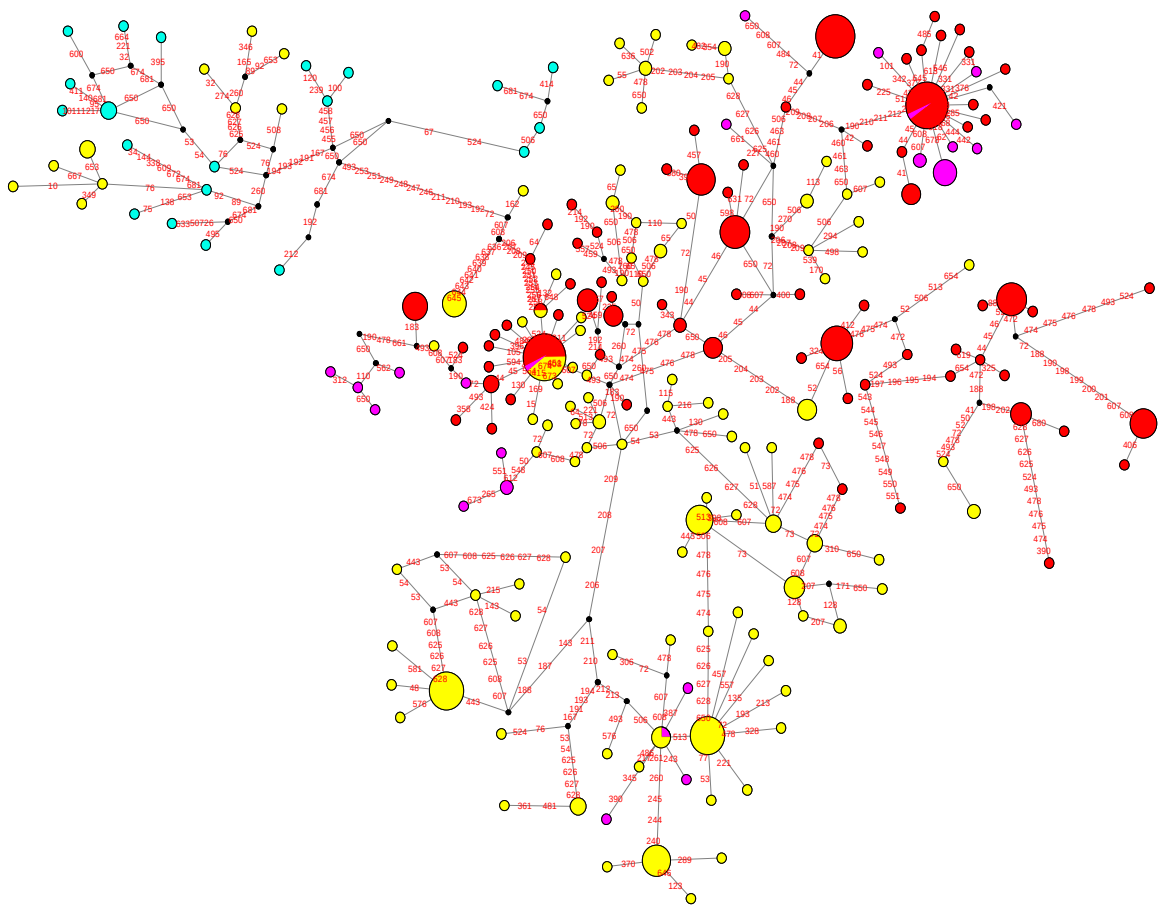
Appendix 1.5 Dissolved oxygen (% saturation) measurements taken every 30 minutes in the back reef (black line) and forereef (yellow line) from 21-March-2006 to 1-April-2006



Appendix 1.6 Relative light intensity (Lumens/sf) measurements taken every 30 minutes in the back reef (black line) and forereef (yellow line) from 21-March-2006 to 1-April-2006



Appendix 1.7 Si(OH)_4 concentration (μM) of water samples taken every four hours for 48 hours starting at midnight on 31-Mar-06 and ending at midnight 2-April-06



Appendix 1.8 Median-joining network construction of entire ITS cloned sequence data set. Mutations are shown in red on the branches (numbers refer to positions in the alignment). The smallest red circles denote unsampled intermediate sequence types. Next size circles and larger are proportional to the number of individual sequences with that sequence type. Red circles are Back Reef samples, Yellow circles are Forereef samples.

CHAPTER TWO:

PATTERNS OF GENE FLOW AND GENETIC STRUCTURE ACROSS HABITAT BOUNDARIES FOR THE REEF-BUILDING CORAL *PORITES LOBATA* IN AMERICAN SAMOA

Abstract

Models of larval dispersal and connectivity patterns in marine organisms have changed dramatically over the past few decades from relatively straightforward considerations of dispersal potential, to complex interactions between behavioral, environmental, and oceanographic influences. Recent advances in the development of highly sensitive genetic markers have shown that connectivity in reef-building coral may be just as elaborate. On the basis of a previous study, we tested the hypothesis that differences in environmental characteristics are the driving force behind population subdivision in the massive coral *Porites lobata* in American Samoa. Genetic analyses of eight nuclear microsatellite and two mitochondrial loci revealed both large scale (<100 km) and small scale (>1 km) population subdivision. A surprising degree of concordance was observed between nuclear and mitochondrial markers, however, there was no clear pattern between habitat designation and population genetic structure. This suggests that factors contributing to maintenance of reduced gene flow between populations are more complex than initially predicted. While a difficult task, determination of the specific mechanisms behind connectivity patterns in reef-building corals is critical for the design of effective management and conservation strategies.

Introduction

Many factors contribute to the degree to which populations of marine invertebrates are connected or subdivided. Life history characteristics such as mode of reproduction, larval development patterns, and settlement cues can often determine how far genetic material can disperse amongst neighboring populations (Levin 2006). Initially oceans were considered to be open, interconnected systems where dispersal was only limited by how long larvae could survive in the water column and how far they could travel via ocean currents during that time-frame (Caley *et al.* 1996; Scheltema 1986). While pelagic larval duration (PLD) does appear to correlate with degree of genetic connectivity in some organisms, many others have a more complex model of physical and biological determinates of population interconnectedness (Levin 2006).

In the case of reef-building corals, which exhibit a variety of reproductive modes and early life-history characteristics, no consistent pattern between dispersal potential and population connectivity is apparent. Some corals that release larvae already competent to settle (brooders) show limited, localized dispersal and high levels of inbreeding (Ayre & Dufty 1994; Ayre & Hughes 2000; Hellberg 1994, 1995); a result that would be predicted based on their reproductive mode. Yet other brooders are capable of dispersing large distances and maintaining population connectivity across hundreds of kilometers (Ayre & Hughes 2000; Ayre *et al.* 1997). Similarly, broadcast spawning corals, which are thought to be capable of dispersing farther than brooders, have been found to exhibit a mix of long and short distance dispersal patterns in a variety of locations (Ayre & Hughes 2000; Baums *et al.* 2005; Miller & Ayre 2008; Severance & Karl 2006). Complimentary

laboratory studies of larval competency and settlement patterns have also revealed that differences in dispersal potential between reproductive strategies may not be as consistent as previously thought (Graham *et al.* 2008; Miller & Mundy 2003; Nishikawa *et al.* 2003).

In addition to dispersal potential, selective forces are likely to play a large role in shaping the biogeography of coral populations. The reef ecosystem is a highly heterogeneous environment with a complex, three-dimensional structure. Most reefs host a multitude of microhabitats in close proximity that differ greatly in environmental characteristics (e.g., flow regime, light levels, temperature profile). Thus, differences in habitat characteristics could affect post-settlement survival success and help shape coral population subdivision. In this study, we chose a population genetics approach using microsatellite and mitochondrial markers to examine the influence of habitat characteristics on population structure of the broadcast spawning, massive coral *Porites lobata*. Characterization of genetic relatedness among sampled populations provides a useful proxy from which inferences about population connectivity, larval dispersal ranges, and migration rates can be inferred (Hellberg 2007; Hellberg *et al.* 2002; Palumbi 1995).

Porites lobata is a massive, reef-building coral that is frequently a dominant species in many near-shore, Indo-Pacific environments (Craig *et al.* 2001; Dollar 1982; Veron 2000). Colonies may be extremely long-lived (>500 yrs), and are among the oldest and largest hermatypic corals known to exist (Brown *et al.* accepted; Fenner 2005; Lough & Barnes 1997; Soong *et al.* 1999). Despite a considerable amount of research on this

species (115 results for a recent Web of Science search), the only two studies that have investigated *P. lobata* genetic structure have focused on testing marker utility (Hunter *et al.* 1997) and discerning broad-scale phylogenetic relationships among sister taxa (Forsman *et al.* 2009). Evidence from a previous study for this species (Barshis Chapter 1), indicates that populations may be significantly subdivided over small geographic distances (<5 km), and between sites with marked differences in habitat characteristics. Here, we use population genetic comparisons to assess the degree to which this pattern is consistent across multiple populations and islands within the territory of American Samoa. The reefs of American Samoa provide an ideal setting in which to investigate environmental influences on genetic structure as they host a variety of habitat types with a suite of different environmental characteristics (Barshis Chapter 1; Oliver & Palumbi 2009; Piniak & Brown 2009; Smith *et al.* 2007; Smith & Birkeland 2003, 2007). We predicted that population subdivision in *Porites lobata* would be low between sites with similar habitat characteristics and comparatively higher between populations in different habitat types. While we did find evidence for high levels of small-scale (<1 km) population subdivision, there was no clear association between level of population differentiation and differences in specific environmental characteristics. This suggests a more complex combination of factors behind maintenance of significant genetic differentiation in populations of *P. lobata* across American Samoa.

Materials and Methods

Study site characterization and sample collection

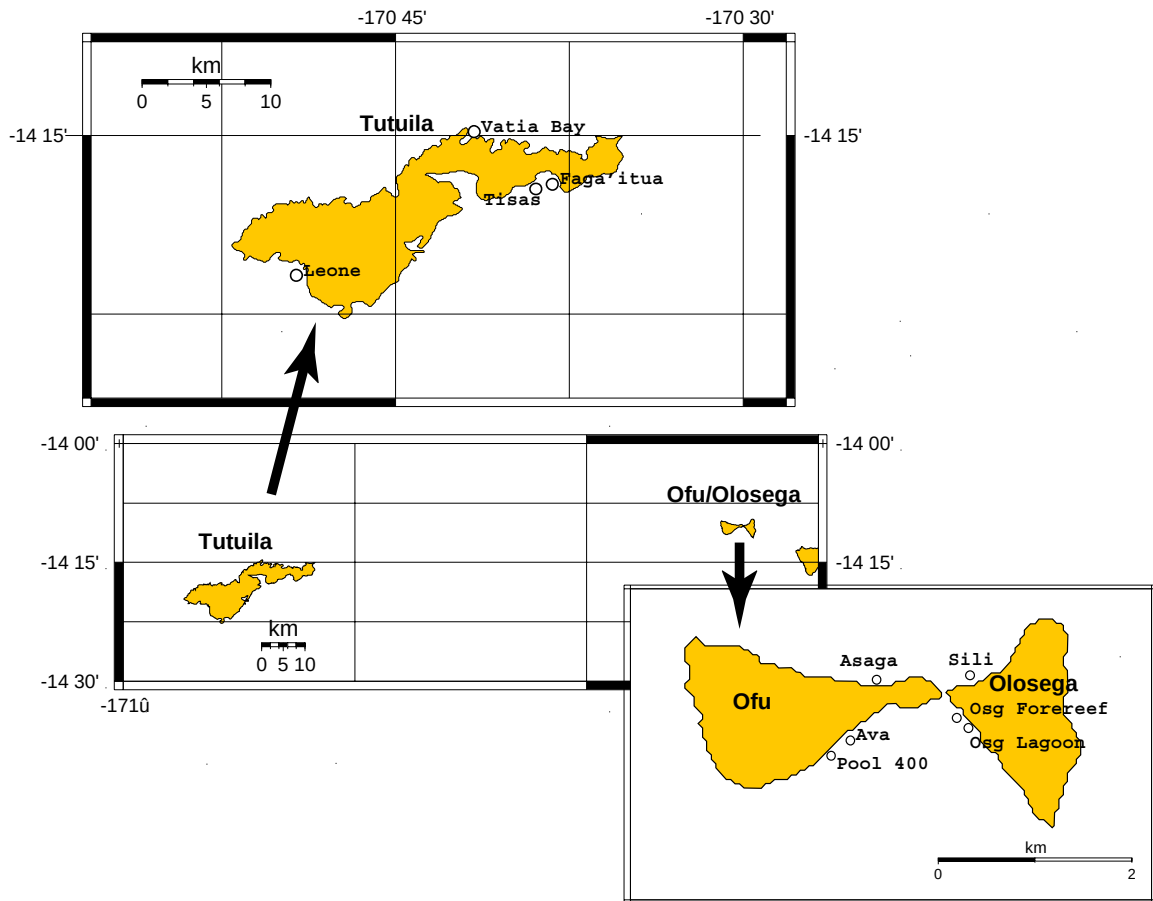


Fig. 2.1 Map of study area denoting sample locations of *Porites lobata*. Map created with OMC (<http://www.aquarius.geomar.de/>)

Samples of *Porites lobata* were collected from three islands in the territory of American Samoa (Fig. 2.1) in 2005 and 2006. Four populations were sampled from Tutuila, and three populations each from Ofu and Olosega islands (Fig. 2.1, Table 2.1). Ofu and Olosega islands lie directly next to each other and are separated only by a small

causeway, while Tutuila lies approximately 100 km to the west (Fig. 2.1). Separation between sites ranged from 500 m to 20 km within islands and 105 -127 km between islands. To investigate population subdivision between contrasting habitats, sampling was intentionally conducted across habitat boundaries within each island. Four back reef sites, one bay, and five forereef sites (Table 2.1) were chosen based on the probability that they represented differing thermal habitats. Coral tissue samples (<10 cm²) were removed from colonies using a hammer and chisel and stored in 95 % ethanol until extraction.

DNA extraction and genotyping

DNA was extracted following a previous study (Concepcion *et al.* 2006) for subsequent use as template in all polymerase chain reactions (PCRs). A 494 base pair (bp) sequence of host coral mitochondrial DNA from the *nad5* intron region was amplified using the primers “NAD_700F” (5’-TGCCGGATGCGATGGAG-3’; Concepcion *et al.* 2006) and “NAD_157R” (5’-VCCATCYGCAAAAGGCTG-3’; Concepcion *et al.* 2006) and a second, 394 bp fragment from the putative control region was amplified with primers “CRF” (5’-GCTTAGACAGGTTGGTTGATTGCCC-3’; Vollmer & Palumbi 2002) and “CO3r” (5’-CTCCCAAATACATAATTTGAACTAA-3’; Vollmer & Palumbi 2002). PCR amplifications and sequencing was performed as described previously (Barshis Chapter 1). In addition to the mitochondrial markers we used eight microsatellite loci (pl0340, pl0780, pl0905, pl1551, pl2069, pl1357, pl1629, pl1868) previously characterized for *Porites lobata* that were amplified and genotyped as previously described (Polato and Baums in prep).

Mitochondrial data analyses

Host mtDNA fragments from the *NAD5* and putative mitochondrial control regions were concatenated into a single sequence for alignment and analysis. Resulting sequences were inspected using Sequencher version 4.5 (Gene Codes Corp., Ann Arbor, MI) and aligned using Bio-edit (Hall 2001) and by eye. Population genetic structure was estimated using an Analysis of Molecular Variance (AMOVA) and pairwise F_{ST} statistics between populations based on Weir and Cockerham (1984), both calculated in Arlequin 2.0 (Schneider 2000). A molecular phylogenetic network was constructed using the median-joining algorithm and maximum parsimony post-processing calculation in NETWORK ver 4.5.0.0 (Fluxus Technology Ltd.; Polzin & Daneschmand 2003). Genetic distances were calculated between pairs of populations and graphically represented using the neighbor-joining option of MEGA version 3.1 (Kumar *et al.* 2004).

Microsatellite genetic diversity

For all nine populations, the number of multilocus genotypes (N) was compared to the number of unique genotypes (N_g), with the ratio of $N_g:N$ a rough estimate of the effects of asexual reproduction. Samples that shared the same diploid genotype across all eight loci were considered to be clonal replicates belonging to the same genet. The probability of identity (P_{ID}) can be used to assess the probability that two individuals with identical genotypes could have arisen by chance rather than descent (Baums *et al.* 2005). Probabilities were calculated in GIMLET version 1.3.2 (Valiere 2002) and combined P_{ID}

was obtained by multiplying individual values for each loci. Biased (Paetkau & Strobeck 1994) and unbiased (Kendall & Stewart 1977) values were 5.03×10^{-11} and 2.74×10^{-11} , respectively (Table 2.2), hence, each identical eight locus genotype was assumed to be an actual clonal replicate (same genet) and included only once in following statistical analyses. Additionally, each potential repetitive clone identified from the microsatellite data was also removed from the mitochondrial data set prior to analysis.

Samples were tested for linkage disequilibrium using GENEPOP (Raymond & Rousset 1995) and significant deviations from Hardy-Weinberg equilibrium (HWE) using GENEALEX version 6 (Peakall & Smouse 2006). Observed and expected heterozygosity (H_O and H_E) and the inbreeding coefficient (F_{IS}), were calculated for each locus and for each population. Additional evidence for occurrence of null alleles was also tested using Micro-Checker (van Oosterhout *et al.* 2004).

Microsatellite genetic structure

A hierarchical AMOVA was performed to assess the amount of genetic variation partitioned by different geographic levels with respect to different alleles (F_{ST}) (Weir & Cockerham 1984). Additionally, all tests were performed using a stepwise mutational model calculating the sum of squared size differences of the alleles (R_{ST}) (Slatkin 1995), however, since results were concordant, only F_{ST} based estimates are presented here. Pairwise F -statistics were calculated based on the Infinite Alleles Model in Arlequin 2.0, in addition to exact tests, performed in GENEPOP, to characterize the significance of genetic differentiation between specific pairs of populations. Cavalli-Sforza genetic

distances (Cavalli-Sforza & Edwards 1967) were calculated for a data set corrected for the influence of null alleles using the GenDIST program in the PHYLIP package (Felsenstein 1993). Cavalli-Sforza distances have been found to be the most robust to the influence of null alleles and correction procedures were performed using FreeNA (Chapuis & Estoup 2007). Genetic distances were represented graphically using the neighbor-joining option of MEGA 3.1.

The significance of the relationship between genetic distance and geographic distance was calculated using an isolation-by-distance test in GENEPOP for both Cavalli-Sforza and Nei's (Nei 1978) unbiased genetic distances. In order to investigate the number of genetically distinct populations found by our sampling, a Bayesian approach was implemented in the program STRUCTURE (Falush *et al.* 2003; Pritchard *et al.* 2000). STRUCTURE employs a Bayesian cluster method that takes sampled genotypes and the assumption of Hardy-Weinberg and linkage equilibrium within subpopulations to determine whether samples are likely part of single population ($K=1$) or multiple populations ($K>1$). STRUCTURE can be run both with and without a-priori population information and both approaches were tested here with the additional following parameters: burn-in = 25,000; replication = 500,000; 'admixture ancestry model'; 'correlated allele frequencies'; and $K=1-9$ w/2 repetitions.

Results

Table 2.1 Sampling locations, habitat, number of colonies genotype

(mtDNA/microsatellite), and number of genets (mtDNA/microsatellite) for *Porites lobata* analyzed in this study; NA, not available

Island	Site	Habitat Designation	Latitude (°)	Longitude (°)	No. of samples	No. of genets
Ofu	Pool 400	Enclosed back reef	-14.17706	-169.65399	28/28	21/19
	Ava	Forereef reef slope	-14.17601	-169.64824	11/10	11/10
	Asaga	Enclosed back reef	-14.16667	-169.63809	22/23	17/18
Olosega	Sili	Forereef reef slope	-14.16721	-169.62549	22/19	22/19
	Osg Lagoon	Enclosed back reef	-14.18003	-169.62392	14/10	13/8
	Osf Forereef	Forereef reef slope	-14.17788	-169.62804	9/6	9/6
Tutuila	Leone	Forereef reef slope	-14.34239	-170.78688	14/12	14/11
	Tisa	Channel to reef slope	-14.28063	-170.63881	9/NA	9/NA
	Faga'itua	Enclosed back reef	-14.27167	-170.61772	24/14	24/14
	Vatia Bay	Inshore slope of bay	-14.24718	-170.675	21/23	17/18

Genetic diversity

Of the 244 samples collected from 10 populations, 174 were successfully genotyped for the two mtDNA fragments (Table 2.1). The two fragments were concatenated to produce an 888bp sequence with nine variable sites, seven of which were parsimony informative. The 174 genotyped samples grouped into 11 unique haplotypes, six of which were shared between two or more habitats. Two haplotypes were unique to Sili forereef, two to Asaga back reef and one to Vatia bay.

For the microsatellite genotyping, 228 samples amplified at one or more loci; however, only 145 samples from nine populations amplified at five or more loci and were able to be used for subsequent analysis (Table 2.2). This greatly reduced our sample size for the microsatellite analysis and prevented us from including the Tutuila Tisa population (only two individuals could have been used; Table 2.1). Out of 252 tests for linkage disequilibrium between microsatellite locus pairs, 41 were found to be significant

($P < 0.05$); however, only seven of these associations, involving six different pairs of loci and three populations were significant after Bonferroni correction (Table 2.3). As no consistent trend was observed across significant associations for specific locus pairs or populations, we considered these loci to be inherited independently in the following analyses.

Significant departures from Hardy-Weinberg equilibria due to heterozygote deficits were detected for at least one locus at all sites (Table 2.2). As suggested recently by Underwood et al. (2009), heterozygote deficits seem to be common features observed in coral and marine invertebrate populations likely arising from biological factors such as spatial and/or temporal admixture and nonrandom mating between sites. However, we cannot determine the specific causes of these heterozygote deficits and rule out the possibility that these patterns were caused by the presence of null alleles. As expected, null allele testing revealed significant evidence for the presence of null alleles at most of the loci in disequilibrium. Hence, data were adjusted for null alleles prior to genetic distance calculations and analyses.

In contrast to our expectations, a number of samples sharing unique eight-locus genotypes were observed for our samples. $N_g:N$ ranged from 0.679 to 1 (0.848 total), and lower ratios appeared to be more common at sites with greater sample sizes (Table 2.2). A total of 123 unique sequence types were observed and only these individuals were included in statistical analyses. Identical, eight-locus genotypes were never observed in samples from different populations; hence the influence of asexual reproduction is thought to be restricted to very small spatial scales. Interestingly, one individual from

Pool 400 on Ofu was found to share identical alleles with an individual 110 km away from Vatia Bay on Tutuila at five of the eight microsatellite loci ($P_{ID} < 1 \times 10^{-6}$), which suggests a high degree of genetic relatedness may occur through means other than asexual reproduction.

Table 2.2 Characteristics of *Porites lobata* microsatellite loci in the nine sampling locations. Given are the number of alleles, observed (H_O) and expected (H_E) heterozygosities, inbreeding coefficients. Significant values for departure from Hardy-Weingberg equilibria after sequential Bonferroni correction are shown in bold. Total number of genotypes (N), unique 8 locus genotypes (N_g), and the ratio of the two are given at the bottom. The probability of identity was also calculated for each locus and for over all loci.

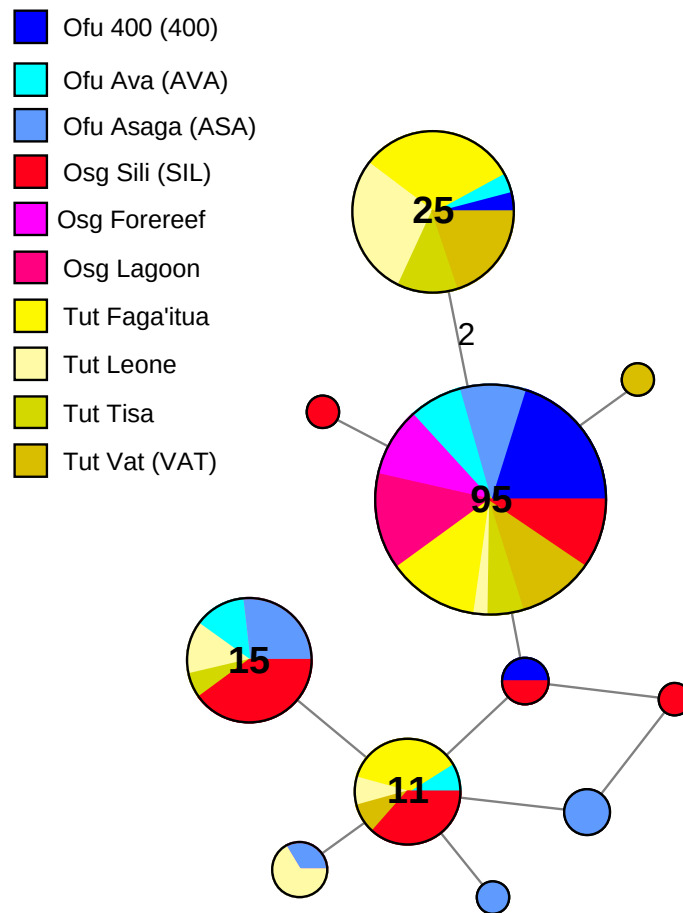
Locus		Pool 400	Ava	Asaga	Osg Lagoon	Sili	Osg Forereef	Faga'itua	Vatia Bay	Leone	P _{ID} Biased	P _{ID} Unbiased
pl0340	# Alleles	7	4	5	3	6	4	7	7	2	0.0688	0.0652
	H _O	0.27778	0.375	0.27778	0	0.36842	0.16667	0.5	0.35714	0		
	H _E	0.65397	0.74167	0.79365	0.48352	0.70697	0.65152	0.84127	0.80159	0.42857		
	F _{IS}	0.563	0.461	0.640	1.000	0.465	0.721	0.384	0.538	1.000		
pl0780	# Alleles	7	9	8	7	7	6	8	10	9	0.0364	0.0336
	H _O	0.52941	0.7	0.8125	0.85714	0.63158	0.66667	0.57143	0.5	0.5		
	H _E	0.7344	0.83158	0.875	0.89011	0.83357	0.87879	0.84656	0.84444	0.85263		
	F _{IS}	0.257	0.114	0.041	-0.037	0.222	0.172	0.300	0.391	0.383		
pl0905	# Alleles	14	9	13	8	12	6	11	12	12	0.0105	0.0089
	H _O	0.61111	0.7	0.66667	0.71429	0.63158	1	0.61538	0.72222	0.54545		
	H _E	0.8746	0.86842	0.89524	0.9011	0.89189	0.86364	0.91385	0.91111	0.92641		
	F _{IS}	0.281	0.152	0.234	0.146	0.273	-0.263	0.300	0.185	0.383		
pl1551	# Alleles	7	6	6	4	6	6	7	6	6	0.0722	0.0687
	H _O	0.57895	0.8	0.38889	0.75	0.68421	0.83333	0.71429	0.47059	0.6		
	H _E	0.77952	0.72632	0.75873	0.675	0.75533	0.87879	0.7672	0.79857	0.81053		
	F _{IS}	0.237	-0.159	0.473	-0.185	0.070	-0.034	0.034	0.393	0.221		
pl2069	# Alleles	5	6	6	3	9	3	5	4	5	0.1012	0.0969
	H _O	0.3125	0.44444	0.6	0	0.42105	0.33333	0.5	0.41176	0.44444		
	H _E	0.59073	0.79085	0.74943	0.48352	0.7724	0.73333	0.80688	0.74153	0.82353		
	F _{IS}	0.454	0.405	0.172	1.000	0.440	0.455	0.357	0.428	0.429		
pl1357	# Alleles	11	8	10	6	12	5	9	10	9	0.0218	0.0194
	H _O	0.78947	0.66667	0.625	0.42857	0.78947	0.66667	0.78571	0.66667	0.81818		
	H _E	0.85775	0.81699	0.86694	0.86813	0.899	0.78788	0.8836	0.89841	0.8355		
	F _{IS}	0.055	0.136	0.256	0.468	0.098	0.077	0.078	0.237	-0.026		
pl1629	# Alleles	4	3	4	3	6	2	3	4	3	0.2696	0.2632
	H _O	0.42105	0.3	0.4375	0.375	0.31579	0.16667	0.5	0.55556	0.18182		
	H _E	0.44239	0.27895	0.61089	0.49167	0.44666	0.16667	0.62169	0.6746	0.17749		
	F _{IS}	0.023	-0.132	0.261	0.186	0.274	-0.091	0.166	0.153	-0.073		
pl1868	# Alleles	7	6	5	6	8	4	9	8	7	0.0444	0.0415
	H _O	0.78947	0.22222	0.1875	0.42857	0.52632	0.66667	0.42857	0.33333	0.36364		
	H _E	0.80228	0.73203	0.76008	0.82418	0.80654	0.77273	0.84921	0.83968	0.84848		
	F _{IS}	-0.011	0.679	0.745	0.440	0.330	0.059	0.477	0.592	0.551		
Total	F _{IS}	0.14932	0.1727	0.30229	0.15545	0.23236	0.08745	0.25587	0.34615	0.31034	5.029E-11	2.739E-11
	N _g :N	0.67857143	1	0.7826087	0.8	1	1	1	0.782608696	0.91666667	0.84827586	

Table 2.3 Significant interlocus associations detected by GENEPOP

Location		
Faga'itua	Vatia Bay	Pool 400
pl0780/pl1629***	pl0780/pl0905***	pl0905/pl1868***
pl0905/pl2069***	pl0905/pl1868***	
pl1551/pl1357***	pl1357/pl1868***	

Population Structure

The genetic structure of *P. lobata* indicated a lack of panmixia between island groupings, with additional subdivision within islands. The mitochondrial and microsatellite markers showed a surprising degree of concordance regarding both between and within island subdivision, with finer scale differences between the two sets of markers evident at the between population level. Neither set of markers supported a pattern of genetic structure based on habitat type (back reef, forereef, or bay), hence populations appear to not be isolated by clearly discernable environmental characteristics.



