Colour polymorphism and its role in stress tolerance in the coral *Acropora millepora* on the Great Barrier Reef

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STATEMENT OF CONTRIBUTION OF OTHERS

This work is the result of collaborations with my supervisors, Prof. Bette Willis, Dr. Line Bay and Dr. Madeleine van Oppen, who provided intellectual and editorial guidance throughout the research project. I also worked in collaboration with Prof. Mikhail Matz and Dr. Dave Francis who provided assistance in data analyses and statistical support of Chapters 2 and 6, respectively. Research funding was provided by the Australian Research Council's Centre of Excellence for Coral Reef Studies and James Cook University. In addition I received a stipend from James Cook University which was co-funded through a Postgraduate Research Scholarship and a Tutorship scholarship from the School of Marine and Tropical Biology. In Chapter 2 Dr. Mikhail Matz provided empirical data on the individual emissions of fluorescent proteins from *Acropora millepora* colonies which were used to validate the model of fluorescent protein concentrations.

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I dedicate this thesis to my parents, Kathleen and Steve. Thank you for instilling in me a sense of curiosity for the world around me, and the autonomy to allow me to pursue my dreams, however far away from home it took me.

General Abstract

The roles of colour and fluorescence in animal species are wide-ranging, but their roles in non-bioluminescent corals remain unresolved, particularly the question of whether or not fluorescent proteins (FPs) in coral tissues have a photoprotective function. Knowledge of the mechanisms corals use to combat stress associated with a changing climate is paramount to predictions of the persistence and management of the coral reef ecosystem. Through a combination of field-based monitoring and controlled experiments, I investigate the role of FPs in corals, using the common coral *Acropora millepora,* which is comprised of four distinct colour morphs, as a model species.

Variation in the proportional abundance patterns of intraspecific colour morphs of *A. millepora* among widely separated populations provides clues about their comparative performance under different environmental conditions. In Chapter 2, I document patterns in the abundance and distribution of the four colour morphs in populations of *A. millepora* located in northern, central and southern Great Barrier Reef (GBR) regions. The highly fluorescent green morph was rare at all sites, despite the postulated advantage of FPs in stress tolerance. Moreover, patterns in the relative abundance of colour morphs were similar across latitudinal regions, depths, and wave regimes. Highly conserved patterns in colour morph abundance across a range of environmental gradients suggest that, in general, environmental parameters are not responsible for patterns in the distribution and abundance of colour morphs in this species.

Results presented in Chapter 2 also quantify, for the first time, relative

concentrations of the three common fluorescent proteins and one non-fluorescent protein in tissues of *A. millepora* colour morphs. Total FP concentrations differed among colour morphs, with the highly fluorescent green morph containing up to 7-fold greater concentrations of the Cyan FP (CFP) than the weakly fluorescent red morph. In all morphs, the most abundant FP was CFP, which was present in concentrations that were up to 1600-fold higher at less turbid northern sites compared to more turbid southern sites, a pattern consistent with a role in photoprotection. Variation in FP concentrations among colour morphs revealed in this study also indicate that colour is a function of the proportional abundance of the 3 FPs rather than a reflection of the FP in highest concentration.

Despite high expression levels of FPs in corals (up to 14% of total protein) and the various important functions ascribed to these proteins, baseline information on temporal variation within coral populations remains undocumented. In Chapter 3, I document FP content in *Acropora millepora* colour morphs in response to temporal (seasonal) environmental variation. CFP was the only FP to significantly fluctuate in abundance among colour morphs and between sampling times, presumably representing cyclical maintenance of optimal protein levels, given the lengthy half-lives of these proteins. Of 150 colonies monitored *in-situ*, five (~3%) changed colour within a four-month timeframe. Colour change from a highly fluorescent green morph to a weakly fluorescent red morph corresponded with declining health, suggesting that maintenance of high concentrations of FPs in coral tissues is energetically costly.

Understanding the mechanisms that underpin variation in bleaching susceptibility in corals is central to the conservation of reefs, particularly in light of predictions of continued ocean warming. In Chapters 4 and 5, I analyse the bleaching condition of three colour morphs of *A. millepora*, each characterised by different FP

levels, following both a moderate bleaching event in the northern GBR and experimental heat stress in the central GBR. In both studies, fragments of the highly fluorescent green morph had greater bleaching resilience to thermal stress compared with the weakly and intermediately fluorescent red and yellow morphs. This is the first demonstration of differential susceptibility to temperature stress among fluorescent colour morphs from similar habitats, and highlights the functional importance of subtle intraspecific differences in FP content. Substantial variation in the response of colour morphs to thermal stress confirmed that high abundance of FPs correlates well with high thermal stress tolerance and highlights the contribution of host-mediated mechanisms in governing the bleaching susceptibility of corals.

Despite the advantages of greater tissue concentrations of coral FPs, the highly fluorescent green morph is consistently rare across a variety of habitat types and following major thermal disturbances. Although coral FPs are long-lived, this unexpected pattern in abundance suggests that maintenance of high FP concentrations may come at the cost of other physiological processes, resulting in the low abundance of the green morph across the GBR. In Chapter 6, I investigated the capacity of *A. millepora* colour morphs to produce FPs under a prolonged period of resource deprivation in the months leading up to their annual reproductive event. Comparisons of investment in reproduction among healthy (control) colour morphs and their starving counterparts leads me to propose that a physiological trade-off exists between investment of resources in the production of FPs versus reproduction. Weakly fluorescent red colonies invested more heavily in reproduction, as indicated by their significantly higher concentrations of wax esters, than highly fluorescent green colonies. Greater investment in reproduction by red colonies corroborates the highly conserved pattern of consistently greater abundance of the red morph in populations

of *A millepora*, and would explain the low abundance of green colonies despite the environmental advantages of containing higher concentrations of coral FPs.

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Chapter 1

An introduction to fluorescence in corals

Corals exhibit prominent colour variation, ranging from fluorescent green and blue to red and non-fluorescent purple (Mazel 1995; Mazel 1997; Veron 2000). Many species display a number of colour types or morphs (Veron 1986; Salih et al. 2000; Field et al. 2006; Leutenegger et al. 2007; Oswald et al. 2007). It is now well-accepted that fluorescent proteins (FPs) produced by the coral host, in combination with the presence of typically brown endosymbiotic dinoflagellates in the genus *Symbiodinium,* produce the characteristically striking hues of reef-building corals (Dove et a. 2001; Kelmanson and Matz 2003; Wiedenmann et al. 2004; Matz et al. 2006; Oswald et al. 2007). FPs comprise a surprisingly high fraction of the total protein complement in coral tissue, ranging from 4.5% in the coral *Montastraea cavernosa,* up to 14% in *Acropora nobilis* (Oswald et al. 2007), suggesting that they fulfil a biologically important role. Although the roles of colour and fluorescence in animal species are wide-ranging (e.g. Shimomura et al. 1962; Wicksten 1983; 1989; McFall-Ngai 1990; Mathger and Denton 2001; Mazel et al. 2004), their role(s) in non-bioluminescent corals is unclear.

1.1 The phenomenon of fluorescence

To understand the roles that fluorescence might have in the coral holobiont, it is first necessary to understand the process of fluorescence. Photoluminescence (PL)

describes the phenomenon of light emission following the absorption of light and is one of many forms of luminescence, or cold light, initiated by the absorption of photons or photoexcitation. Following photoexcitation, various relaxation processes occur before photons are re-radiated (or light is emitted). The energy of an exciting photon with respect to its emission is a parameter used to classify PL processes. For example, when a system undergoes internal energy transitions before re-emitting the energy initially absorbed, some of the original energy is dissipated so that the emitted light photons have lower energy and longer wavelength than those absorbed, giving rise to the phenomenon known as fluorescence. Fluorescence occurs when fluorescent molecules, called fluorophores, are excited from the ground state (S_0) into a higher energy state (such as S_1, S_2, etc., depending on the amount of energy absorbed) (Fig. 1.1A). Because fluorophore excitation is unfavourable, dissipation of the excess energy must occur before it can return to ground-state. Most commonly, energy is released by emission of a photon equivalent in energy to the difference between the lowest excited state (S_1) and the ground state (S_0). Through vibrational relaxation and internal conversion (I_c), the fluorophore returns to the lowest excited state (S_1) before emitting a photon (Fig. 1.1A). Hence the emitted photon loses some energy compared with the absorbed energy and is thus of lower energy and a longer wavelength than the absorbed photon (Fig. 1.1B). Eventually, the remaining energy is released in the form of a photon and the fluorophore returns to ground state (Fig. 1.1).

In chlorophyll fluorescence, light energy absorbed by a chlorophyll molecule can be used to undergo photosynthesis (photochemistry), dissipated as heat (non-photochemical quenching), or re-emitted as light (as per description above). These three processes directly compete with one another so that an increase in one results in a decrease in another. Therefore the measurement of the yield of chlorophyll

fluorescence can reveal information about photochemistry and non-photochemical quenching (Maxwell and Johnson 2000).

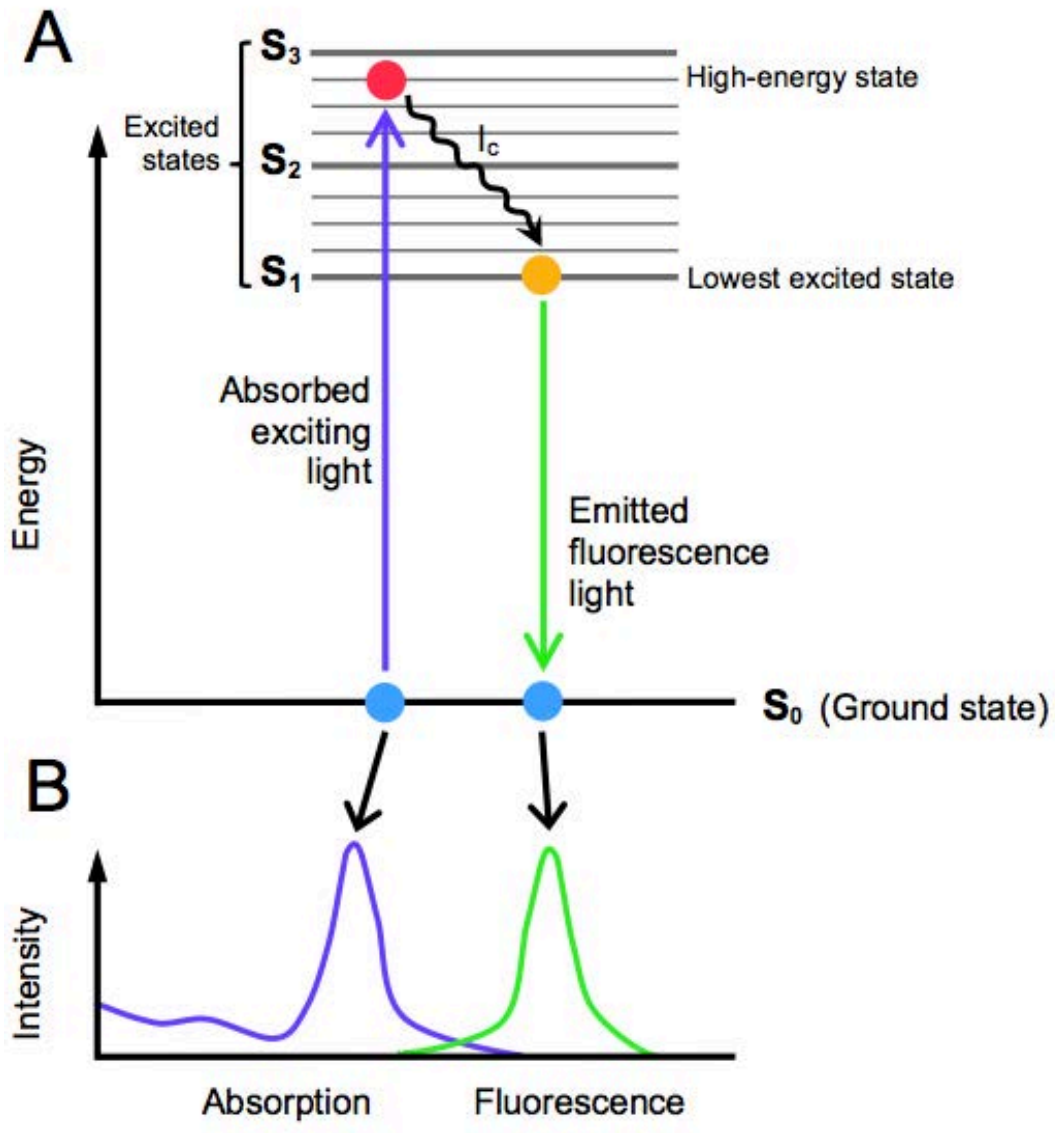

Figure 1.1 Schematic representation of a Jabloński (1933) diagram showing: (A) changes in the energy level of fluorophore giving rise to fluorescence, and (B) corresponding spectra. The coloured circles represent the energy state of the fluorophore, where blue depicts the normal energy level and red the maximum energy level. Non-radiative energy transitions are represented by a black wavy arrow and radiative energy transitions by coloured, straight arrows. The vibrational ground states of each excited state are represented with thick lines and the higher vibrational states with thinner lines. Ic = internal conversion. Adapted from Jablonski (1933).

1.2 GFP, GFP-homologs and chromoproteins

Green fluorescent protein (GFP), first identified from the bioluminescent jellyfish *Aequorea victoria*, is a spontaneously fluorescent protein that absorbs blue light and re-emits it as green fluorescence (Shimomura et al. 1962, Johnson et al. 1962). What makes GFP unique is its ability to catalyse the synthesis of the pigment (chromophore) on its own, thus acting both as a substrate and an enzyme (Matz et al. 2002). In contrast to all other cases of natural pigmentation, where many different enzymes are involved in pigment production, colour attributable to GFP is essentially determined by the nucleotide sequence of a single gene. The only external agent required to complete the pigment biosynthesis is atmospheric oxygen (Heim et al. 1994; Gross et al. 2000). Therefore, expression of the gene coding for GFP leads directly to the appearance of a fluorescent signal. Insertion of the GFP gene prior to the stop codon for a gene of interest would result in the production of GFP any time that gene is produced. As fluorescence intensity is proportional to concentration (Desha 1920), GFP fluorescence has been used widely as a reporter gene to investigate a number of biological events through gene expression (Chalfie et al. 1994; Harper et al. 1999), gene silencing (Ruiz et al. 1998; Hammond et al. 2000), and the exploration of developmental and quantitative properties of promoter genes (Marton et al. 2005; Tany and Newton 2004). Additionally, colours produced by GFPs are easily detected with the naked eye, GFP spectra and fluorescence are easily measured and compared under different experimental conditions, and GFPs are readily expressed and extraordinarily resistant to proteolysis and denaturing agents, such as high temperature, detergents, and extremes of pH (Ward and Cormier 1979; Baird et al. 2000; Ward et al. 2000).

Bioluminescence, the emitting of light through chemical reactions, differs from fluorescence, the emission of light following absorption of a photon without involving a catalytic or enzymatic chemical reaction. Many of the bioluminescent pathways investigated in organisms like hydroid polyps, comb jellies and copepods (Matz et al. 1999) are not homologous to that of *A. victoria* (Matz et al. 2002). These systems evolved recently and independently within different lineages, and their enzymes were recruited from biochemical pathways completely unrelated to light production (Hastings 1983; Hastings 1995; Rees et al. 1998). Speculation that the recruitment of GFP might similarly be recent, gave rise to the notion that GFP-like proteins (or GFP homologs) might exist among non-bioluminescent organisms. Indeed, fluorescence has been shown to be due to the presence of a diverse group of GFP homologs within non-bioluminescent, reef-building, corals (class Anthozoa, order Scleractinia) (Matz et al. 1999; Matz et al. 2002; Alieva et al. 2008). There are at least 40 different GFP homologs, of which only 6 are from bioluminescent organisms; the rest are from fluorescent and coloured, non-bioluminescent representatives of Anthozoa (Labas et al. 2002; Matz et al. 2002). To date, scleractinian corals represent the largest known repository of spectroscopic diversity of GFP homologs (Alieva et al. 2008).

Many genetically enhanced GFP variants have been developed through directed mutagenesis of the GFP nucleotide sequence, and range in emission spectral profiles from the blue (Yang et al. 1998), to cyan (Heim and Tsien, 1996), green (Heim et al. 1995), yellow (Wachter et al. 1998), and red (Matz et al. 1999) regions of the visible spectrum. GFPs and their homologs each have unique major excitation and emission maxima (Tsein 1998), indicating that each pair of excitation and emission spectra found represent individual GFP proteins, enabling easy identification from

spectral profiles (Dove et al. 2001). The four most common fluorescent protein (FPs) types are cyan, green, yellow and red, each with unique excitation and emission spectra (Fig 1.2). Additionally, non-fluorescent chromoproteins (CPs), also called pocilloporin, only shows intense absorption capabilities and does not fluoresce (Alieva et al. 2008).