① ② ③

Fig. 2.2 Maximum parsimony phylogenetic network reconstruction of mitochondrial DNA haplotypes. Lines between nodes represent single substitutions; multiple substitutions are denoted using single black digits located next to each line. Diameter of circle at each node is proportional to the number of individuals with identical sequences. Nodes with greater than 3 sequences have the corresponding number of sequences marked within the circle.

The phylogenetic network construction based on the mtDNA sequences illustrated a lack of strict division between sites with 95 samples, at least two from each population, sharing a single haplotype (Fig. 2.2). One haplotype, shared by 23 individuals, was dominated by samples from all Tutuila populations and shared by only two samples from Ofu Pool 400 and Ava populations respectively. It appears that this haplotype, in combination with the five haplotypes unique to Ofu and Olosega may be driving the significant differences between island groupings discussed below.

Table 2.4 Analysis of molecular variance (AMOVA) among islands and populations for *Porites lobata* using (A) 8 microsatellite loci and (B) the NAD5 and putative control regions of mitochondrial DNA. Significance tests were based on 50,000 permutations.

(A)						
Source of Variation (F_{ST})	DF	Sum of squares	Variance components	Percentage of variation	Fixation index	P-Value
Among Island Groupings	1	10.189	0.0556	2.41	0.02409	0.0119+-0.00049
Among Populations	7	26.701	0.04029	1.75	0.01789	0.00195+-0.00020
Within Populations	114	313.435	0.53732	23.28	0.2429	0.00000+-0.00000
Within Individuals	123	206	1.6748	72.56	0.27435	0.00000+-0.00000
(B)						
Source of Variation (F_{ST})	DF	Sum of squares	Variance components	Percentage of variation	Fixation index	P-Value
Among Island Groupings	1	9.377	0.09843	12.16	0.12164	0.01409+-0.00058
Among Populations	8	13.997	0.07283	9	0.10247	0.00104+-0.00015
Within Populations	147	93.773	0.63791	78.84	0.21165	0.00000+-0.00000

AMOVA revealed significant between island and within island population structure (Table 2.4) for both mtDNA and microsatellite markers, with 2.41 and 12.16 percent of the variation attributable to island groupings (for microsatellite and mtDNA respectively). The between island structure was significant only when Ofu and Olosega islands were combined as a single island grouping (data not shown). Hence, for the following discussion we will refer to these two islands as a single group termed Ofu/Olosega. Ofu and Olosega are separated physically only by a shallow causeway (<3m max depth). Indeed, pairwise F_{ST} comparisons found that some populations on Ofu were more similar to populations on Olosega than they were to other populations on Ofu (Table 2.5)

Pairwise F_{ST} s and Exact tests showed finer-scale population subdivision within and between islands (Table 2.5). Within-island population subdivision did not seem to correlate with habitat designation; with only one significant pairwise F_{ST} comparison between adjacent back reef and forereef sites. For F_{ST} based analyses, there were more significant pairwise comparisons from the mtDNA data set (20 initial, 7 after Bonferroni correction) than the microsatellite data set (15 initial, 6 after Bonferroni correction). Notably, sample sizes for the microsatellite data set were consistently smaller than those of the mtDNA set which may have had an impact on the sensitivity of the microsatellite tests. In contrast, exact tests of the microsatellite data showed much higher levels of population subdivision than the mtDNA data with 27 and 7 significant pairwise comparisons after Bonferroni correction respectively.

Table 2.5 Pairwise population comparisons using F_{ST} values calculated based on (A) multilocus microsatellite and (B) mitochondrial genotypes of sampled *Porites lobata*. Significant levels of divergence pre and post Bonferroni correction are indicated by asterisks (* $P < 0.05$) and (** $P < 0.0014$)

(A)	Pool 400	Ava	Asaga	Osg Lagoon	Sili	Osg Forereef	Faga'itua	Vatia Bay	Leone
Pool 400									
Ava	0.03917*								
Asaga	0.04842**	0.03733							
Osg Lagoon	0.02404	0.02883	0.00815						
Sili	0.01974	-0.00867	0.01349	-0.00694					
Osg Forereef	0.03296	0.01847	0.03751	0.02768	0.00117				
Faga'itua	0.07597**	0.07294*	0.06131*	0.06442*	0.05887**	0.08216*			
Vatia Bay	0.03032*	0.03472	0.02024	0.00832	0.02237	0.02806	0.02492		
Leone	0.08626**	0.05198*	0.09181**	0.08564*	0.05961**	0.05384	0.03888	0.05561*	

(B)	Pool 400	Ava	Asaga	Osg Lagoon	Sili	Osg Forereef	Faga'itua	Vatia Bay	Leone	Tisa
Pool 400										
Ava	0.13657*									
Asaga	0.29918**									
Osg Lagoon	-0.02477	0.16329*	0.29124*							
Sili	0.33481**	0.0161	-0.04152	0.33259*						
Osg Forereef	-0.04825	0.11294	0.24553*	0	0.2925*					
Faga'itua	0.14813*	0.04513	0.19323*	0.1811*	0.22625**	0.14676*				
Vatia Bay	0.11382	0.07392	0.24183**	0.16102	0.28113**	0.12105	-0.02976			
Leone	0.33517**	0.08398	0.15807*	0.34038**	0.18989*	0.288*	0.03137	0.09204		
Tisa	0.16865*	0.00753	0.16596*	0.22772*	0.20738*	0.16667	-0.06887	-0.07217	-0.0029	

The lack of a distinct pattern to within island structuring is further evident in the distance analyses. There was significant evidence for isolation by distance in the microsatellite data across all populations for all distance measures (F_{ST} , R_{ST} , Cavalli-Sforza chord, and Nei's D ; $p = 0.03190$, 0.03143 , 0.0043 , and 0.0060 respectively). This pattern is likely driven by the significant geographic and genetic separation between the Tutuila and Ofu/Olosega populations as no significant evidence for isolation by distance was observed within-island groupings (data not shown). The distance dendrograms illustrate two distinct clades, one for Tutuila populations and a second for Ofu/Olosega populations, but no consistent within clade patterns (Fig 2.3). The distinct separation of

Tutuila and Ofu/Olosega clades is evident for both the microsatellite and mitochondrial markers, even though the mtDNA test for isolation by distance was not significant.

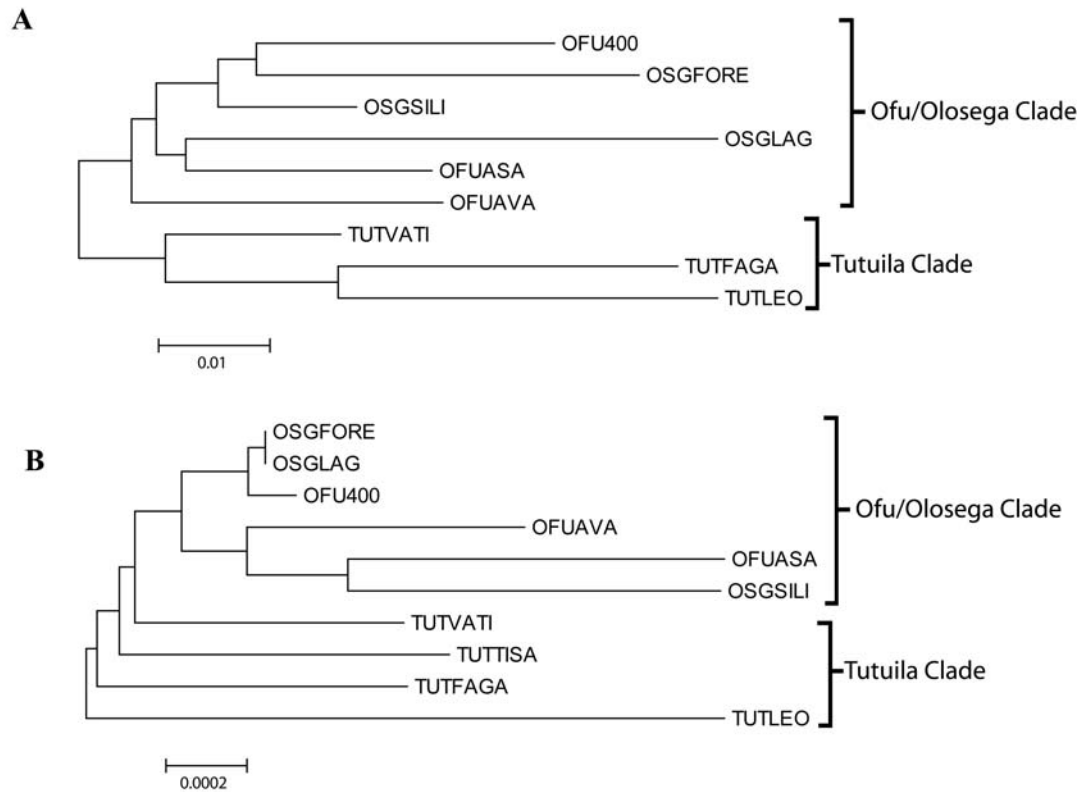


Fig. 2.3 Neighbor joining dendrograms showing relationships among sampled populations based on (A) Cavalli-Sforza and Edwards chord distances for microsatellite and (B) strict pairwise genetic distances for mitochondrial loci constructed in Mega ver 3.1.

The Bayesian analysis performed in STRUCTURE proved to be the most sensitive of all, finding highest support for K=9 populations ($\text{LnP(D)} = -2878.6$) when no a-priori population information was available and equal probability for K=2 and K=9

populations ($\text{LnP(D)} = -1$) when population information was taken into consideration. For $K=2$ populations, the average membership coefficient for Tutuila populations was actually higher for the Ofu/Olosega cluster than it was for the Tutuila cluster (0.404 and 0.596, respectively), suggesting weak support for this model. In contrast, when $K=9$ populations were designated, all populations (except Faga'itua) had highest membership coefficients in the cluster from which they were sampled (termed original population coefficient; Fig. 2.4).

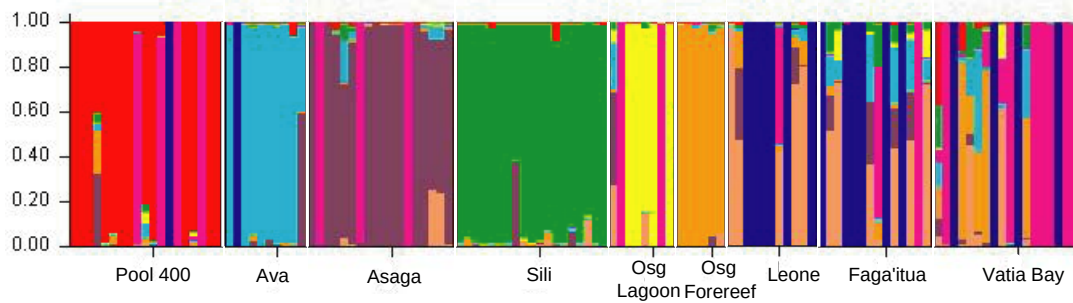


Fig. 2.4 Geographical clustering of *Porites lobata* populations inferred using STRUCTURE, Each vertical bar represents an individual coral sample, the color of the bar signifies the population to which it was assigned using the model, solid black bars separate samples from different populations and each population origin is noted below the graph.

Discussion

We used mitochondrial DNA and microsatellites to assess the population structure of *Porites lobata* within the territory of American Samoa. Evidence from a previous study (Barshis Chapter 1) suggested that strong differences in environmental

characteristics may play a role in shaping fine-scale population structure. This study was an attempt to investigate the consistency of that pattern across a wider-sampling of populations. While significant population structure was observed over small (<600 m) and large (>100 km) spatial scales with both sets of markers, there appeared to be no clear correlation between habitat types and level of population differentiation. The most consistent population subdivision observed was between populations from different island groups separated by ~100km.

A significant departure from Hardy-Weinberg equilibrium was observed for all loci, due primarily to consistent heterozygote deficits. The employment of genetic markers to test population structure in corals has shown heterozygote deficits to be a common feature of many scleractinian populations (Ayre & Hughes 2000; Ayre & Miller 2004; Baums *et al.* 2005; Mackenzie *et al.* 2004; Magalon *et al.* 2006; Miller & Ayre 2004, 2008; Ridgway *et al.* 2008; Sherman *et al.* 2006; Underwood *et al.* 2007, 2009). Heterozygote deficiency may result from high levels of inbreeding caused by self-fertilization and/or limited gamete dispersal. While no data exist for reproductive mode of *Porites lobata* in American Samoa, this species has been found to be a gonochoric, broadcast-spawning coral at various other sites across the indo-pacific (Richmond & Hunter 1990). The separate sexes of different colonies limits the potential for selfing, although clonal individuals in close proximity could cross fertilize if clonemates were of opposite sexes. The fine-scale population subdivision discovered by this study suggests that inbreeding with closely related individuals may be the significant contributor to deviations from HWE. Alternatively, heterozygote deficits can result from the admixture

of separate populations or temporally distinct recruitment cohorts (Lenfant & Planes 2002) thereby creating a Wahlund effect by sampling individuals from non-randomly mixed gene pools. While we cannot distinguish between the specific mechanisms contributing to these deficits, the level of small-scale population differentiation observed in this study is consistent with other, similar findings of restricted dispersal and inbreeding (Mackenzie *et al.* 2004; Miller & Ayre 2004, 2008; Whitaker 2006).

Initially surprising, was our finding of a high number (~15% of all samples) of apparently clonal genotypes (ramets) within our sampled populations. Other, recent studies have expressed similar surprise at the prevalence of clonality in massive coral populations (Foster *et al.* 2007; Miller & Ayre 2008; Severance & Karl 2006). Intuitively, it is difficult to comprehend that such massive coral colonies would be as susceptible to breakage and redistribution of fragments as their more fragile branching relatives. However, asexual reproduction via fission and fragmentation has long been observed to be a significant contributor to reproduction in massive corals, including *Porites*, in a variety of habitats (DeVantier & Endean 1989; Done & Potts 1992; Highsmith 1980, 1982; Kojis & Quinn 1981). Done and Potts (1992) suggested that asexual reproduction via fission and fragmentation may also be more common in back reef versus forereef environments, where *Porites* colonies often reach large (>3 m diameter) sizes. This appears to be the case from our data, as clones were observed for three of four back reef and only two of five non-back reef sites: Vatia Bay (clones only observed between samples taken in the nearshore and back reef sections of the bay), and Leone (only one clone pair was detected). This pattern is suggestive, although additional sampling is

necessary as inconsistent sample sizes between sites likely influenced our ability to quantify the degree of clonality.

Another, often overlooked method of asexual reproduction involves the “bail-out” of individual polyps from a parent colony that settle anew in different nearby locations (Sammarco 1982). It is admittedly more difficult to directly assess the contributions of “bail-out” to the clonal structure observed in this study. Although this strategy has not been observed for *Porites*, significant contributions of “bail-out” would be predicted to generate an array of size classes, with colonies resulting from resettled polyps being consistently smaller than parental colonies over time. An examination of the size relationship of clonal genotypes revealed no specific pattern, with clones detected equally between large (>1 m diameter) colony pairs, small colony (<1 m) pairs, and large and small colony pairs (data not shown). The lack of size association pattern, in addition to the lack of recorded observation of “polyp bail-out” in *Porites*, lead us to conclude that fission and fragmentation are likely the major contributor to asexual reproduction in this study area. The degree of clonal structure observed here, in conjunction with that of other studies using similar, highly polymorphic markers (Foster *et al.* 2007; Miller & Ayre 2008; Severance & Karl 2006), is beginning to provide the genetic verification of long-standing observations regarding asexual reproduction in massive corals.

The high level of agreement observed between our mitochondrial and microsatellite data sets is a novel finding that was also unexpected. Rates of mitochondrial DNA evolution are known to be significantly slower in corals and select marine invertebrates than other organisms (Shearer *et al.* 2002; van Oppen *et al.* 1999).

While successfully employed in a variety of organisms as markers of population-level divergence (*e.g.* Avise 2004; Bowen *et al.* 2006; Bowen *et al.* 2005; Rocha *et al.* 2007), their utility in coral is thought to be restricted to species or genus level distinctions (Concepcion *et al.* 2006; Shearer *et al.* 2002). Agreement between mitochondrial and nuclear markers has been shown previously in the genus *Porites* at the species level, although nuclear markers exhibited a higher degree of resolution of some interspecific boundaries (Forsman *et al.* 2009). Microsatellites are thought to be among the most rapidly evolving sections of the genome and are employed in a variety of ways including diagnosis of population divergence of closely related conspecifics (Avise 2004; Jarne & Lagoda 1996; Oliveira *et al.* 2006). Hence, the degree to which the mtDNA data set supported the same conclusion of between island and within island population subdivision illustrates the utility of both sets of markers for diagnosing population level divergence within this species. Additionally, all analyses except the microsatellite F_{ST} comparison, clearly defined Pool 400 and Sili as separate populations. This adds an additional layer of agreement, as previous work using the Internal Transcribed Spacer (ITS) region of nuclear ribosomal DNA supported the same conclusion (Barshis Chapter 1).

It is important to note that some disagreement between the two marker sets was apparent. At a finer-scale, pairwise F_{ST} comparisons between some population pairs were significant for one marker and not the other (Table 2.5). However, differences in sample sizes and specific population composition due to unsuccessful amplification of particular individuals make strict comparisons between the two data sets difficult. Exact tests using

the microsatellite data set also appeared more sensitive than those with mtDNA; although as these tests are based on allele frequency distributions, this was to be expected given the greater allelic diversity within microsatellite loci.

Population structuring between Tutuila and Ofu/Olosega also followed the pattern of isolation by distance; although the pattern was not significant between sites within island groupings (data not shown). The distance dendrograms (Fig. 2.3) for both marker sets also illustrate the division between island groups. Vatia bay appears to be the most closely related site to the Ofu/Olosega populations and may be the source of connection between the two areas. This hypothesis was further supported by the STRUCTURE analyses which assigned seven of the 80 Ofu/Olosega samples back to the Vatia bay population (Fig. 2.4). STRUCTURE analyses also proved strongest for K=9 populations when *a priori* information taken into consideration. Membership coefficients were highest for all but one (Faga'itua) population in the cluster from which samples were originally taken (Fig. 2.5). Interestingly, the average original population coefficient for Ofu/Olosega populations was over double that for Tutuila populations (0.796 and 0.393, respectively). This suggests that Ofu/Olosega populations may maintain stronger levels of subdivision than Tutuila populations despite their closer geographic proximity (average within-island geographic distances between populations were 2.06 and 11.31 km for Ofu/Olosega and Tutuila respectively).

As a whole, data from both marker sets and a variety of approaches support the description of *P. lobata* populations in American Samoa as highly subdivided at within and between island spatial scales. As asexually produced clones were not included in

these analyses, this pattern appears to be driven by localized differences in larval dispersal and settlement success. A similar combination of larger and smaller scale population subdivision has been observed in coral populations previously, and appears to be a common feature of many coral communities (Ayre & Hughes 2000; Magalon *et al.* 2005; Miller & Ayre 2008; Severance & Karl 2006; Sherman *et al.* 2006; Underwood *et al.* 2007, 2009; Whitaker 2006). Complex, stochastic interactions between spawning success, ocean microcurrents, environmental heterogeneity, and many other factors have been invoked as explanations of these difficult to interpret patterns of coral population structure. Large variability in size and effective settlement of recruitment cohorts is well known in corals and other coral reef organisms (Coles & Brown 2007; Penin *et al.* 2007a; Penin *et al.* 2007b). However, predicting how these recruitment events and patterns will manifest themselves over evolutionary time can be difficult, especially for such long-lived animals as massive corals. Additionally, large scale disturbance events may non-randomly structure certain coral populations, the signals of which may persist for some time (Underwood *et al.* 2007).

On the basis of a previous study (Barshis Chapter 1), we attempted to assess the relationship between population structure and specific habitat type for *P. lobata* in American Samoa. While our sampling did not demonstrate clear environmental influence on population subdivision, our back reef and forereef habitat designations may have been too general to uncover a pattern. Selective forces likely play a large part in the structuring of coral reef communities, yet untangling the signature of these forces from the multitude of other behavioral, hydrodynamic, and temporal influences is a challenge we are only

beginning to meet in this system. While no easy task, developing a functional understanding of how these factors influence genetic connectivity of coral populations is crucial for the design of effective management strategies and ensuring the conservation of these ecosystems for future generations.

Appendix 2.1 Exact tests of population differentiation based on (A) microsatellite and (B) mitochondrial loci for sample *Porites lobata* in American Samoa. Significant values are designated by * and ** for significance pre (<0.05) and post (<0.00143) Bonferroni correction

(A)	Pool 400	Ava	Asaga	Osg Lagoon	Sili	Osg Forereef	Faga'itua	Vatia Bay	Leone	
Pool 400										
Ava	0.00001**									
Asaga	Highly sign**.	0.01707*								
Osg Lagoon	0.00004**	Highly sign.**	0.00103**							
Sili	0.00055**	0.35435	0.00239*	Highly sign.**						
Osg Forereef	0.05063	0.13537	0.10657	0.00027**	0.74112					
Faga'itua	Highly sign.**	0**	0**	0**	Highly sign.**	0**				
Vatia Bay	0**	0.00111**	0**	0**	0**	0.04119*	0.00003**			
Leone	Highly sign.**	0.00026**	0**	Highly sign**.	0**	0.00617*	0.00023**	0**		
(B)	Pool 400	Ava	Asaga	Osg Lagoon	Sili	Osg Forereef	Faga'itua	Vatia Bay	Leone	Tisa
Pool 400										
Ava	0.07016									
Asaga	0.00273*	0.61590								
Osg Lagoon	1.00000	0.03328*	0.03050*							
Sili	0.00038**	0.73717	0.14301	0.00918*						
Osg Forereef	1.00000	0.32190	0.20454	NA	0.06066					
Faga'itua	0.00293*	0.09740	0.00026**	0.00483*	0.00107**	0.02180*				
Vatia Bay	0.03919*	0.24724	0.00344*	0.01696*	0.00257*	0.14092	0.48446			
Leone	0.00000**	0.02659*	0.00150*	0.00002**	0.00064**	0.00036**	0.01841*	0.02188*		
Tisa	0.02823*	0.59828	0.14524	0.01514*	0.08096	0.08486	0.31780	0.78724	0.27087	

CHAPTER THREE:

TEMPORAL, SEASONAL AND LONG-TERM DIFFERENCES IN STRESS PROTEIN RESPONSE OF THE CORAL *PORITES LOBATA* TO CONTRASTING HABITAT TYPES IN AMERICAN SAMOA

Abstract

The influence of abiotic factors on organismal physiology has important implications for ecological processes such as species range distributions, niche occupation and susceptibility to global climate change. While upper physiological stress tolerance limits often follow clear patterns, an increasing understanding of sublethal environmental influences at the cellular and molecular levels is revealing a more complex story. This study describes seasonal, temporal and long term variability of the stress biomarker protein response of the coral *Porites lobata* to contrasting environmental conditions. A series of transplants were conducted between a highly variable back reef and a more stable forereef habitat on Ofu and Olosega islands in American Samoa. The most consistent differences observed were elevated levels of ubiquitin-conjugated proteins in back reef source corals versus their forereef conspecifics, suggesting a greater amount of or capacity to process irreversible protein denaturation. A significant reduction in ubiquitin-conjugates was observed during winter versus summer seasons when daily extremes in back reef characteristics were lowest. All markers showed evidence for genetic/epigenetic influence on protein response, suggesting significant limitations to phenotypic response in the face of novel environmental conditions. As a whole, these data suggest that the fluctuating environmental characteristics of the back reef have significant influence on cellular stress responses across seasons and time scales. The degree to which the fitness of these corals may be affected in the long term remains uncertain, however, this initial insight suggests that small scale differences in habitat

characteristics could play an important role in shaping responses to future environmental change.

Introduction

The effects of environmental variability on organismal physiology can influence processes at the smallest molecular levels to the largest ecological and evolutionary scales. For marine ectotherms, changes in abiotic factors such as temperature, pH, pO₂, and irradiance often have drastic consequences on growth, survival, and physiological function (e.g., Helmuth *et al.* 2006; Menge *et al.* 2002; Somero 2002; Stillman 2003). In the case of reef-building corals, slight changes in these factors can trigger a breakdown of the coral-dinoflagellate symbiosis, termed “coral bleaching”, frequently resulting in widespread mortality (Baird & Marshall 1998; Fitt *et al.* 2001; Glynn 1993), increased incidence of disease (Jones *et al.* 2004), and shifts in community composition (Done 1992; Hughes *et al.* 2007). Yet while much attention has been focused on large scale environmental changes and their impacts, an emerging area of research aims to develop a better understanding of how small-scale heterogeneity in abiotic factors shapes physiological responses and tolerance limits (e.g., Anthony & Hoegh-Guldberg 2003; Helmuth & Hofmann 2001; Leichter *et al.* 2006; Smith *et al.* 2007).

Many corals and other marine organisms are living at or near their upper tolerance limits of environmental conditions (Carpenter *et al.* 2008; Stillman 2003; Tomanek 2008), hence slight increases in abiotic factors such as temperature and ocean acidification can often have lethal consequences (Anthony *et al.* 2008; Jokiel & Coles 1990). In contrast, sublethal stress exposures, particularly in corals, may actually enhance future stress tolerance and resilience through a number of mechanisms. For instance, it has been shown that a history of exposure to higher temperatures (Griffin *et al.* 2006) and

irradiance (Brown *et al.* 2002b) correlates with increased upper thermal tolerance thresholds in two reef-building coral species. Additionally, beneficial aspects of exposure to fluctuating versus constant temperatures such as increases in growth (Les *et al.* 2009; Spigarelli *et al.* 1982; Wang *et al.* 2007) and thermal tolerance limits (Feldmeth *et al.* 1974; Feminella & Matthews 1984; Hines *et al.* 1980) have been observed for a variety of aquatic organisms.

One mechanism of acquired thermotolerance following sublethal stress exposure is linked to the induction of a family of molecular chaperones called the heat shock proteins (HSPs; reviewed in Kregel 2002). Members of the HSP family assist in preventing irreversible protein denaturation by promoting correct refolding and maintenance of protein structural integrity (Feder & Hofmann 1999). Induction of the heat shock response via a conditioning exposure has been shown to decrease the energetic load of additional stress protein synthesis in subsequent thermal exposures, thus providing a degree of acquired thermotolerance (reviewed in Kregel 2002). Another potential mechanism of increased cellular stress defense in corals involves the capacity of the coral host and symbiont to deactivate reactive oxygen species (ROS) that are formed during photosynthesis and oxidative stress. Previous research has shown that symbiotic forming cnidarians may contain novel forms of superoxide dismutase (SOD) that are not found in the tissues of aposymbiotic congeners (Richier *et al.* 2005). Further characterization of how these cellular processes respond to environmental characteristics can provide insight into the mechanistic basis behind increases in stress tolerance due to previous experience.

This study investigates the responses of a group of four stress biomarker proteins in the coral *Porites lobata* from two distinct nearshore habitats in American Samoa. The reefs of American Samoa provide an ideal setting in which to investigate environmental influences on genetic structure as they host a variety of habitat types with a suite of different environmental characteristics (described in detail previously; Barshis Chapter 1; Oliver & Palumbi 2009; Piniak & Brown 2009; Smith *et al.* 2007; Smith & Birkeland 2003, 2007). A set of reciprocal transplant experiments (RTEs) were performed to test potential environmental (acclimation) versus fixed (epigenetic/genetic) influences on coral response in a highly fluctuating back reef versus a more stable forereef habitat. The biomarkers were chosen based on their functional roles in stress defense and stress-caused damage: heat shock protein 70 (hsp70) is a molecular chaperone that helps maintain protein structure and function under heat related denaturation, functionally serving as a cellular defense against temperature related stress (Feder & Hofmann 1999); Manganese Superoxide Dismutase (MnSOD) deactivates the reactive superoxide anion formed under oxidative stress before it can damage other proteins involved in cellular processes (Halliwell & Gutteridge 1999); 4-Hydroxynonenal (4HNE) is a reactive lipid peroxide that causes oxidative protein damage (Halliwell & Gutteridge 1999), and ubiquitin is a cellular protein tag that is bound to damaged proteins, marking them for degradation (Hawkins 1991). As protein synthesis can account for more than 16% of basal energy requirements of some marine organisms (Hawkins 1985), characterization of the stress protein response provides valuable insight into the relative impact of environmental influence on maintenance of cellular homeostasis.

Porites lobata, a massive, broadcast spawner, was selected as our study species because it is common in American Samoa, as well as other locations in the Indo-Pacific, is frequently a dominant member of the nearshore reef community, and gross colony morphology appears to differ between forereef and back reef habitats (Craig *et al.* 2001; Smith *et al.* 2007). The purpose of this study was to determine how previous exposure to the large, daily fluctuations in various environmental characteristics (e.g., temperature, flow rate, dissolved oxygen saturation, etc.) of the back reef shapes protein biomarker response. To this end, three transplant experiments were conducted within and between the back reef habitat and a more stable, neighboring forereef that has a significantly lower amount of environmental variability. Transplants were conducted during the southern winter (June to August) and summer (December to March) seasons, and over a range of time scales ranging from five to 261 days. We predicted that the environmental fluctuations of the back reef environment would either: (a) confer some type of increased stress tolerance based on previous exposure history, or (b) contribute to chronic environmental stress loading that may impact back reef coral and transplant fitness. Clear seasonal and temporal differences in biomarker response attributable to both environmental and genetic influence were observed in all of the transplant experiments. While patterns of response were highly variable, these data provide an initial insight into the characterization of differences between the influence of forereef and back reef environmental conditions on coral physiological response.

Materials and Methods

Study site

The research was carried out between 2004 and 2008 on Ofu and Olosega islands in the territory of American Samoa (14°11'S, 169°40'W). These islands host diverse communities of ~85 shallow reef-building coral species, many of which are consistently exposed to seawater temperatures (Craig *et al.* 2001) and irradiances (Smith & Birkeland 2007) much higher than neighboring areas. The extreme conditions on Ofu are caused by a prominent reef crest that blocks circulation between the waters of the back reef and the open ocean, limiting mixing of back reef waters during midday low tides (Smith & Birkeland 2003). This limited mixing coupled with high midday solar irradiance produces large (up to 6 °C) daily fluctuations in temperature (Barshis Chapter 1; Smith & Birkeland 2003, 2007). In addition, frequency of large storm events, corresponding rainfall, and potential groundwater release sporadically cause drops in back reef salinity (up to 5 ppt; Barshis Chapter 1; Smith *et al.* 2007). In comparison, the shallow (<3m low tide depth) forereef of Olosega island represents a more typical, average reef environment with a more constant flow regime, significantly lower temperature, irradiance, and salinity variability, and extremes of each (Barshis Chapter 1; Smith *et al.* 2007; Smith & Birkeland 2003, 2007). These two habitats provide an ideal field laboratory in which to investigate coral physiological responses to a suite of contrasting environmental characteristics.

Sample collection and experimental design

Replicate cores of *P. lobata* were collected using a pneumatic drill and hole saw (19 mm diameter) attachment and affixed to the substrate as described previously (Barshis Chapter 1). Three separate transplant experiments were conducted as follows: a time series, a Winter reciprocal transplant experiment (RTE) and a long-term RTE.

Time series

A first transplant experiment conducted only within Pool 400 on the Ofu back reef was designed as a time series profiling of natural variability in protein response. 38 cores were removed from six separate *P. lobata* source colonies on 20-Jan-2005. Of these 38, two were immediately frozen for baseline analysis and the rest were arranged into 18 separate rows and affixed to a shallow (1 - 1.5 m low-tide depth), flat piece of exposed rock using Z-Spar, Splash Zone marine epoxy (Carboline Company, St Louis, MO). After a 32 day period to recover from coring/transplantation stress, cores were assumed to have returned to a natural physiological state and were destructively sampled every four hours from 0:00 hrs on 22-Feb-05 to 20:00 hrs on 24-Feb-05 for a total duration of 68hrs. Samples were immediately placed on pre-incubated -80 °C ice packs until placement in the freezer and remained frozen at -80 °C until analysis.

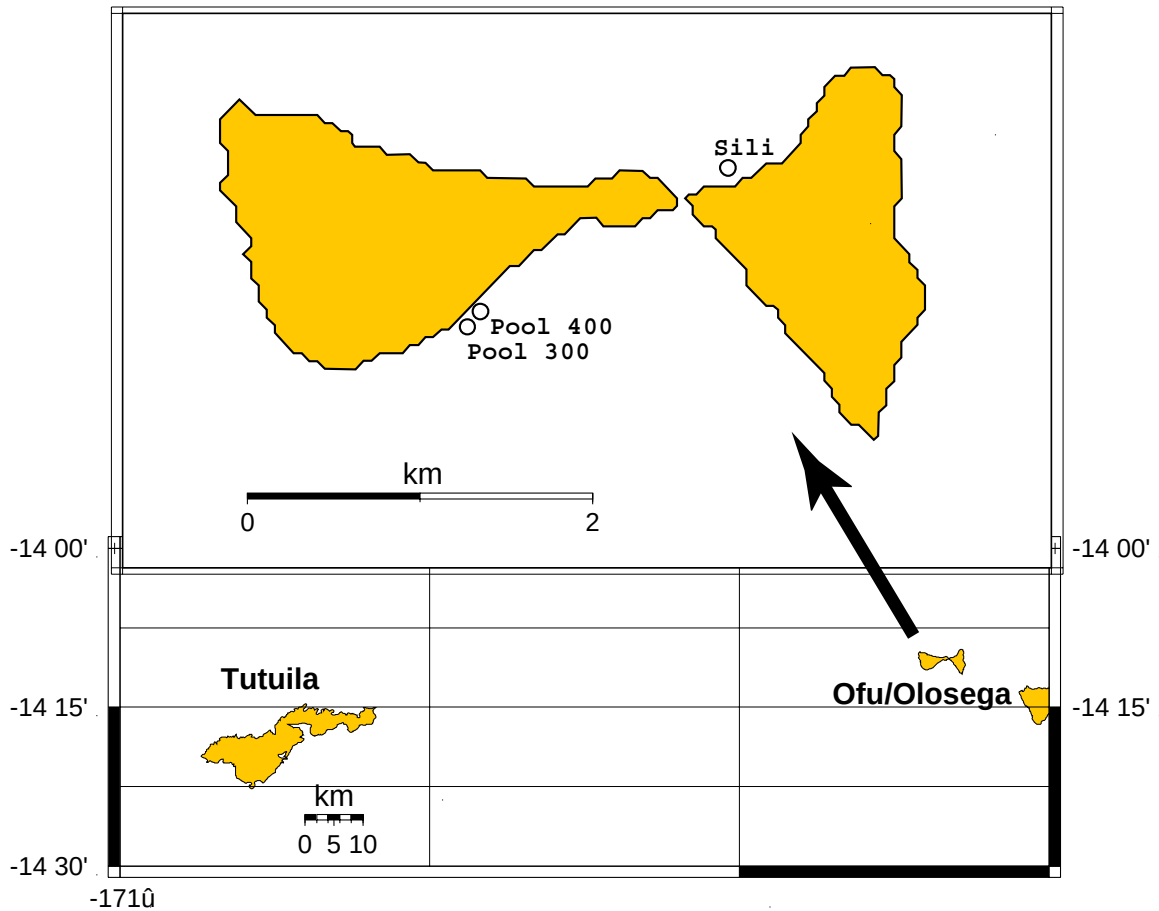


Fig. 3.1 Map of the study areas and transplant sites on Ofu and Olosega Islands. Map created with OMC (<http://www.aquarius.geomar.de/>)

Winter RTE

A total of six *P. lobata* source colonies from two locations were selected for use in a reciprocal transplant experiment (RTE) performed during the southern winter season in August of 2004. The RTE design utilized four replicate groups that were transplanted between a back reef (Pool 400) on Ofu and a forereef (Sili) on Olosega: From Pool 400 to Pool 400 (400 native), from Pool 400 to Sili (400 cross), from Sili to Sili (sili native) and

from Sili to Pool 400 (sili cross; Fig. 3.1). 24 cores were obtained from each source colony and selected for either cross or native transplantation. Transplants were affixed to the substrate in three separate grids (one per original source colony) in each location that were separated by > 5 m to minimize the potentially confounding influence of differences in microenvironment. The winter RTE was initiated on 11-Aug-04 at approximately 13:00 hrs. After a 24 hr recovery period, all samples were destructively sampled for four consecutive days (13-Aug to 16-Aug; Days 2 – 5) between 13:45 and 14:15 and immediately placed on pre-incubated -80 °C ice packs until placement in the freezer. All samples remained frozen at -80 °C until analysis.

Long-term RTE

A second, long-term RTE was conducted between 21-May-2007 and 6-Feb-2008 for a total of 261 days. An additional back reef site (Pool 300) was included in the long-term RTE as this site is known to undergo even greater environmental fluctuations than Pool 400 (Craig *et al.* 2001; Piniak & Brown 2009; Smith & Birkeland 2003; Smith *et al.* 2008). A total of 12 *P. lobata* source colonies (four from each of three sites) were selected for use in this transplant experiment comprising nine unique transplant groups: Pool 300 to Pool 300 (300 – 300), Pool 300 to Pool 400 (300 – 400), Pool 300 to Sili (300 – sili), Pool 400 to Pool 300 (400 – 300), Pool 400 to Pool 400 (400 – 400), Pool 400 to Sili (400 – sili), Pool Sili to Pool 300 (sili – 300), Pool Sili to Pool 400 (sili – 400), and Pool Sili to Sili (sili – sili). 12 replicate cores were removed from each source colony and selected for native (same location) or cross (new-location) transplantation. 5

cores from each source colony were affixed to the substrate in each location in May-07 and collected in Feb-08. Transplants were arranged in four separate grids (one per original source colony) separated by > 5 m in each location to minimize the potentially confounding influence of differences in microenvironment. Percent survival of each core was assessed visually in the field by estimating percent surface area of live vs. dead tissue. All samples were destructively sampled between 10:00 and 12:30 and immediately placed on pre-incubated -80 °C ice packs until placement in the freezer. All samples remained frozen at -80 °C until analysis.

Within an RTE framework, comparisons between transplant groups are designed to assess environmental versus genetic/epigenetic control over the response variable. Comparisons between native and transplanted groups examine variability associated with transplant destination (i.e., Pool 400 native vs. Pool 400 cross, Sili native vs. Sili cross) and with source colony origin (i.e., Pool 400 native vs. Sili cross, Sili native vs. Pool 400 cross; Smith *et al.* 2007). Variability by transplant destination is indicative of environmental influence on response (i.e., phenotypic plasticity), while variability by source colony origin indicates potential genetic or epigenetic influence on the response variable (DeWitt & Scheiner 2004; Schluter 2000; Smith *et al.* 2007).

Protein Analysis

Samples were extracted and levels of the four stress-biomarker proteins were assessed via SDS-PAGE and Western Blotting as described in Chapter 1. Antibody specificity was verified by molecular weight comparisons with known standards (Barshis

Chapter 1). For the MnSOD and hsp70 antibodies, a single band of the predicted molecular weight was visualized, while for the ubiquitin and 4HNE antibodies, a variety of bands were observed due to the conjugation of these markers to a wide range of proteins (Barshis Chapter 1). Antibody reactivity against *Symbiodinium* protein was verified using protein extracts from 3 different *Symbiodinium* cultures (KB8, AipB, and *Montipora Capitata*) graciously provided by the lab of Dr. Robert Kinzie III at the Hawai'i Institute of Marine Biology (Barshis Chapter 1). These cultures are believed to represent *Symbiodinium* types from taxonomic clades A, B, and D respectively. Antibody recognition of Cnidarian host protein is assumed based on manufacturers recommendations and previous study (Downs *et al.* 2000). Hence, the data presented herein are from holobiont homogenates comprised of both symbiont and host proteins.

Experimental protein concentration values were calculated relative to a standard coral sample dilution curve for each blot image based on integrated optical density using National Institute of Health software Image J. Statistical analyses were performed using Minitab[®] 15 (Mintab Inc., State College, PA), JMP Ver. 8 (SAS Institute Inc., Cary, NC), and SPSS Ver. 10.0 (SPSS Inc., Chicago IL). For the winter RTE, a repeated measures analyses of variance (ANOVA) was performed with marker concentration as the response variable for each transplant day compared against fixed factors of source colony origin and transplant destination, and the random factor of source colony number nested within origin. A repeated measures framework was deemed to be the most appropriate as measurements taken on replicate samples from the same source colony on different days were not independent from one another. A Tukey's post-hoc pairwise comparison was

conducted on individual days for the significant source colony origin by transplant destination interaction observed for hsp70 in order to elucidate underlying patterns behind the interaction. Data were natural log transformed for hsp70, square root transformed for 4HNE-conjugates, and a weighted least squares correction was used for MnSOD and ubiquitin-conjugates prior to the repeated measures analysis to meet assumptions of normality and homoskedasticity. In order to test possible seasonal differences in response levels, a two sample Mann-Whitney test was performed between Pool 400 native transplants from the winter RTE versus all samples from the summer time series. In an attempt to correct for potential effects of different sample sizes the tests were rerun on ten random samples of 48 values from the time series data set, although no difference in statistical outcome was observed. Unfortunately, no such comparison was able to be performed for the sili native transplant group as the only corresponding sample set from the summer RTE (Barshis Chapter 1), was analyzed using a different reference coral protein standard.

For the long-term RTE, a multi-factorial ANOVA was performed with marker concentration and the response variable compared against fixed factors of source colony origin and transplant destination, and the random factor of source colony number nested within origin. A Tukey's post-hoc pairwise comparison was conducted as above for the significant source colony origin by transplant destination interaction observed for the ubiquitin-conjugates in order to elucidate underlying patterns behind the interaction. No transformation was needed for hsp70 and 4HNE-conjugate data, and data were inverse square root transformed for ubiquitin-conjugates and a weighted least squares correction

was used for MnSOD to meet assumptions of normality and homoskedasticity. An additional ANOVA was conducted as above with untransformed percent survival as the response variable.

Two types of analyses were conducted on the time series experiment. First, a repeated measures ANOVA was performed with marker concentration as the response variable, source colony number as a fixed factor, and time as the repeated measure. No transformation was needed for hsp70 data, a square root transform was performed for ubiquitin-conjugates and 4HNE-conjugates, and a natural log transform for MnSOD data to meet assumptions of normality and homoskedasticity. In addition, a cross-correlation analysis was performed in Minitab[®] 15 between biomarker concentration and recorded temperature to investigate the potential influence of seawater temperature on protein response. Average values for biomarker levels were calculated for each time point using all six source colonies. Additional cross-correlations were calculated for each source colony individually, however, as trends were similar for both approaches only the average values are reported here. Baseline samples were taken 31 days prior to the start of the time series and are included in Figure 2 for reference, however they were removed from the statistical analysis to minimize confounding influence on the results.

Results

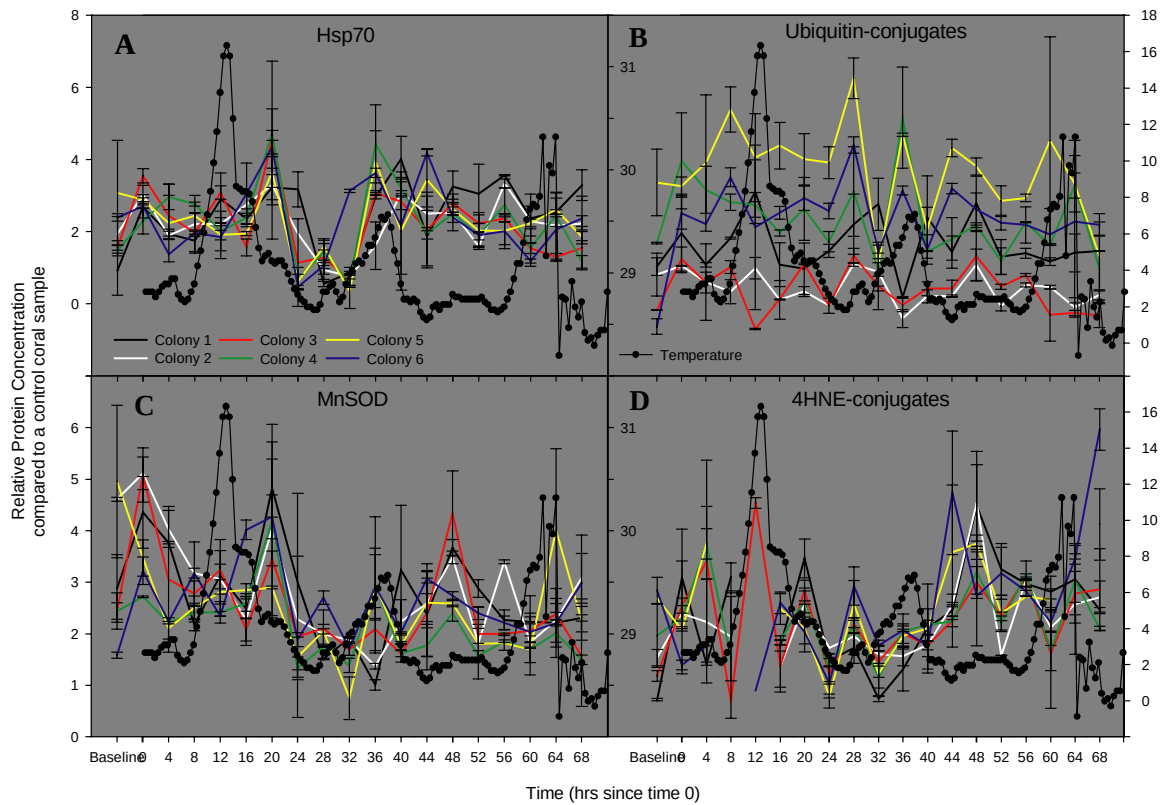


Fig. 3.2 Temperature profile and molecular marker concentrations (**A**) Hsp70, (**B**) Ubiquitin-conjugates, (**C**) MnSOD, and (**D**) 4HNE-conjugates calculated from western blots of host and symbiont homogenates relative to a standard coral sample (optical density of marker concentration per 2 μ g total soluble protein) for baseline and subsequent 4 hr sampling intervals of the time series. Means of two replicate nubbins for each individual source colony $\pm$ SE, source colony numbers are distinguished by color as designated in panel A, center axis is temperature in $^{\circ}$ C.

Table 3.1 Repeated measures ANOVA (source colony individual) for protein biomarker concentrations (ubiquitin conjugates, hsp70, MnSOD, and 4HNE) repeated over the 68 hr course of the time series experiment. Significant values are designated by bold-face type

Factor	df	MS				F				p			
		ubiq	hsp70	MnSOD	4HNE	ubiq	hsp70	MnSOD	4HNE	ubiq	hsp70	MnSOD	4HNE
Time	17	0.487	6.19	0.693	1.303	3.50	10.67	8.73	6.24	<0.001	<0.001	<0.001	<0.001
Source colony indiv	5	10.43	0.645	0.364	0.126	50.50	0.714	3.21	0.255	<0.001	0.614	0.011	0.936

Time series

A total of 228 samples were analyzed, comprising 19 time points and two replicate cores for each of six original source colonies per time point (Fig. 3.2). As described previously, antibody recognition of proteins extracted from cultured *Symbiodinium* was observed for MnSOD, ubiquitin-conjugated proteins, and 4HNE-conjugated proteins but not for hsp70 (Fig. 1.3 in Barshis Chapter 1). The repeated measures ANOVA revealed a significant effect of time for all four biomarkers and a significant effect of source colony only for ubiquitin-conjugate and MnSOD levels (Table 3.1). Visibly, the ubiquitin-conjugate profiles exhibited the largest variability between source colonies with over a three-fold difference between some source colonies on the same day (Fig. 3.2B). All biomarker levels of baseline samples (pre-coring), fell within the range observed during the course of the profile suggesting that cores had recovered from any handling associated stress prior to the beginning of the experimental sampling (Fig. 3.2B). Additionally, all corals showed signs of new tissue growth approx. 1-2 weeks after transplantation (pers. observation), which was further illustration of a rapid recovery from handling induced stresses.

Cross-correlation analyses indicated consistent increases in protein levels at various times following thermal increases for hsp70, MnSOD, and ubiquitin-conjugate profiles with a weaker, more inconsistent association for the 4HNE-conjugate profile (Fig. 3.3). MnSOD response responded quickest to temperature variability with positive associations observed at 0, 4 and 8 hr lags, followed by hsp70 (4 and 8 hr lags) and ubiquitin-conjugates (12 and 16 hr lags; Fig. 3.3). No clear association between temperature and 4HNE conjugates was apparent from the cross-correlation analysis. The other three markers also showed evidence for a longer delay in response with the largest correlation coefficients observed at 24 and 32 hr lags for hsp70 and ubiquitin-conjugates respectively, and a smaller coefficient for MnSOD at the 24 hr lag (Fig. 3.3).

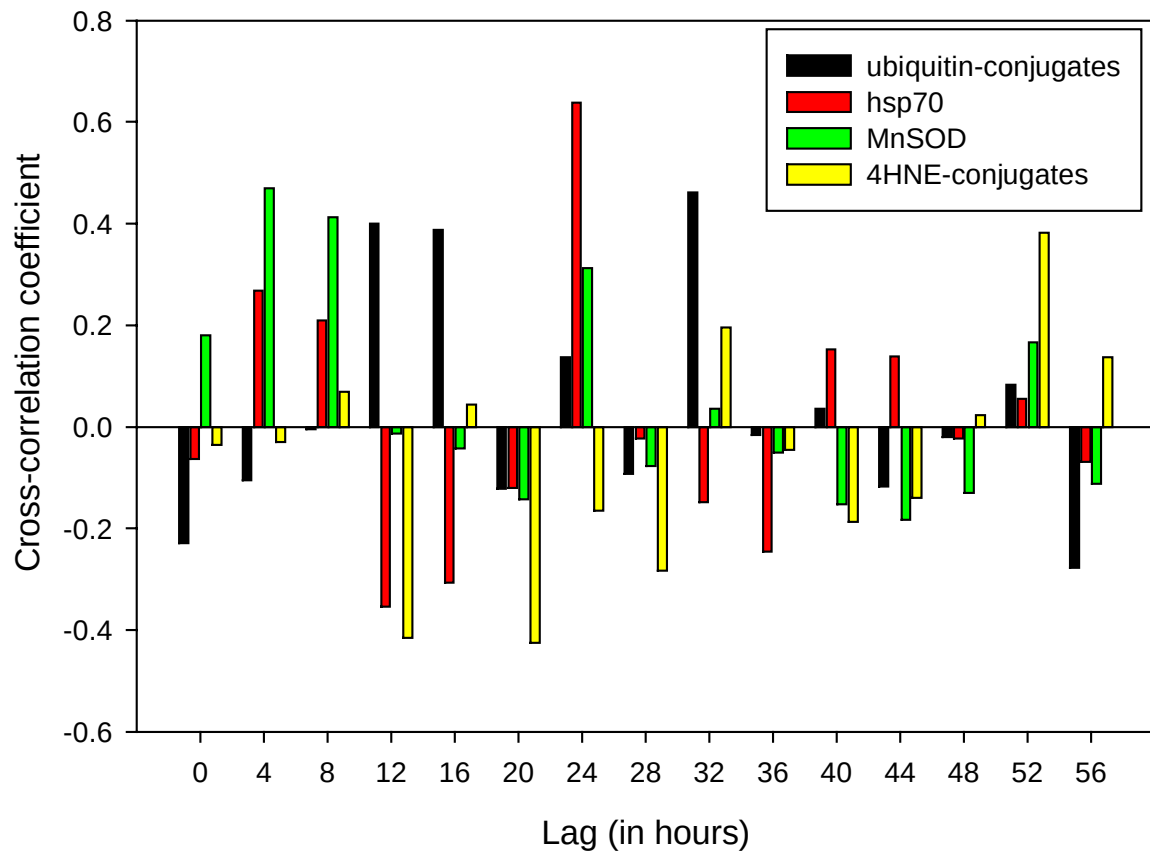


Fig. 3.3 Cross correlation coefficients between temperature and average marker levels across all source colonies as calculated in Minitab® 15. Each marker is assigned a unique color as specified in the figure panel.

Winter RTE

A total of 96 samples were analyzed, comprising two replicates for each of six source colonies, two transplant treatments, and four experimental days (Fig. 3.4). There was a significant effect of day on all four biomarker responses (Table 3.2). Source colony origin affected ubiquitin-conjugate and 4HNE-conjugate responses, with elevated levels of ubiquitin-conjugates found in Pool 400 source colonies for days 2, 3 and 4, and higher

levels of 4HNE-conjugates in Pool 400 source colonies on day 3 (Fig. 3.4). Source colony individual affected three of the four biomarkers (ubiquitin-conjugates, hsp70, and MnSOD) suggesting an additional level of genetic or epigenetic influence on marker response (Table 3.2). No significant effect of transplant destination was observed for any marker, however a significant source colony origin by transplant destination effect was observed for hsp70 (Fig. 3.4). Hsp70 levels of sili cross transplants were greater than those of native transplants on day 3 opposite in relationship day 4 ($p < 0.05$; Fig. 3.4). Pool 400 source colonies had over 2 fold greater levels of ubiquitin-conjugates than the Sili source colonies regardless of transplant destination ($p < 0.001$; Table 3.2; Fig. 3.4). Ubiquitin-conjugate levels were also the only marker that showed a significant difference between seasons, with all tests showing lower levels of ubiquitin conjugates in Pool 400 native transplants versus those samples taken during the summer time series (Mann-Whitney, $p < 0.001$).

Table 3.2 Three-way repeated measures ANOVA (Source colony origin, transplant destination, and source colony individual) for protein biomarker concentrations (ubiquitin conjugates, hsp70, MnSOD, and 4HNE) repeated over the five day course of the experiment

Factor	num df	den df	MS				F				p			
			ubiq	hsp70	MnSOD	4HNE	ubiq	hsp70	MnSOD	4HNE	ubiq	hsp70	MnSOD	4HNE
Day		15	1.723	2.105	1.700	1.880	8.62	11.22	9.07	10.02	0.0015	0.0003	0.0010	0.0006
Source colony origin	3	15	2.240	0.553	0.282	1.534	11.22	2.95	1.51	8.18	0.0004	0.0643	0.2509	0.0016
Transplant destination	3	15	0.083	0.193	0.319	0.218	0.41	1.03	1.70	1.16	0.7461	0.4067	0.2069	0.3539
Source colony indiv	6	30	0.306	0.461	0.289	0.935	4.04	2.52	4.58	0.18	0.0044	0.0410	0.0018	0.9800
Origin x destination	3	15	0.141	1.919	0.391	0.477	0.70	10.23	2.09	2.54	0.5645	0.0005	0.1423	0.0927

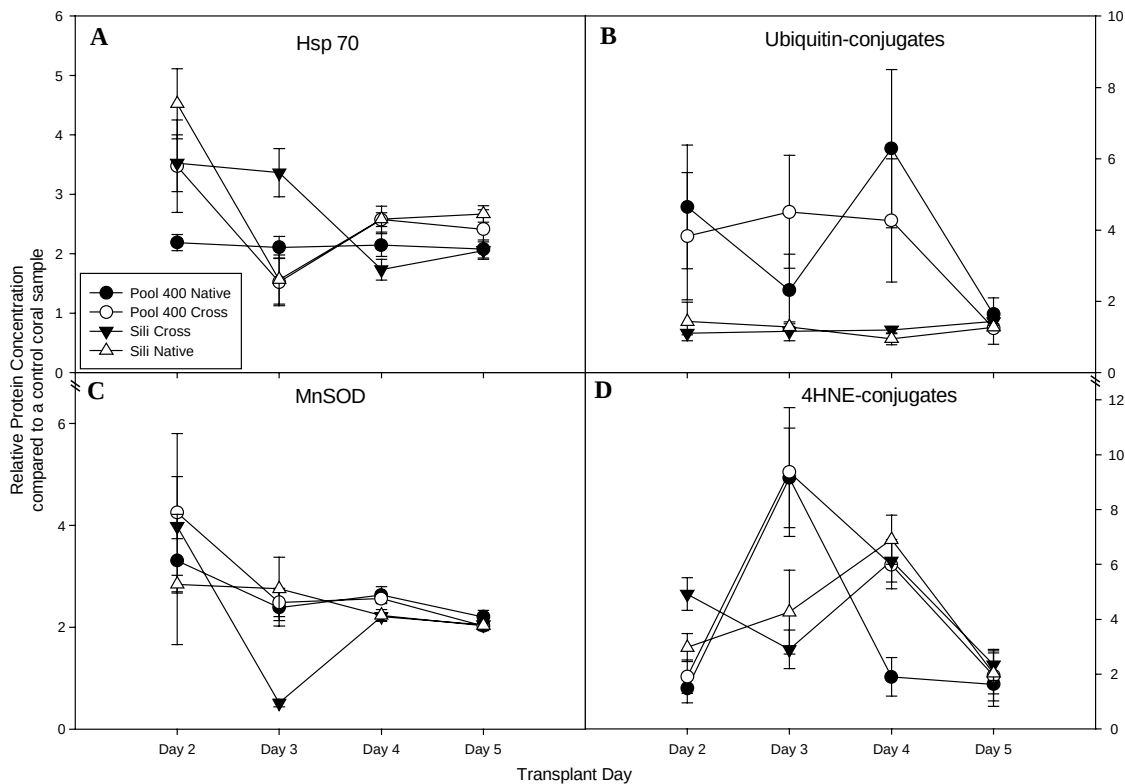


Fig. 3.4 Molecular marker concentrations (A) Hsp70, (B) Ubiquitin-conjugates, (C) MnSOD, and (D) 4HNE-conjugates calculated from western blots of host and symbiont homeogenates relative to a standard coral sample (optical density of marker concentration per 2 μ g total soluble protein) for each day of the winter RTE. Means $\pm$ SE. Black and white triangles and black and white circles represent Pool 400 native and cross transplants and Sili cross and native transplants respectively.