Phylogenetic analyses of GFP-like nucleotide sequences suggest evolutionarily newer cyan and red FPs evolved under positive natural selection (Field et al. 2006), strongly indicating that colour diversity serves a biological function. Multiple examples of parallel evolution, whereby the same colours have evolved in cnidarians as diverse as Actiniaria, Corallimorpharia and Alcyonaria, strongly corroborates this interpretation (Alieva et al. 2008). However, the adaptive significance of this colour diversity remains unresolved (Matz et al. 2002; Kelmanson and Matz 2003).

1.3 Coral colouration and the role of coral FPs

Since early observations of fluorescence in corals (Phillips 1927; Kawaguti 1944; Shibata 1969), patterns in the distribution and abundance of fluorescent morphs have suggested that FPs function in photoprotection, either of the coral host or their photosynthetic algal symbionts (Kawaguti 1944; Salih et al. 1998, 2000, 2006; Dove et al. 2001). However, based on the premise that a FP would only be suitable for photoprotection of algal symbionts if its excitation wavelength was the same as UV light and/or its presence exerted influence on chlorophyll excitation/fluorescence, the finding that GFP absorption did not reduce the chlorophyll excitation spectrum in Caribbean corals (Mazel et al. 2003) raises questions about the role of FPs in algal

photoprotection. Confounding the issue is that many coral species exhibit multiple FPs, in addition to GFP, simultaneously (Mazel et al. 2003). The combined range of excitation and emission spectra that would occur in the presence of multiple FPs

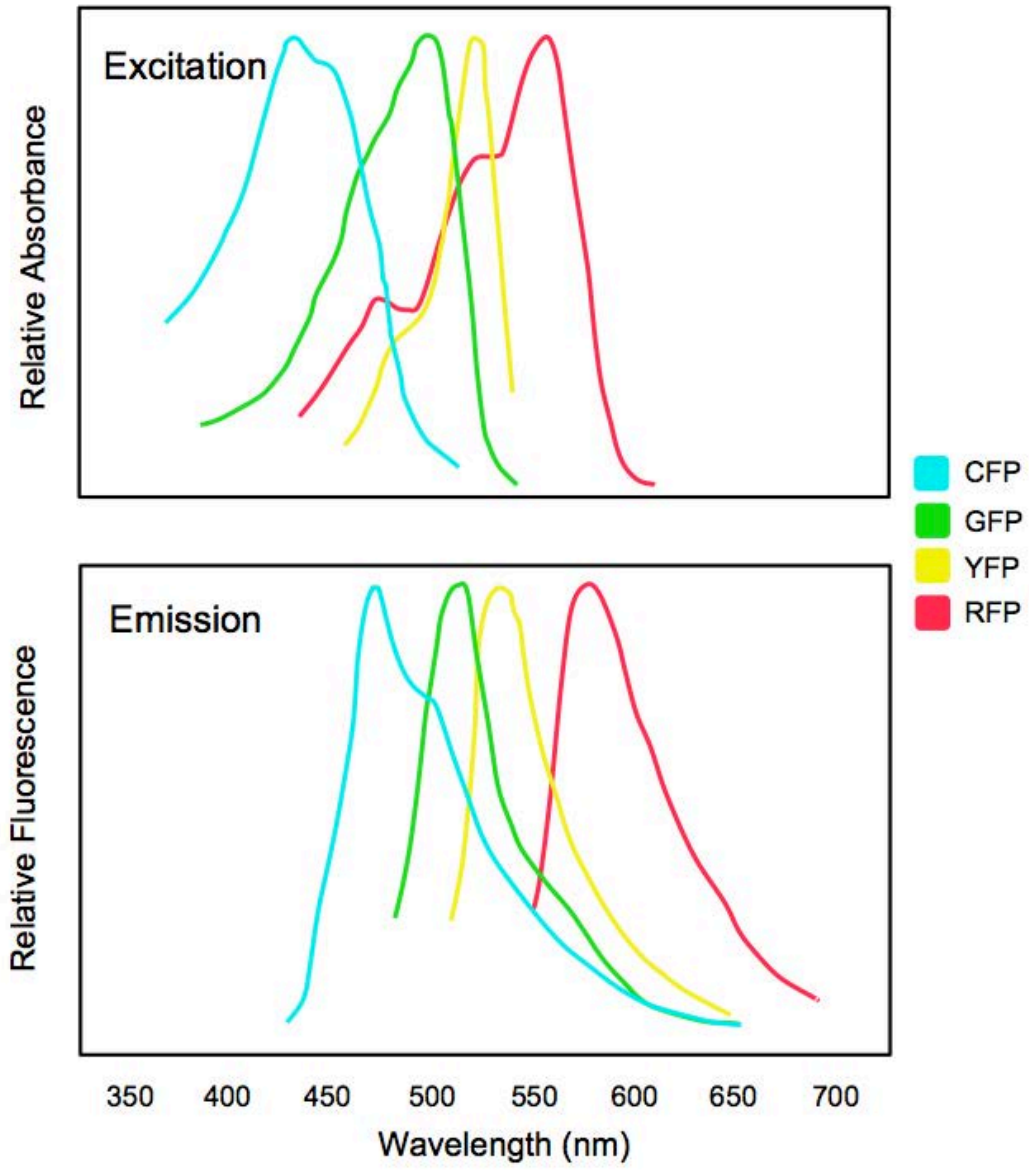

Figure 1.2 Schematic representation of absorption/excitation and emission spectra for four common fluorescent proteins (FPs) found in corals: cyan FP (CFP), green FP (GFP), yellow FP (YFP) and red FP (RFP).

suggests possible energy transfer (Salih et al. 2000). Förster resonant energy transfer (FRET) describes the process of energy transfer between fluorophores, where in an

excited state, a donor fluorophore may radiatively (or non-radiatively) transfer energy to an acceptor fluorophore. The efficiency of this transfer is inversely proportional to the distance between donor and acceptor and can be used to measure interaction distances (in nm) between fluorophores (Herman et al. 2001). Light energy transfer between FPs could lead to the absorption and re-allocation of energy into less photosynthetically active pigments, thereby reducing damage to PSII of a coral's zooxanthellae (Cox et al. 2007). Although FRET did not occur in isolated pigments of *Acropora millepora,* imaging of the entire emission spectrum from reef-collected *A. millepora* samples revealed that up to 93% of the FPs were involved in FRET pairs, implying their use in photoprotection (Cox et al. 2007). It was postulated that fluorescence kinetics and FPs could lead to the absorption and re-allocation of energy into less photosynthetically active pigments, thereby reducing damage to PSII of a coral's zooxanthellae (Cox et al. 2007). However fluorescence kinetics and excitation versus emission mapping indicated that FP emission plays only a minor role, if any, in chlorophyll excitation (Gilmore et al. 2003), therefore our understanding of the photoprotective mechanism of FPs remains ambiguous.

A number of lines of evidence further argue against a role for FPs in photoprotection, including observations of different coloured morphs (containing different types/amounts of FPs) co-occurring (side-by-side) in the field (Takabayashi and Hoegh-Guldberg 1995), the possession of green-to-red photoconverting FPs by azooxanthellate corals (Wiedemann et al. 2004; Oswald et al. 2007; Schnitzler et al. 2008), and lack of correlation between FP abundance and water depth (Mazel et al. 2003). Alternative roles that have been proposed include that FPs (1) function as antioxidants ameliorating the coral stress response (Bou-Abdallah et al. 2006), (2) control zooxanthellae population densities (Field et al. 2006; Dove et al. 2008), (3)

comprise part of an innate immune response (Palmer et al. 2009), and (4) that they act to camouflage corals from predatory fishes (Matz et al. 2006) (summarised in Table 1.1). It is likely that coral FPs have multiple functions, as has been suggested recently by several authors (Field et al. 2006; Alieva et al. 2008, D'Angelo et al. 2008, 2012).

1.4 Coral colour polymorphism and stress tolerance

There are four common GFP-based colours in corals, the fluorescent colours cyan, green, red and a non-fluorescent purple-blue (Alieva et al. 2008), and a less common yellow pigment (C. Palmer pers. comm. in: Alieva et al. 2008). Colour polymorphism refers to the occurrence of multiple colour morphs within a single species. The underlying cause of colour variation can be either genetic or environmental. Genetic polymorphism refers to phenotypic variation in colour appearance that is heritable, largely determined during zygote formation, and corresponds to allelic variations at certain loci. In contrast, colour polyphenism occurs when the colour is not genetically determined, and is instead influenced by environmental cues. Thus colour expression can be either plastic (polyphenism) or stable (genetic polymorphism). Knowledge of factors underlying colour variation allows colour to be interpreted in light of that organism's life history or condition (Kelmanson and Matz 2003). However, coral colour diversity may reflect a combination of both models, where both allelic polymorphism and environmentally controlled differential gene expression play a role in determining colour appearance.

Several approaches can be followed to unravel the underlying basis for colour polymorphism in corals, including the evaluation of temporal patterns in the stability versus flexibility of colour morphs, and the identification of environmental influences

on the relative proportion of colour morphs within populations. Studies of temporal patterns in coral colour are limited (but see Todd et al. 2002 a, b; R. Rowan pers. comm. in: Kelmanson and Matz 2003; Leutenegger et al. 2007), and understanding of the mechanism(s) responsible for colour change is poor. Variation in colour and the associated expression levels of FPs/CPs in corals are generally thought to be genetically determined rather than regulated by the environment, although there is some evidence of non-genetic variation. For example, a green-to-red change in a deep water specimen appears to be due to photoconversion (Leutenegger et al. 2007), and there is evidence of phenotypic plasticity in FP expression induced by light (Takabayashi and Hoegh-Guldberg 1995; Kelmanson and Matz 2003), that resukts in variation in colour. Todd et al. (2002a,b) observed discrete changes in coral colour 7 months after colonies of *Favia speciosa* and *Diploastrea helipora* were transplanted to different habitats. Their findings suggested a significant genotype × environment interaction, however, patterns of colour changes were independent of changes in depth/habitat (Todd et al. 2002a, b). Similarly, R. Rowan (pers. comm. in: Kelmanson and Matz 2003) described how a single specimen of *Fungia fungites* changed from brown to purple, to fluorescent green in response to changing light conditions. Kelmanson and Matz (2003) also demonstrated that colour variation of *Montastraea cavernosa* was most likely environmentally regulated, indicating coral colour in this species was polyphenic. However, allelic variation in the genes that regulate FP transcription and/or differences in the regulatory regions of coral FP genes may exist and were not investigated.

Knowledge of spatial patterns in the distribution and abundance of coral colour morphs and of differences in physiological performance among colour variants is limited. In the Caribbean, green morphs of Porites asteroides were initially found

Table 1.1 Summary of the literature in support of and against a photoprotective role for coral FPs / CPs and other suggested roles.

In Support of a Photoprotective Role	
Reference	Finding
Kawaguti 1944, 1969	First suggested coral FPs found in epithelial cells may act photoprotectively
Wiedenmann et al. 1999	Proposed that coral FPs fulfill a photoprotective function in some Anthozoans like sea anemones
Salih et al.1998, 2000, 2006	Coral FPs regulate light environment by screening UV light: Position of coral FPs relative to zooxanthellae (e.g. above, in high-light vs. below, in low-light) indicates a photoprotective role
Dove et al. 2001	The molecular properties of coral CPs indicate functions from converting high-intensity UV light to photosynthetically active radiation to shielding of *chlorophyll a*, *c* from high-intensity light
Gilmore et al. 2003	Coral FPs may indirectly act to absorb, screen and scatter excess UV light to protect the photosynthetic mechanism of Photosystem II (via FRET)
Dove 2004	Experimentally, coral FPs were photoprotective at normal temperatures
Klueter et al. 2006	Strongly fluorescent colonies of *Montipora digitata* contain greater densities of algal symbionts
Cox et al. 2007	Up to 93% of coral FPs investigated were involved in FRET pairs indicating their involvement in photoprotection
Leutenegger et al. 2007	Slow deacy rates and high stability of coral FPs indicates their usefullnes in functions requiring high concentrations, like photoprotection
Oswald et al. 2007	Coral FPs contribute between 5 and 14% of soluble protein in ectodermal coral tissues which indicates their usefullnes in functions requiring high concentrations, like photoprotection
D'Angelo et al. 2008	FPs and CPs are upregulated at the transcriptional level in response to increased exposure to light
Dove et al. 2008	Corals increase fluorescence during temperature stress and bleaching
Roth et al. 2010	Experimentally, corals photoacclimate by changing FP concentrations
D'Angelo 2012	High accumulation of coral FPs in growth zones provide photoprotection for incoming zooxanthellae yet to populate areas exposed to high-light intensity
Smith et al. 2013	Screening by coral CPs leads to reduced chlorophyll excitation
Against a Photoprotective Role	
Reference	Finding
Dove et al. 1995	Absorption spectra of coral CPs are not likely to protect coral symbionts from photobleaching
Takabayashi and Hoegh-Guldberg 1995	Different coloured morphs exist side-by-side in the field, no correlation between coral FP concentration and depth, and zooxanthellae densities are lowest in most pigmented regions of coral colonies
Fang et al. 1989 and D'Angelo et al. 2008	Low numbers of zooxanthellae are present in highly pigmented branch tips
Schlichter and Fricke 1990	Coral FPs provide photo-enhancement in low-light conditions rather than photoprotection
Mazel et al. 2003	Coral FPs neither photo-enhance or -protect algal symbionts: FP concentration does not correlate well with the removal of photons from chlorophyll excitation. Bathymetric distribution of coral FPs did not change with depth
Wiedenmann et al. 2004 and Schnitzler et al. 2008	Coral FPs are found in azooxanthellate coral species in low-light habitats
Gleason 1993	Green coral FPs did not provide protection against UV light
Vogt et al. 2008	Corals with FPs found in low-light, deep-sea habitats between 500-600m
Other Suggested Roles	
Reference	Finding
Ward 2002	Coral FPs act as visual trigger for predatory fish
Bou-Abdallah et al. 2006	Coral FPs provide supplementary antioxidant protection by deactivating reactive oxygen species
Field et al. 2006 and Dove et al. 2008	Coral FPs regulate zooxanthellae populations
Matz et al. 2006	FPs provide camouflage from herbivorous fishes
Palmer et al. 2008 and D'Angelo et al. 2012	Coral FPs function as a part of coral innate immune response
Palmer et al. 2009	Antioxidant properties found in coral FPs

to be more abundant in shallow water habitats, whereas their brown counterparts were more abundant at depth, but when the spatial scale of the study was expanded, the pattern became inconsistent (Gleason 1998). In some cases, the pattern was reversed in areas of high sediment load, with brown colonies of *P. asteroides* better at shedding sediment from colony surfaces and suffering less sediment-induced tissue death than green morphs (Gleason 1998). On the Great Barrier Reef (GBR), fluorescent morphs were found to be more common in shallow water, and FPs were more concentrated in colony areas exposed to greater light-intensity (Salih et al. 2000). Pink colonies of *Pocillopora damicornis* showed reduced growth rate in comparison to brown colonies, prompting the authors to suggest that CP production, which only occurs in pink colonies, is energetically costly (Takabayashi and Hoegh-Guldberg 1995). These studies indicate great variation in the physiological performance and in the distribution patterns of coral colour morphs, but there has been little advance over the last ~15 years in understanding the biological implications of such variation or how such variation relates to the expression of coral FPs.

Although the presence of colour polymorphism is well documented in reef-building corals (Veron 1986; 2000), few studies have systematically described colour morphs within a species and the FP complexes that characterize them (but see Kelmanson and Matz 2003 and Oswald et al. 2007). Previously, the FP in greatest concentration was thought to govern visual appearance (Cox et al. 2007). However Mazel and Fuchs (2003) have explained how the visual impact of FPs may not correlate well with abundance and perceived colour as assessed by the human eye.

As ocean warming (Hughes et al. 2003) and the frequency of coral bleaching events (Hoegh-Guldberg 1999) escalate, several studies have examined the varying response of colour morphs to changes in environmental temperature (Salih et al. 2000;

Dove 2004; Smith-Keune and Dove 2008 etc). Following the 1998 mass coral bleaching event on the GBR, bleaching resistance was found to be highly correlated with the concentration of FPs within coral tissues (R^2 = .09471; $p < 0.0001$; Salih et al. 2000). The same authors observed that non-fluorescent morphs were significantly more photoinhibited and recovered to pre-inhibition rates slower than fluorescent morphs (Salih et al. 2000). Conversely, when Dove (2004) experimentally heated branches of three distinctly-coloured morphs of the coral *Acropora aspera*, bleaching was most severe in the most heavily pigmented blue morph, and even subtle temperature increases had detrimental effects on the photoprotective mechanism. Additionally, Smith-Keune and Dove (2008) observed a down regulation in an FP coding gene following exposure to experimental heat stress, which they suggested would lead to a decline in FP concentration. Consequently there are major gaps in current understanding of how (1) colour variation and coral FP concentration are linked (2) variations in colour morph physiology are linked to environmental stimuli, and (3) FPs influence coral susceptibility to heat stress.