Long-term RTE

A total of 30 surviving samples were analyzed, comprising twelve total replicate source colonies from three different habitats. Transplants to Pool 400 from half of the original parent colonies experienced 100 % mortality, limiting comparisons in this site to

only two representative source colonies from each habitat (Pool 300, Pool 400, and Sili). Average transplant survival over all sites was 65 % (78 % when the two Pool 400 sites were excluded). There were no significant differences in transplant survival based on source colony origin, individual or transplant destination (Appendix 3.1). There was a significant effect of source colony origin and source colony individual on ubiquitin-conjugates only, suggesting a degree of genotypic influence on biomarker responses (Table 3.3). Transplant destination significantly affected three of the four biomarkers (ubiquitin-conjugates, hsp70 and 4HNE), suggesting a strong influence of environment after the longer experimental duration.

Table 3.3 Three-way ANOVA (Source colony origin, transplant destination, and source colony individual) for protein biomarker concentrations (ubiquitin conjugates, hsp70, MnSOD, and 4HNE) at the end of the 261 day long-term RTE

Factor	df	MS				F				p			
		ubiq	hsp70	MnSOD	4HNE	ubiq	hsp70	MnSOD	4HNE	ubiq	hsp70	MnSOD	4HNE
Source colony origin	2	0.275	0.712	1.475	20.930	6.40	2.49	1.34	2.47	0.0170	0.1250	0.2990	0.1240
Transplant destination	2	0.101	1.662	1.197	50.096	8.23	5.92	1.08	4.84	0.0060	0.0160	0.3690	0.0290
Source colony indiv	9	0.048	0.288	0.862	8.176	3.93	1.03	0.78	0.79	0.0150	0.4730	0.6390	0.6310
Origin x destination	4	0.127	0.162	1.006	5.890	10.34	0.58	0.91	0.57	0.0010	0.6840	0.4880	0.6900

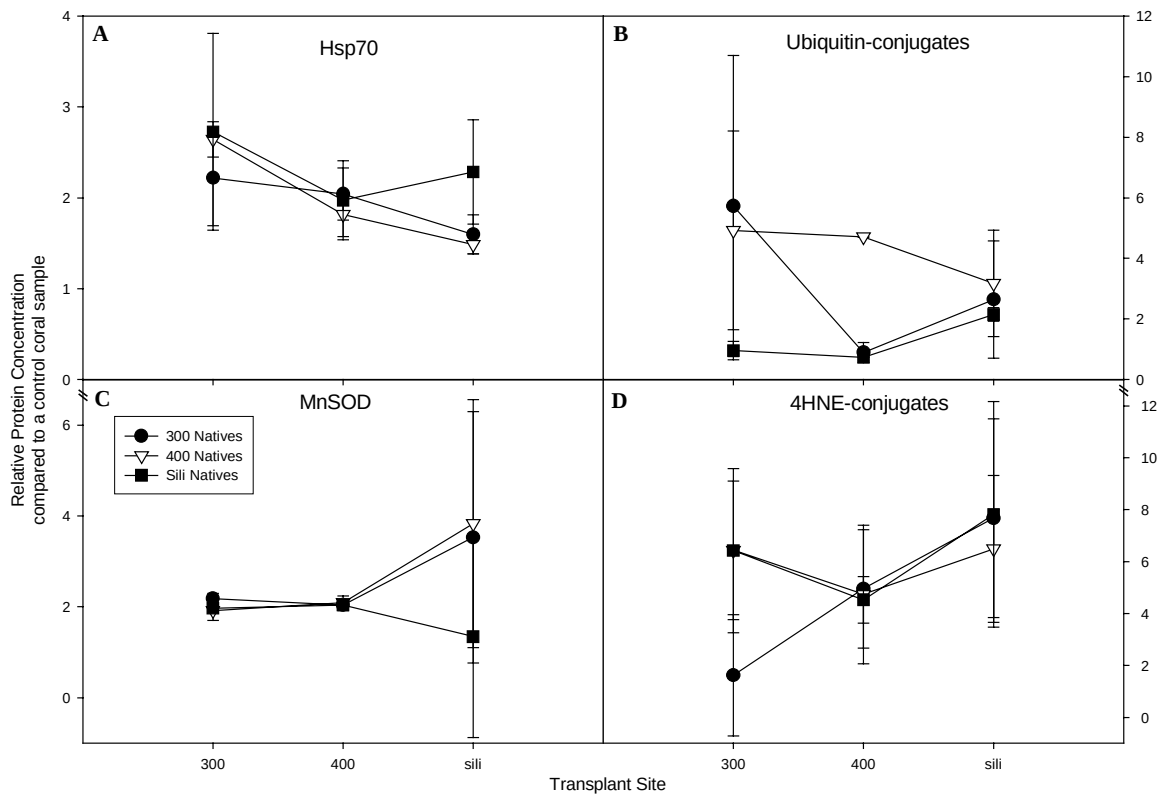


Fig. 3.5 Molecular marker concentrations (A) Hsp70, (B) Ubiquitin-conjugates, (C) MnSOD, and (D) 4HNE-conjugates calculated from western blots of host and symbiont homeogenates relative to a standard coral sample (optical density of marker concentration per 2 μ g total soluble protein) after the 261 day RTE duration. Means $\pm$ SE. Transplant site is shown across the bottom of the panel with each source origin identified by a unique symbol.

A significant source colony origin by transplant destination interaction was only observed for ubiquitin-conjugates. This interaction appeared to be driven by the contrasting response of Pool 300 cross transplants to Pool 400 (300 – 400; Fig. 3.5). The ubiquitin-conjugate levels of 300 – 400 were significantly lower than the 300 – 300

native transplants, 400 – 400 natives, and the 400 – 300 crosses (Fig. 3.5). That the 300 – 400 group only consisted of two of the four original source colonies may have had an impact on this trend. In general, as with the Winter RTE, the most dramatic differences were observed in the ubiquitin-conjugate responses, with elevated levels of ubiquitin-conjugates in back reef source colonies (Pool 300 and 400) transplanted to back reef sites in comparison to forereef (Sili) source colonies transplanted to the back reef (Fig. 3.5).

Discussion

We investigated temporal, seasonal and long-term variability in protein biomarker responses to the contrasting environmental conditions found on the nearshore reefs of Ofu and Olosega islands in American Samoa. Previous research had identified potential genetic and environmental influences on the response of these markers to the variability of the back reef and forereef environments (Barshis Chapter 1). The results of this study agree with those found previously, best characterizing the protein pools of these corals as dynamic, temporally variable, and influenced by a combination of genetic or epigenetic and environmental factors. We report three important findings: (i) consistent genotypic influences on biomarker responses were observed across all experiments and multiple markers, (ii) biomarker responses were correlated with fluctuations in temperature, and (iii) data from all experiments indicate ubiquitin-conjugates to be the most informative marker.

Temporal variability in biomarker profiles

Large (up to ten-fold) differences in biomarker concentrations were observed between individual source colonies and between time points over the three day sampling period of the time series experiment (Fig. 3.2). The majority of this variation can be attributed to fluctuations over time, however, the significant effect of source colony individual on ubiquitin-conjugates and MnSOD response suggests a degree of genetic influence as well. While there also appeared to be a relationship between temperature fluctuations and biomarker response, this relationship was different for each marker under investigation (Fig. 3.3).

We present the first attempt to describe short-term responses of protein biomarkers to either controlled or *in situ* temperature variability in reef-building corals. Although the uniqueness of our study makes direct comparison to other studies difficult, significant increases in biomarker levels following temperature exposure have been observed as quickly as four hours post-exposure in the coral *Goniopora djiboutiensis* (Sharp *et al.* 1997); and for a number of coral species and other marine invertebrates at similar or longer durations (Brown *et al.* 2002a; Choresh *et al.* 2007; Downs & Downs 2007; Downs *et al.* 2002; Downs *et al.* 2000; Dutton & Hofmann 2008; Hofmann & Somero 1995, 1996a, b; Sorte & Hofmann 2005).

We had initially expected to observe a stronger pattern of stress response and recovery, with biomarker levels following a similar profile as the daily fluctuations in environmental characteristics associated with back reef tidal changes (described in Barshis Chapter 1; Craig *et al.* 2001; Smith & Birkeland 2007). A gradual decline in

ubiquitin-conjugate levels during recovery from tidal emersion has been observed in the intertidal mussel *Mytilus trossulus* (Hofmann & Somero 1996b); although this recovery was after mussels were removed from the field and held under constant conditions in a laboratory seawater table. The reduced levels of hsp70, MnSOD, and 4HNE conjugates observed during the relatively lower temperatures between 24 – 32 hrs suggest that there may be periods of reduced stress loading (Fig. 3.2); however, a greater experimental duration is needed to see if this pattern is consistent over longer timeframes.

Additionally, the lack of such a pattern in the ubiquitin-conjugate profile indicate that the environmental conditions of the back reef may not consistently stabilize for time periods long enough to elicit a recovery pattern in these corals.

Seasonal differences in biomarker response

The significant effects of source colony origin (ubiquitin-conjugates, 4HNE-conjugates) and source colony individual (ubiquitin, hsp70, and MnSOD) indicate that genotypic influences on marker response are consistent across seasons (Table 2). In contrast, the only evidence for an environmental influence was the significant origin by transplant destination interaction for hsp70 (Table 2). This interaction was driven by significant differences between Sili cross and native transplants on days 2 and 3, with a reverse trend observed on day 3 compared to day 2 (Fig. 3.4). The weak evidence for environmental influence is likely due to the more similar temperature profiles of forereef and back reef habitats during the winter season (Appendix 2). The cloudy weather, stronger winds, and cooler water temperatures of winter weather patterns all contribute to

reduced variability in the environmental fluctuations of the back reef (Craig *et al.* 2001; Smith *et al.* 2007; Smith & Birkeland 2003); making the distinction between forereef and back reef habitats less clear. The effects of reduced environmental variability were apparent within transplant groups as well, with significantly lower ubiquitin-conjugate levels in Pool 400 native transplants in the winter RTE versus the summer time-series (Mann-Whitney, $p < 0.001$). Seasonal variability in a variety of biomarkers has been documented previously in corals (Downs *et al.* 2002); and for ubiquitin-conjugates specifically in intertidal mussels (Dutton & Hofmann 2008; Hofmann & Somero 1995).

While ubiquitin-conjugate levels were lower for Pool 400 natives in the winter, they were still significantly higher than Sili cross transplants (Fig. 3.4 days 2-4) despite the lower temperatures of Pool 400 during the RTE (Table 3.2, Appendix 3.2). These elevated levels were observed for both Pool 400 cross and native transplants, which may indicate a potential response to transplantation and associated handling stresses.

Additionally, the decline in ubiquitin-conjugate levels on day 5 would be consistent with diminishing response to any effects caused by transplantation. Alternatively, some other environmental variable common to both sites besides temperature could have caused the elevated response (e.g., increased irradiance, freshwater runoff). Regardless of the specific force behind the elevated ubiquitin-conjugates in the Pool 400 source colonies, the lack of a response shown by both Sili cross and native transplants suggests either a reduced sensitivity to the specific stressors or limitations to the stress response in these source corals.

Response differences still evident after long-term transplantation

The results of the long-term transplant support the hypothesis that forereef source colonies are limited in their capacity to respond to the environmental fluctuations on the back reef. Despite the longer transplant duration, back reef source colonies had significantly higher levels of ubiquitin-conjugates than their forereef conspecifics in both Pool 300 and Pool 400 (Table 3.3, Fig. 3.5). Notably, Pool 300 cross transplants to Pool 400 showed a similar response to Sili – 400 transplants, although whether this is an artifact of the decreased sample size or indication of greater tolerance in Pool 300 source colonies is uncertain. Additionally, significant effects of source colony origin and individual were still apparent after the 261 day transplant for ubiquitin-conjugates as well (Table 3). There was also significant evidence for environmental influence on ubiquitin-conjugate, hsp70 and 4HNE-conjugate response, demonstrating the capacity for physiological acclimatization (i.e., phenotypic plasticity) in these markers. Yet for ubiquitin-conjugates, acclimatization potential was still not enough to overcome the genetic influence of source colony origin.

Conclusions

Here, we present data on the changing responses of protein biomarkers in the coral *Porites lobata* across different habitats, time scales, and seasons. We observed a high degree of variability in biomarker responses due to many different factors (e.g., time, habitat, individual, season) which agrees with a previous observations questioning the utility of these markers in field-collected corals (van Oppen & Gates 2006). Our

consistent findings of significant genotypic influence across all experiments, however, in addition to the positive correlations between temperature and protein levels observed during the time series, suggest that despite their drawbacks, valuable information can be obtained when these markers are employed comprehensively and carefully.

The most promising results were found within the ubiquitin-conjugated protein levels, which appeared to be highly sensitive to genotypic, environmental, and seasonal influences. The elevated levels of ubiquitin-conjugated proteins found in back reef source colonies are consistent with the hypothesis that the back reef environment may be more stressful to protein stability, yet the lack of differences in transplant survival, in addition to previous findings of increased growth in back reef source colonies and back reef transplants (Smith *et al.* 2007) suggest the relationship may be more complicated. Given the significantly lower values observed in winter ubiquitin-conjugate levels, it is possible that the impact of environmentally induced protein denaturation is seasonally dependent and the reduced stress exposure during cloudy weather and cooler seasons may counteract the effect observed during the mid-day low tides of the southern summer. Additionally, the increased levels of ubiquitylation in back reef source colonies could be indicative of non-stress related functions of the ubiquitin pathway such as protein turnover, translocation or chaperoning (Li & Ye 2008; Welchman *et al.* 2005), or perhaps demonstrate a greater capacity for the ubiquitin proteasome pathway of the back reef source colonies to respond to an increased level of protein denaturation without additional consequences. Whichever the case, the lack of a corresponding increase in

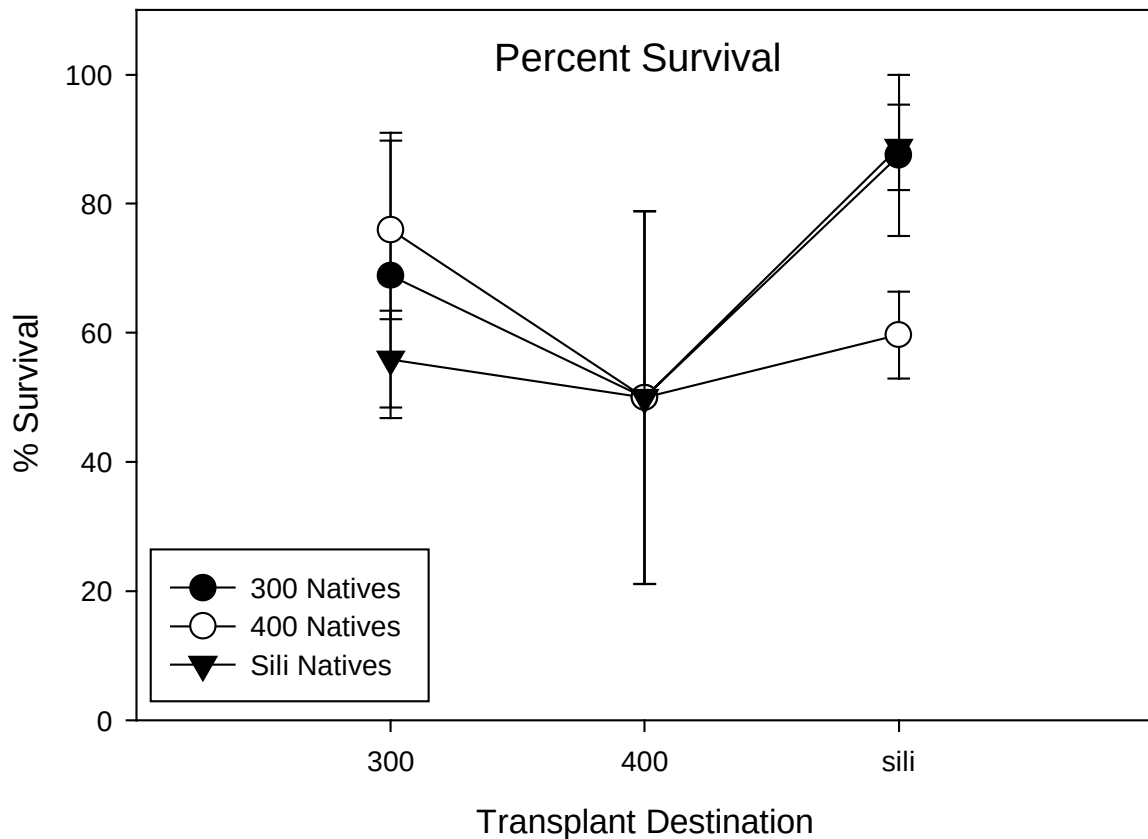
ubiquitin response from forereef cross transplants even after 261 days of acclimation indicates potential limitations to phenotypic plasticity in forereef source colonies.

As a whole, these data demonstrate that natural, sublethal stress responses of *Porites lobata* on Ofu and Olosega are highly variable, yet follow seasonal, environmental, and temporal patterns. The reduced environmental variability of the back reef during the southern winter corresponds with lower levels of protein denaturation, however distinct response differences attributable to source colony origin remain persistent. Previous characterization identified these two populations as comprised of genetically distinct cnidarian hosts with similar algal endosymbiont types (Barshis Chapter 1, Chapter 2). Importantly, the methods employed during the current study lack the sensitivity to distinguish between host and symbiont protein types, yet the lack of differences in algal symbiont identity suggest a host genetic mechanism behind the significant source colony origin effects observed across time scales and seasons (this possibility will be examined in detail in the following chapter).

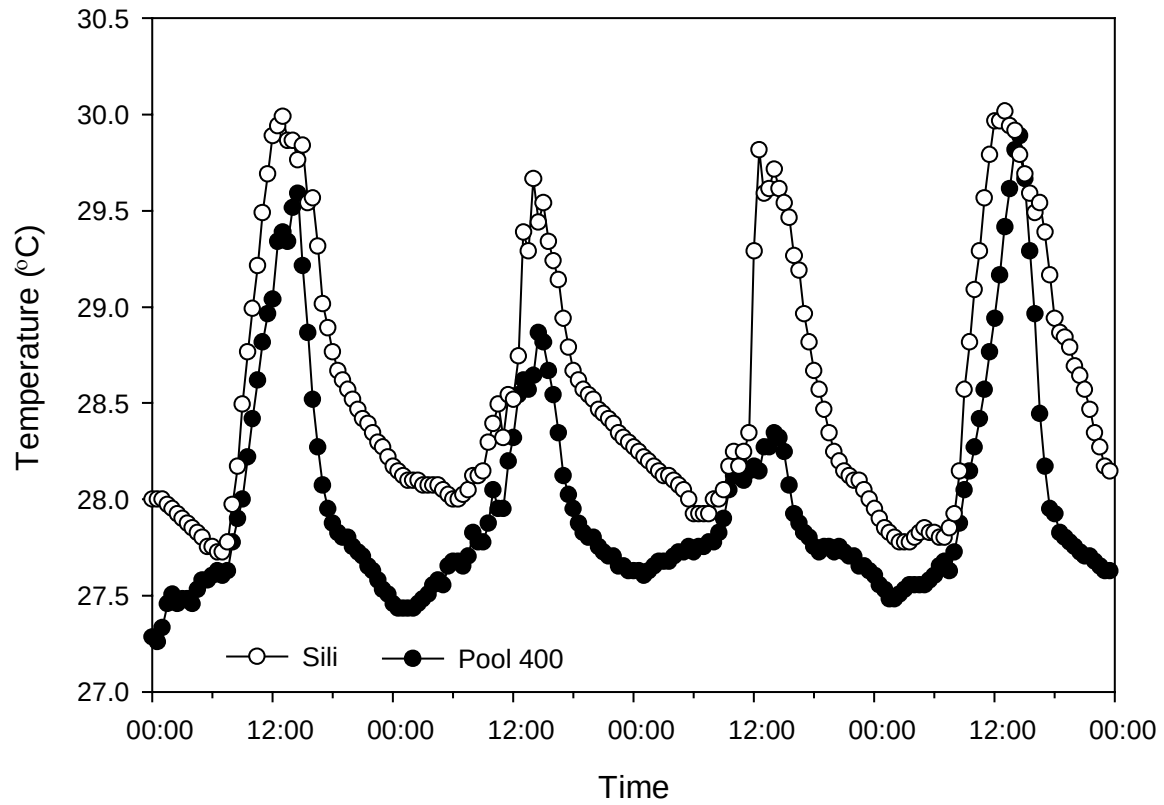
Small scale environmental variability often imposes significant challenges to species at the physiological level (e.g., Helmuth & Hofmann 2001); however, understanding the complex interactions between environment and physiology has important implications for our ability to predict how populations will respond to future environmental changes (Helmuth 2009). This study attempts to understand how small scale differences in habitat characteristics may shape the physiological and genetic structure of a dominant member of the reef-building coral community, *Porites lobata*. The characterization of naturally occurring differences in coral stress responses presented

here is hoped to provide insight into the potential of these corals to survive future environmental impacts and aid in their protection and conservation before the opportunity is lost.

Appendix 3.1 Percent survival calculated by visual estimation of live tissue coverage after the 261 day RTE duration. Transplant site is shown across the bottom of the panel with each source origin identified by a unique symbol. Means $\pm$ SE. Transplant site is shown across the bottom of the panel with each source origin identified by a unique symbol.



Appendix 3.2 Temperature recorded every 30 minutes in Pool 400 (solid circles) and Sili (open circles) during the 5 day duration of the winter RTE



CHAPTER FOUR:

ACCLIMATION POTENTIAL AND THERMAL STRESS TOLERANCES OF CORALS FOLLOWING EXPOSURE TO DIFFERING DEGREES OF TEMPERATURE VARIABILITY: IMPLICATIONS FOR RESILIENCE OF CORALS FROM FLUCTUATING ENVIRONMENTS

Abstract

In response to the continued degradation of coral reef ecosystems, researchers and managers alike have begun to integrate characteristics of ecological resilience into the design of marine protected area networks and conservation strategies. However, accurate assessment of the resilience for specific coral reef populations remains a challenging task that demands a variety of approaches. Here, we present a multi-parameter investigation of short- and long-term thermal acclimation potential of the reef coral *Porites lobata* from three environments with contrasting thermal stress profiles. Corals were subjected to a 35 day acclimation period to either a fluctuating versus a more stable temperature profile. Heat stress tolerances were evaluated after exposure to multiple static temperatures (28, 31, 34, and 37 °C) and consecutive increases in temperature variability (27 to 31, 29 to 33, 31 to 35, 33 to 37, and 35 to 39 °C). Physiological performance (tissue growth rates, photosynthetic efficiency) and molecular biomarkers (heat shock proteins, ubiquitin proteasome labeling, superoxide dismutase activity) showed effects of source population origin, individual colony, and acclimation treatment on both host and symbiont physiological response. Corals from the back reef environments exhibited the greatest tolerance to increasing temperatures suggesting that prior conditioning to environmental variability has strong epigenetic or adaptive influence on the physiology of these corals. These results indicate that corals found in naturally variable environments could possess enhanced resilience characteristics that may promote their survival of future impacts of environmental disturbance.

Introduction

An emerging concept in marine ecosystem conservation is the integration of characteristics of ecological resilience into the design of marine protected area networks and management strategies (Game *et al.* 2008; Levin & Lubchenco 2008; West & Salm 2003). Resilience, as used here, is defined according to Levin and Lubchenco (2008) as “... the capacity of a system to absorb stresses and continue functioning.” Resilience-based management aims to assess the potential benefits of protecting more resilient populations from avoidable impacts in hopes that they may be better suited than more susceptible populations to withstand anomalous impacts beyond human control (Game *et al.* 2008; Levin & Lubchenco 2008). In the case of coral reefs, resilience components include such things as high levels of larval recruitment and connectivity, prevalence of herbivorous fishes and invertebrates, diversity in populations of the coral endosymbiotic dinoflagellate *Symbiodinium sp.*, and contributions of coral host tissues to intrinsic environmental stress resistance (e.g., Baird *et al.* 2009; Hofmann & Gaines 2008; Hughes *et al.* 2003; Hughes *et al.* 2005; Hughes *et al.* 2007; Levin & Lubchenco 2008; van Oppen & Gates 2006). Yet the challenges facing researchers and managers alike are the accurate assessment of resilience characteristics of a particular area, selecting which parameters are most important for consideration, and effectively integrating resilience considerations into a management framework.

Recent technological advances are allowing coral reef researchers to assess these characteristics with some degree of reliability (Hofmann & Gaines 2008), yet the emerging pattern is often complex. Once thought to be relatively open and panmictic,

larval dispersal capacity and connectivity of coral populations now appears patchy and localized, involving complex stochastic interactions between reproductive strategies, ocean microenvironments, recruitment events, disturbance artifact, and a variety of other selective forces and life history characteristics (e.g., Levin 2006; Miller & Ayre 2008; Underwood *et al.* 2009). Considerable evidence also points towards certain types of the algal endosymbiont of the genus *Symbiodinium* as conveying increased stress tolerance to the coral host (e.g., Baker 2001, 2003; Rowan 2004), yet new investigations have revealed fine-scale patterns that suggest that past generalizations about stress tolerant symbiont “types” may not be as applicable as previously thought (Abrego *et al.* 2008; Sampayo *et al.* 2008). Additionally, it has been shown that a history of exposure to sublethal environmental stressors such as temperature (Coles 1978; Griffin *et al.* 2006) and irradiances (Brown *et al.* 2002b) correlates with increased upper thermal tolerance thresholds in three reef-building coral species; however, the prevalence of this phenomenon in other species, as well as the long term effects of such exposures under controlled conditions remain untested.

The goal of this study was to use a variety of metrics to assess the relative contributions of thermal exposure history and acclimation potential to upper stress tolerance limits and physiological capacity of the reef-building coral *Porites lobata*. To this end, corals were selected from three sites that differ greatly in variability in environmental characteristics that are described in detail below and in the previous chapters (Barshis Chapter 1, Chapter 3). Corals were subjected to two controlled temperature acclimation treatments approximating the ranges of variability of thermal

variability observed in their native environments. Our hope was to determine whether acclimation to fluctuating environmental conditions and/or a history of long-term exposure to such conditions could be a reliable diagnostic of coral resilience to future environmental perturbation. While we did find significant effects of acclimation treatments, the effects of coral origin remained strong, with corals from the more variable environments exhibiting increased thermal tolerance versus those from the more stable environments. These findings suggest that corals from dynamically fluctuating environments may be better suited to withstand the impact of future environmental changes and may warrant consideration during the design and implementation of resilience-based management strategies.

Materials and Methods

Study site, sample collection and transport

Corals were collected during May 2007 from three sites on Ofu and Olosega islands in the territory of American Samoa ($14^{\circ}11'S$, $169^{\circ}40'W$). These islands host diverse communities of ~85 shallow reef-building coral species, many of which are consistently exposed to seawater temperatures (Craig *et al.* 2001) and irradiances (Smith & Birkeland 2007) much higher than neighboring areas. The extreme conditions on Ofu are caused by a prominent reef crest that blocks circulation between the waters of the back reef and the open ocean, limiting mixing of back reef waters during midday low tides (Smith & Birkeland 2003). This limited mixing coupled with high midday solar irradiance produces large (up to $6^{\circ}C$) daily fluctuations in temperature (Barshis Chapter

1; Smith & Birkeland 2003, 2007). In addition, frequency of large storm events, corresponding rainfall, and potential groundwater release sporadically cause drops in back reef salinity (up to 5 ppt; Barshis Chapter 1; Smith *et al.* 2007). In comparison, the shallow (<3m low tide depth) forereef of Olosega island represents a more typical, average reef environment with a more constant flow regime, significantly lower temperature, irradiance, and salinity variability, and extremes of each (Barshis Chapter 1; Smith *et al.* 2007; Smith & Birkeland 2003, 2007).

Two back reef sites (Pool 300 and Pool 400) and one forereef site (Sili) were selected based on general differences in environmental characteristics. These differences are described in greater detail elsewhere (Barshis Chapter 1; Craig *et al.* 2001; Piniak & Brown 2009; Smith *et al.* 2007; Smith & Birkeland 2007; Smith *et al.* 2008). Briefly, Pool 300 is smaller, shallower, more thermally variable and experiences higher current velocities than Pool 400, while the Sili forereef is relatively more stable than both back reef sites. *Porites lobata*, a massive, broadcast spawner, was selected as our study species because it is common in American Samoa, as well as other locations in the Indo-Pacific, is frequently a dominant member of the nearshore reef community, and gross colony morphology appears to differ between forereef and back reef habitats (Craig *et al.* 2001; Smith *et al.* 2007).

A pneumatic drill and hole saw attachment was used to remove 30 cores, 19 mm diameter, from the upward facing surfaces of each of five source colonies in each site. Cores were affixed to nylon bolts with Z-Spar, Splash Zone marine epoxy (Carboline Company, St Louis, MO), arranged in grids and placed in Pool 400 for a seven day

recovery period prior to shipping. Cores were wrapped in plastic bags and wet paper towels with a minimal amount of seawater, shipped in insulated coolers to our aquarium facility at San Francisco State University's Romberg Tiburon Center for Environmental Studies in Tiburon, CA and immediately placed in experimental aquaria.

Coral acclimation conditions and experimental analyses

The cores from each source colony were divided into two groups of 15 and held in separate experimental aquaria at a constant temperature of 28 °C and average irradiance of 260 $\mu\text{mol quanta m}^{-2} \text{sec}^{-1}$ (12hr light/dark cycle) for a 28 day recovery period. After the recovery period, the temperature profile of one of tanks was changed to fluctuate between 27 and 32 °C while the other tank was set to remain stable at 28.5 °C for 35 days of acclimation to the contrasting conditions. Temperature was recorded at 1 minute intervals (Fig. 4.3). During the acclimation period some heaters failed and so temperature profiles of the stable tank were not always perfectly stable, nor did the fluctuating tank have the same temperature spikes each day (Fig. 4.3). The specific temperatures and amplitude of the fluctuating treatment were chosen to reflect average water temperature and daily range extremes of natural summer temperature profiles of the forereef and back reef sites. After the 35 day acclimation period, coral physiological performance was assessed using a variety of assays.

Host genetic analyses

To assess the potential influence of host genotype on physiological responses, the internal transcribed spacer region (ITS) of nuclear ribosomal DNA was amplified and sequenced from each individual source colony. Primer sequences, polymerase chain reaction conditions, and sequencing methods were performed as described previously (Barshis Chapter 1, Chapter 2). Resulting sequences were inspected using Sequencher version 4.5 (Gene Codes Corp., Ann Arbor, MI) and aligned using Bio-edit (Hall 2001) and by eye. Population genetic structure was estimated using an analysis of molecular variance (AMOVA) in Arlequin 2.0 (Schneider 2000). A molecular phylogenetic network was constructed using the median-joining algorithm and maximum parsimony post-processing calculation in NETWORK ver 4.5.0.0 (Fluxus Technology Ltd.; Polzin & Daneschmand 2003).

Growth

New tissue growth was measured as the distance the tissue had extended down the sides of each coral core. Tissue extension was measured linearly down four sides of each core using calipers. The four measurements were then averaged and resulting analysis was performed using a single average value for each individual core.

Photophysiology

Chlorophyll *a* fluorescence of coral zooxanthellae was measured using a pulse-amplitude modulated (PAM) fluorometer (DIVING-PAM, Walz GmbH, Germany). PAM

fluorometry is a rapid, non-invasive technique which assesses the photosynthetic efficiency of photosystem II (PSII) reaction centers which can be used as a proxy for assessing the health of the symbiotic association (Fitt *et al.* 2001). DIVING-PAM parameters and measurements were made following a previous study (Piniak & Brown 2009). Briefly, initial chlorophyll a fluorescence measurements (F) were between ~150–400 units, maximum fluorescence ($F'm$) was measured using a saturating light pulse (0.8 s, $\sim 8000 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$), and the change in fluorescence ($\Delta F = F'm - F$) was used as effective quantum yield ($\Delta F / F'm$) for light-adapted corals (Genty *et al.* 1989). Maximum quantum yield [$(Fm - Fo)/Fm$, or Fv / Fm] was measured for dark-adapted samples at the end of each experimental day using the same parameters after all lights had been turned off for more than 20 min.

Superoxide dismutase activity assay

Superoxide Dismutase (SOD) enzyme activity was assessed using a modification of the methods of Beauchamp and Fridovich (1971). SOD catalyzes the dismutation of the superoxide anion (O_2^-) into hydrogen peroxide and molecular oxygen; and is one of the most important enzymes involved in first defense against oxidative stress (Fridovich 1975). Each coral core was flash frozen in liquid nitrogen before the tissue layer (up to 1 cm below surface) was removed with bone cutting pliers and placed in a pre-frozen, 50 ml stainless steel mixing jar (Glennmills, Clifton, NJ). The tissue and skeleton was crushed using a TissueLyser[®] (Qiagen, Valencia, CA) at 25 rpm for 5 s. Resulting sample was transferred to individual 2.5 ml cryovials and stored at -80°C until further analyses.

Between 280-380 mg of crushed tissue was added to a prechilled 2 ml microcentrifuge tube to which 750 μ L of chilled 50 mM of phosphate buffer (pH 7.8) was added. Samples were vortexed and then centrifuged at 2000 x *g* for 5 min to separate out host and algal endosymbiont tissue fractions. The supernatant (host fraction) was removed and placed on ice while the remaining pellet (skeletal debris and symbiont fraction) was washed three times with fresh phosphate buffer before resuspended in a final volume of 500 μ L of phosphate buffer, sonicated for 5 min, and briefly centrifuged to remove skeletal debris. Aliquots were removed from both host and symbiont fractions and stored at -80 °C until further analyses.

Samples were assayed for total protein content using the Bio-Rad RC/DC protein concentration assay (Bio-Rad, Hercules, CA). Samples were then diluted to a concentration of 0.15 μ g/ μ L in phosphate buffer prior to the assay. 10 μ L of sample and 10 μ L of xanthine oxidase solution were added to 280 μ L of assay buffer for a final concentration of 50 mM phosphate buffer, 0.1 mM xanthine, 0.025 mM nitroblue tetrazolium (NBT), and 0.1 mM EDTA. Xanthine oxidase solution was diluted in phosphate buffer to yield a rate increase of the uninhibited sample (blank) absorbance between 0.015 and 0.020 min^{-1} . Assay buffer was made fresh every few days and stored at 4 °C in the dark when not in use. Absorbance of experimental versus blank solutions was monitored at 560 nm and 25 °C every 9 sec for 5 min in a SpectraMax M2 Microplate Reader (Molecular Devices, Sunnyvale, CA). All experimental and uninhibited samples were run in triplicate in sets of eight (7 experimental and 1 uninhibited). The linear rate absorbance increase was compared between experimental

and uninhibited samples to yield the percent inhibition. These values were compared against a standard curve using known amounts of purified SOD that was generated for each batch of assay solution (Fig. 3.1). Final values of experimental samples are given as U of SOD per mg of total sample protein (U/mg).

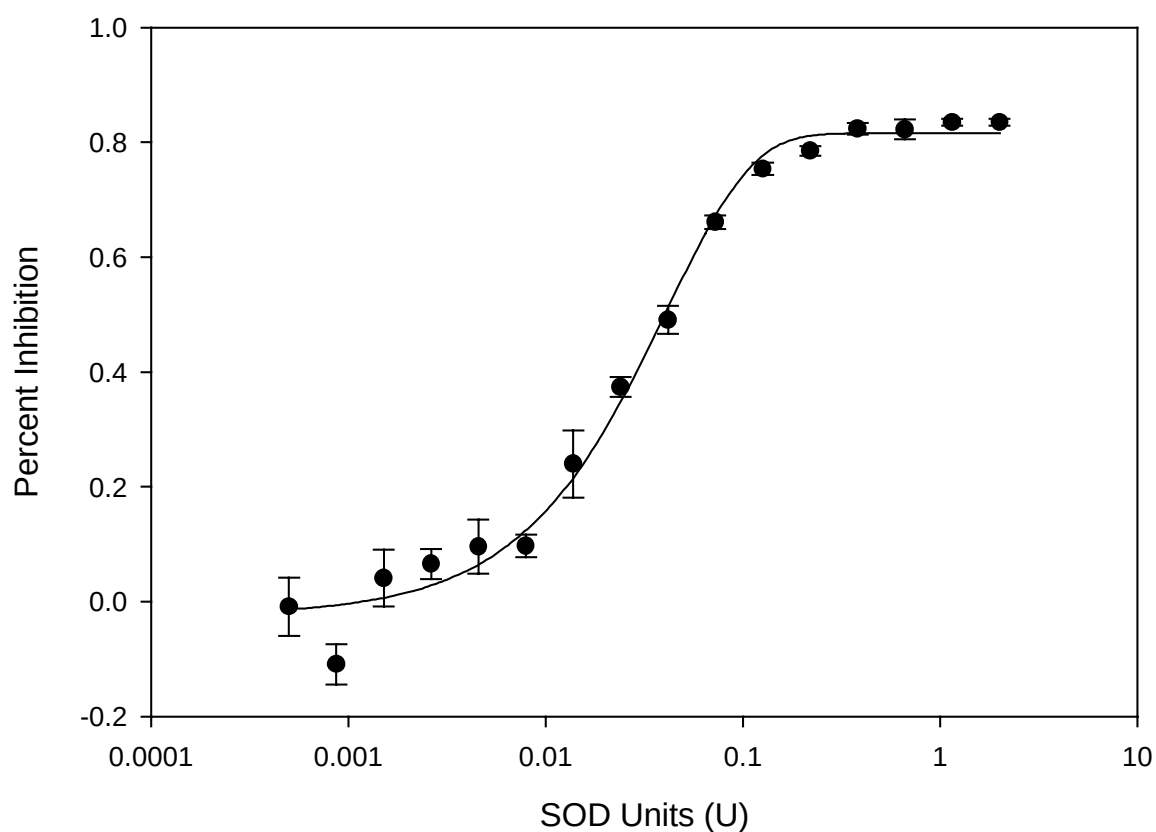


Fig. 4.1 Superoxide dismutase activity standard curve. Points represent known amounts of purified SOD enzyme (Cat # 574593, EMD Biosciences, Inc. La Jolla, CA), the curve was generated using a four parameter sigmoid model in SigmaPlot. Predicted values that fell within the linear range of the curve were used to generate a logarithmic regression equation for calculation of experimental SOD Unit amounts.

Protein biomarkers: Hsp70 and ubiquitin-conjugates

Levels of heat shock protein 70 (hsp70) and ubiquitin conjugated proteins were assessed via western blot for both host and symbiont protein fractions. After the SOD activity and total protein assay a 1:10 volume of 10x extraction buffer was added to the remaining sample suspensions in phosphate buffer and boiled and extracted as described previously (Barshis Chapter 1, Chapter 3). A second protein assay was performed on the western blot extractions and all samples were assayed in triplicate following previous study (Barshis Chapter 1, Chapter 3).

Stress experiments

After the 35 day acclimation period, two stress experiments were performed to test potential effects of the different source populations and acclimation treatments on upper thermal stress tolerances. The first experiment, termed “static temp,” consisted of four static temperature treatments of 28, 31, 34, and 37 °C. One core from each source colony and treatment was placed in four 3 L aquaria and held at a constant temperature for 8.5 hrs before they were returned to the larger stable acclimation tank for a 1.5 hr recovery period before freezing. PAM measurements were taken on all cores prior to the start of the experiment, at nine intervals during the temperature exposure under light-adapted conditions, and during the recovery period after 45 min of dark-adaptation. Corals in the 37 degree tank showed rapid signs of bleaching and were removed after 6 hrs, placed in a recovery tank for 1 hr and dark adapted for 45min before final PAM

measurement and freezing. Samples taken after the full recovery periods were analyzed for SOD activity and hsp70 and ubiquitin-conjugate levels as outlined above.

The second stress experiment termed “ramping temp” consisted of placing five replicate cores from all source colonies and treatments in the fluctuating temperature treatment tank (~27 to 32 °C) for one day and subsequently raising the upper and lower limits of the fluctuation by two degrees every 24 hrs for four additional days with a final temperature fluctuation of (~35 to 40 °C) and experimental duration of 120 hrs. PAM measurements were taken each day at 16:45 under light-adapted conditions and at 21:15 after 45 min of dark-adaptation. A single core from each source colony and acclimation treatment was sacrificed for protein analyses following PAM measurements each night and stored and analyzed as above.

Statistical analyses

Within a common garden framework, comparisons between transplant groups are designed to assess acclimation potential versus genetic/epigenetic control over the response variables. Comparisons between acclimation treatments examine variability associated with acclimation conditions (i.e., phenotypic plasticity), while comparisons between source colony origins and individuals examines potential genetic or epigenetic influence on the response variables (DeWitt & Scheiner 2004; Schluter 2000; Smith *et al.* 2007). To this end, univariate analyses of variance (ANOVAs) were used to test the influence of source colony origin, acclimation treatment, and source colony individual on the various response variables measured in this study.

Growth, photosynthetic efficiency, SOD activity, and western blot biomarker levels were assessed following the acclimation to the differing temperature profiles of the two experimental aquaria. Statistical analyses were performed using Minitab[®] 15 (Mintab Inc., State College, PA), and SPSS Ver. 10.0 (SPSS Inc., Chicago IL). Each variable was tested against the fixed factors of source colony origin and acclimation treatment, and the random factor of source colony individual nested within origin in a three-way ANOVA. Data were first tested for normality and homoskedasticity and a Box-Cox transformation was applied as needed to meet the assumptions of the model. No transformation was needed for PAM, linear extension, and host SOD activity measurements; Box-Cox exponents were 0.6 and 0.2 for host hsp70 and ubiquitin-conjugates respectively, 0.5 and 0.3 for symbiont hsp70 and ubiquitin-conjugates, and a natural log transform was used on symbiont SOD activity data.

Photosynthetic efficiency, host and symbiont hsp70 and ubiquitin-conjugate levels, and SOD activity were assessed for both the static and the ramping temperature stress experiments. For the PAM measurements taken during the static temperature exposure, a repeated measures ANOVA was used within each temperature treatment group to test for significant effects of source colony origin, individual, acclimation treatment and an origin by acclimation interaction. All other variables were tested against the fixed factors of source colony origin and acclimation treatment, and the random factor of source colony individual nested within origin in a three-way ANOVA. Data were tested for normality and homoskedasticity and a Box-Cox transformation was applied as needed to meet the assumptions of the model. No transform was needed for host SOD

activity, static temperature PAM, and day 1 and 4 ramping temperature PAM measurements; Box-Cox exponents were 1.7, 1.2 and 0.5 for PAM ramping temperature days 0, 2, and 3, 0.5 for host and symbiont hsp70 ramping temperature and symbiont hsp70 static temperature, 0.2 for host ubiquitin ramping and static temperature, 0.3 for symbiont ubiquitin ramping and static temperature, 0.6 for host h70 static temperature, and -0.4 for symbiont SOD activity from the ramping temperature exposures respectively, and a natural log transform was used for symbiont SOD activity measurements from the static temperature exposures.

Results

Host genetic analyses

A 368 base pair (bp) fragment of the internal transcribed spacer region (ITS) was amplified from 15 individuals for a total of 77 cloned sequences. These 77 sequences were comprised of 28 unique haplotypes: one shared between the Pool 300 and 400 populations, one shared between the Pool 300 and Sili populations, and eight, nine and nine unique to the Pool 300, 400 and Sili populations respectively. The analysis of molecular variance (AMOVA) revealed significant population subdivision between all three populations ($F_{ST} = 0.20609$, $p < 0.0001$). Pairwise F_{ST} comparisons were highest between Pool 400 and the other two populations ($F_{ST} = 0.24829$ and 0.23190 , $p < 0.0001$ for Pool 300 and Sili respectively), while Pool 300 and Sili showed less significant subdivision ($F_{ST} = 0.05091$, $P < 0.03$). This was evident in the phylogenetic network

construction as well which showed a more explicit separation between Pool 400 haplotypes versus Pool 300 and Sili haplotypes (Fig. 4.2).

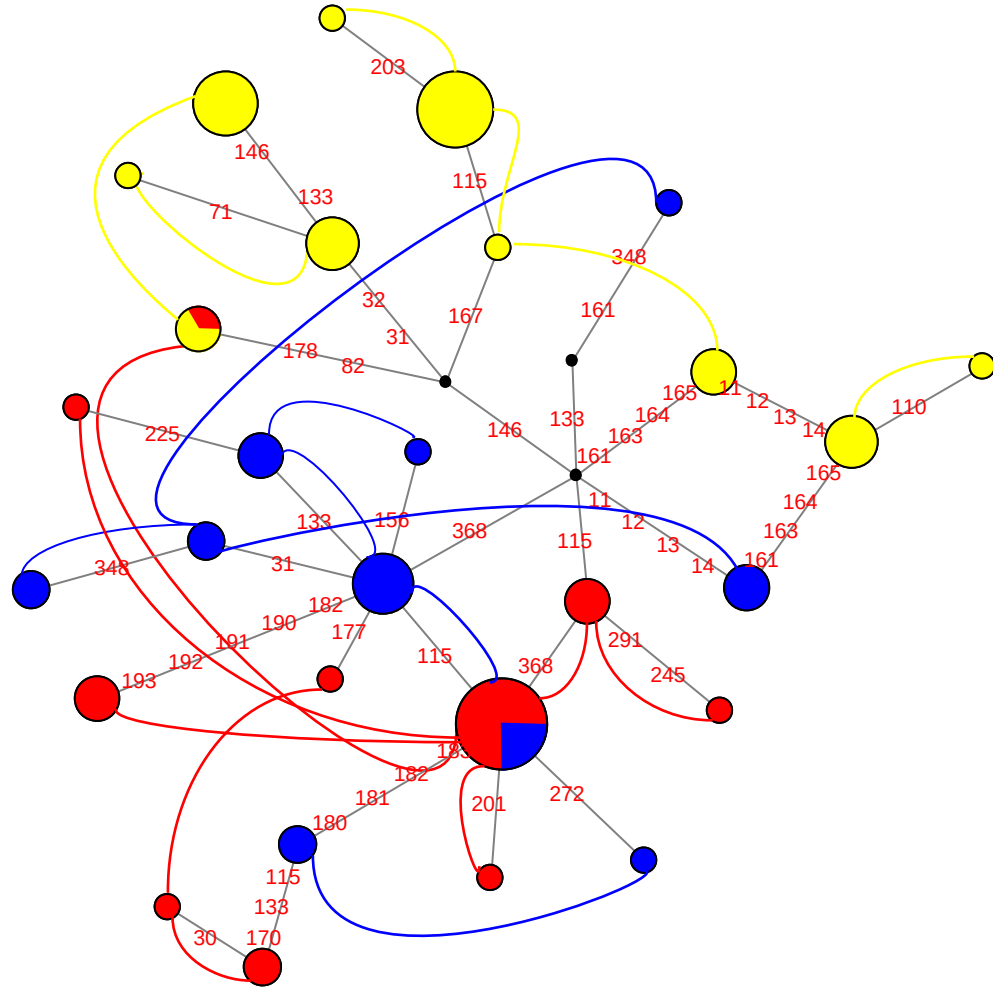


Fig. 4.2 Maximum parsimony phylogenetic network reconstruction of ITS rDNA haplotypes drawn in NETWORK ver 4.5.0.0 (Fluxus Technology Ltd.; Polzin & Daneschmand 2003)). Haplotypes shown in red, yellow, and blue are from Pool 300, 400, and Sili populations respectively. Diameter of circles at each node is proportional to the number of individuals with identical sequences. Haplotypes that co-occur in the same individual are connected by colored curves. Mutations are shown in red on each branch

with the number corresponding to the base pair position, hypothetical intermediate haplotypes are designated by black circles.

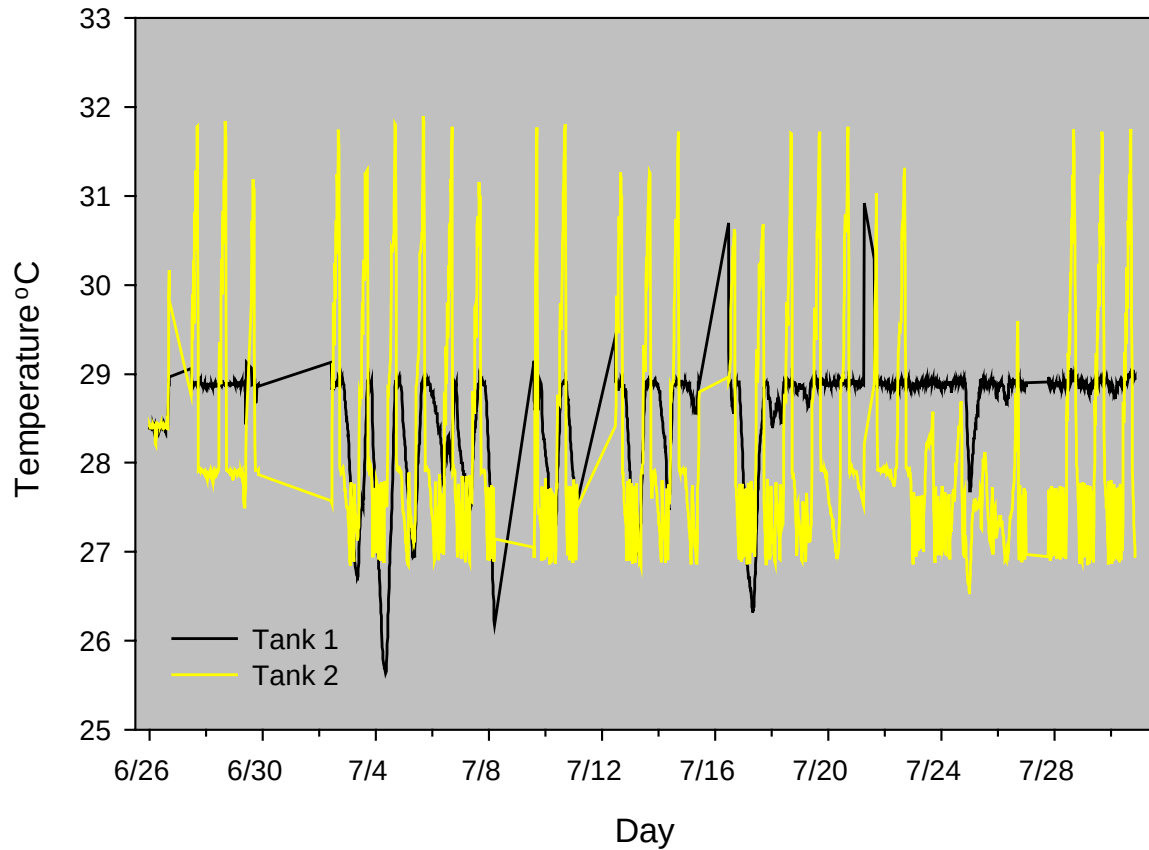


Fig. 4.3 Temperature profiles of the two experimental acclimation tanks during the 35-day acclimation period, temperatures were recorded every 14 sec, the yellow line corresponds to tank #2 (designated as the “fluctuating” tank) and the black line corresponds to tank #1 (the “stable” tank).

Acclimation responses

Temperature profiles for the two acclimation tanks are shown in Fig. 4.3. Tank 1 had a slightly higher mean temperature and lower standard deviation than tank 2 (28.54

+/- 0.63 and 28.15 +/- 1.20 °C for tanks 1 and 2 respectively). On average, the daily range of tank 2 was 11.84 times greater than the daily range of tank 1. Of the 33 acclimation days for which temperature records were available, tank 2 had a daily range greater than 3 °C on 24 days (73%), while the daily range of tank 1 exceeded 3 °C on only one day. Irradiance levels did not appear to differ between the two tanks with an average irradiance of 262.6 and 258.6 $\mu\text{mol quanta m}^{-2} \text{sec}^{-1}$ for tanks 1 and 2 respectively.

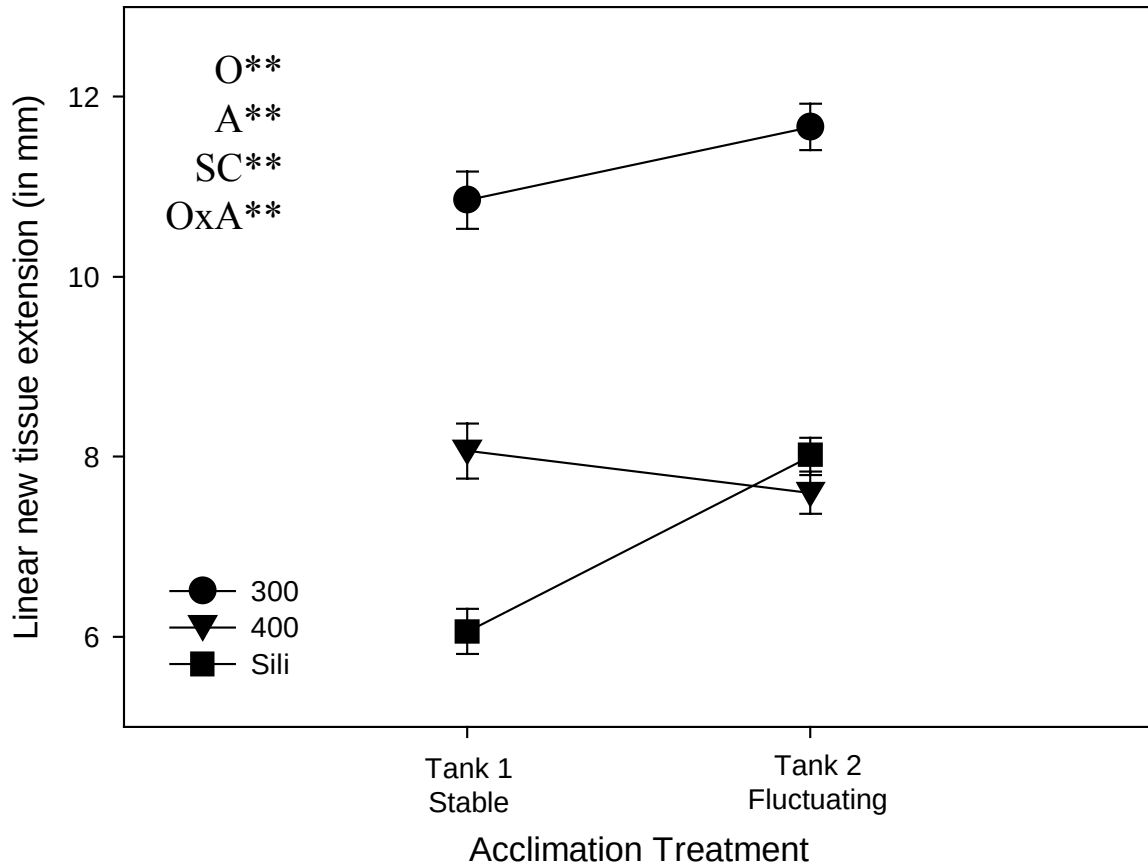


Fig. 4.4 Linear tissue extension measurements taken after the 35-day acclimation period.

Circles, triangles, and squares represent Pool 300, 400 and Sili source colonies

respectively. Values are category means +/- SE, tank 1 is the stable acclimation tank and

tank 2 is the fluctuating acclimation tank. Statistical significance at $p < 0.05$ (*) and $p < 0.01$ (**) is presented in the table below each graph for comparisons of source colony origin (O), acclimation treatment (A), and source colony individual (SC).