1.5 *Acropora millepora:* a model coral

Characterization of spatial and temporal patterns in protein expression and the distribution of colour types within a polymorphic coral species possessing the full complement of coral FPs and CPs is needed to advance current understanding of the biological significance of colour variation in corals (Mazel and Fuchs 2003; Alieva et al. 2008). The common species *Acropora millepora* is a member of the speciose and ecologically important coral genus *Acropora,* and has frequently been used as a model coral in a diverse range of studies (e.g., Miller et al. 2000; van Oppen et al. 2006;

Alieva et al. 2008). It is a common coral on Indo-Pacific reefs, with a distribution that ranges from Sri Lanka and Thailand east to the Marshall Islands and Tonga, including Australia (Veron and Wallace 1984; Veron 1995). Its compact-branching, corymbose colonies exhibit a wide range in tissue colours (Veron and Wallace,1984; Wallace and Willis 1994; Veron 1995; Mackenzie et al. 2004). At least four colour morphs have been described in this species (Veron 2000, Cox et al. 2007, Alieva et al. 2008), and they commonly co-occur on shallow reef flats, lagoons, and upper reef slopes less than 4 metres deep (Fig. 1.3). The occurrence of multiple distinct colour varieties within populations of *A. millepora,* combined with the well-characterised nature of its FP/CP complement (Cox et al. 2007; Alieva et al. 2008; D'Angelo et a. 2008; Smith et al. 2013), make this species ideal for addressing questions about how colour variation and FP concentration are linked. To date, no studies have documented patterns in the distribution, abundance, spectral characteristics, tolerance to changes in environmental conditions including temperature, or colour × symbiont genotype associations for colour morphs within the coral species *A. millepora*.

Anecdotal evidence indicates that a green morph with orange axial corallites is a common colour type in this species (Veron 1995), although no abundance or distribution data have been reported. Colonies of *A. millepora* exhibit a 'complementary' pattern of fluorescence, with green and red FPs found in different anatomical regions of the coral, often with distinct boundaries between them (Gruber et al. 2008). Common FP colours in anthozoa are found in the cyan, green, and red spectral range (Wiedenmann 1997; Matz et al. 1999; Oswald 2007; Alieva et al.

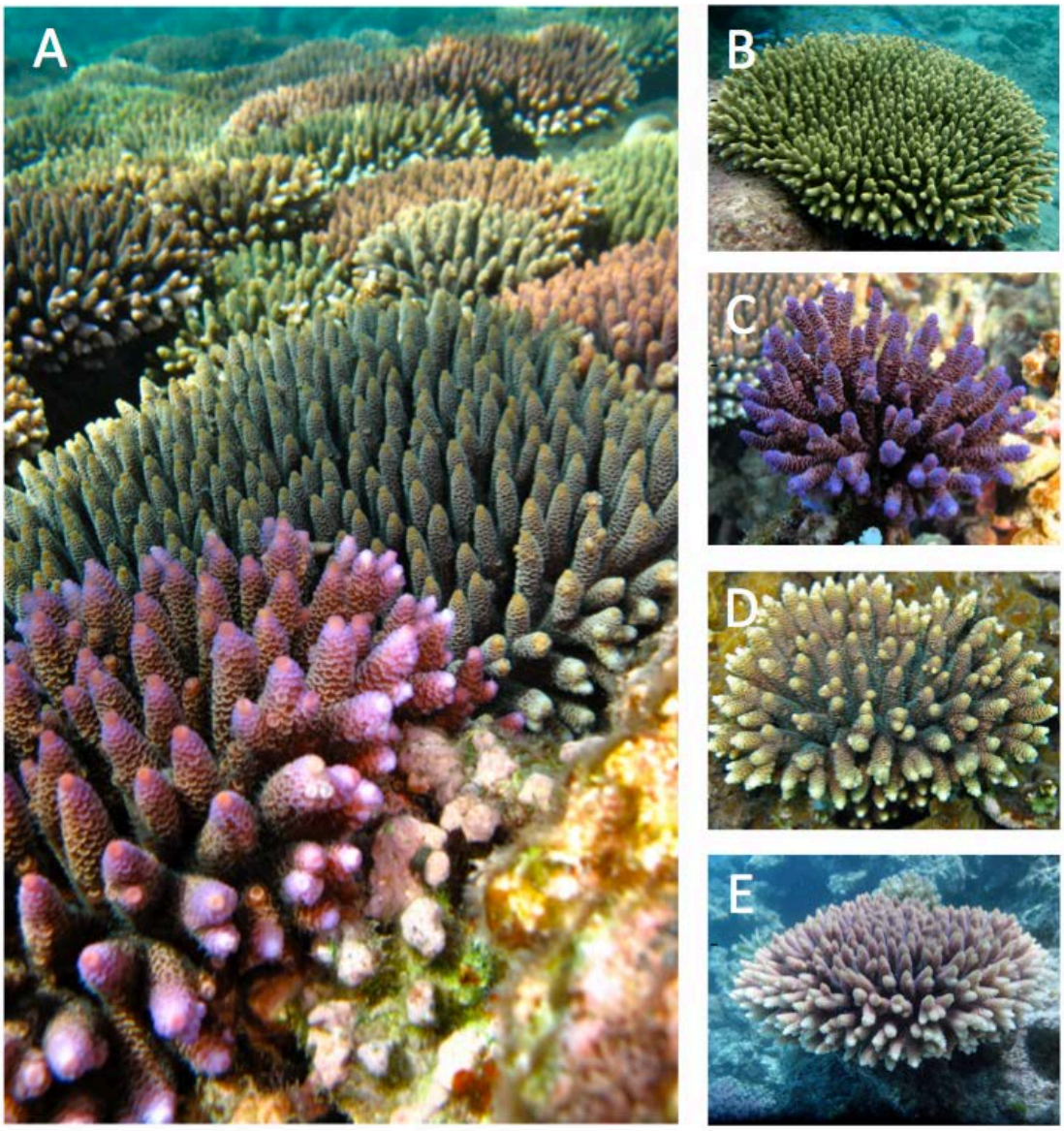

Fig. 1.3 Common colour morphs of the coral *Acropora millepora* from the Great Barrier Reef. (A) *A. millepora* colour morphs living side-by-side in the field, and (B) green, (C) blue, (D) yellow, and (E) red colonies pictured individually. Green colonies exhibit striking green fluorescence under natural daylight conditions and are easily distinguished from the brown to orange appearance of yellow colonies. Blue colonies (which often photograph purple) exhibit deep pigmentation that is visually distinct from red colonies.

2008). These major colour types are encountered in *A. millepora* (D'Angelo et al. 2008; Alieva et al. 2008), although the specific emission wavelengths reported vary between these reports. The Cyan FP emits between 484 and 489 nm, green FP emits near ~512nm, Red FP emits between 593 and 597, and CP is excited at 588 nm (Alieva et al. 2008; D'Angelo et al. 2008).

1.6 Study aims and objectives

The over-arching goal of research described in this thesis will be to determine the significance of intraspecific colour polymorphism for stress tolerance of reef-building corals. Field and experimentally-based comparisons of the responses of colour morphs to changes in environmental conditions were designed to resolve ambiguities in our current understanding of the functional role of coral florescent proteins. Using *Acropora millepora*, a common scleractinian coral on the Great Barrier Reef, as a model organism, I address the following five specific objectives to achieve this goal:

1. **To establish if FP/CP types and/or concentrations differ between intraspecific colour morphs.** Although coral colour morphs are visually distinguishable in the field, an understanding of how fluorescent and non-fluorescent protein content compares among intraspecific colour morphs remains lacking. Results presented in Chapter 2 quantify, for the first time, relative concentrations of the three common fluorescent proteins and one non-fluorescent protein in tissues of colour morphs. A novel approach that combines measurements of the relative

contributions of coral FPs to total fluorescence through multiple regression analysis will be used.

2. To document the abundance and distribution of colour morphs across habitats of the Great Barrier Reef. Variation in the proportional abundance of colour morphs among habitats is a good indicator of their comparative performances under differing environmental regimes. To investigate the possible ecological roles of coral FPs, I quantify visible colour polymorphism by comparing the relative proportions of colour morphs across three latitudinal regions of the Great Barrier Reef and correlate their distributions with environmental parameters (Chapter 2).

3. To determine if patterns in FP/CP concentration are temporally variable under non-stressful, seasonal environmental patterns. Comparisons of temporal variability in the patterns of FP/CP content among colour morphs through a spring-summer season (Chapter 3) will provide a valuable opportunity to explore the role of FPs in coral responses to environmental change, and are previously undocumented.

4. To determine if thermal stress, either under natural field or experimental laboratory conditions, induces change in the patterns of FP/CP concentrations expressed by colour morphs that is related to thermal tolerance. Comparisons of bleaching susceptibility among colour morphs of *A. millepora* during a natural, moderate bleaching event in the northern Great Barrier Reef (Chapter 4) provide a unique opportunity to test hypotheses about the role of FPs in the thermal stress response of corals. Because temperature elevation was moderate in the natural bleaching event, comparisons of bleaching susceptibility to severe thermal stress

induced by experimental heating among colour morphs will shed further light on the role of fluorescence in corals (Chapter 5). These studies will enable predictions of environmental tolerance for colour morphs of this common coral species.

5. To compare the response of colour morphs to prolonged periods of experimental starvation. Coral FPs are long-lived and thought to be relatively inexpensive energetically to produce and maintain. However, great disparity in the abundance of colour morphs, from the uncommon but highly fluorescent morph to the common but weakly fluorescent morph, suggests that maintaining high FP concentrations comes at the expense of performance in other physiological parameters. Comparisons of coral tissue condition, investment in reproductive effort and FP concentrations among colour morphs experimentally exposed to a prolonged period of resource deprivation will address questions about the energetic costs of FP production (Chapter 6).

6. To synthesise the results presented in this thesis and offer an improved perspective of the advantages and consequences associated with coral FPs. In chapter seven, results from the previous chapters will be discussed in an ecological context to gain insights into the importance of coral colour and coral fluorescent proteins as part of the suite of adaptive or acclimatory mechanisms corals possess in their response to future climate scenarios.

Chapter 2

The causes of colour in the common Indo-Pacific coral *Acropora millepora* on the Great Barrier Reef

This chapter forms the basis for the publication:

Paley AS, Matz MV, Willis BL, van Oppen MJH, Bay LK (accepted and in revision) The causes and consequences of colour in the common Indo-Pacific coral *Acropora millepora* on the Great Barrier Reef. PLOS One.

Green and red A. millepora morphs, Halfway Island, Keppel Islands, GBR

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2.0 Abstract

To enhance understanding of possible roles of fluorescent proteins (FPs) in corals, we examined the relative concentrations of three FP types (Cyan, Green, Red) in four colour morphs of *Acropora millepora* (red, yellow, green, blue) on the Great Barrier Reef (GBR), and assessed colour morph abundances in relation to latitude, depth, wave exposure and colony size. Total FP concentrations differed among colour morphs of *A. millepora,* with the highly fluorescent green morph containing 1.6- to 7-fold greater concentrations of the Cyan FP than the weakly fluorescent red morph. In all morphs, the most abundant FP was Cyan, which was present at concentrations of two-fold to more than 1600-fold greater than the other FPs. Total FP levels in the northern Great Barrier Reef (GBR) were nearly 2.5 and seven times higher than in the central and southern GBR, respectively. However, relative abundances of colour morphs were similar across regions, depths, and wave regimes with two exceptions: the blue morph was more abundant in the southern GBR, and the yellow morph was less abundant at deep sites in the central region versus other regions. The highly fluorescent green morph was rare at all sites, despite the postulated advantage of FPs in stress tolerance. Colour in *A. millepora* is determined by the relative concentrations of long versus short wavelength-emitting FPs. 1600-fold higher concentrations of the Cyan FP at less turbid northern sites is consistent with its role in photoprotection. Similarity in the distribution and abundance of colour morphs across strong latitudinal, depth and wave exposure gradients indicates that, overall, fluorescence has a limited role in the organism-environment interactions in this species. We hypothesise that rarity of the highly fluorescent green morph in all environments

highlights a trade-off between investment in high levels of Cyan FP versus physiological processes like growth and reproduction.

2.1 Introduction

Sessile marine invertebrates commonly exhibit intra-specific colour polymorphisms (Wicksten 1989), with the distribution and abundance of colour morphs varying in response to environmental stimuli (Harley et al. 2006; Kenkel et al. 2011) and/or genetic factors (Kelmanson and Matz 2003; Leutenegger et al. 2007) Colour diversity is extensive among cnidarians (Wicksten 1989), particularly reef-building corals (Veron and Pichon 1976; Veron 1986), largely due to a suite of fluorescent proteins (FPs) related to the green fluorescent protein (GFP) within coral tissues (Dove et al. 2001; Mazel and Fuchs 2003; Dove 2004; Alieva et al. 2008). Scleractinian corals harbour the largest known spectroscopic diversity of homologs to these GFPs (Alieva et al. 2008; Matz et al. 1999), yet the relationship between visual colour, FP abundance, and environmental parameters is poorly described for reef corals in their natural habitat.

The substantial contribution (14%) that FPs make to total proteins in coral tissues (Leutenegger et al. 2007; Oswald et al. 2007) suggests that they are functionally important. Although it is commonly assumed that FPs are photoprotective because of their ability to dissipate light energy (Kawaguti 1944; Salih et al. 2000; Dove et al. 2001; Bhagooli and Hidaka 2003; Dove 2004; Khang and Salih 2005; Enriquez et al. 2005), their roles in photoprotection and other potential functions within the coral holobiont are still under considerable debate. Corresponding with a photoprotective role, FPs are common in shallow water corals

(Alieva et al. 2008; Salih et al. 2000), and their expression increases with increasing light levels and symbiont densities (Klueter et al. 2006; Bay 2009 a, b). However, only the Cyan FP spectrally matches the wavelength absorption spectra of chlorophyll *a* and *c*, making it the only FP suitable for shielding *Symbiodinium* cells *in hospite* (D'Angelo et al. 2008). However, more recent evidence that light usage by *Symbiodinium* is less discriminative spectrally than studies of wavelength absorption based on diluted pigment solutions indicate (Smith et al. 2013) suggest that screening may be provided by FPs other than Cyan FPs. The expression levels of FPs may be genetically determined, which may be in addition to being modulated by light environments (Kelmanson and Matz 2003; Leutenegger et al. 2007). Further, the existence of fluorescently pigmented symbiont-free corals in low-light habitats suggests functions other than photoprotection for FPs (Wiedenmann et al. 2004; Schnitzler et al. 2008). Finally, corals have other, potentially much more efficient mechanisms of photoprotection (Mazel et al. 2003), including the capacity to adjust their complements of UV-screening mycosporin-like amino acids (MAAs) and antioxidant molecules (Falkowski and Dubinsky 1981; Hoegh-Guldberg and Smith 1989). Therefore, the roles of FPs in corals and other cnidarians appear to be manyfold (Alieva et al. 2008; D'Angelo et al. 2008; Roth and Deheyn 2013), as is the case in other fluorescent organisms (Bomati et al. 2009).

Despite a considerable number of studies directed at describing the diversity (Wiedenmann et al. 2004; Cox et al. 2007; Smith-Keune and Dove 2008; Alieva et al. 2008), evolution (Matz et al. 2002; Kelmanson and Matz 2003; Ugalde et al. 2004; Field et al. 2006) and function (Kawaguti 1969; Salih et al. 2000; Mazel et al. 2003; Palmer et al. 2009; D'Angelo et al. 2012) of coral FPs, a basic understanding of variation in FP levels among intra-specific colour morphs of corals, and the

distribution and abundance of colour morphs within reef habitats, remains limited. Given the numerous proposed functions of FPs, analyses of variation in the expression of the FP complements that underlie colour polymorphism (Field et al. 2006; Cox et al. 2007) in a range of ecological conditions should provide insights into their roles. For example, if the Cyan FP is indeed photoprotective, a correlation between Cyan FP concentration and photosynthetic stress is expected. Four visually distinguishable colour morphs (blue, green, yellow and red) occur within the common, shallow-water Indo-Pacific coral, *Acropora millepora,* on the Great Barrier Reef (GBR) (Veron 2000; Cox et al. 2007; Alieva et al. 2008; Smith-Keune and Dove 2008). Colonies of *A. millepora* contain the full complement of the fluorescent and non-fluorescent proteins that have been characterised in corals, (i.e., cyan, green, and red FPs and non-fluorescent blue chromoprotein; (Alieva et al. 2008)). Cyan FPs are excited at 441nm and have an emission peak between 484-489 nm (Alieva et al. 2008). Green FPs are excited at 503nm and have an emission peak around 512nm (Alieva et al. 2008). The red FP is excited at 560nm and has an emission peak between 593 and 597 producing colouration from orange to red (Mazel and Fuchs 2003), while CPs, are characterized by intense absorption at 588 nm (Alieva et al. 2008) but do not fluoresce (Wiedenmann et al 1999; Lukyanov et al. 2000; Dove et al. 2001). To address their possible ecological roles, we quantified visible colour polymorphism and relative proportions of different FP types in natural populations of *Acropora millepora* at multiple spatial scales. Specifically, we test how colour morphs are distributed among three latitudinal regions of the GBR and whether their distributions are correlated with depth, wave exposure and/or colony size.