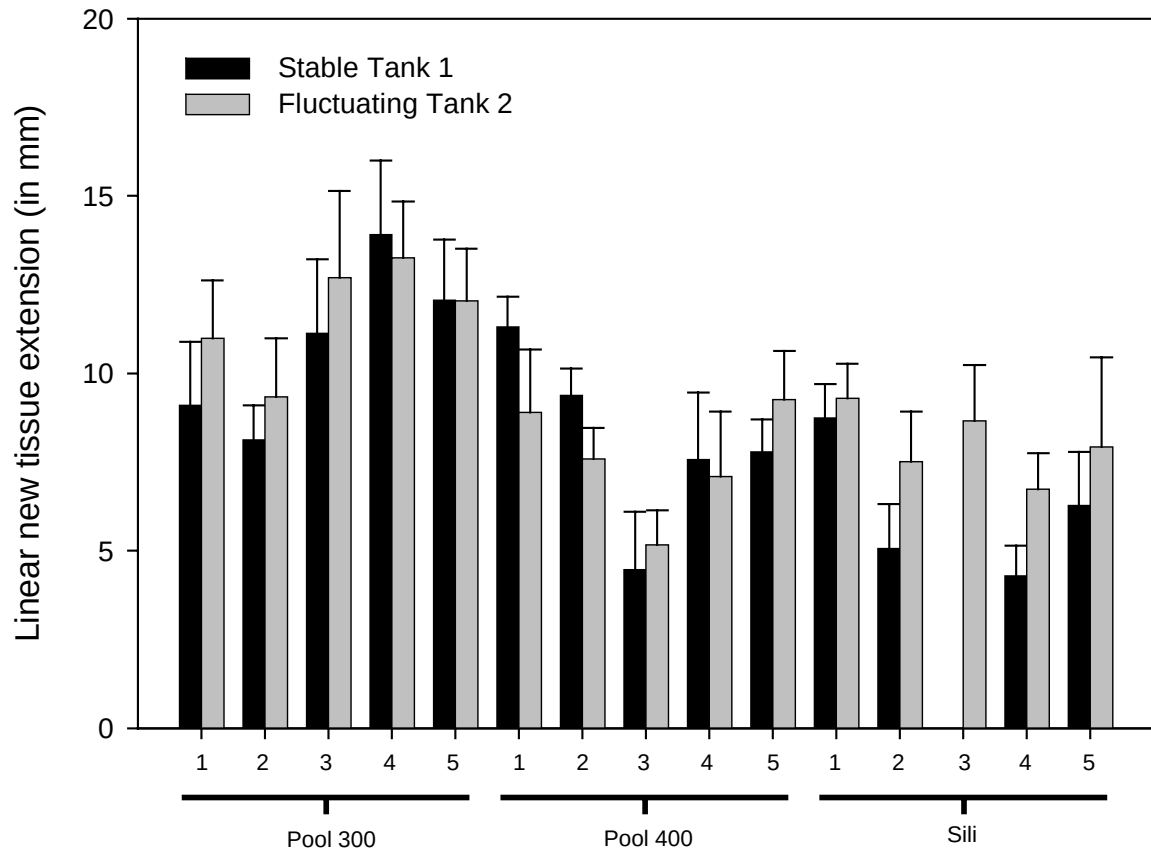


Fig. 4.5 Linear tissue extension measurements as described above but separated by individual source colony, dark bars represent tank 1 acclimated samples, light bars represent tank 2 acclimated samples.

Growth

New tissue growth was significantly affected by source colony origin, individual and acclimation treatment (Fig. 4.4). Pool 300 source colonies had an average tissue extension of 10.87 mm in the stable tank and 11.67 mm in the fluctuating tanks; Sili source colony values were 6.24 and 8.03 mm for the stable and fluctuating treatments; and Pool 400 source colony values were 8.08 and 7.60 for the stable and fluctuating tank treatments respectively. Pool 300 and Sili source colonies exhibited a 7.5 and 32.1% increase in growth in the fluctuating temperature tank (Fig. 4.4; $p < 0.001$) with the highest growth overall observed in Pool 300 source corals. There was not a strong difference in growth between acclimation treatments for Pool 400 source colonies as a whole, although different individual Pool 400 source colonies exhibited contrasting responses to the acclimation treatments, as evidenced by the significant affect of source colony origin on growth response ($p < 0.001$; Fig. 4.5).

Photophysiology

Measurements of effective quantum yield were significantly affected by source colony individual and acclimation treatment in addition to a significant source colony origin by acclimation treatment interaction (Fig. 4.6). Sili source colonies showed the highest yield in the stable acclimation tank and exhibited a strong decline in the fluctuating acclimation treatment, whereas Pool 300 and Pool 400 source colonies remained relatively unchanged between the two tanks. While there was a significant

effect of source colony individual, there did not appear to be a strong relation between individual yield and linear tissue growth measurements (Fig. 4.7).

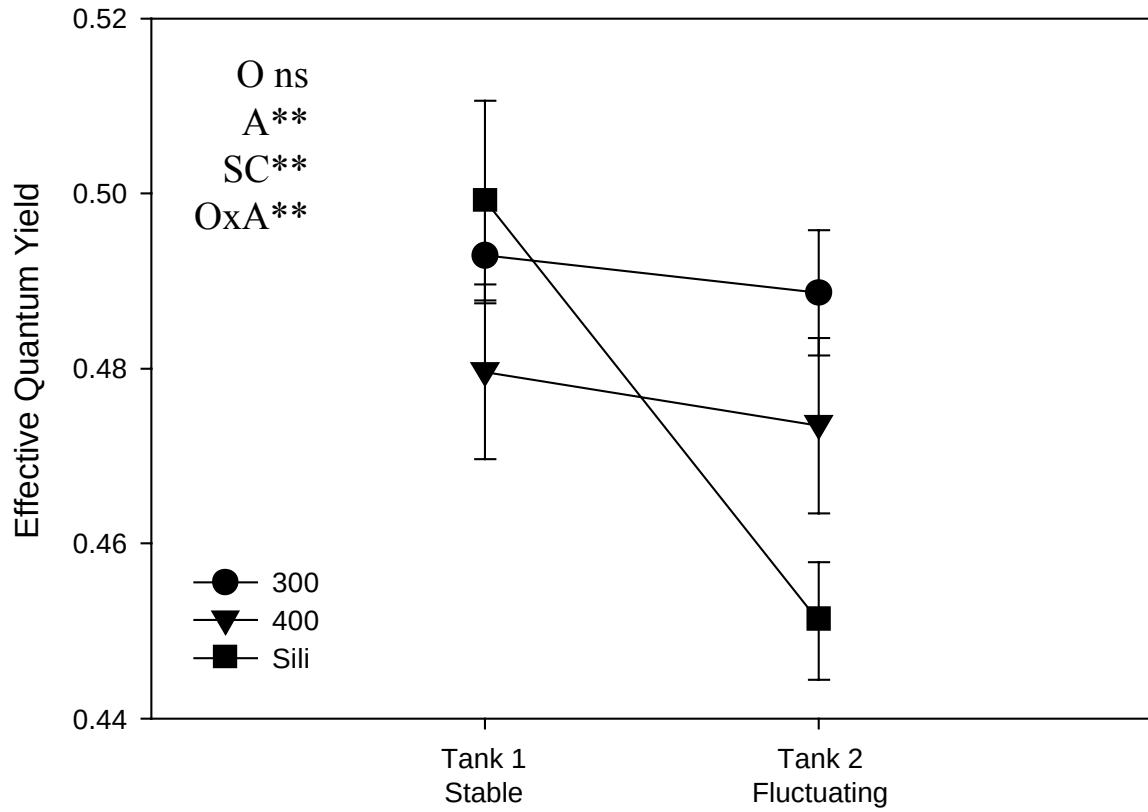


Fig. 4.6 PAM fluorometry measurements taken after the 35-day acclimation period of dark-adapted quantum yield following 45 min of dark-adaptation, for a description of symbols and significance levels see Fig. 4.4 legend.

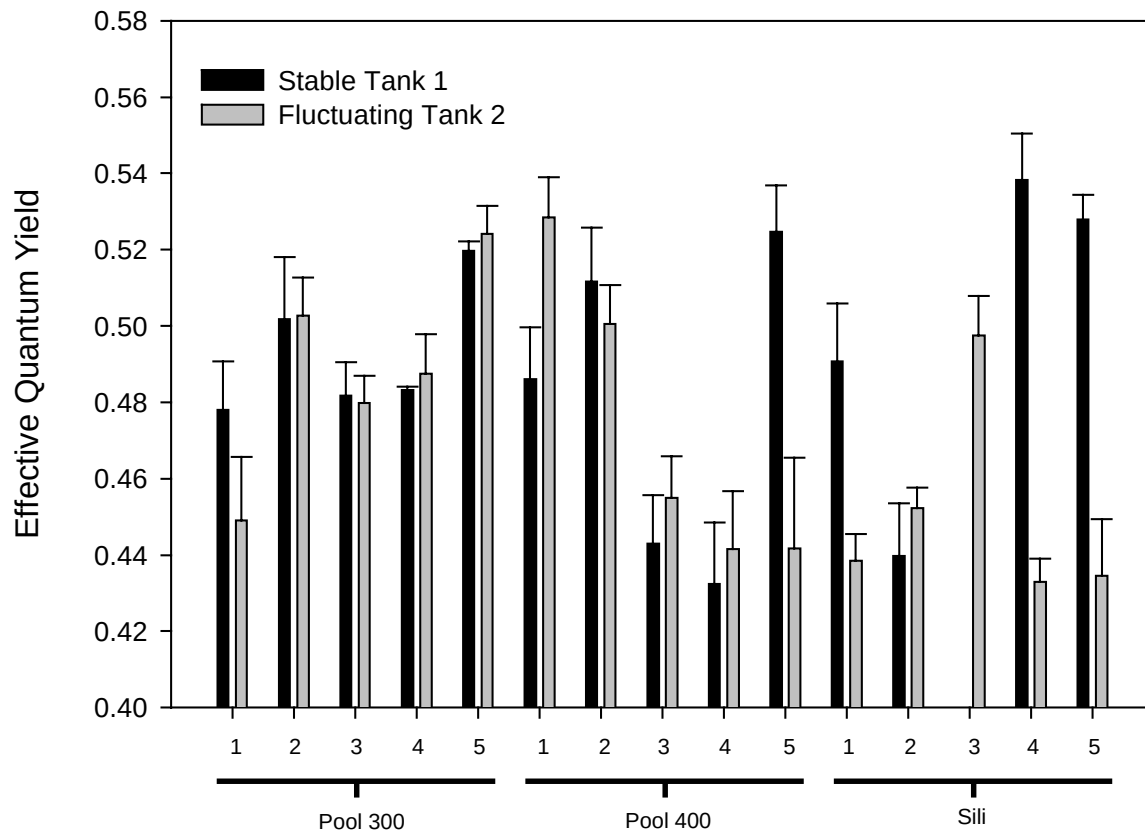


Fig. 4.7 PAM fluorometry measurements taken after the 35-day acclimation period separated by individual source colony, dark bars represent tank 1 acclimated samples, light bars represent tank 2 acclimated samples.

Protein biomarkers: Hsp70 and ubiquitin-conjugates

Host hsp70 levels were consistently higher for all source populations than symbiont hsp70 levels (Fig. 4.8). Significant effects of acclimation treatment and source colony individual were observed for hosts while only source colony origin, acclimation

treatment and the interaction between the two were significant for symbionts. Both hosts and symbionts had significantly lower hsp70 levels in the fluctuating treatment tank when compared to stable tank and field baseline levels (Fig. 4.8). Ubiquitin-conjugate response was opposite for hosts with increased levels of ubiquitin-conjugates in the fluctuating versus the stable acclimation tank as evidenced by the significant acclimation effect (Fig. 4.9). Levels also appeared higher in symbiont protein fractions, with only a source colony origin effect largely due to the reduced levels of ubiquitin-conjugates found in sili source colonies (Fig. 4.9). Additionally, host response was influenced significantly by source colony individual, suggesting a fine-scale genotypic influence on this response variable.

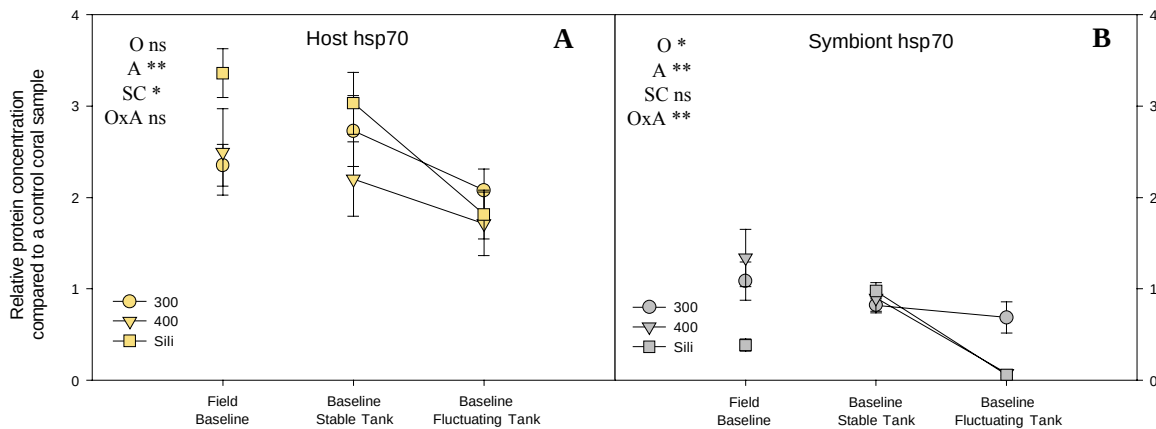


Fig. 4.8 (A) host and **(B)** symbiont hsp70 measurements taken after the 35-day acclimation period, yellow symbols represent host protein fractions and grey symbols represent symbiont protein fractions, for a description of symbols and significance levels see Fig. 4.4 legend.

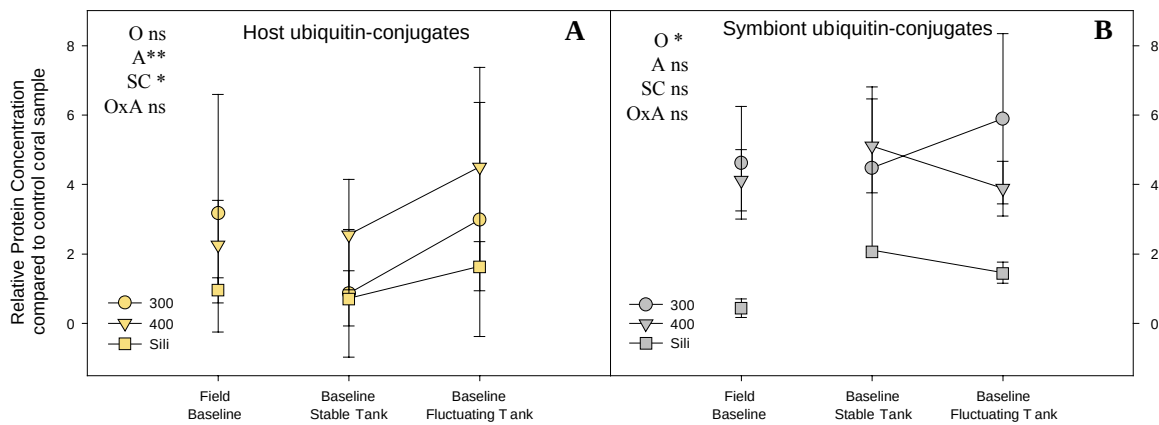


Fig. 4.9 (A) host and (B) symbiont ubiquitin-conjugate measurements taken after the 35-day acclimation period; for a description of symbols and significance levels see Fig. 4.4 legend.

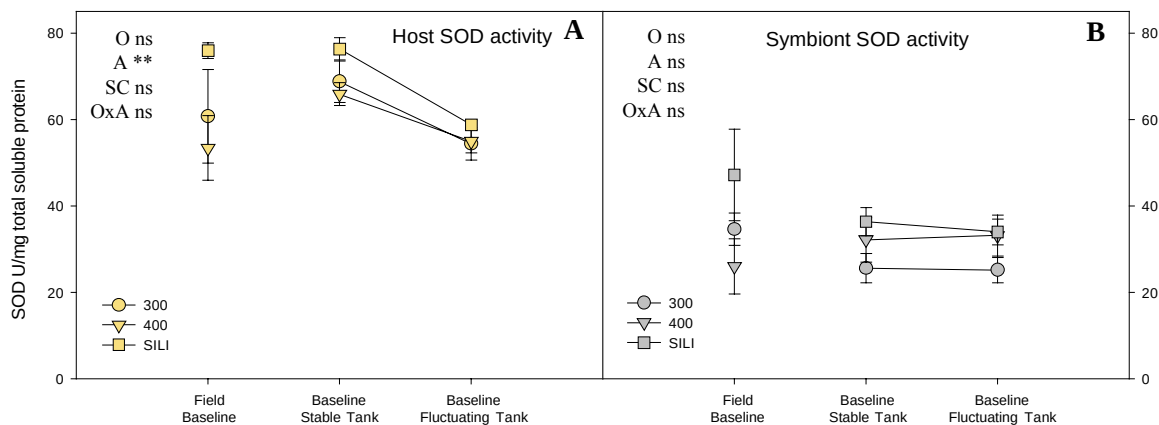


Fig. 10 (A) host and (B) symbiont SOD activity measurements taken after the 35-day acclimation period; for a description of symbols and significance levels see Fig. 4.4 legend.

Superoxide dismutase (SOD) activity

SOD activity appeared to be influenced much less than hsp70 and ubiquitin-conjugate response with the only significant effect found in the reduced host acclimation response to the fluctuating tank treatment (Fig. 4.10). As a whole, SOD activity was higher for hosts than for symbionts suggesting that host tissues play a significant role in the oxidative stress defense of the coral holobiont (host and symbiont symbiotic unit).

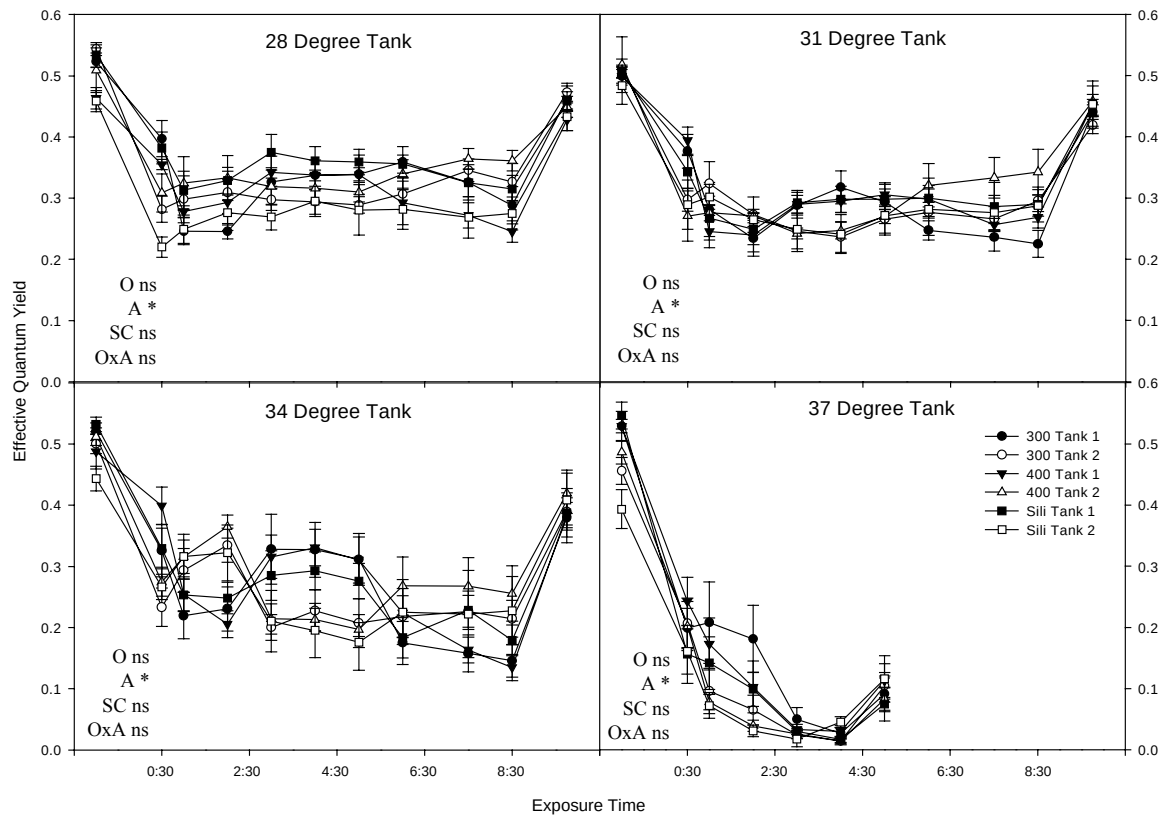


Fig. 4.11 PAM fluorometry measurements of dark-adapted quantum yield taken throughout the time course of the four static temperature treatments, for a description of symbols and significance levels see Fig. 4.4 legend.

Static temperature experiment

Photophysiology

Measurements of effective quantum yield were significantly affected by acclimation treatment for all four static temperature exposures (Fig. 4.11). Reduction in yield was highest for the 37 °C exposure, second for the 34 °C exposure and similar between the 28 and 31 °C exposures (Fig. 4.11). The overall effect of acclimation treatment may have been driven by higher effective quantum yields from corals in the stable temperature acclimation tank, although the pattern was not very consistent across the duration of the experiment (Fig. 4.11).

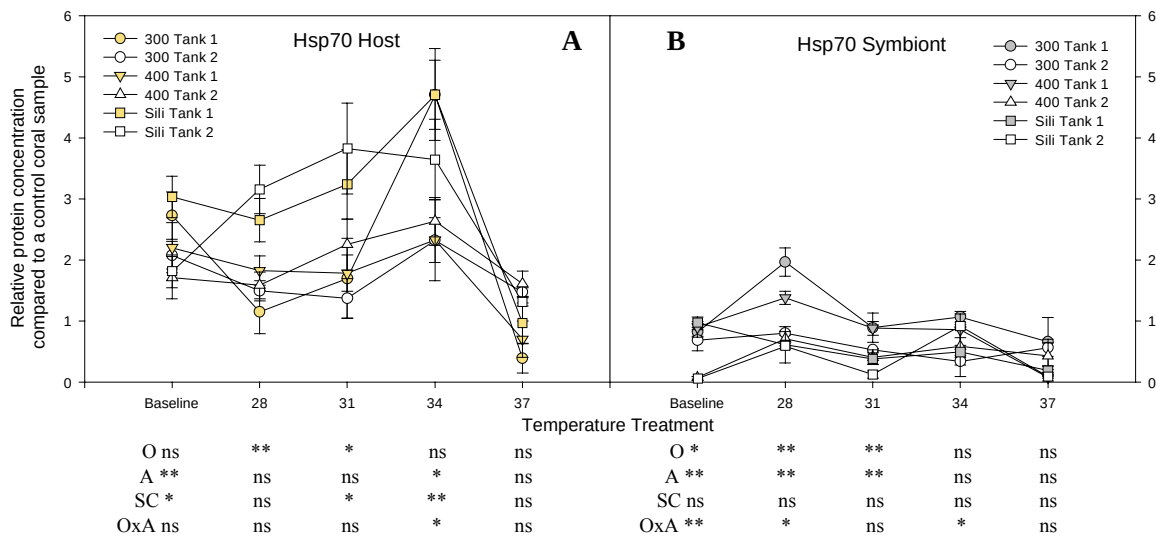


Fig. 4.12 Measurements of (A) host and (B) symbiont hsp70 levels taken during the four static temperature treatments; yellow and grey symbols represent host and symbiont protein fractions respectively for stable (tank 1) acclimated samples while open symbols represent fluctuating (tank 2) acclimated samples in each panel respectively and for a description of significance levels see Fig. 4.4 legend.

Protein biomarkers: Hsp70 and ubiquitin-conjugates

Host hsp70 levels showed an increased response correlated with increasing temperature exposure for all source populations (Fig. 4.12). A significant effect of source colony origin was evident at the 28 and 31 °C exposures, driven by the higher levels of hsp70 found in sili source colonies regardless of acclimation treatment (Fig. 4.12). Induction of the hsp70 response was also quickest for sili source colonies from the fluctuating temperature treatment (Fig. 4.12 Sili Tank 2, 28 °C). The highest hsp70 levels were found in the 34 °C treatment, where significant effects of acclimation, source colony individual, and an origin by acclimation interaction were evident. Symbiont hsp70 levels were markedly reduced compared to those in the host ($p < 0.05$) and peaked at lower values, with significant effects of source colony origin and acclimation treatment evident at the 28 and 31 °C exposures (Fig. 4.12). A weak origin by acclimation interaction term was observed in the 34 °C exposure, while no other tests were significant (Fig. 4.12).

Host ubiquitin-conjugate levels did not show a marked increase over baseline levels for any of the temperature treatments (Fig. 4.13). Significant acclimation and source colony individual effects were observed for the 28, 31, and 34 °C treatments; although it is unclear as to which samples are behind these effects as multiple comparisons were not significant for any treatment grouping (data not shown). Symbiont ubiquitin-conjugate levels were significantly affected by source colony origin (23, 31, and 37 °C treatments), acclimation tank (all treatments) and source colony individual (all treatments; Fig. 4.13). Generally, back reef source colonies exhibited higher levels of ubiquitin-conjugates than source colonies from the Sili forereef, regardless of transplant

treatment. Corals from the stable acclimation treatment also exhibited higher ubiquitin-conjugate levels than those corals from the fluctuating acclimation treatment, a trend that was not significant in the baseline acclimation samples prior to the stress exposure (Fig. 4.13).

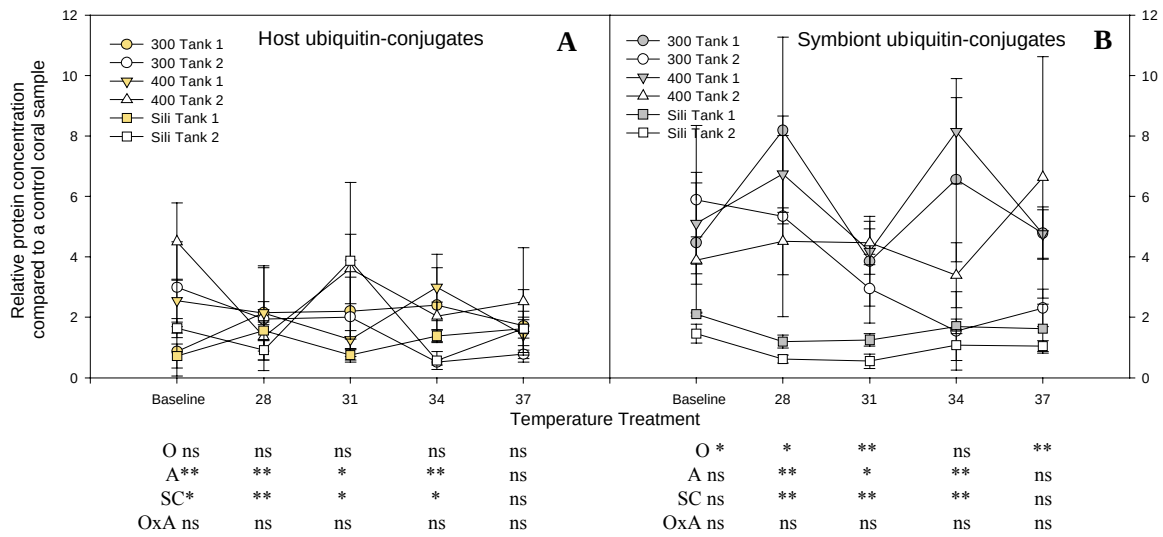


Fig. 4.13 Measurements of (A) host and (B) symbiont ubiquitin-conjugate levels taken during the four static temperature treatments; for a description of symbols and significance levels see Fig. 4.4 legend.

Superoxide dismutase (SOD) activity

Host SOD activity was significantly affected by source colony origin and individual in the 28 and 34 °C treatments (Fig. 4.14). A significant acclimation effect was observed in three of the four treatments with higher levels of SOD activity in corals from the stable versus the fluctuating acclimation treatment (Fig. 4.14). Symbiont SOD activity was generally lower than that of the host tissue fraction ($p < 0.05$). When source colony

origin was significant, Sili forereef source corals appeared to have higher levels of SOD activity compared to corals from the two forereef sites. There did not appear to be a large change in SOD activity response between baseline and temperature treatments, with an apparently decreasing trend in symbiont SOD activity following temperature exposures.