2.2 Materials and Methods

2.2.1 Sampling Design

This study was carried out on fringing reefs of the Lizard Island group (14°40'S 145°28'E), the Palm Islands (18^{o}35’S, 146^{o}29’E), and the Keppel Islands (23°10’S, 150°57’E), which span a latitudinal gradient in temperature approximating a 2^{o}C increase in mean summer temperatures from the Southern to the Northern GBR. Reefs of the Lizard Island group are located in the middle of the continental shelf, approximately 30 km off the far north Queensland coast (mean winter and summer temperatures are 24^{o}C and 30^{o}C [all temperature data courtesy of the Australian Institute of Marine Science: http://data.gov.au/aimsrtds], respectively). Reefs in the Palm and Keppel Islands are located on the inner continental shelf, and lie between approximately 15 and 18 km off the central and southern Queensland coast (mean winter temperatures: 21 and 19^{o}C, respectively; mean summer temperatures: 30^{o}C and 28^{o}C, respectively). Ten study sites were selected in both the Lizard and Palm Island groups: five on each of the leeward and windward sides of the islands (Fig. 2.1A-B). In the Keppel Islands, nine study sites were selected: four on the leeward and five on the windward sides of the islands (Fig. 2.1C). Windward sites are exposed to stronger annual southeasterly trade winds compared to leeward sites (Larcombe and Woolfe 1999) and are hereafter termed exposed and sheltered, respectively.

Coral branches were collected between September 2007 and October 2009. Three vertically oriented branches were sampled from each of 7 to 15 haphazardly chosen colonies of each colour morph from each site (blue colonies were only

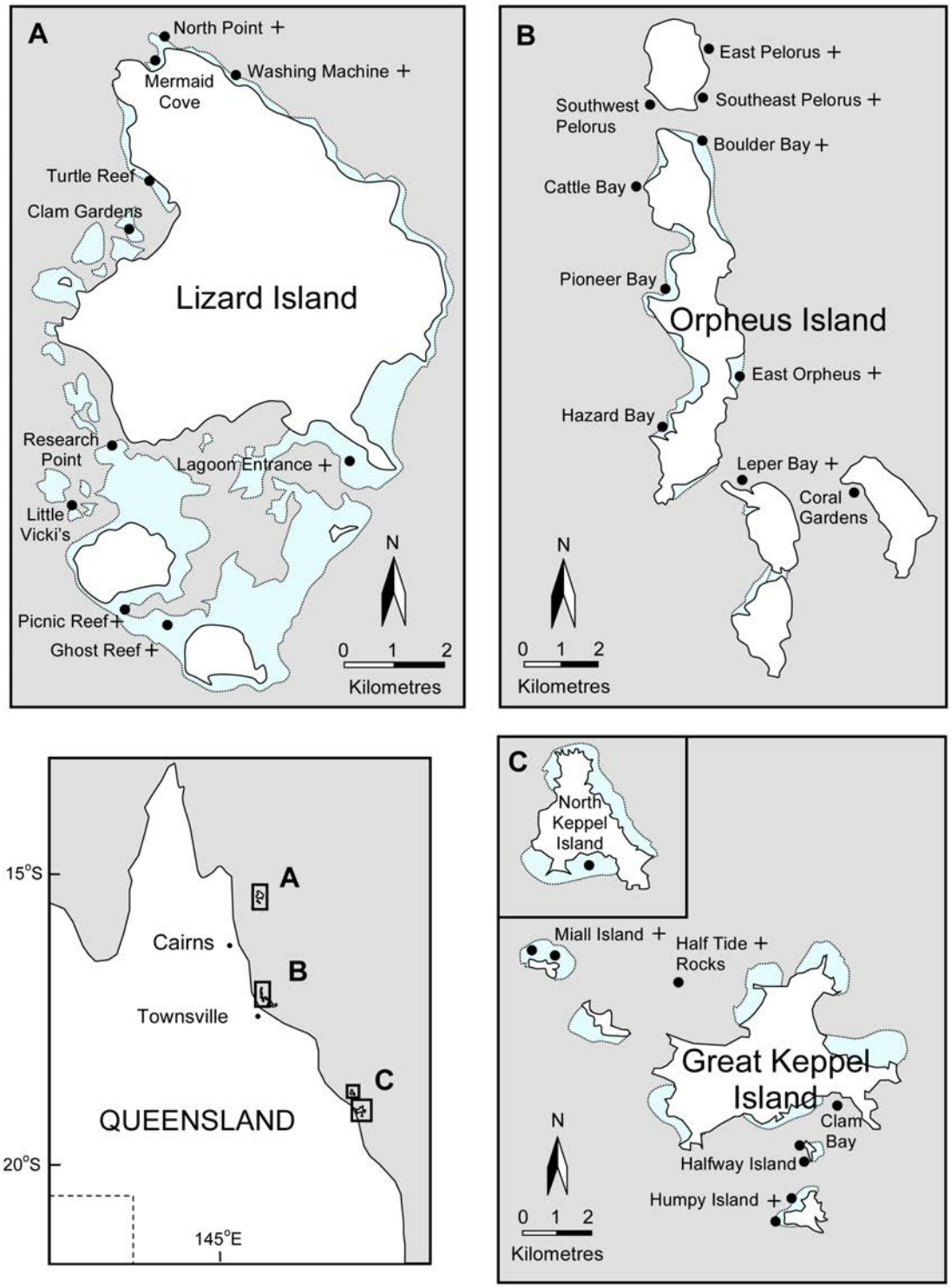

Figure 2.1 Location of sites where colour morphs of *Acropora millepora* were surveyed. Sites were located in the northern (A), central (B), and southern (C) Great Barrier Reef. +: denotes an exposed site.

sampled in the southern region as they are rare elsewhere). Branches were selected from the middle sections of colonies (> 5 cm from colony centres, or > 5 cm from peripheries for small colonies) to control for possible effects of colony position on the abundance of FPs. Branches were immediately snap frozen in liquid nitrogen (LN2) and stored at -80^{o}C until analysis.

2.2.2 Surveys of Colour Morph Distribution and Abundance

The distribution and abundance of colour morphs were assessed between August 2008 and June 2009. Surveys consisted of three replicate, random 50 x 10 m belt transects aligned parallel to the shoreline and along reef contours at ~2 m depth at each site. Deeper transects (between 5 and 12m) were conducted where suitable habitat was available (3 sites each in the northern and southern regions and 1 site in the central region). Within transects, all colonies of *A. millepora*, partially or wholly within the survey area, were counted, measured and visually categorized as belonging to one of four colour groups: red, yellow, green or blue using the CoralWatch Coral Health Chart (University of Queensland) as a guide. This chart approximates the well-established Munsell soil colour chart (New York, Year 2000 Revised Washable Edition). The side of the chart that most closely matched a corals' pigmentation was aligned next to the colony and a category of 1 to 6 was chosen to best represent the overall appearance, where 1 = completely bleached and 6 = healthy/darkly pigmented using the chart's colour fields as a reference. For analysis, number categories were pooled into three bleaching categories (1 - 2 = Bleached, 3 - 4 = Pale and, 5 - 6 = Healthy (darkly pigmented)). In total, 2145 *A. millepora* colonies were surveyed (Northern: 308, Central: 437, Southern: 1350). Green morphs have neon green

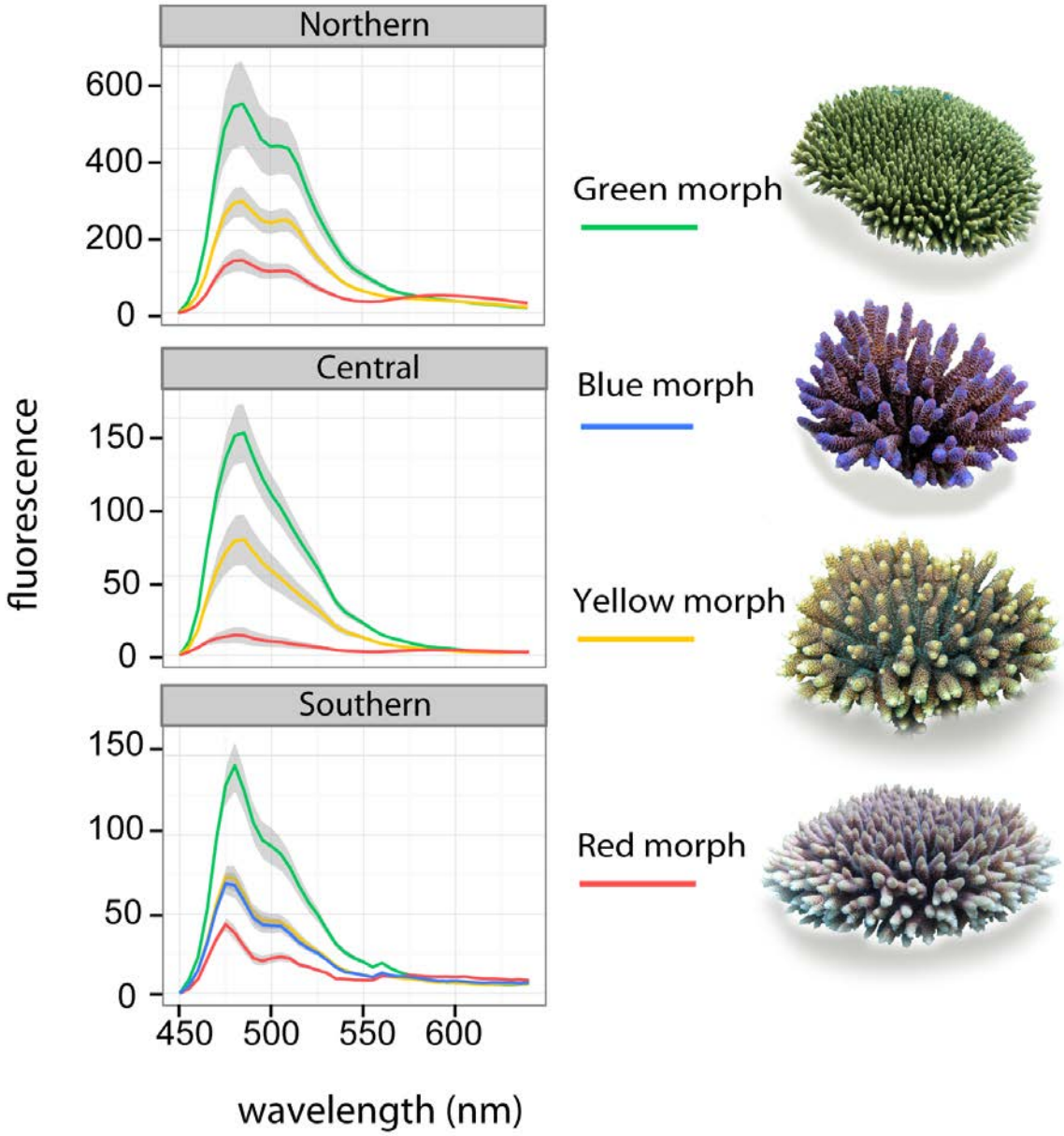

Figure 2.2 Fluorescence emission spectra (excitation 280 nm) of three colour morphs of the coral *Acropora millepora,* after background subtraction. Mean fluorescence (± SE) expressed by green (n = 41 colonies), blue (n=10), yellow (n = 46), and red (n = 47) morphs from the northern, central and southern GBR regions. Grey shading indicates ± SE. Three emission peaks are identified at 485 nm, 510 nm, and 590 nm representing Cyan, Green and Red FPs.

fluorescence on the edges of corallites (Fig. 2.2). Blue morphs are a vivid pale blue or bright violet, and are clearly distinct from red colonies underwater (Fig. 2.2). Yellow morphs range from brown to orange pigmentation on corallite edges and have neither red nor green colouration (Fig. 2.2). Red morphs have pink to crimson pigmentation on corallite edges (Fig. 2.2). To account for irregular colony shapes, colony size was expressed as the mean of the greatest length and greatest perpendicular width, both measured in the horizontal plane.

2.2.3 Sample Preparation and Spectroscopy

Coral tissue was removed from each sample on ice using a high-pressure air-gun directly into 15 ml of 1 micron filtered seawater. Tissue slurries were homogenized for 30 s in a glass beaker (Janke and Kunkel, IKA-Labortechnik) and subsequently centrifuged (5 min) at 3,500 rpm at 4^{o}C to remove cellular debris. The supernatant was immediately frozen at -30^{o}C until analysis. Total protein concentration was measured for each sample using a modified Peterson's-Lowry total protein standard assay (Peterson 1977); Sigma-Aldrich TP0300) using a five-fold dilution series of bovine serum albumin (BSA) as a standard. Twenty µl aliquots of each tissue sample were added in triplicate to 90 µl of MilliQ water, followed by 100 µl of Lowry reagent following 20 min reaction time, with 50 µl of a Phenol solution in a Costar 96-well UV transparent, plastic microtitre plate. Plates were read after 3 min in a SpectraMax M2 (Molecular Devices) spectrophotometer for endpoint absorbance at 750 nm using SoftMax Pro 5.2. Additional aliquots of 20 µl from each tissue sample were added in triplicate to a Greiner 384-well black/clear plastic bottom

microtitre plate, excited at 280 nm on the spectrophotometer and read as a fluorescent spectrum in increments of 5 nm from 400 to 700 nm. To detect the presence of non-fluorescent chromoproteins (CP), the plate was immediately re-read for endpoint absorbance excited at 588 nm (Alieva et al. 2008).

2.2.4 Relative Quantification of Fluorescent Proteins

Relative contributions of individual FPs (cyan [485 nm], green [510 nm], and red [590 nm] CFP, GFP and RFP, respectively) to total fluorescence were determined using multiple regression analysis (implemented in R (Team RDC 2008)) of the fluorescence spectra of coral protein extracts excited at 280 nm (Fig 2.2). The analysis was based on and validated by the emission spectra of known concentrations of purified *A. millepora* FPs expressed in bacteria (Alieva et al. 2008). The exponentially decaying background was calculated using the formula $B = F_{450} / \exp(k*(w-450))$, where F_{450} is the fluorescence reading at 450 nm (where no FP emission is observed), w is wavelength in nanometers, and k is the empirically determined coefficient resulting in a good fit of the multiple regression model. The parameter k was kept the same ($k = 0.1$) for all samples analysed. The relative proportions of FPs were calculated based on the area of the background-subtracted fluorescence spectrum attributable to each FP component, multiplied by the coefficient reflecting the relative excitability of purified *A. millepora* FPs at 280 nm (CFP: 0.85, GFP: 0.92, RFP: 1) (Fig. 2.3). The relative amounts of each FP thus determined were then multiplied by the background-subtracted total fluorescence and standardized to total protein concentration determined using the Lowry method (as above). The resulting values are directly proportional to the concentration of each FP in the sample. Due to their

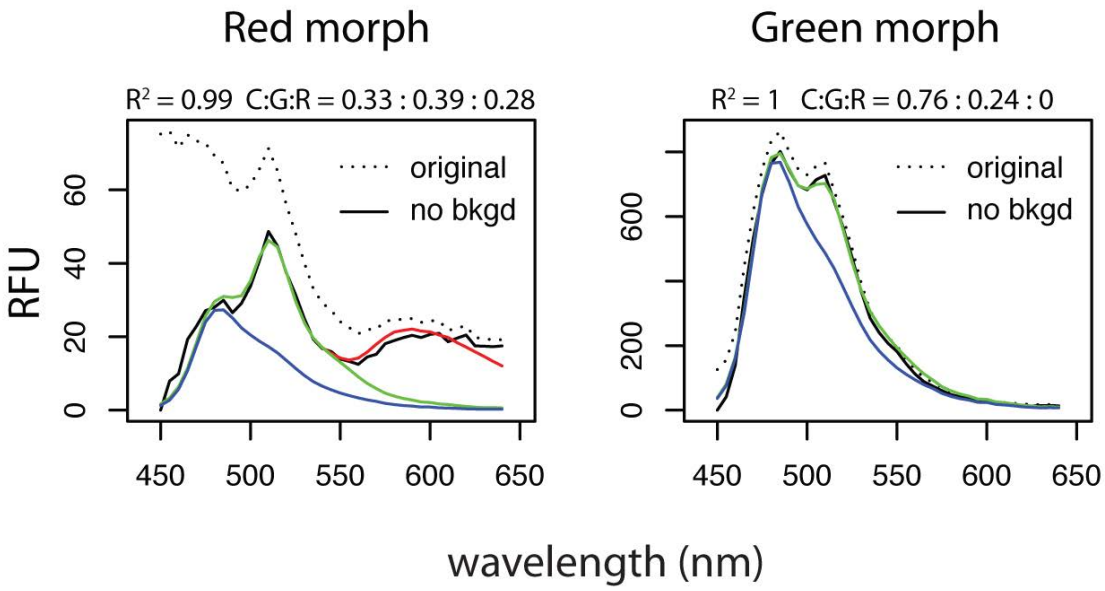

Figure 2.3 Examples of multiple regression analysis for red and green morphs to determine relative amounts of different FP types in protein extracts. On both panels, the dotted line is the original emission spectrum of the extract at 280 nm excitation. The blue, green and red lines indicate the calculated contributions of cyan, green, and red FPs, respectively, into the background-subtracted spectrum (solid black line, mostly obscured due to good fit). The relative proportions of each FP type, corrected for their excitability at 280 nm, are listed above each plot. Horizontal axis – wavelength in nanometers, vertical axis – relative fluorescence units.

extreme rarity, blue colonies were not collected from the northern or central regions and, are therefore not included in the analyses of FP and CP concentrations.

2.2.5 Statistical Analyses

FP and CP Concentrations: Concentrations of the three FPs (CFP, GFP, RFP) from three colour morphs (red, yellow, green) were compared between regions (northern, central, southern) in a 2-way multivariate ANOVA (Wilks test), where colour and region were treated as fixed effects. CP concentrations in colour morphs were compared in a 2-way ANOVA, with both colour and region treated as fixed factors. Post-hoc, Fisher's LSD multiple comparisons of FP and CP concentrations among colour morphs were interpreted with a Benjamini-Yekutieli (2011) type I error correction at the false discovery rate of $\alpha = 0.012$. All analyses were performed using STATISTICA 10 (StatSoft) with $\log_{(10)}$ transformed data, which satisfied assumptions of normality.

Colour Morph Abundances: The influences of regional location and wave exposure on colour morph abundances in shallow (< 5m) and deep (> 5m) water were investigated with a series of linear mixed effects models implemented in R (Team RDC 2008) using the package *lme4* (Bates 2005). We observed large differences in colour morph abundance across study sites and regions, therefore, we assessed the relative abundance of colour morphs between different reef habitats using proportional colour morph data. Colour (red, yellow, green), region (northern, central, southern), exposure condition (exposed, sheltered) and depth (shallow, deep) were fixed effects, while site within region was treated as a random effect. To assess the validity of these mixed effects analyses, we performed likelihood ratio tests by

comparing the models with fixed effects to the null model that only considered the random effect. Significance of fixed factors was estimated using Markov Chain Monte Carlo (MCMC) simulations at $\alpha = 0.05$ with the R packages MCMCglmm (Hadfield 2010) and MCMC.qpcr (Matz et al. 2013), and plotted with ggplot2 (Wickham 2009).

Colony Size: $Log_{(2)}$ of mean colony diameter (size) was compared among colour morphs (fixed), and between northern and central GBR regions (fixed) relative to the southern region as a control using MCMCglmm (Hadfield 2010), with site as a random factor in R (Team RDC 2008). Because few blue colonies were measured ($n_{blue} = 40$) compared to other morphs ($n_{red} = 623$, $n_{yellow} = 555$ and $n_{green} = 126$), and because this morph is very rare in the northern and central regions n = 2 and n = 1, respectively), it was omitted from analysis. Significance of fixed factors was estimated using Markov Chain Monte Carlo (MCMC) simulations at $\alpha = 0.05$ with R packages MCMCglmm (Hadfield 2010) and MCMC.qpcr (Matz et al. 2013). In addition to the linear mixed model above, mean colony diameter (size) was also compared among colour morphs from the three regions using a 2-way ANOVA between colour morph (fixed) and region (fixed) to determine whether the variation in size among colour morphs varied significantly.

2.3 Results

2.3.1 Coral fluorescence

Patterns in the total concentration of fluorescent proteins present in *A. millepora* differed among the three common colour morphs (green, yellow, red) in a

manner that was dependent on region ($F_{12,207}$ = 1.9, p = 0.036; Table 2.1). Overall, total fluorescent protein concentration in the northern region were nearly 2.5 times greater than levels in the central region and nearly 7 times greater than in the southern region (Fig 2.4). Total FP concentrations were consistently highest in the green morph in all regions, by between 1.6 and 7 times compared to the red morph, which had the lowest total FP concentrations (Fig. 2.4). CFP was, by far, the dominant FP in all colour morphs, ranging from concentrations 2-fold greater in the red morph up to concentrations 1600-fold greater in the green morph, in comparison to levels of the two other FPs. Irrespective of region, green colonies contained significantly more CFP than red colonies (Table 2.2), while

Table 2.1 Two-way multivariate ANOVA (WILKS test) of $\log_{10}$ abundance of the three FPs among GBR regions and colour morphs of *Acropora millepora*. *: denotes significance at α = .05.

Model	Test	Value	F	df	Error	p	
Region	Wilks	.187	34.1	6	156	.000	*
Morph	Wilks	.435	13.4	6	156	.000	*
Region*Morph	Wilks	.759	1.90	12	207	.036	*

Table 2.2 Fisher LSD for the concentration of each of the three FPs compared among GBR regions and colour morphs of *A. millepora*. *: denotes significance at α = .012.

Cyan FP

Region	Region	Northern			Central			Southern		
	Colour	Red	Yellow	Green	Red	Yellow	Green	Red	Yellow	Green
Northern	Red									
	Yellow	**.001***								
	Green	**.000***	.028							
Central	Red	**.000***	**.000***	**.000***						
	Yellow	.489	**.001***	**.000***	**.000***					
	Green	.135	.187	**.003***	**.000***	.063				
Southern	Red	**.000***	**.000***	**000***	**.006***	.018	**.000***			
	Yellow	**.005***	**.000***	**.000***	**.001***	.082	**.000***	.481		
	Green	.385	**.000***	**.000***	**.000***	.938	.037	**.012***	.067	

Green FP

Region	Region	Northern			Central			Southern		
	Colour	Red	Yellow	Green	Red	Yellow	Green	Red	Yellow	Green
Northern	Red									
	Yellow	.488								
	Green	.077	.249							
Central	Red	**.000***	**.000***	**.000***						
	Yellow	**.000***	**.000***	**.000***	.589					
	Green	**.000***	**.000***	**.000***	.284	.594				
Southern	Red	.519	.210	.029	**.000***	**.000***	**.000***			
	Yellow	.333	.117	.014	**.000***	**.000***	**.000***	.766		
	Green	.290	.098	**.011***	**.001***	**.000***	**.000***	.704	.935	

Red FP

Region	Region	Northern			Central			Southern		
	Colour	Red	Yellow	Green	Red	Yellow	Green	Red	Yellow	Green
Northern	Red									
	Yellow	.013								
	Green	**.000***	.164							
Central	Red	**.000***	.043	.487						
	Yellow	**.000***	**.000***	.025	.137					
	Green	**.000***	**.000***	**.002***	.020	.386				
Southern	Red	**.012***	.794	.288	.091	**.001***	**.000***			
	Yellow	**.001***	.206	.876	.392	.015	**.001***	.352		
	Green	**.000***	.142	.975	.495	.023	**.002***	.262	.847	

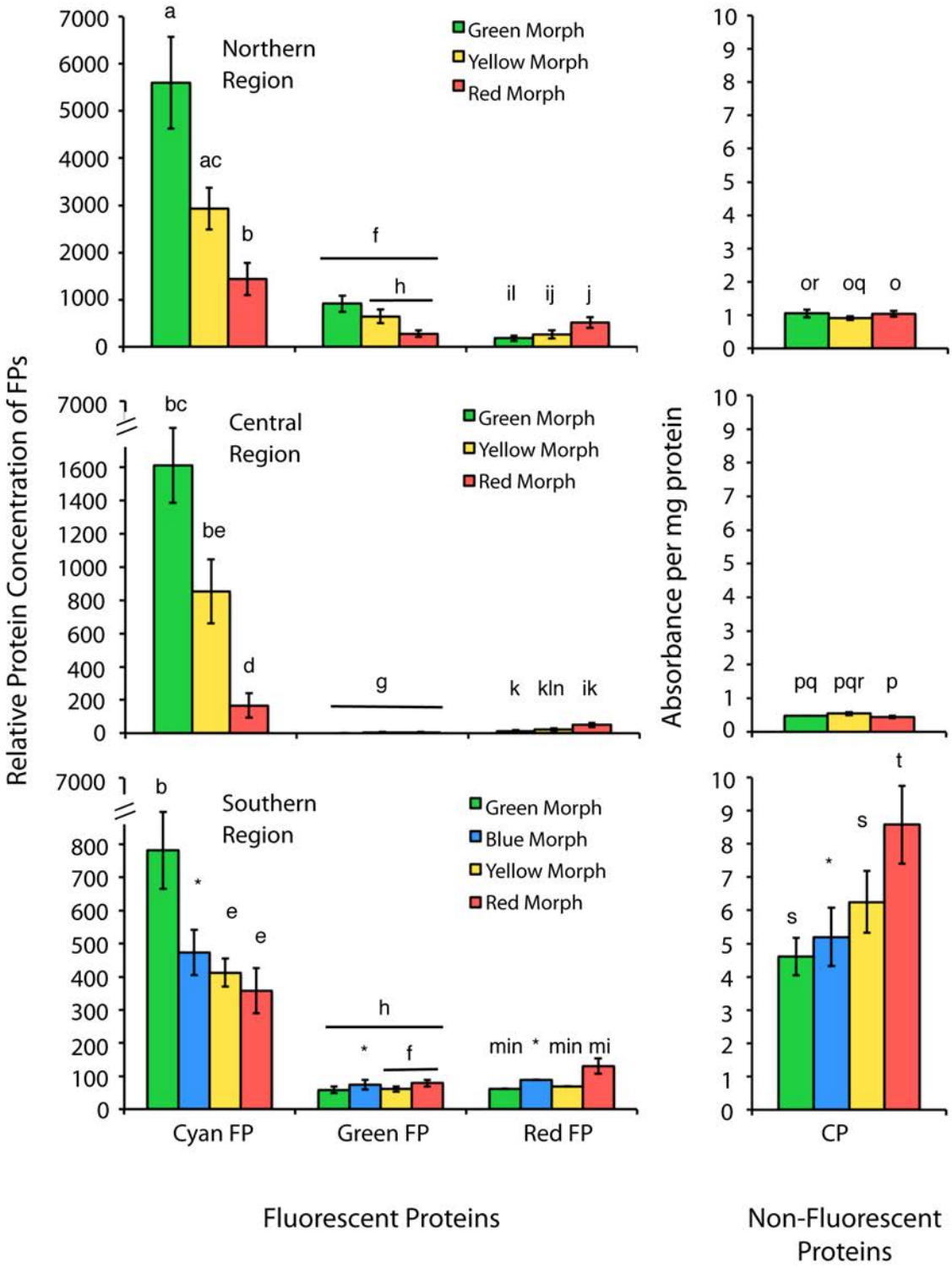

Figure 2.4 Fluorescent and non-fluorescent protein concentrations compared among coral colour morphs and regions. Mean relative protein concentration (± SE) of fluorescent and non-fluorescent proteins among four colour morphs from the northern, central and southern GBR regions. Letters sets 'a' through 't' signify similar concentrations, as determined by Fisher's LSD post-hoc analyses and evaluated using an FDR corrected $\alpha = 0.012$. *: denotes data not included in statistical comparisons.

yellow (and blue, in the southern region) colonies contained intermediary CFP concentrations. However, whereas CFP concentrations in the green and yellow morphs did not differ significantly between the southern and central regions, mean CFP concentrations in the red morph were approximately double in the southern region compared to the central region, explaining the significant morph x region interaction term (Fig. 2.4; Table 2.2).

Non-fluorescent CP concentrations also differed significantly among colour morphs depending upon region ($F_{4,80} = 4.42$, $p = .003$; Table 2.3). In contrast to the pattern of high FP concentrations in the northern region, CP concentrations were at least 4 times greater in the southern region compared to the northern and central regions, a pattern that was consistent for all three colour morphs (Fig. 2.4; Table 2.4). However, whereas CP concentrations were similar across all three morphs in each of the northern and central regions, mean CP concentrations in the southern region were between approximately 1.5 and 2-fold greater in the red morph compared with the green and yellow morphs, which had similar concentrations (Table 2.4).

2.3.2 Colour morph distributions

For green, yellow and red morphs, patterns in the abundance of colonies were constant among regions, i.e., the abundance of green colonies did not differ significantly among regions in either shallow or deep water, as was the case for red and yellow colonies (Table 2.5). In contrast, only three blue colonies were observed in the northern and central regions combined, with the remaining 81 colonies comprising

Table 2.3 Two-way factorial ANOVA of $\log_{10}$ CP content among GBR regions and colour morphs of *A. millepora*. *: denotes significance at α = .05.

Model	SS	df	F	p	
Region	7.05	2	355	.000	*
Morph	.086	2	4.31	.017	*
Region*Morph	.176	4	4.42	.003	*
Error	0.795	80			

Table 2.4 Fisher LSD for the concentration of CPs between GBR regions and *A. millepora* colour morphs. *Denotes significance at α = .012.

	Region	Northern			Central			Southern		
Region	Morph	Red	Yellow	Green	Red	Yellow	Green	Red	Yellow	Green
Northern	Red									
	Yellow	.460								
	Green	.969	.496							
Central	Red	**.002***	.012	**.004***						
	Yellow	**.011***	.053	.019	.605					
	Green	**.005***	.024	**.009***	.816	.776				
Southern	Red	**.000***	**.000***	**.000***	**.000***	**.000***	**.000***			
	Yellow	**.000***	**.000***	**.000***	**.000***	**.000***	**.000***	**.006***		
	Green	**.000***	**.000***	**.000***	**.000***	**.000***	**.000***	**.000***	.033	

Table 2.5 Markov Chain Monte Carlo (MCMC) simulations for the comparison of colour morph abundance among regions for (A) shallow and (B) deep sites.

A Shallow

Region	Northern	Central	Southern
Red Morph			
Northern			
Central	.944		
Southern	.758	.768	
Yellow Morph			
Northern			
Central	.848		
Southern	1.000	.828	
Green Morph			
Northern			
Central	.614		
Southern	.408	.658	

B Deep

Region	Northern	Central	Southern
Red Morph			
Northern			
Central	.534		
Southern	.944	.556	
Yellow Morph			
Northern			
Central	.210		
Southern	.666	.312	
Green Morph			
Northern			
Central	.172		
Southern	.388	.464	

Table 2.6 Raw colour morph abundance data by site and transect for each GBR region surveyed.

Northern

Site	Trsct	Count Abundance			
		Green	Blue	Yellow	Red
Mermaid	1	0	0	4	19
	2	2	0	8	18
	3	3	0	24	36
	4	0	0	1	1
Lagoon Entrance	1	0	0	2	3
	2	1	0	5	8
	3	0	0	4	8
	4	1	0	1	5
Washing Machine	1	0	0	0	2
	2	0	0	0	1
	3	0	0	1	2
North Point	1	0	0	2	2
	2	0	1	1	2
	3	0	0	3	7
Picnic Beach	1	1	0	4	8
	2	1	0	1	2
	3	1	0	3	4
Station Entrance	1	4	0	7	23
	2	4	0	6	12
	3	2	0	7	11
Ghost Beach	1	1	0	1	0
	2	1	0	6	3
	3	0	0	1	4
Turtle Beach	1	0	0	3	7
	2	0	0	4	3
	3	0	0	3	4
Little Vicki's	1	3	0	19	31
	2	4	0	16	29
	3	3	0	6	6

Central

Site	Trsct	Count Abundance			
		Green	Blue	Yellow	Red
Cattle Bay	1	0	0	2	6
	2	0	0	3	12
	3	2	0	1	10
	4	2	0	6	15
Hazard Bay	1	0	0	11	15
	2	1	0	4	6
	3	0	0	1	3
Pioneer Bay	1	1	0	10	11
	2	0	0	8	16
	3	0	0	2	4
SW Pelorus Is.	1	0	0	4	11
	2	0	0	3	11
	3	0	0	6	9
Leper Bay	1	0	0	7	10
	2	1	0	0	2
	3	0	0	5	3
Coral Gardens	1	0	0	2	2
	2	0	0	1	2
	3	1	0	2	3
SE Pelorus Is.	1	0	0	0	4
	2	0	1	2	7
	3	0	0	4	5
E Orpheus Is.	1	0	0	3	8
	2	1	1	3	6
	3	0	0	2	22

Southern

Site	Trsct	Count Abundance			
		Green	Blue	Yellow	Red
Humpy Is. 1	1	1	6	16	3
	2	2	4	18	7
	3	2	6	14	3
Humpy Is. 2	1	3	2	8	4
	2	0	1	4	3
	3	4	7	24	8
Humpy Is. Deep	1	0	0	0	4
	2	0	0	0	1
	3	0	0	0	0
Halfway Is. 1	1	2	11	43	23
	2	7	12	50	16
	3	4	9	37	11
Halfway Is. 2	1	0	10	30	10
	2	5	4	45	8
	3	4	2	40	6
	4	5	9	56	20
	5	10	7	41	7
Miall Is.	1	3	2	0	17
	2	7	8	0	16
	3	4	3	0	15
Miall Is. Deep	1	0	0	12	3
	2	0	0	9	6
	3	0	0	5	4
Clam Bay	1	3	17	26	55
	2	4	8	22	43
	3	4	13	19	46
Halftide Rocks	1	0	3	8	5
	2	0	0	2	0
	3	0	0	4	2
S. North Keppel Is.	1	2	8	22	11
	2	3	12	45	28
	3	2	5	27	34

~6% of the population in the more densely populated southern region (Table 2.6).