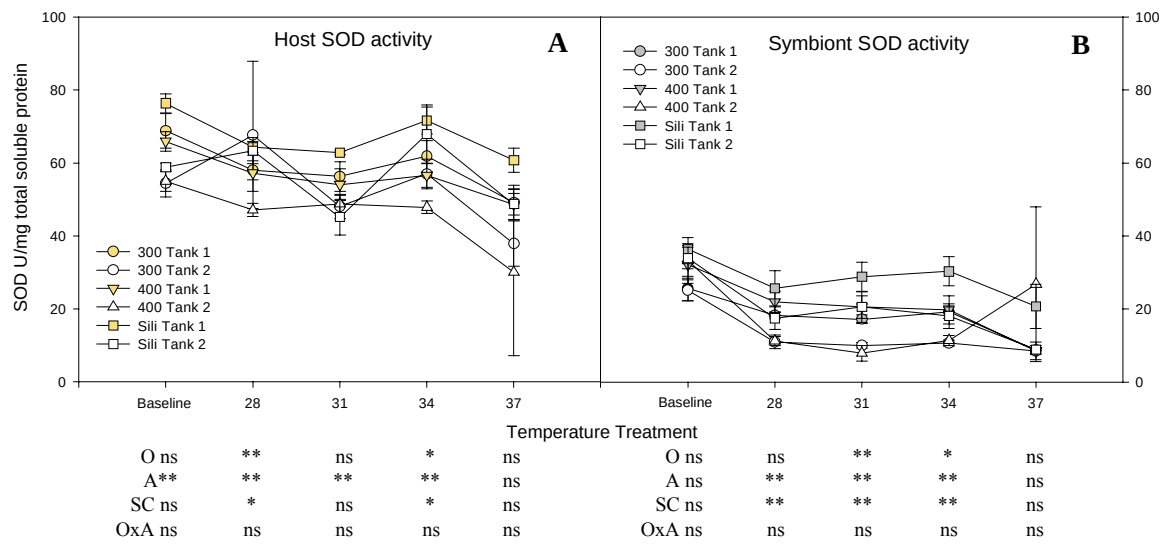


Fig. 4.14 Measurements of (A) host and (B) symbiont SOD activity taken during the four static temperature treatments; for a description of symbols and significance levels see Fig. 4.4 legend.

Ramping temperature experiment

Photophysiology

Effective quantum yield responded strongly to the ramping temperature treatment with a sharp decline in observed yield in the Sili forereef source colonies following the day 2 and day 3 exposure (Fig. 4.15). The initial acclimation effect observed on days 0 and 1 was quickly overshadowed by this source colony origin influence on days 2 and 3.

Interestingly, source colony individual effects remained significant until the final day of the experiment when yield measurements were close to zero, suggesting a strong influence of individual on yield response (Fig. 4.15).

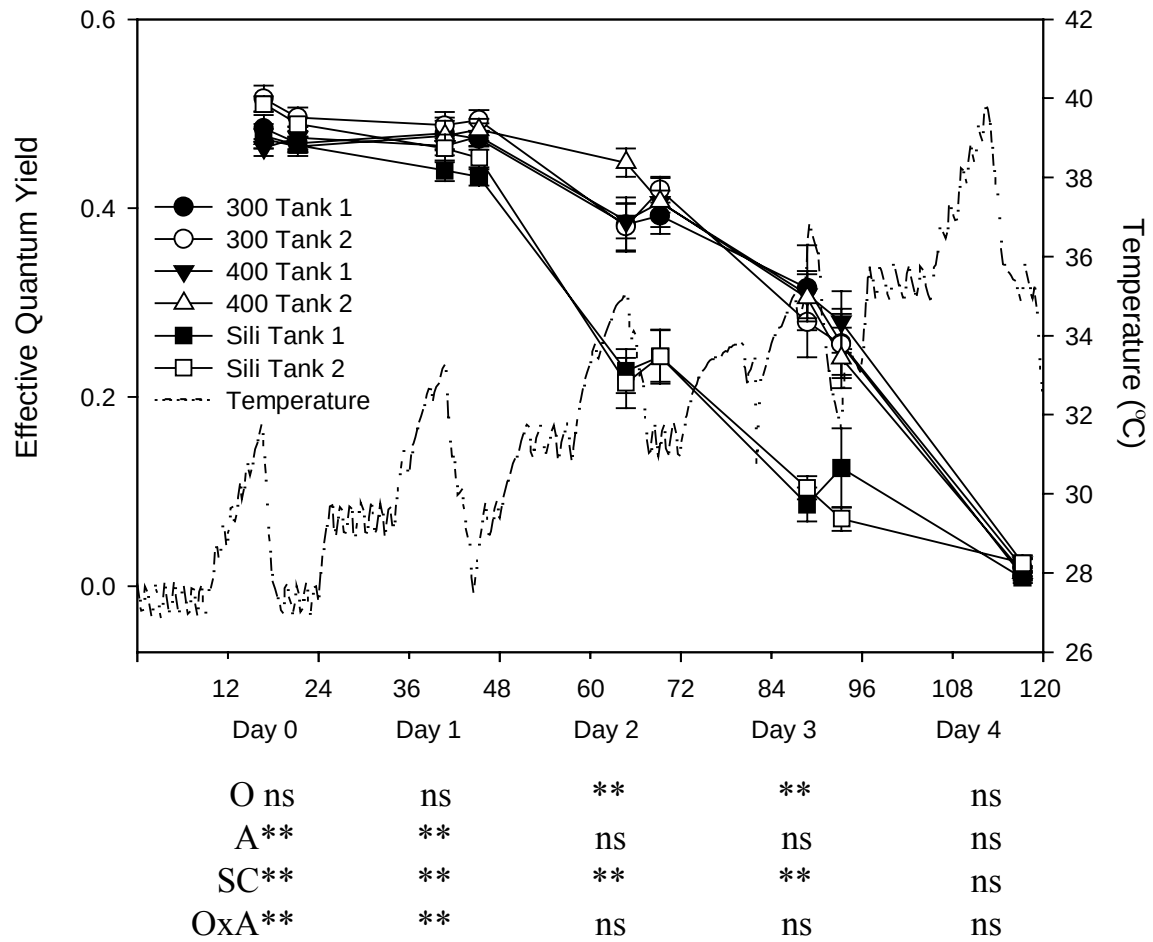


Fig. 4.15 PAM fluorometry measurements and temperature profile of the ramping temperature experiment, for a description of symbols and significance levels see Fig. 4.4 legend.

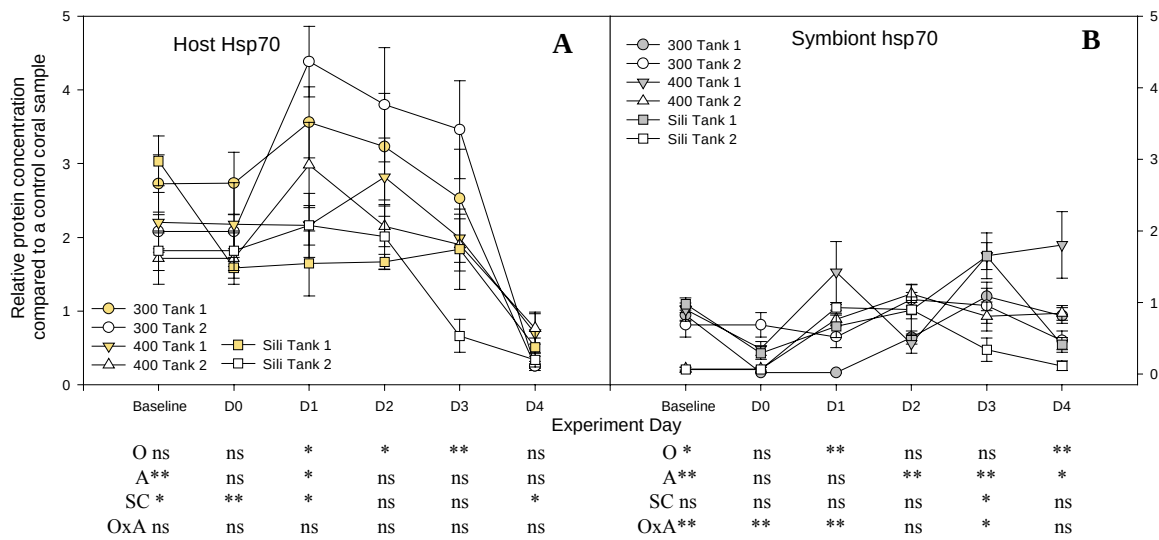


Fig. 4.16 Measurements of (A) host and (B) symbiont hsp70 levels taken during the five day ramping temperature treatment; for a description of symbols and significance levels see Fig. 4.4 legend.

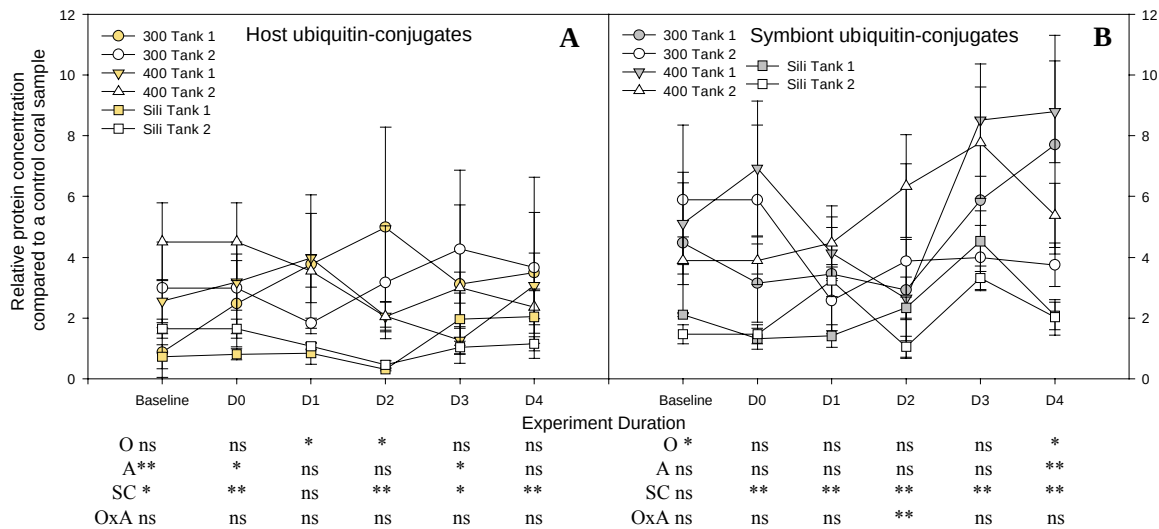


Fig. 4.17 Measurements of (A) host and (B) symbiont ubiquitin-conjugate levels taken during the five day ramping temperature treatment; for a description of symbols and significance levels see Fig. 4.4 legend.

Protein biomarkers: Hsp70 and ubiquitin-conjugates

The highest levels of hsp70 were observed in Pool 300 source colonies while levels in Sili source colonies were lowest (days 1-3, Fig. 4.16). There was a large induction in host hsp70 levels in both Pool 300 and 400 source colonies from both acclimation treatments while little increase in response was seen from Sili source colonies (Fig. 4.16). A significant effect of acclimation treatment was observed on day 1, with greater hsp70 levels in corals from the fluctuating versus the stable treatment (Fig. 4.16). No clear pattern was apparent in symbiont hsp70 levels, with a mix of acclimation and origin effects and interactions throughout the duration of the experiment (Fig. 4.16).

A significant effect of source colony origin was observed in host ubiquitin-conjugates levels on days 1 and 2 of the ramping temperature treatment, with Sili source colonies exhibiting lower levels of protein ubiquitination than their back reef conspecifics (Fig. 4.17). Source colony individual effects were also observed for four of the five experimental days (days 0, 2, 3, 4 Fig. 4.17). Variability was much higher for symbiont ubiquitin-conjugate levels with the most consistent effect caused by source colony individual over all experimental days (Fig. 4.17). Source colony origin and acclimation effects were only observed on day 4, although an origin by acclimation interaction was observed on day 2.

Superoxide dismutase (SOD) activity

Host SOD activity was influenced by a mix of source colony origin, individual and acclimation effects (Fig. 4.18). Generally, higher levels of SOD activity were observed in

Sili source colonies versus their back reef conspecifics. The strong acclimation effect observed in baseline samples was not consistent throughout the temperature experiment with some source locations showing contrasting patterns on different days (Fig. 4.18). Symbiont SOD activity followed a grossly similar trend to that during the static temperature treatments, although no significant effects were observed aside from a single effect of source colony individual on day 1 (Fig. 4.18).

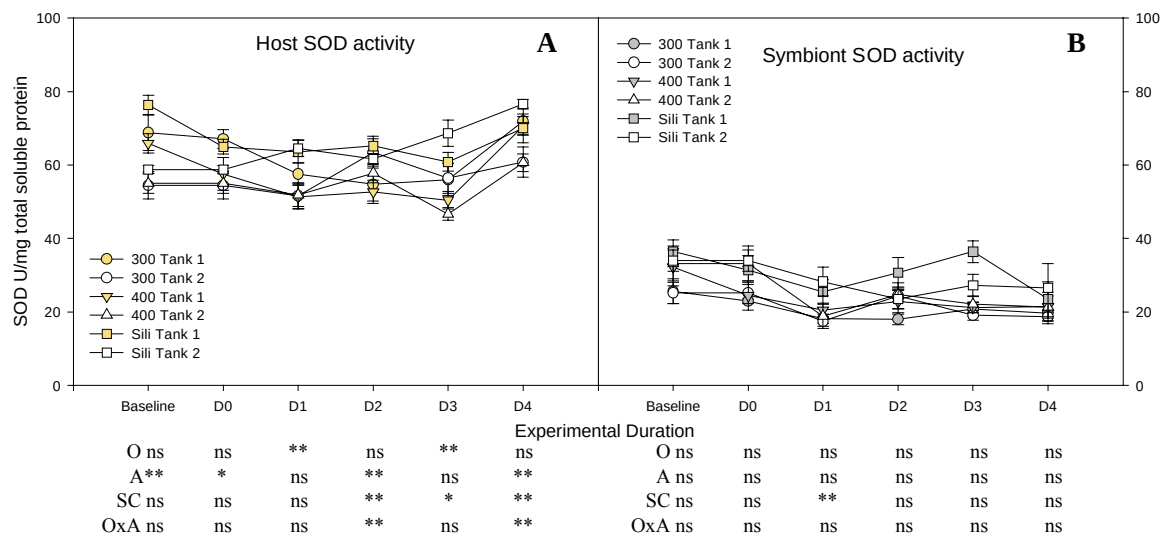


Fig. 4.18 Measurements of (A) host and (B) symbiont SOD activity taken during the five day ramping temperature treatment; for a description of symbols and significance levels see Fig. 4.4 legend.

Discussion

This study investigated the thermal acclimation potential and upper stress tolerance limits of corals acclimatized to three contrasting environments in American Samoa, where a host of physicochemical differences (e.g., temperature, salinity, oxygen,

pH) are present. Previous studies of these coral populations in field transplantation studied identified strong physiological differences and potential limitations in their acclimatization responses (Barshis Chapter 1, Chapter 3; Smith *et al.* 2007). In this study we have performed laboratory experiments to isolate temperature as an important driving force behind differences in coral physiology.

We report four salient findings: (i) increased growth was observed in response to fluctuating versus more stable temperature conditions, (ii) there was a consistent influence of source colony individual on all host and symbiont physiological responses, (iii) the consistent source colony origin influence on ubiquitin-conjugate levels previously observed appeared stronger in symbiont protein fractions than in those of the host, and (iv) acclimation treatments did not overcome strong influences of source colony origin on physiological responses and upper tolerance limits. Our results were consistent with previous characterizations of limitations to the acclimation potential of corals from these source populations. Each of these findings is discussed in detail below.

Growth responses

The demonstration of increased rates of new tissue extension in response to fluctuating temperature conditions provides some of the first evidence that variability in temperature may positively affect coral growth. Additionally, corals that came from the more thermally variable environments (Pool 300 and 400) exhibited increased rates of tissue extension compared to those from the more stable forereef (Sili). While linear tissue extension rate only represents one aspect of coral growth, this measurement has

previously been used successfully as an approximation of growth rate and response to induced environmental stressors (Lirman & Manzello 2009).

Although coral growth responses to fluctuating temperatures have not previously been characterized, the relationship between stable temperatures and coral growth over both short and long time periods has been well established (Buddemeier & Kinzie 1976; Clausen & Roth 1975; Edmunds 2005). Edmunds (2005) found that three day, sub-lethal temperature exposures up to 6 °C above natural mean water temperatures significantly increased coral growth rates. The effects of long-term exposure to such a treatment, however, would likely prove detrimental, as increases of only 1 to 2 °C above mean summer maximum seawater temperatures have been shown to regularly elicit coral bleaching and mortality over several weeks (Jokiel & Coles 1990). Yet the results of Edmunds (2005), in conjunction with those from the present study, are provocative in that they indicate that short-term and fluctuating temperature exposures may actually impart beneficial influence on coral growth characteristics. Additionally, the increased rates of tissue extension, and, presumably, corresponding skeletal accretion, seen in back reef corals (e.g., Pool 300 and 400) versus those from more thermally stable forereef (Sili; Fig. 4.4), raise the possibility of fixed, long-term benefits of exposure to temperature variation. This agrees with previous studies that have also shown a variety of beneficial aspects of exposure to fluctuating versus constant temperatures for various aquatic organisms including increases in growth (Les *et al.* 2009; Spigarelli *et al.* 1982; Wang *et al.* 2007) and thermal tolerance limits (Feldmeth *et al.* 1974; Feminella & Matthews 1984; Hines *et al.* 1980).

An important caveat to address concerns the specific level of temperature control exhibited in our particular aquarium system. Strict temperature manipulation of large amounts of seawater over short timeframes (minutes to hours) remains a difficult task to achieve in a laboratory setting; likely one of the reasons behind the lack of controlled fluctuating temperature studies in the literature. The temperature acclimation profiles shown in Fig. 4.3 illustrate some of the difficulties experienced during the initial run of this particular experimental system. While the variability of the two temperature treatments were roughly contrasting in nature (i.e., tank 1 more stable versus tank 2), an amount of fluctuation was unavoidably present in tank 1 due to equipment malfunction (Fig. 4.3). However, as a strictly constant temperature environment is unlikely to be found in nature, the higher daily maxima as well as the larger variance associated with the acclimation conditions of tank 2 provide a valuable first insight into the effects of fluctuating versus more stable temperature conditions on coral acclimation response.

In addition to the effects of acclimation treatment, significant effects of source colony individual were observed consistently throughout the experiments and for all variables under investigation. During the acclimation treatment, different source colonies showed completely opposite growth responses to the two tank conditions, with some colonies increasing growth in the fluctuating tank and some exhibiting decreased growth (Fig. 4.5). These results are consistent with the prevailing thought that individual colonies can have very different physiological responses under the same environmental stresses and acclimation conditions (Gates & Edmunds 1999). The specific mechanism behind this individual variation could be related to individual host genotype, as four out of five

individuals from Pool 400 and Sili, and all five individuals from Pool 300 had at least one unique sequence type not shared with any other sample (Fig. 4.2). Although this certainly raises the possibility of host genotypic influence on individual response, similar levels of variation between individuals have also been observed for replicates of the same host clone (Bruno & Edmunds 1997; Edmunds 1994). Hence, variation in physiological responses between individuals likely involves a complex interaction between host and symbiont genotype and many environmental influences on cellular and physiological processes.

Protein biomarkers

Previous research on corals from these populations had identified a strong source colony origin signal behind differences in ubiquitin-conjugate levels between groups (Barshis Chapter 1, Chapter 3). Within a common garden framework, effects of source colony origin are thought to indicate potential genetic/epigenetic influence on the response variable (DeWitt & Scheiner 2004; Schluter 2000). Prior studies examined protein extracted from tissue homogenates comprised of both host and symbiont material and were unable to distinguish between responses of the two symbiotic partners. As genetic examinations of host and symbiont population structure revealed significant host subdivision and no strong pattern in symbiont type corresponding to source colony origin, a host genetic mechanism behind differences in ubiquitin-conjugate levels was hypothesized (Barshis Chapter 1, Chapter 2, Chapter 3).

Surprisingly, the present study revealed strong influence of source colony origin on symbiont ubiquitin response after acclimation and during both exposure experiments despite the lack of corresponding differences in genotypic composition (Figs. 4.9, 4.13 and 4.17). We discuss two possible explanations for this observed response which are not mutually exclusive. First, accurate characterization of the diversity of algal endosymbionts within the genus *Symbiodinium* remains a challenging task. Taxonomic designations have largely been based on molecular markers that show high degrees of intragenomic variability, often making clear genotypic boundaries hard to distinguish (e.g., (Apprill & Gates 2007; Stat *et al.* 2006; Thornhill *et al.* 2007)). Recent progress in methodological analyses are beginning to overcome this hurdle (Correa & Baker 2009; Sampayo *et al.* 2009), yet a functional understanding of the relationship between *Symbiodinium* genotype and physiology remains elusive. The results of our previous work revealed a number of unique sequence types of *Symbiodinium* in Pool 400 and Sili coral populations, with the dominant sequence type in both environments determined to be ITS2 type C15 (Barshis Chapter 1). It is possible, however, that fine-scale diversity within C15 may exist as has been found with other ITS2 genotypes (Santos *et al.* 2004). Hence, there could be some physiological influence behind the source colony origin effects observed in this study of the unique ITS2 sequences types previously observed or of cryptic diversity within the C15 symbiont type itself.

Alternatively, a host genetic influence could still be behind the observed ubiquitin response of the symbiont protein fraction. A recent study demonstrated that holobiont stress tolerance not only depends on symbiont genotype, but that different host genetic

backgrounds exhibit contrasting responses to temperature and light stress despite similar symbiont types (Abrego *et al.* 2008). Host genetic influence on photophysiological response has also been demonstrated for corals that occupy large depth ranges and contrasting light environments (Frade *et al.* 2008). Thus, it is possible that the source colony origin and individual effects observed in symbiont ubiquitin-conjugates may be indicative of host mediated differences in the intracellular symbiotic environment. This would also be supported by the significant genetic differentiation observed in coral hosts from the three separate populations ($F_{ST} = 0.20609$, $p < 0.0001$; Fig. 4.3). Future research focusing on fine-scale genotypic diversity of the symbiont and differences in acclimation potential within host clonal genotypes could provide further insight into the relative roles of host and symbiont genotype on ubiquitin response.

Acclimation treatment effects appeared strongest for growth, host and symbiont hsp70, host SOD activity, host ubiquitin-conjugates, and effective quantum yield measurements immediately following the acclimation period (Figs. 4.4, 4.6, 4.8-4.10). As discussed above, growth measurements were higher in the fluctuating versus stable temperature tank suggesting a degree of increased productivity associated with variable temperature exposure (Fig. 4.4). This is consistent with the reduced host and symbiont hsp70 levels and host SOD activity which support a hypothesis of reduced stress response induction in the fluctuating temperature treatment (Figs. 4.8, 4.10). Yet the increased levels of host ubiquitin-conjugates and decreased effective quantum yield observed in tank 2, would suggest greater rates of irreversible protein damage and decreased photosynthetic efficiency in response to the temperature fluctuations (Figs. 4.6, 4.9).

Further, these relationships hold true throughout the various stress exposures (Figs. 4.11-4.18), with the addition of higher symbiont ubiquitin-conjugates in tank 2 acclimated corals (Fig. 4.13). These results paint a contrasting picture of potentially beneficial physiological effects such as increased growth and reduced heat and oxidative stress defense response, alongside putative negative effects of increased thermal protein denaturation and reduced photosynthetic efficiency. While the specific ecological value of acclimation to the contrasting conditions remains elusive, some insight may be gained from examination of the additional influences on physiological response observed during this study.

Despite the significant effects of acclimation treatment on all response variables, strong effects of source colony origin were also observed during the course of the study. Nowhere was this more evident than in the quantum yield measurements from the ramping temperature experiment (Fig. 4.15). Significant acclimation effects were observed for baseline and days 0 and 1 measurements, yet these were quickly overshadowed by origin effects on days 2 and 3 of the ramping exposure (Fig. 4.15). This was due to the dramatic reduction in yield in Sili source colonies from both acclimation treatments. Conventional interpretation of reduced photosynthetic yield in response to temperature exposure would lead us to conclude an increased susceptibility to thermal stress and reduced thermotolerance in these source corals (e.g., Bhagooli & Yakovleva 2004; Fitt *et al.* 2001; Genty *et al.* 1989). Hence, these results suggest that despite the acclimation to fluctuating temperature conditions, Sili source colonies are less tolerant of repeated exposure to increasing temperatures compared to the back reef conspecific

populations of Pool 300 and 400. This raises the possibility that long-term exposure to the fluctuating conditions of the back reef environment has positively affected the upper thermotolerance limits in Pool 300 and 400 source populations. Further, the consistently higher levels of ubiquitin-conjugates found in back reef source colonies in the current and previous studies (Barshis Chapter 1, Chapter 3) may actually indicate an increased capacity for stress tolerance, rather than a classical interpretation of chronic levels of stress-induced protein damage. The lack of a similar pattern during the static temperature experiments adds an additional layer of complexity in that repeated exposure to increasing thermal fluctuation may reveal finer-scale differences in coral thermotolerance than extended exposure to static temperature increases. Indeed, it was the goal of the ramping temperature experiment to attempt a simulation of natural thermal anomalies where a pool of warmer water slowly approaches a location and establishes residency over an extended timeperiod, e.g., the natural variability associated with daily temperature fluctuations is preserved while a slow increase in the mean water temperatures relative to previous levels is caused by the thermal anomaly.

As a whole, data from this study illustrate that the coral stress response is a dynamic, complex process involving a suite of interactions between host and symbiont genotype, thermal acclimation history and exposure to environmental variability. Sub-lethal effects of acclimation to fluctuating versus constant temperatures appear to drive increased growth, while significant limitation related to source colony origin and individual restrict acclimation potential. Separation of specific host and symbiont contributions to acclimation response and upper stress tolerance proved difficult, with

significant effects of cryptic symbiont genetic diversity and/or host mediation of the intracellular symbiotic environment likely influencing the restricted plasticity in *Symbiodinium* proteasome function. Lastly, acclimation treatments were not enough to overcome strong signals of source colony origin, further supporting the hypothesis that the greater environmental variability of the Ofu back reef has far-reaching effects on coral physiological response; and may be behind epigenetic or adaptive increases in stress tolerance of back reef versus forereef coral populations. These findings have important implications for the resilience of fluctuating reef environments in the face of future rates of global climate change. The results presented in this study suggest that corals that are found in naturally occurring extreme environments may be most critical in ensuring the persistence of coral reefs into the future.

GENERAL SUMMARY AND CONCLUSIONS

The objective of my dissertation research was to assess the effects of fluctuating environmental conditions on the physiological mechanisms of stress tolerance and resilience of the reef-building coral *Porties lobata*. I addressed questions related to if, and how exposure to large variability in environmental characteristics enhances coral resilience via either acclimatization or adaptive processes. To this end, molecular stress biomarkers were examined in a series of field transplant experiments (Chapters 1 and 3), host and symbiont genetic studies (Chapters 1 and 2), and controlled laboratory manipulations (Chapter 4) of *P. lobata* from contrasting environments on the forereef and back reefs of Ofu and Olosega Islands.

The ‘Research Questions and Experimental Results’ section below summarizes how each question was investigated using a set of experiments, synthesizes the results of the experiments, and provides a final summary of how each experiment fits together as a whole. Finally, a brief section entitled ‘Future Research Areas’ summarizes potential future research foci identified during the course of this research.

Research Questions and Experimental Results

Experiments were designed to address each of the seven research questions described in the general introduction (and repeated below) using *Porites lobata* as the study species and a variety of experimental approaches. Results are summarized below according to each group of research questions and corresponding experiments:

Q_1 : Do the different environmental conditions of the forereef and back reef cause intrinsic physiological differences between forereef and back reef populations of *P. lobata*?

Q_2 : If there are physiological differences between corals from the two environments, are they fixed (e.g., due to adaptation) or plastic (e.g., due to acclimatization) in nature?

To address Q_1 and Q_2 , a Reciprocal Transplant Experiment (RTE) was performed between one of the back reef pools (Pool 400) and the neighboring Sili forereef during the southern summer season when back reef environmental variability is greatest (Chapter 1). A suite of stress biomarkers was investigated in an attempt to characterize differences in the stress response of corals from different source populations and transplant destinations. Effects of transplant destination, source colony origin, and source

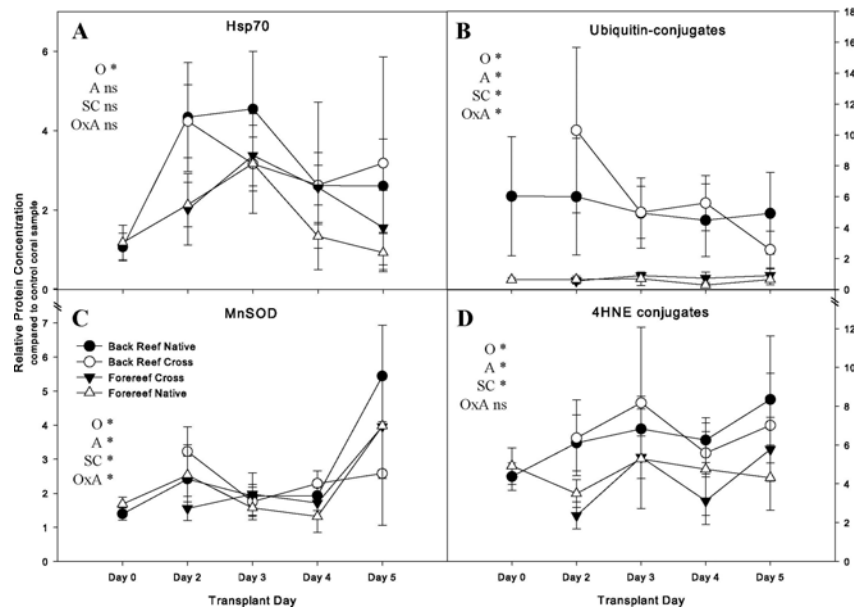


Figure 1.4 Molecular marker concentrations for forereef (triangles) and back reef (circles) native (open symbols) and cross (black symbols) transplants before (day 0) and

during (days 2-5) the summer RTE, significant effects of source colony origin (O), transplant destination (A), source colony individual (SC) and the interaction of origin and destination (OxA) are denoted with an asterisk *.

colony individual were observed for all biomarkers and most consistently for ubiquitin-conjugated proteins (Fig. 1.4). These results suggest that there are indeed intrinsic differences between these two populations of corals (Q_1), and despite evidence for plasticity in the phenotypic response, strong source colony origin and individual effects suggest fixed limits to acclimatization potential (Q_2). These results provoked Q_3 in order to determine potential sources of adaptive influence.