In shallow water, the red morph was consistently the most abundant, the green morph the least abundant, and the yellow morph occurred at intermediate levels of abundance across all three regions (Fig. 2.5). This pattern was also consistent among regions in deeper water, except in the central region, where abundances of yellow and green morphs were similar (Fig. 2.5). This decrease in the abundance of the yellow morph in the central region in deeper water explains the statistically significant difference found in the linear mixed effects model when the proportional abundances of the three colour morphs were compared among regions and depths ($Chi^2_6 = 30.6$, $p = 3E^{-05}$; Table 2.7). It is worth noting that sample sizes of the yellow morph at deep sites were low in the central region (n=5 colonies, 1 site sampled) compared to the other regions (northern: n=136 colonies, 3 sites; southern: n=647 colonies, 3 sites). Overall, the red morph was between 3.5- and more than 14-fold more abundant than the rare green morph at both shallow and deep sites across the three GBR regions (Fig. 2.5). Although similar at deep sites in the central region, overall proportional abundance of yellow colonies was between 2 and 32 times greater than green colonies (Fig. 2.5). Green colonies comprised between ~4% and 10% of the total population in deep and shallow water, respectively (Fig. 2.5). The blue morph (not analysed) was the least abundant, with only 84 (~4%) colonies observed in shallow water and one (0.8% of colonies) observed in deep water. No differences in the proportional abundances of morphs were detected between exposed and sheltered habitats, i.e. when morph, depth and exposure were included in the linear mixed effects model (Table 2.8).

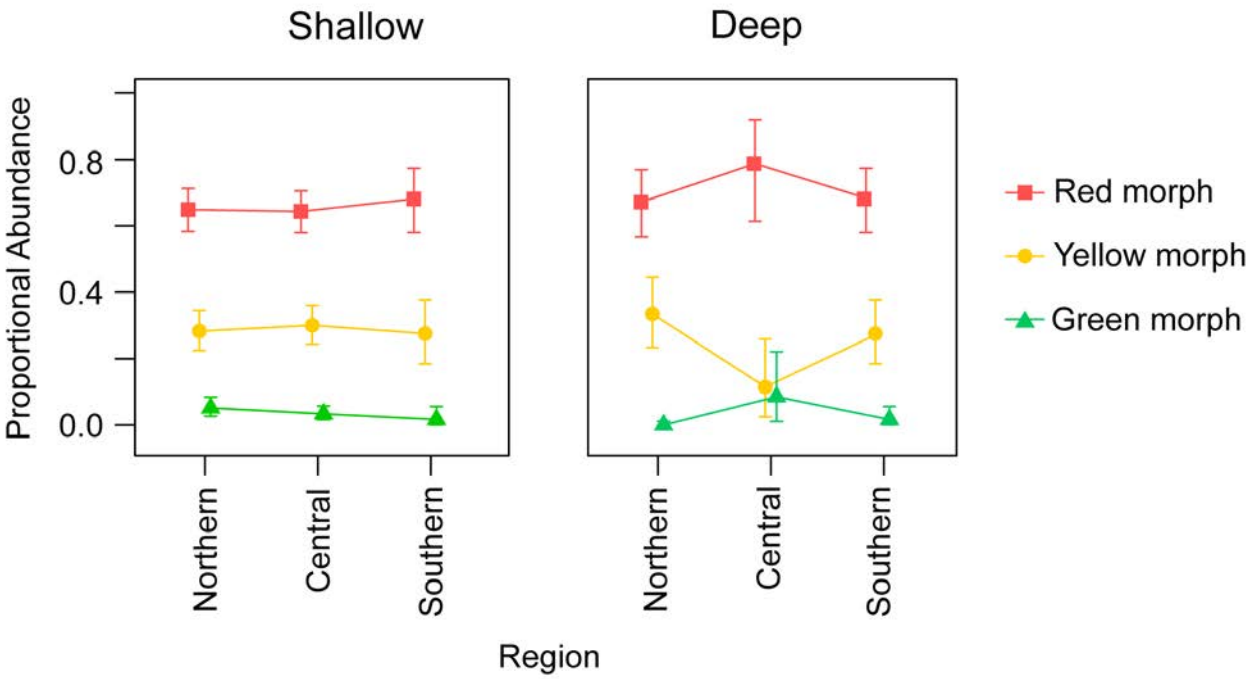

Figure 2.5 Proportional abundances of red (red squares), yellow (yellow circles) and green (green triangles) morphs across the northern, central and southern GBR regions in shallow and deep water. The whiskers denote 95% credible intervals of the posterior distribution.

Table 2.7 Linear mixed effects model on colour morph abundance between depths and among regions, where morph = Mor, depth = Dep, and region = Reg. *: denotes significance at α = .05.

Model	LogLik	df	Chi2	Pr(>Chi2)	
Mor	8.96	2	82.4	<2e-16	*
Mor + Mor*Dep	12.9	3	7.95	.047	*
Mor + Mor*Dep + Mor*Reg	28.2	6	30.6	3E-05	*
Mor + Mor*Dep + Mor*Reg + Mor*Reg*Dep	35.2	6	14.1	.029	*

Table 2.8 Linear mixed effects model on colour morph abundance between depth and exposure. *: denotes significance at α = .05.

Model	*LogLik*	*df*	*Chi2*	*Pr(>Chi2)*	
Morph	8.96	2	82.4	<2e-16	***
Morph + Morph*Dep	12.9	3	7.95	.047	*
Morph + Morph*Dep + Morph*Exp	13.6	3	1.24	.744	

2.3.3 Colony size

Overall, colony size was similar among colour morphs ($F_{2,1296}$=2.47, p=0.085; Figure 6; Table 2.9), with all colour morphs of *A. millepora* typically being small, e.g. greater than 50% of colonies were smaller than 30 cm in diameter (data not presented). On average, mean colony diameter tended to be ~7 cm larger in the southern region compared with the northern and central regions (Fig. 2.6), although regional differences were only statistically significant when mean yellow morph size was compared between the central and southern regions (Table 2.10). Further analysis using Markov Chain Monte Carlo simulations revealed that mean colony sizes of the red and yellow morphs were marginally greater in the southern than in the central region (red: p = 0.048, yellow: p = 0.046) (Table 2.11). In contrast, mean colony diameter (size) of green morphs was similar across all GBR regions (Fig. 6; Table 2.11).

2.4 Discussion

2.4.1 Coral fluorescence is highly variable among colour morphs and GBR regions

The common coral *Acropora millepora* occurs on the Great Barrier Reef as four colour morphs (red, yellow, green, blue) that differ in the relative concentrations of three FPs (cyan, green red) and a chromoprotein. Of the three morphs typically found in all three regions (red, yellow, green), the green morph is the most strongly

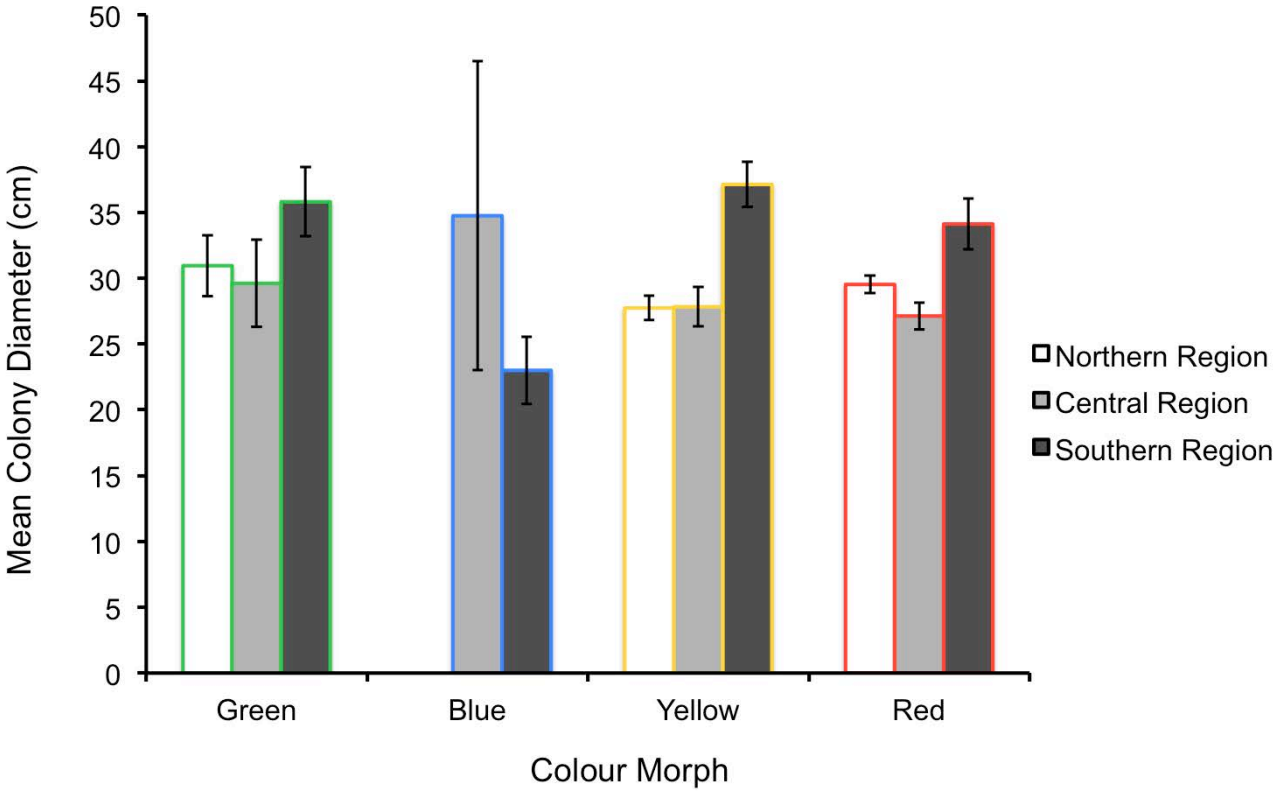

Figure 2.6 Mean colony size (± SE) of green, blue, yellow and red morphs across the three regions of the GBR.

Table 2.9 Two-way factorial ANOVA of mean colony diameter among GBR regions and *A. millepora* colour morphs. *: denotes significance at α = 0.01.

Model	SS	df	F	p	
Region	1.09	2	8.07	.000	*
Morph	.333	2	2.47	.085	
Region*Morph	.221	4	.82	.514	
Error	87.6	1296			

Table 2.10 Linear mixed effects model on colour morph colony size (diameter) among regions. Comparison made to control region (Southern). *: denotes significance at α = .05.

Region	*Morph*	*Posterior Mean*	*Lower 95% CI*	*Upper 95% CI*	*N*	*pMCMC*
Northern	*Red*	-.163	-.449	.117	1000	.268
	Yellow	-.231	-.504	.076	1000	.144
	Green	-.200	-.546	.141	1000	.256
Central	*Red*	-.302	-.590	.023	1000	.054
	Yellow	-.327	-.646	-.031	1000	.044 *
	Green	-.179	-.619	.280	1176	.444

Table 2.11 Markov Chain Monte Carlo (MCMC) simulations for the comparison of colony size (diameter) among regions. *: denotes significance at α = 0.05.

Region	Northern	Central	Southern
Red Morph			
Northern			
Central	.354		
Southern	.224	**.048***	
Yellow Morph			
Northern			
Central	.554		
Southern	.096	**.046***	
Green Morph			
Northern			
Central	.936		
Southern	.256	.436	

fluorescent but the rarest across a latitudinal gradient spanning more than 1100 km from the northern to the southern GBR. Conversely, the red morph is the least fluorescent but the most abundant across the same latitudinal gradient. The strong fluorescence of the green morph is attributable to high concentrations of CFP, which range from being two-fold to more than 1600-fold higher than concentrations of the other two FPs in this morph. Although levels of CFP varied among morphs, it was, by far, the most abundant FP in all four colour morphs, thus variation in levels of the dominant CFP was responsible for differences in overall patterns of fluorescence among morphs. The red morph had the lowest concentrations of CFP, hence their status as weakly fluorescent. Intermediate levels of fluorescence of yellow and blue morphs reflected their intermediary concentrations of CFP.

Overall higher concentrations of FP proteins in the northern GBR compared to the central and southern GBR, notably the 2- to more than1600-fold higher concentrations of the dominant CFP and the 3- to more than 900-fold higher concentrations of GFP in the red, yellow and green morphs, suggest that a latitudinal environmental gradient at least partially governs FP concentrations in *A. millepora*. Although sites in the three regions vary in mean summer and winter temperatures by a few degrees (AIMS), differences in light levels and water quality between clear-water, offshore sites in the northern region and turbid inshore sites in the central and southern regions create a more striking environmental gradient. The inshore fringing reefs of both the Palm and Keppel Islands are seasonally affected by terrestrial run-off from the Burdekin and Fitzroy River basins, the two largest catchments draining into the GBR lagoon (Brodie 1996; King et al. 2002). During wet seasons, suspended particles, including sediment in the water (turbidity) dramatically reduce light penetration required for photosynthesis by endosymbiotic algae (Risk and Sammarco

1991; Anthony 2006). In contrast, the offshore Lizard Group in the northern GBR is relatively unaffected by terrigenous influences and is characterized by high water clarity and lower levels of turbidity year-round (Brodie et al. 2007; Cooper et al. 2007; De'ath and Fabricius 2008). Thus local light regimes, even at similar depths, are potentially very different at northern compared to central/southern sites.

Similarity in the levels of CFP in green and yellow morphs in the two inshore regions, combined with the fact that CFP in these two morphs was significantly lower in inshore regions than in the northern offshore region, provide further evidence that differences in light levels and/or water quality at least partially govern differences in FP concentrations between these regions. While declining fluorescence in coral species distributed along vertical gradients in light attenuation has been reported (e.g. Takabayashi and Hoegh-Guldberg 1995; Klueter et al. 2006), variation in coral fluorescence has not been reported across latitudinal gradients. Our interpretation that 2-fold to 1600-fold higher levels of CFP in northern offshore colour morphs represent a response to higher light levels is supported by a number of studies which have suggested that coral FPs act photoprotectively by dissipating light energy (Salih et al. 2000; Dove et al. 2001; Bhagooli and Hidaka 2003; Dove 2004; Enriquez et al. 2005), and by a recent study demonstrating that only CFP has the spectral properties required to shield *Symbiodinium* (D'Angelo et al. 2008). We hypothesise that latitudinal patterns in FP concentrations reflect turbidity differences between the central/southern inshore versus the northern offshore reef locations rather than other potential environmental correlates of the north-south latitudinal gradient, and up/down regulation of FPs in response to altered light levels offers a potential mechanistic explanation for these patterns (D'Angelo et al. 2008).

In contrast with patterns found for FP concentrations, levels of the non-fluorescent CP were highest and most variable in the southern GBR but did not differ between colour morphs within other GBR regions. The nearly four-fold difference in CP levels between the two inshore reef regions, both areas of lower light and water quality, suggests that gradients in these two environmental parameters do not govern their distributions. Comparisons of FP and CP concentrations across inshore-offshore gradients within a single region would provide further insights into potential environmental correlates of colour in *A. millepora*.

2.4.2 Relative concentrations and visual impacts of FP types determine colony colour

Our results indicate that it is the relative concentration of long wavelength-emitting FPs that determine colour in *A. millepora,* rather than the FP in highest abundance, as previously postulated (Cox et al. 2007). For example, Alieva et al. (Alieva et al. 2008) found that green morphs had dominant emission spectra peaking at 506 nm (GFP), but did not report emissions corresponding to RFP or CFP for this morph. We show that CFP was consistently the most abundant FP in all colour morphs, despite visually distinct appearances under natural daylight conditions. Mazel and Fuchs (2003) suggested that colours with longer emission wavelengths have a stronger visual effect than colours with shorter emission wavelengths. Consequently, Red FPs may disproportionally affect visual colour compared to FPs that emit at shorter wavelengths, such as CFP (Mazel and Fuchs 2003). Our results support this hypothesis, demonstrating that when the relative abundance of short wavelength CFPs is reduced, for example so that CFP is only two-fold higher than RFP, long

wavelength RFPs govern visual appearance and produce the red morph. In contrast, when short wavelength CFPs are in very high abundance compared with other FPs, they mask the powerful contribution of longer wavelength RFPs and result in the green morph. Our study confirms that it is the combined effect of the relative concentrations and the visual impacts of FP types present in tissues that govern colour in the coral *A. millepora*.

2.4.3 Colour morph abundance and distribution patterns are similar across regions and depths

Similarity in the abundances of the three most common colour morphs on shallow and deep transects and across all three regions indicates that environmental parameters exert little control on colour morph distributions of *A. millepora* on the GBR. The only variation in colour morph abundance detected was that yellow morphs were proportionally less abundant in deep compared to shallow water in the central GBR, roughly equalling the abundance of the consistently rare, green morph. Similarly, the rare blue morph was observed with much greater frequency in shallow water compared with deep, confirming the findings of Salih et al. (2006) that colonies with high concentrations of CPs were more abundant in shallow sites. However, these trends should be viewed with caution because of the comparatively small sample sizes of colonies at depth (Yellow colonies: n_{deep} = 119 and $n_{shallow}$ = 2,002 colonies; Blue colonies n_{deep} = 1 and $n_{shallow}$ = 84). The lack of correlation between colour morph distribution patterns and environmental gradients in our study differs from correlations reported in other studies, however, a variety of factors could explain these differing results, including a different or unknown basis for colour variation

(Takabayashi and Hoegh-Guldberg 1995; Todd et al. 2002a; Todd et al. 2002b), or the potential for differences in colour morph distribution patterns to reflect species-level differences (Stobart and Benzie 1994; Klueter et al. 2006). In another study, colour varied with both depth and sediment load, thus while green morphs of *Porites astreoides* were typically found in shallow water and brown morphs in deeper water, this relationship was reversed at locations where sediment load was high, with brown morphs removing sediment better than green morphs (Gleason 1993; Gleason 1998). Our study, which is the first to compare the relative abundances of fluorescent colour morphs within a single coral species across a comprehensive latitudinal and depth distribution, strongly suggests that the distribution patterns of fluorescent colour morphs are the result of heritable variation in colony colour rather than an environmental basis for *A. millepora.*

2.4.4 The consequences of colour in corals

In light of benefits commonly associated with FPs, particularly photoprotection (Salih et al. 2000; Dove 2004 etc.) and antioxidant properties (Bou-Abdallah et al. 2006), it is surprising that weakly and intermediately fluorescent morphs (i.e., red and yellow) are 3.5- to more than 4-fold more abundant than the highly fluorescent but ecologically rare green morph. The postulated photoprotective properties of FPs (Kawaguti 1944; Salih et al. 2000) have been suggested to enhance bleaching resistance (Salih et al. 2000), but our finding that the highly fluorescent green morph is rare in locations where mortality from bleaching was high raises questions about the extent of photoprotection afforded. Mass bleaching events occurred on the GBR in 1998 and 2002, with bleaching more severe on inshore than

on offshore reefs (Berkelmans and Oliver 1999; Berkelmans et al. 2004). The 1998 bleaching event had the greatest impact on Palm Island reefs in the central GBR, where an estimated 70% of corals died (Berkelmans and Oliver 1999), followed by further mortality after the 2002 event (Berkelmans et al. 2004; van Oppen et al. 2005). As a consequence, higher abundances of highly fluorescent colonies were predicted for the central GBR. The similarity in abundances of each of the colour morphs across the northern, central and southern GBR, combined with the rarity of the highly fluorescent green morph in all regions including locations where mortality from bleaching was high, suggests that any photoprotective advantage conferred by the Cyan FP has limited ecological relevance and may come at the expense of other colony fitness traits, resulting in rarity of the green morph. Although the rare green morph bleached less than co-occurring yellow or red morphs during a moderate bleaching event in the northern GBR (Paley and Bay 2012), the rarity of the highly fluorescent green morph across all regions and depths suggests that tradeoffs between bleaching resistance and other physiological processes limit the overall ecological benefits of high fluorescence. We hypothesise that the need to invest in physiological processes like somatic growth and reproduction may result in low investment of resources into the production of FPs. Further studies comparing growth rates and other physiological processes among morphs differing in fluorescence are required to test the veracity of this hypothesis.

2.4.5 Conclusions

Our study demonstrates that colour in four morphs of the common Indo-Pacific coral *A. millepora* is determined by the relative concentrations of long versus

short wavelength-emitting FPs, rather than by the FP present in highest abundance. Two-fold to more than 1600-fold greater concentrations of the dominant CFP on northern offshore reefs, where light levels are comparatively higher than those on turbid inshore central and southern reefs, in colour morphs typically found in all regions (red, yellow, green), suggests that CFP plays a role in photoprotection. However, the extent of photoprotection afforded may be limited, as distribution and abundance patterns of these colour morphs across extensive latitudinal and depth gradients and across sites differing in thermal histories provide no evidence that overall fluorescence plays a role in governing the ecology of this species. We hypothesise that the maintenance of high FP content comes at the expense of other key, life-history energetic investments (e.g. somatic and reproductive tissue growth). Testing this hypothesis will be crucial to resolving our understanding of the importance of FPs to corals.

Chapter 3

Seasonal variability in fluorescent and non-fluorescent protein levels in *Acropora millepora* colour morphs

Table of Contents

3.0. Abstract

Despite the generally high expression levels of FPs in coral (up to 14% of total protein) and the various important functions ascribed to these proteins, baseline information on temporal/seasonal variation within coral populations remains undocumented. This study is the first to investigate FP content in *Acropora millepora* colour morphs on the Great Barrier Reef (GBR) in response to temporal environmental variation. Colonies remained healthy (as indicated by high symbiont density and visual appearance) and local bleaching temperature thresholds were not exceeded for more than 3 consecutive days over the 7 month monitoring period. Amongst the four FP concentrations monitored, the Cyan FP (CFP) was the only FP to significantly fluctuate in abundance among colour morphs and between monthly sampling time points ($F_{12,4}$ = 3.4; p = 0.016). Although FP tissue concentrations were depressed in the warm summer months in the red morph, a pattern consistent with down-regulation under thermal stress, it is unlikely that fluctuating changes in CFP concentrations in the green and yellow morphs were the result of warm temperature stress. Instead, I suggest that fluctuating CFP concentrations in these morphs are the result of cyclical maintenance of optimal protein levels given the lengthy half-lives of these proteins. Of the 150 colonies monitored, five (~3%) changed colour within a four month timeframe. Two colour change directions were observed: (1) red to green, and (2) green to red. Changes from the highly fluorescent red morph to the weakly fluorescent green morph signified declining health, whereas a colour change in the opposite direction did not correspond with any other visually perceivable change in coral health/condition. Hence, coral colony colour change is

possible over short timeframes and corresponds to changes in the abundance of fluorescent pigments within host-coral tissues.

3.1 Introduction

A variety of roles have been ascribed to fluorescent proteins (FPs) in sessile marine invertebrates, including photoprotection (Kawaguti 1944; Salih et al. 2000), antioxidant quenching (Bou-Abdallah et al. 2006), dinoflagellate regulation (Dove et al. 2008; Field et al. 2006), innate immunity (Palmer et al. 2009) and camouflage (Matz et al. 2006), but despite extensive studies over more than 65 years, the debate continues. Biochemical studies of anthozoan FPs have shown that these GFP-homologs often occur as tightly packed tetramers that potentially confer higher stability to molecular structures than those found in other organisms (Leutenegger et al. 2007), which is consistent with a role for GFPs in thermal tolerance in anthozoans. For example, GFPs found in sea pansies (*Renilla reniformis*) are surprisingly resistant to proteolysis and various denaturing agents like high temperature (Ward and Cormier 1979), their stability largely owing to their rigid and unique ß-can fold (Yang et al. 1996; Tsien 1998). Other studies, however, provide contradictory evidence. The ability of immature GFP found in bioluminescent jellyfish (*Aequorea victoria*) to fold correctly has been shown to decrease with increasing temperatures, and reduced levels of GFP maturation have been observed *in vivo* at temperatures as low as 30^{o}C (Lim et al. 1995). Ecological studies of seasonal patterns in FP content would contribute significantly to discussions about the stability of FPs in marine invertebrates and provide insights into their function.

Gene expression studies highlight further inconsistencies regarding the potential role of FPs in enabling marine invertebrates to respond to environmental stress. It has been shown that changes in environmental conditions can result in rapid down-regulation of GFP-homologs in corals (Bay et al. 2009a, b; Smith-Keune and Dove 2008). In contrast, other studies suggest that expression levels of fluorescent and non-fluorescent GFP-homologs are primarily genetically determined, rather than modulated by environmental factors (Kelmanson and Matz 2003; Leutenegger et al. 2007). Scant but recent work demonstrating that pigment concentrations and changes in colour can occur when corals are acclimated to new habitats with contrasting or even similar environmental parameters (Todd et al. 2002a, b; D'Angelo et al. 2008; Roth et al. 2010), suggests that colour is at least partially environmentally controlled. While changes in FP concentration produced in the laboratory are tightly linked to changes in experimental light conditions, the stability of FP expression levels under different *in-situ* light and temperature regimes has not been examined. Therefore ambiguity exists about the extent to which colour is phenotypically plastic in wild corals, and whether changes in colony colour also correlate with changes in FP concentrations in the field. Similarity in the relative abundances of colour morphs of the coral *Acropora millepora* across depth gradients, wave regimes and regional scales (Chapter 2) suggests a genetic component to such spatial distribution patterns. However, concentrations of the three primary FPs (CFP, GFP, RFP; see definitions in Chapter 2) differed among regions, with up to 7-fold greater concentrations of the CFP in the northern compared to the southern region, suggesting that levels of FPs within a colour morph play a role in responding to environmental differences (Chapter 2).

The occurrence of a range of colour morphs that differ greatly in levels of fluorescent and non-fluorescent protein (chromoprotein (CP)) concentrations within a single coral species, and indeed, within a single population, provides a unique opportunity to explore the role of FPs in responding to environmental change. To date, there are no studies that document natural variability in FP levels in coral tissues among intraspecific colour morphs or that evaluate how FP levels are affected by seasonal changes in temperature. An understanding of natural levels of variation in FP content among coral colour morphs is important for identifying baselines against which stress responses can be compared. Potential changes in coral colour and underlying FP concentrations may signify important information about coral condition that could serve as early warning of declining health.

In this study, my objectives are to determine if FP/CP content, visual coral condition and *Symbiodinium* density vary in three common colour morphs of the coral *Acropora millepora* as sea surface temperatures increase over a spring-summer season. By monitoring tagged colonies of each colour morph, I evaluate if colonies change colour with seasonally changing temperatures, and if so, if colour changes correspond to change(s) in FP concentration. By monitoring these parameters throughout a spring-summer season lacking thermal anomalies, my study provides baseline temporal patterns in colony colour and FP concentrations against which patterns in years with thermal anomalies can be compared.

3.2 Materials and Methods

3.2.1 Study sites and sampling design

The study was carried out on the fringing reefs of Orpheus, Pelorus and Fantome Islands in the Palm Island group (18°35'S, 146°29'E) (Fig. 3.1). Six study sites were selected: four on the leeward side of Orpheus Island (Cattle Bay, Pioneer Bay sites 1 and 2, and Hazard Bay), one on the leeward side of Pelorus (southwest Pelorus) and one on the windward side of Fantome Island (Leper Bay) (Fig. 3.1).

Surveys of colour morph abundance and bleaching condition were conducted at each of the six sites between November 2007 and May 2008, as described in Chapter 2 (see section 2.2.2). Additionally, 30 colonies falling within transect dimensions were tagged at all sites but Pioneer Bay site 2, and three branches sampled, following the protocols outlined in Chapter 2 (see Section 2.2.1), monthly for four months, from November 2007 through February 2008. At Pioneer Bay site 2, 15 colonies (five of each colour morph) were specifically selected for tagging and sampled monthly for seven months, from November 2007 through May 2008. All but three of the colonies at this latter site were located within a 10 m^2 quadrat. Due to their rarity, three green colonies outside the quadrat, approximately 20 and 40 m south of the quadrat, were included in this study. Branches were snap frozen in liquid nitrogen (LN_2) within 15 min of collection and later transferred to a -80°C freezer. Colony bleaching condition was determined using the CoralWatch Coral Health Chart (University of Queensland) as a guide, as described in Chapter 2 (see section 2.2.2).

Two temperature loggers were positioned in Pioneer Bay, such that their proximity to each colony within the quadrat was within 10 metres. Temperatures were collected from Nov 25, 2007 through May 19 2008 (the first and last sampling periods). Daily averages were calculated from daily readings at 10:00, 12:00 and 14:00 and averaged between the two loggers.

3.2.2 Fluorescent and non-fluorescent protein and spectral analyses

Protein concentration and relative FP/CP abundance measurements from Pioneer Bay site 2 colonies (n = 15 colonies) were obtained for each branch collected using the methods outlined in Chapter 2 (see Sections 2.2.3. and 2.2.4.). Bleaching status surveys revealed that five colonies across all study sites (~3%; n = 157 colonies surveyed) changed colour, with three changing from red to green and two changing from green to red (Fig. 3.2). FP levels throughout the four-month study period were analysed to reveal whether visual changes in colour were reflected in the abundance of FPs. FP content for samples from these five colonies was compared to that of four red colonies and four green colonies that were identified as healthy and showed no colour change throughout the study. As colonies were seen to either change from red to green or green to red, only green and red colonies were chosen for comparison.

3.2.3 *Symbiodinium* density

To determine temporal patterns in the density of *Symbiodinium* and evaluate if such patterns varied with coral colony colour, algal cell densities were quantified for each colony from Pioneer Bay site 2 (n = 15 colonies) from November 2007 through May 2008. Frozen branches were air-brushed to remove tissues from intact coral skeletons using a modified air-gun attached to a cylinder of compressed air. Fifteen mL of 500 μM filtered seawater was poured over each frozen branch and allowed to accumulate at the bottom of a plastic bag containing the branch. Tissue was

airbrushed from the skeleton and collected in the bottom of the bag with the filtered seawater creating a tissue slurry. Once all tissue was removed from a branch, the resultant slurry was homogenized for 1 minute in a glass beaker (Janke and Kunkel, IKA-Labortechnik) to lyse coral tissue cells. Nine ml of slurry was then immediately added to 1 ml of formalin to fix *Symbiodinium* cells and prevent lysis. *Symbiodinium* cell counts were conducted using an improved Neubauer haemocytometer with two counting chambers filled with 1 mm^2 or 100 µl of tissue slurry sub-sample. Three replicate cell counts of each haemocytometer counting chamber were averaged for each sub-sample of fixed tissue slurry.

The surface area of each branch was determined using a wax coating technique (Stimson and Kinzie 1991; Vytopil and Willis 2001). Bleached branches (dipped in 20% chlorine solution for approximately 2 minutes) were dipped in paraffin wax melted at 100^oC and lightly shaken to remove drips so that an even coating remained. This sealed the surface area of the coral skeleton ensuring that each branch had a surface of identical adhesion quality. Once the skeleton surface was primed, branches were weighed, re-dipped, and re-weighed to determine the increase in mass due to the second surface coat. To relate the increase in mass of wax coating to surface area, a calibration curve was established using paraffin cylinders of known surface area and weight ranging from 207.5 to 125,66.4 cm^2 and 0.29 to 1.44g. The relationship between increments of mass and surface area of the cylinders was used to predict the skeletal surface area of a coral branch knowing its increase in mass between wax coatings (Fig. 3.3). Using the regression equation, surface area can then be calculated for each branch as follows:

Equation 3.2.1 $SA\ (cm^2) = Z\ x\ W,$

where *Z* is the regression equation for the calibration curve *(cm^2*mg^{-1})* and *W* is the difference in weight between coatings *(mg)*. The average concentration of zooxanthellae per coral branch was then calculated as follows:

Equation 3.2.2 $[Avg.\ zoox] = N \times 10^4 \times D / SA$,

where *N* is the average number of *Symbiodinium* cells counted, *D* is the volume of water added to the bag (i.e. 10 ml) and SA is the surface area (cm^2) of the branch.

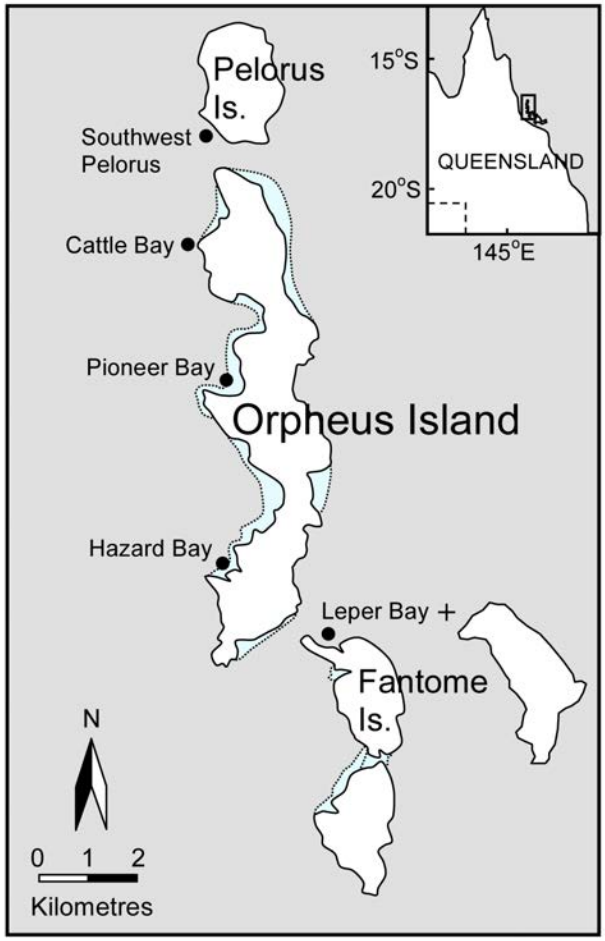

Fig. 3.1 Colour morph survey locations in the Palm Island Group, central Great Barrier Reef. Southwest Pelorus, Cattle Bay, Pioneer Bay and Hazard Bay are sheltered locations; Leper Bay is more exposed to seasonal southeasterly trade winds.