Q_3 : Do the forereef and back reef populations of *P. lobata* differ in the genetic makeup of their algal endosymbionts?

A genetic investigation into symbiont community identity was conducted on a larger sample of Pool 400 and Sili source colonies in an attempt to determine if different symbiont types were hosted by the two populations (Chapter 1). While a degree of sequence diversity was uncovered, no clear pattern separating the forereef and back reef symbiont communities was apparent (Fig. 1.5). A sequence identical to the ITS2 type C15 was the dominant sequence type found in all source colonies, a type known to associate with Pacific *Porites* species (LaJeunesse *et al.* 2003). An increasing association with the more thermally tolerant clade D *Symbiodinium* has been observed in other coral species in the back reef pools (Oliver & Palumbi 2009; Smith 2008), yet *Porites lobata* is thought to transmit its symbionts vertically from parent to offspring which potentially restricts its ability to associate with additional symbiont genotypes (Richmond & Hunter

1990; Stat *et al.* 2008a). This raises the possibility of a host adaptive mechanism behind the observed physiological differences between source populations.

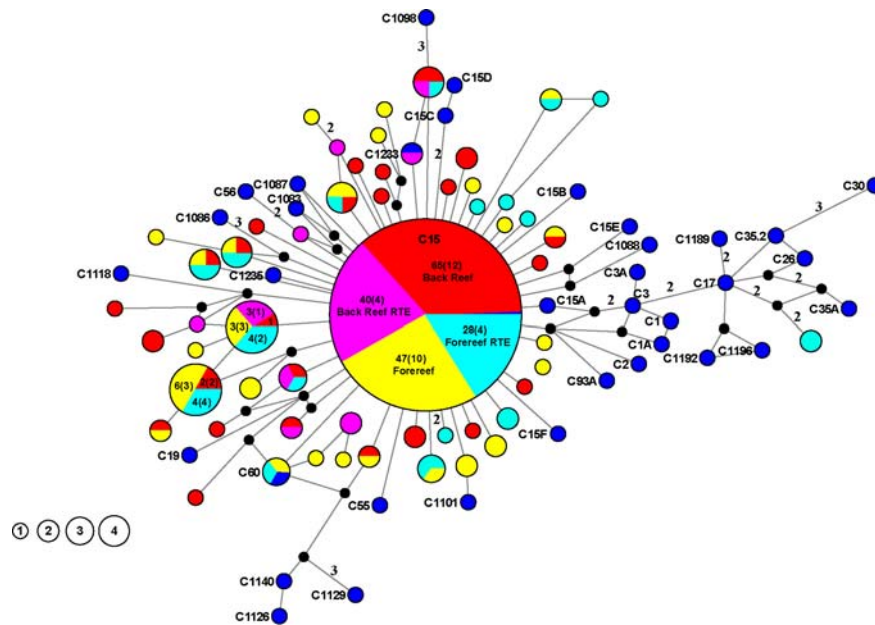


Figure 1.5 Maximum parsimony phylogenetic network reconstruction of *Symbiodinium* sp. ITS2 isolates from symbiont genetic survey compared to previously published sequences. Sequences from back reef individuals are shown in red, those from colonies used in the reciprocal transplant experiment shown in light purple, forereef individuals shown in yellow and forereef RTE colonies shown in light blue, and previously published sequence types shown in dark blue.

Q₄: How well are host *P. lobata* populations genetically subdivided within American Samoa, are there patterns of genetic subdivision that correlate with habitat designations?

An in-depth analyses of the coral host genetic structure of the two populations used in the summer RTE was performed as part of Chapter 1 in addition to a larger-scale survey of multiple forereef and back reef populations on Ofu, Olosega, and Tutuila Islands (Chapter 2). Significant population structure was found at both within island and between island spatial scales suggesting a high degree of subdivision within these *P. lobata* populations (Fig. 2.4). No clear association between population subdivision and habitat type was observed, as individual forereef and back reef habitats were equally different from other populations from both similar and contrasting habitat types. The greater degree of population differentiation potentially found in Ofu and Olosega populations versus Tutuila populations (Fig. 2.4) suggests the possibility of stronger limitations to dispersal in the Ofu/Olosega reef system.

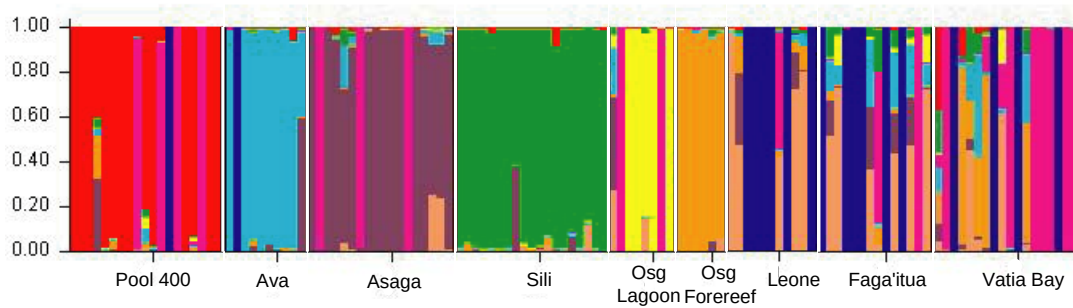


Fig. 2.4 Geographical clustering of *Porites lobata* populations inferred using STRUCTURE. Each vertical bar represents an individual coral sample, the color of the bar signifies the population to which it was assigned using the model, solid black bars separate samples from different populations and each population origin is noted below the graph.

Q₅: Are there seasonal or temporal differences in coral physiological responses that correlate with temporal and seasonal variability in environmental conditions?

To address the seasonal component of Q₅, an additional RTE between Pool 400 and Sili was conducted during the southern winter (Chapter 3) when environmental variability is known to be less than in the summer season (Craig *et al.* 2001; Smith & Birkeland 2003). There was evidence for a seasonal reduction in the level of ubiquitin-conjugates of back reef source colonies during the winter, although they were still significantly elevated compared to forereef source colonies in both transplant locations (see Fig. 3.4). Additionally, source colony origin and individual effects were still observed during the winter, although less consistently than during the summer RTE.

A profiling of natural variability in biomarker response was conducted over a 72 hr time period for six source colonies in Pool 400 to investigate the temporal component of Q₅ (Chapter 3). Biomarker levels were shown to fluctuate significantly over time and evidence was found for a positive association between temperature and biomarker variability (Fig. 3.2). Both short (0, 4 and 8hr) and long-term (12, 16, 24 and 32hr) lags in biomarker response were observed, suggesting that cellular responses may be significantly delayed following temperature exposure (Fig. 3.3).

Q₆: What is the long term acclimatization potential of forereef and back reef *P. lobata* populations?

Since the summer and winter RTEs (Chapters 1 and 3) and the time series (Chapter 3) were conducted over relatively short time scales (3 – 5 days), two additional experiments were performed over longer durations to address Q_6 . These consist of the long-term field RTE in Chapter 3 that lasted a total of 261 days and the tank acclimation treatments in Chapter 4 that lasted 36 days. Results from the long-term RTE showed a stronger influence of transplant destination on biomarker response than observed previously, suggesting that long term exposure to the different environmental conditions has serious potential to shape cellular processes. Yet significant effects of source colony origin, individual, and an origin by destination interaction were still observed for ubiquitin-conjugated protein levels (Table 3.3).

Table 3.3 Three-way ANOVA (Source colony origin, transplant destination, and source colony individual) for protein biomarker concentrations (ubiquitin conjugates, hsp70, MnSOD, and 4HNE) at the end of the 261 day long-term RTE

Factor	df	MS				F				<i>p</i>			
		ubiq	hsp70	MnSOD	4HNE	ubiq	hsp70	MnSOD	4HNE	ubiq	hsp70	MnSOD	4HNE
Source colony origin	2	0.275	0.712	1.475	20.930	6.40	2.49	1.34	2.47	0.0170	0.1250	0.2990	0.1240
Transplant destination	2	0.101	1.662	1.197	50.096	8.23	5.92	1.08	4.84	0.0060	0.0160	0.3690	0.0290
Source colony indiv	9	0.048	0.288	0.862	8.176	3.93	1.03	0.78	0.79	0.0150	0.4730	0.6390	0.6310
Origin x destination	4	0.127	0.162	1.006	5.890	10.34	0.58	0.91	0.57	0.0010	0.6840	0.4880	0.6900

Results were similar in the acclimation treatments performed in Chapter 4 with a combination of origin, individual, and acclimation treatment effects observed for most variables under investigation (Chapter 4). As a whole, these experiments demonstrate that long term acclimatization to contrasting conditions is possible, yet distinct response

differences remain between back reef and forereef *P. lobata* populations even after extended periods of time.

Q₇: Is temperature one of the driving variables behind observed physiological response differences between back reef and forereef populations of *P. lobata*?

Controlled temperature manipulations were performed on corals from two back reef (Pools 300 and 400) and one forereef (Sili) sites holding all other variables constant to address Q₇ (Chapter 4). Acclimation treatments were intended to mimic the larger daily temperature fluctuations of the back reef environment versus the reduced temperature variability of the forereef. Significant effects of acclimation to constant versus fluctuating temperatures were observed for a variety of variables including tissue growth, photosynthetic efficiency, and antioxidant enzyme activity (Chapter 4). The results of increased growth, and reduced heat shock and antioxidant response in response to acclimation to the fluctuating temperature profile suggest a beneficial effect of this acclimation treatment. However, this contrasts with the reduced quantum yield and increase in ubiquitin-conjugated proteins which suggest detrimental effects of the treatment. While the specific value of each set of acclimation conditions remains difficult to assess, these results confirm the important influence of differences in temperature variability on coral physiological response.

Final Summary

The focus of my dissertation research was to assess the physiological impacts of short (e.g., acclimatization) and long-term (e.g., adaptation) exposures to the fluctuating

environmental conditions of the Ofu back reef. Essentially, I was interested in the question of whether exposure to greater levels of environmental variability contributes to enhanced coral resilience. Resilience is used here as the capacity for a system to withstand disturbance while continuing to function (Holling 1973; Levin & Lubchenco 2008). In the current time of rapid coral reef degradation, it is increasingly realized that resilient populations, species, and areas will likely provide the foundation for the stability of reefs and ensure their recovery in the near future (Grimsditch & Salm 2006; Hughes *et al.* 2005; Levin & Lubchenco 2008; Nystrom *et al.* 2000). The results of this dissertation support previous observations that the back reef environment on Ofu island may promote coral resilience and contain diverse populations of resilient corals (Smith 2008).

Initial investigation of the back reef environment revealed that a wide array of coral species apparently tolerate extreme conditions without visible signs of chronic impact, e.g., reduction in species diversity, consistent bleaching, or increased mortality (Craig *et al.* 2001). Follow-up studies demonstrated that specific abiotic parameters such as intermittent high water motion and reduced irradiance may alleviate potential harmful impacts of thermal exposures (Smith 2008; Smith & Birkeland 2007); yet an understanding of how intrinsic coral responses were being affected by the same variability remained undeveloped. The results of this dissertation suggest that the internal picture of coral resilience is far more complicated than initially thought, although resilient characteristics in back reef populations of *P. lobata* and consistent effects of exposure to back reef conditions were found.

Measures of holobiont physiological capacity such as growth and photosynthetic efficiency appear to be clearest in illustrating the differences between back reef and forereef source populations. Smith and colleagues (2007) demonstrated that the back reef environment was conducive to elevated growth and that back reef corals outperformed their forereef conspecifics regardless of transplant location. The acclimation experiments in described in Chapter 4 confirm this to be true, and caused, in part, by the differences in temperature variability between the two locations. Since back reef source colonies exhibited superior growth characteristics, one might also expect them to exhibit increased stress tolerance as well. This was clearly the case based on the measurements of photosynthetic efficiency taken during the ramping temperature exposure in Chapter 4, with Sili source colonies exhibiting a dramatic reduction in effective quantum yield by the 2nd day of elevated temperature exposure (Fig. 4.4).

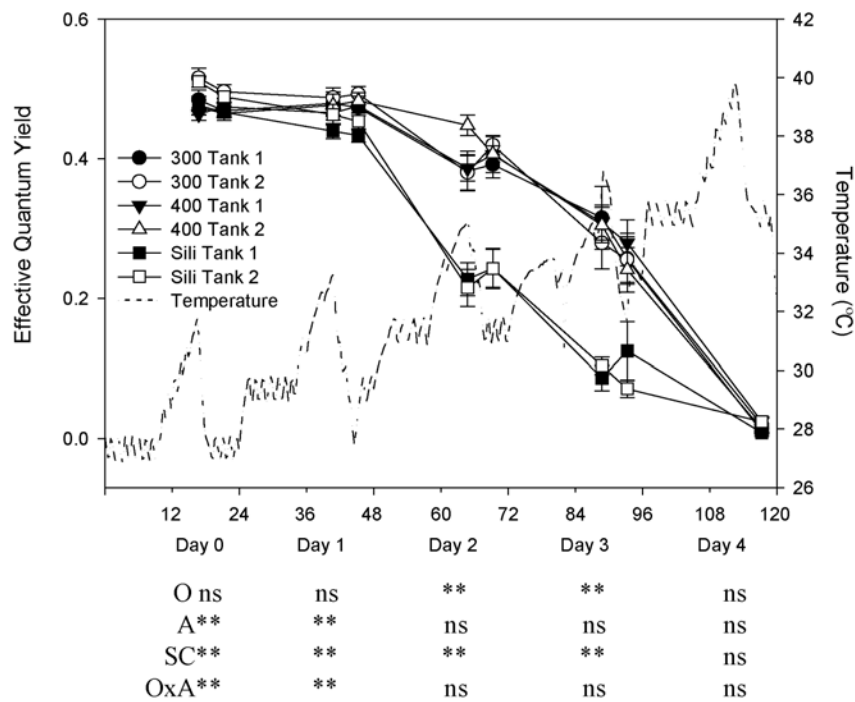


Figure 4.15 PAM fluorometry measurements and temperature profile of the ramping temperature experiment of Chapter 4, Tank 1 denotes the stable acclimation treatment, Tank 2 the fluctuating acclimation treatment, symbols are as designated within the figure, significance values are denoted beneath the figure as in Fig. 4.4.

However, a clear, corresponding pattern was not observed for any of the molecular biomarker or enzymatic activity results from the same samples (Chapter 4). Strong responses to elevated temperatures have been observed in these biomarkers previously in corals (Brown *et al.* 2002a; Downs *et al.* 2002; Gates & Edmunds 1999; Hayes & King 1995; Robbart *et al.* 2004; Sharp *et al.* 1997; Yum 2006), yet the results presented here indicate that the cellular stress response of these particular corals is highly variable (Chapters 1, 3, and 4). This finding is not necessarily surprising, as the molecular assays measure physiological response on a smaller spatial and mechanistic scale than the gross indicators of skeletal extension and effective quantum yield. The growth rate of a given coral colony reflects the combined interaction and operation of all cellular and metabolic processes of the cnidarian host and its algal endosymbionts. Any one of these processes examined in isolation, details the response of that particular mechanism by itself; which is decoupled, in part, from the combined physiological function of the organismal unit. Podrabsky and Somero (2004) for instance, found that acclimation conditions caused fundamentally different heat shock response in the annual killifish, with small heat shock proteins induced more strongly in animals in a fluctuating versus an elevated temperature treatment; where larger chaperones (hsp70 and hsp90) were

transcribed more readily. In this example, similar physiological function at the organismal level may have been maintained by different mechanisms at the molecular level of the heat shock response.

The most consistent differences at the molecular level observed for *P. lobata*, involved the higher levels of ubiquitin-conjugated proteins of back reef source colonies from all experimental manipulations (Chapters 1, 3, and 4). Increases in the pool of ubiquitin-conjugated proteins are classically thought to represent greater rates of irreversible protein damage in response to environmental stressors (Brown *et al.* 2002a; Downs & Downs 2007; Dutton & Hofmann 2008; Hofmann & Somero 1995, 1996a, b; Yum 2006). In contrast, the results presented here demonstrate increased growth and stress tolerance of back reef corals alongside higher levels of ubiquitin-conjugated proteins (Chapters 1, 3 and 4); apparently contradictory findings. However, it is increasingly realized that ubiquitin conjugation to proteins is involved in many processes (e.g., cross membrane translocation and chaperoning), not all of which are directly related to stress induced protein damage (Hicke 2001; Li & Ye 2008; Welchman *et al.* 2005). Additionally, the methods used here lack the sensitivity to distinguish between the various types of ubiquitin conjugation, impeding the ability to definitively assess the source of the elevated signal. Hence, the increased levels of ubiquitylation in back reef source colonies could be indicative of non-stress related functions of the ubiquitin pathway, or perhaps demonstrate a greater capacity for the ubiquitin proteasome pathway of the back reef source colonies to respond to an increased level of protein degradation without additional consequences. Essentially, the back reef corals may be better able to

maintain cellular function and protein homeostasis in the face of increased levels of environmental stress.

It is possible that the environmental variability of the back reef environment has shaped the *P. lobata* population through selective forces that promote increased resilience to protein damage, either via enhanced capacity for protein turnover or elevated function of the ubiquitin proteasome pathway. The observed increased growth performance of back reef corals versus forereef corals in both back reef (native) and forereef (non-native) environments (Chapter 4;(Smith *et al.* 2007) indicate that this could have occurred without a corresponding fitness trade-off. Additionally, the lack of symbiont community differentiation would shift the selective pressures of the environmental variability of the back reef upon the coral host, further increasing the potential for an adaptive response.

Theory suggests that ecological factors predicted to promote local adaptation include limited gene flow between populations and strong selection against genotypes adapted to other habitats with more moderate selection against intermediate genotypes (Kawecki & Ebert 2004). The significant subdivision between host *P. lobata* populations demonstrates that some type of reduction in gene flow is maintained despite close proximity (Chapters 1 and 2), yet the lack of a clear association between populations from similar versus contrasting habitat types suggests the picture is not so clear. Additionally, the increased growth of forereef source colonies cross transplanted to the back reef (Smith *et al.* 2007) and subjected to the fluctuating acclimation treatment (Chapter 4), suggest that the back reef environment may be more conducive for growth and stress resilience for many genotypes. Regardless of the specific mechanism behind

maintenance of population differentiation of Samoan *P. lobata*, the high degree of subdivision has important implications for dispersal potential and survival in such a heterogeneous environment as the nearshore reefs of Ofu and Olosega.

As a whole, the results of my dissertation support the hypothesis that the environmental variability of the back reef enhances characteristics of coral resilience through a complex combination of acclimatization and adaptive mechanisms. Exposure to fluctuating versus more constant temperatures increased coral growth and decreased heat shock and antioxidant response yet did not significantly enhance the upper thermal tolerance limits of forereef source corals. Back reef source colonies consistently outperformed forereef source colonies, which appeared to be related to enhanced function of the ubiquitin proteasome pathway. Similar symbiont types among populations contrasted sharply with significant population subdivision of the cnidarian host, although no clear influence of habitat was apparent at a broad scale. Lastly, this research serves to illustrate that only with integrated approaches and concurrent use of a variety of tools can we begin to understand the complex interaction of multiple genetic partners, environmental influences, and cellular processes that comprise the coral stress response.

Future Research Areas

1. The particular significance of the increased ubiquitin-conjugates in back reef source colonies remains enigmatic, particularly the source colony origin effect on symbiont ubiquitin levels observed in Chapter 4. Future studies could investigate rates of protein turnover and metabolism, proteasome function, and genotypic

- variation of genes related to the ubiquitin pathway in an attempt to better elucidate the mechanistic basis behind this response.
2. While the significant differentiation of coral host populations across the territory could indicate a host adaptive mechanism behind source population influences, the lack of a clear association with habitat type questions the hypothesis of local adaptation. Genetic investigations of within “type” *Symbiodinium* diversity as well as of functional genomic regions in the coral host may identify targets of selection and/or cryptic symbiont diversity behind source population effects.
 3. The high degree of variability observed in these particular biomarkers questions their utility as a diagnostic tool. Broader screening of the holobiont proteome could reveal more consistent indicators of stress tolerance, resilience, and resistance.
 4. The temporal resolution of sampling was limited by the amount of coral material available for collection, e.g., attempts were made to avoid significantly impacting the study populations through over-harvesting. Hence, these data could be used to focus sampling resolution around informative time points to enhance our understanding of temporal variability in the stress response.
 5. The significant effects of source colony individual observed throughout the studies could be also used to purposely select coral colonies with strongly contrasting physiologies; investigating the genotypic influence on coral response in greater detail.

MANUSCRIPT PUBLICATION OUTLINE

This outline is intended to describe the publication plans for each research chapter of my dissertation as well as additional modifications that will be made to prepare each chapter for submission as individual manuscripts.

Chapter One:

Chapter one is currently in final form and will be resubmitted to *Molecular Ecology* in the month following final dissertation submission.

Chapter Two:

Dissertation edits have been incorporated into the current chapter two with small additional edits required prior to submission to *Marine Ecology Progress Series* or *Coral Reefs* for publication. The main text, analyses, figures, and conclusions will remain the same with minor edits to wording and phrasing to be performed before submission in late summer or early fall.

Chapter Three:

Dissertation edits have been incorporated into the current version of the text, with slight, additional restructuring required before submission to the *Journal of Experimental Biology* or similar. The three experiments performed as part of chapter three will not be broken up individually as they considerably relate to one another and no single

experiment is deemed substantial enough to stand on its own. Significant revision has already been incorporated into the Results and Discussion sections, in addition to added qualifications related to genetic versus epigenetic control, and a consistent organizational scheme for the sections following the introduction. Revision for manuscript submission will include minor reduction in length as well as incorporation of the appendices into a supplementary information section. These edits will be performed during the upcoming fall with a potential submission after the first of the year.

Chapter Four:

Chapter four will require division before it is suitable for publication. Significant revision has been incorporated into the current version consisting of: a more straightforward and descriptive results section, subheadings related to each specific analysis, additional discussion and overall consistent organizational scheme. Prior to publication the chapter will initially be broken into two main parts, with potential for a third shortened publication as a separate novel finding/note/brief communication. Upon this first split, I will reevaluate the cohesiveness and impact of the separate papers to determine if additional splitting is required.

Chapter 4.1:

The first draft manuscript will consist solely of the acclimation experiment and post-acclimation response variables. Below is an outline of the components of manuscript 4.1:

- **Main Focus:** Potential for plasticity in the thermal acclimation responses of corals from contrasting temperature environments.
- **Revised Introduction:**
 - In depth discussion of the acclimatization vs. adaptation debate concerning coral stress tolerance
 - Epigenetic versus genetic contributions to acclimation limits
 - Site descriptions
 - Metric Introductions
- **Data to be used:** All data from the following metrics will consist of the post-acclimation measurements taken prior to any additional stress experiments
 - Host genetic analyses (mtDNA and hopefully microsatellite additions to the current data set depending on release of primer sequences)
 - Tissue extension measurements (including additional discussion of the roughness of this metric)
 - PAM photophysiology
 - Western blotting: host and symbiont hsp70 and ubiquitin-conjugates
 - SOD activity
- **Figures:**
 - Figures 2, 4, 6 and 10 will be included as main figures
 - Figures 8 and 9 will be combined into a multi panel figure
 - Figures 1, 3, 5 and 7 will be incorporated into a supplementary information section.

- **Major conclusions:**
 - Host genotypes appear differentiated between individuals as well as sources (low sample size limits power, need to validate with additional loci)
 - Fluctuating temperature acclimation promotes coral growth (verifying previous findings of Smith, Barshis, and Birkeland 2008), while fixed limits to acclimation potential suggest epigenetic or genetic influence on growth physiology
 - Quantum yield decreases under fluctuating conditions (discussion of paradox between reduced yield and increased growth)
 - Significant influence of source colony individual consistent across multiple variables (potential integration with host genotypic results)
 - hsp70 decreases while ubiquitin-conjugates increase under fluctuating exposures (potential metabolic changes related to turnover, reduced hsp70 pool)
 - source origin signal only significant in symbiont protein fraction (potential for host mediation of symbiont physiology), consistently higher back reef source colony ubiquitin response is consistent with previous observations
 - SOD activity higher in host fractions, consistent between individuals and origins, and reduced in fluctuating temperature treatments (agreement with hsp70 responses, implications for “stress” levels under fluctuating temperatures)

- **Overall:** Acclimation to fluctuating temperatures appears beneficial, yet limitations to acclimation capacity persist associated with epigenetic or genetic influence on holobiont physiology.
- **Additional considerations:** The Methods, Results, and Discussion will present the five metrics as individual subsections to improve clarity and flow of the paper. Additional statistic comparisons of host and symbiont biomarker and activity responses will also be performed (these were only referenced for one comparison in the current text due to space limitations). Possible incorporation of the host genetic data into statistical analyses will be explored, although it is unlikely that sample sizes are large enough to gain sufficient power for any quantitative genetics calculations. Sequencing of the host mtDNA regions is currently underway and I have am awaiting a response concerning the microsatellite primer sequences.

Chapter 4.2:

Chapter 4.2 will be constructed as a brief communication, suitable in form for a brevia submission to Science. This chapter will consist solely of the PAM fluometry data taken during the ramping temperature stress exposure (Fig. 15). The title will consist of something like “Environmental variability promotes coral stress tolerance.” The main focus and conclusion of the chapter will be that acclimation potential of corals is limited and that long-term exposure to environmental variability promotes stress tolerance via epigenetic or genetic mechanism. While this idea has been suggested before and

correlatively shown in many field studies, studies that directly test thermal acclimation potential in corals from contrasting environmental sources are few and far between. I think these data perfectly summarize some of the major conclusions of my research and are worthy of an attempt at a high level, short publication.

Chapter 4.3:

Chapter 4.3 will consist of the responses measured during the static and ramping temperature stress experiments. Below is an outline of manuscript 4.2:

- **Main Focus:** Upper thermal tolerance responses of corals from contrasting acclimation treatments and source environments.
- **Revised Introduction:**
 - Upper thermal tolerance limits of corals and ecological implications
 - Summary of previous research concerning sub-lethal stress responses of corals from these locations
 - Acclimation potential results
 - Metric Introductions
- **Data to be used:** All data from the following metrics will consist of the post-acclimation measurements taken during the static and ramping temperature stress experiments
 - PAM photophysiology (PAM data will be included from the static temperature experiment and referenced for the ramping temp experiment conditional on prior submission of those data as Chapter 4.2)

- Western blotting: host and symbiont hsp70 and ubiquitin-conjugates
- SOD activity
- **Figures:**
 - Figure 11 will be included as a main figure
 - Figures 12, 13 and 14, and 16, 17 and 18 will be combined into two separate multi panel figures
- **Major conclusions:**
 - fine-scale differences in coral response more apparent in ramping temperature rather than static temperature exposures
 - greater host hsp70 induction in back reef corals during ramping temp yet greater in forereef corals during lower static temperatures (lower induction threshold for forereef corals, higher induction/greater response capacity of back reef source corals)
 - hsp70 induction greater in back reef fluctuating tank corals than stable tank corals on day 1 then remained high (increased response capability in tank 2 acclimated corals)
 - Origin effect on symbiont protein appeared more prominent during static than ramping temperature exposures (additional evidence for phenotypic differences between similar symbiont genotypes or host mediated symbiont response)
 - **Overall:** Acclimation to fluctuating temperatures enhances coral stress tolerance, possibly through faster and greater hsp70 induction. Yet significant

limitations to acclimation potential, related to epigenetic or genetic response to long-term exposure to the back reef exert greater influence over thermal tolerance than acclimation.

- **Additional considerations:** The Methods, Results, and Discussion will present the two experiments as individual subsections with the three metrics separated within each subsection to improve clarity and flow of the paper.

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