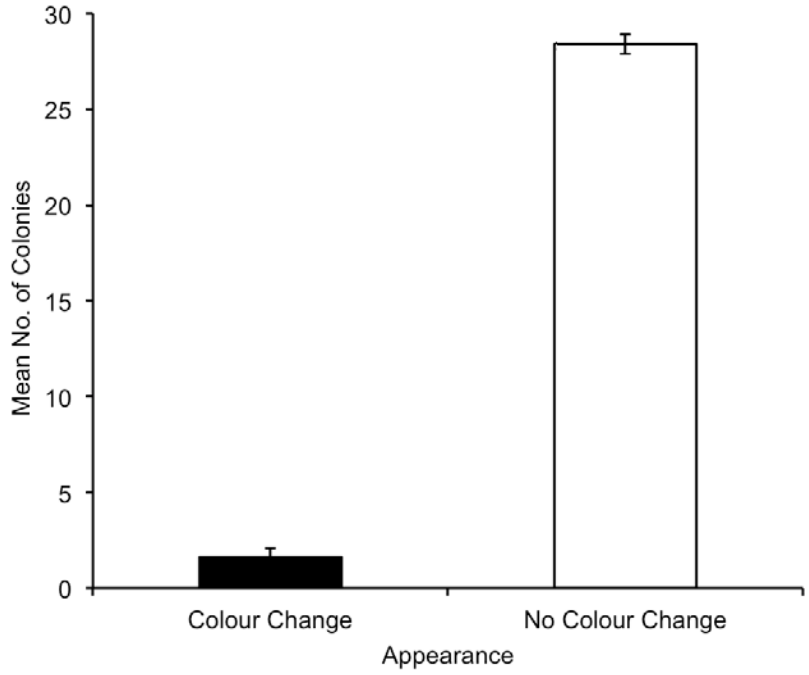

Fig. 3.2 Comparison of the mean number of colonies (±SE) that changed versus did not change colour in each month across all study sites between November 2007 and February 2008. N = 4 months, 157 corals surveyed each month.

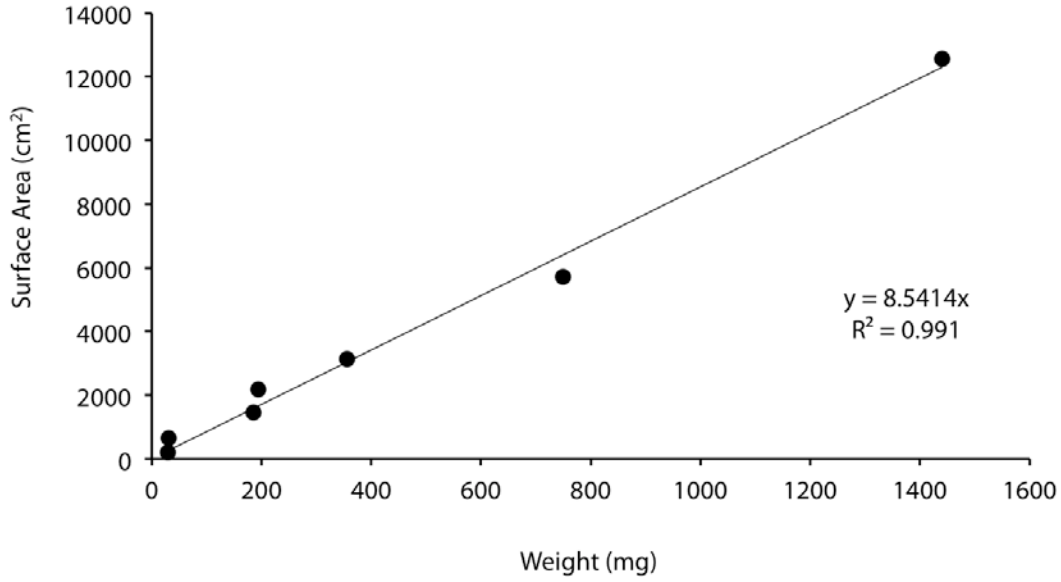

Fig. 3.3 Calibration curve for determining the relationship between weight and surface area based on the paraffin wax dipping procedure (see Section 3.2.3).

3.2.4 Statistical analyses

Bleaching Condition: The proportions of healthy, pale, bleached, and dead colonies in each month were compared among colour morphs in a 2-way ANOVA, where colour morph and health status were treated as fixed effects. Because not all study sites were monitored for the full 7 month time-frame, the total number of colonies surveyed differed between sampling times. To identify variation in condition among colour morphs independent of changes in sample size, the variable compared in the two-way ANOVA was the proportion of colonies in each health status category at each sampling time. A Kolmogorov-Smirnov test for normality ($p < 0.1$) and a Cochran's test ($p = 1.0$) for equality of variances confirmed conformation to ANOVA assumptions when data were arcsin-squareroot transformed. Planned, post-hoc multiple comparisons of bleaching status among colour morphs were interpreted with Tukey's HSD tests. Additionally, the proportion of healthy, pale, bleached, and dead colonies were compared throughout the 7-month timeframe irrespective of colour. On 11 March 2008, Pioneer Bay received strong westerly winds averaging 30 knots (AIMS remote weather station 2008), which caused uncharacteristically rough waters and large swell on the leeward sides of the Island (Orpheus Island Research Station Manager, pers. com.). This disturbance resulted in smothering by anoxic sediments and eventual mortality of two of the green colonies tagged at Pioneer Bay site 2 and is likely to have caused mortality of five other colonies located at less frequented sites. Because this mortality was most likely due to an unusual environmental conditions associated with the location of colonies within the bay (see section 3.2.1 above),

rather than thermal stress or colour difference, mortality was not compared among Pioneer Bay site 2 colour morphs.

Symbiodinium Density and Water Temperature: Changes in mean *Symbiodinium* density over the 7-month study were compared among colour morphs from Pioneer Bay site 2 using a univariate repeated-measures ANOVA. The main effects tested were sampling time (fixed) and colour morph (green, yellow, red), with the colony term (random) nested in colour morph. Patterns in local water temperature were visually compared with mean *Symbiodinium* density data from the seven sampling periods.

FP and CP Concentrations: To examine temporal expression patterns of fluorescent proteins, three multivariate (Pillai's test) repeated measures ANOVA tests were conducted to individually compare levels of Cyan, Green, and Red FPs (hereafter referred to as CFP, GFP and RFP) from the seven sampling times among the three colour morphs (green, yellow, red). Post-hoc, multiple comparisons of Cyan FP content compared among colour morphs from sampling times were interpreted with Tukey's HSD tests. Additionally, a contingency table Chi^2 test was used to compare the relative concentrations of the three FPs among morphs (green, yellow, red) from the seven sampling times. Changes in the absorbance of the non-fluorescent CP were analysed using a univariate repeated-measures ANOVA, with sampling time and colour morph (green, yellow, red) as fixed factors, and colony a random factor nested within colour morph. Lastly, to determine if changes in colour corresponded with changes in FP content, three multivariate repeated measures ANOVA tests were conducted to individually compare levels of Cyan, Green and Red FPs from the four sampling times, from November 2007 through February 2008, during which colour

changes were observed. Post-hoc, multiple comparisons of Cyan, Green and Red FP concentrations from sampling times were interpreted with Tukey's HSD tests.

3.3 Results

3.3.1 Temporal variation in colony condition

In total, 14 colonies of the green morph, 25 colonies of the yellow morph and 121 colonies of the red morph of *A. millepora* were monitored for between five and seven months across all six sites. The primary temporal pattern in colony health status detected over the timeframe of the study was that the proportion of pale colonies declined from the initial sampling time point in November, when approximately one-third of colonies were pale (i.e. 52 colonies, representing 35% of red colonies, 35% of yellow colonies, 18% of green colonies), to 0% appearing pale at the end of the study in May (Fig. 3.4). At each of the seven sampling times in the study, between 65 and 100% of colonies monitored appeared visually healthy (Fig. 3.4). Only three (~2%) of the 157 colonies monitored (two yellow, one green) were observed white bleaching throughout the 5-7 month study period (Fig. 3.5); bleaching was only recorded in the warm summer months (November, December, and February; Fig. 3.4).

Overall, patterns in the mean proportion of colonies in each of the four bleaching/health categories (healthy, pale, bleached, dead) were consistent among the three colour morphs ($p = 0.915$; Table 3.1). Within each of the three colour morphs, between 79 and 83% of colonies remained healthy in appearance throughout (Fig 3.5). Seven colonies (~5%) died between March and May 2008 (two of each of the red and green morphs, and three yellow colonies) following severe storm disturbance

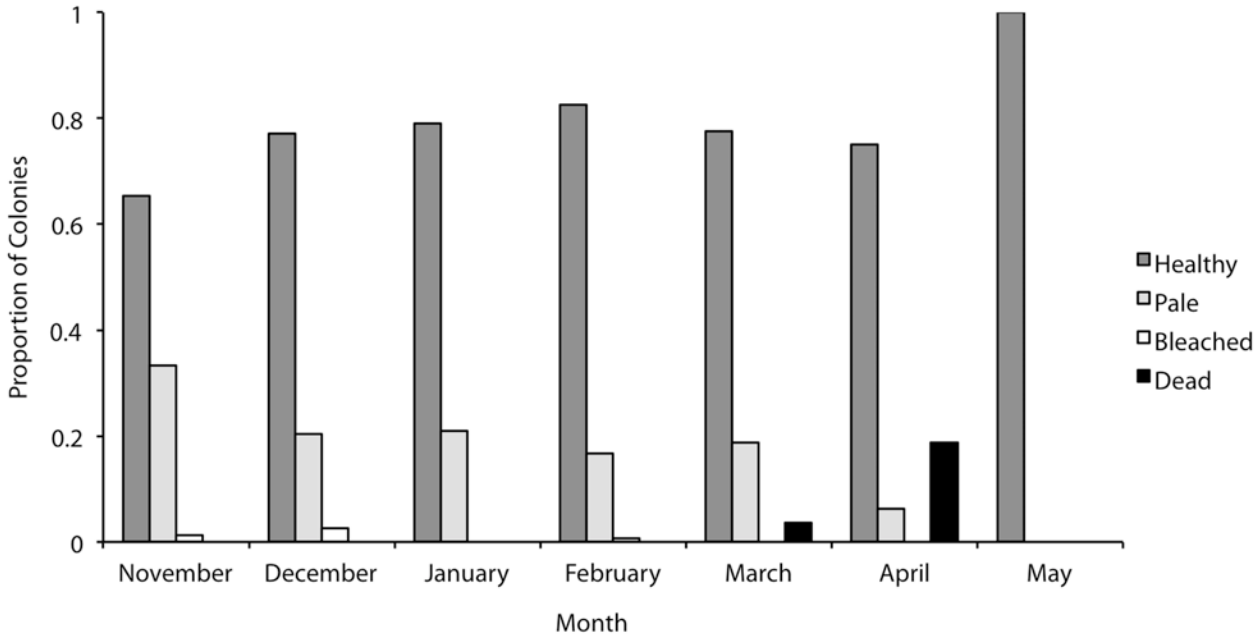

Fig. 3.4 Comparison of the mean proportion (±SE) of colonies in four bleaching / health status categories among colour morphs throughout a 7-month sampling period (November 2007 through May 2008). Two red and green colonies and three yellow colonies died between the February and April sampling times. n =157 colonies monitored over the sampling period.

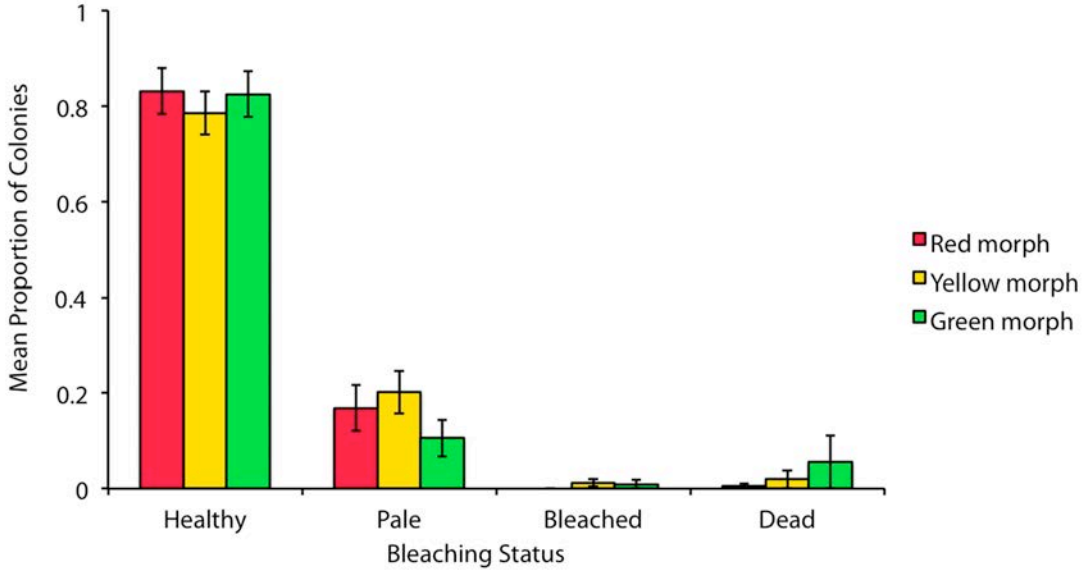

Fig. 3.5 Proportion of colonies in each of four bleaching / health status categories (healthy, pale, bleached, dead) throughout the sampling period (November 2007 through May 2008). n = 157 colonies monitored

Table 3.1 Two-way factorial ANOVA comparing the proportion of colonies in each of four bleaching / health status categories among colour morphs of *A. millepora* at seven sampling times (from November 2007 through May 2008) at Orpheus Island. *: denotes significance at α = 0.05.

Source of Variation	SS	df	MS	F	p	
Morph	.001	2	.000	.001	.999	
Condition	15.9	3	5.3	24.4	.000	*
Morph*Condition	.441	6	.074	.338	.915	
Error	15.9	73	.218			

Table 3.2 Tukey's HSD tests comparing bleaching / health status among colour morphs of *A. millepora* from Orpheus Island. *: denotes significance at α = .05.

Colour	Colour	Red				Yellow				Green			
Colour	Condition	Healthy	Pale	Bleached	Dead	Healthy	Pale	Bleached	Dead	Healthy	Pale	Bleached	Dead
Red	Healthy												
	Pale	**.000 ***											
	Bleached	**.000 ***	1.0										
	Dead	**.000 ***	1.0	1.0									
Yellow	Healthy	.565	**.000 ***	**.000 ***	**.000 ***								
	Pale	**.000 ***	.565	.565	.565	**.000 ***							
	Bleached	**.000 ***	1.0	1.0	1.0	**.000 ***	.565						
	Dead	**.000 ***	1.0	1.0	1.0	**.000 ***	.565	1.0					
Green	Healthy	.170	**.000 ***	**.000 ***	**.000 ***	1.0	**.000 ***	**.000 ***	**.000 ***		**.000 ***	**.000 ***	**.000 ***
	Pale	**.000 ***	1.0	1.0	1.0	**.000 ***	.565	1.0	1.0	**.000 ***		1.0	.170
	Bleached	**.000 ***	1.0	1.0	1.0	**.000 ***	.565	1.0	1.0	**.000 ***	1.0		.170
	Dead	**.000 ***	.170	.170	.170	**.000 ***	1.0	.170	.170	**.000 ***	.170	.170	

(see Section 3.2.4). In summary, the majority of colonies within each colour morph were healthy over the timeframe of the study, with significantly lower and declining proportions of colonies in the pale, bleached and dead categories (Fig. 3.5; Table 3.2).

3.3.2 Temporal variation in *Symbiodinium* density and water temperature

Temporal patterns in mean *Symbiodinium* density did not differ among the three colour morphs during the 4-7 month study period ($p = 0.866$; Table 3.3; Fig. 3.6A). Mean *Symbiodinium* density increased in all colour morphs, from 3.2 $x10^4$ cells per mm^2 in November 2007 to more than 1.5 $x10^5$ cells/mm^2 in May 2008 ($p = 0.000$; Table 3.3; Fig. 3.5A). Mean midday daily water temperatures ranged from 33.3°C in December to 23.9°C in May (Fig. 3.4B). Temperatures exceeded 31°C, the 5-day bleaching threshold for a congeneric species at Orpheus Island (Berkelmans and Willis 1999), for 27 days (~15 % of days) between November 2007 and May 2008, and did so consecutively for a period of 8 days in December (Fig. 3.6B). In general, as temperatures cooled between December and May, mean *Symbiodinium* density increased to a maximum of ~170,000 algal cells per mm^2 (Fig. 3.6A&B). It should be noted that in November, these colonies appeared paler in appearance compared with later observations throughout the seven month study (Fig. 3.4). This observation partially explains the order of magnitude lower densities recorded in comparison to densities typically found for this species (ref), and the 2.5 to 3-fold increase in *Symbiodinium* densities between the November and December samples.

Table 3.3 Univariate repeated measures ANOVA of *Symbiodinium* density among colour morphs of *A. millepora* from November 2007 through May 2008.

Source of Variation	SS	df	MS	F	p	
Morph	1.1E+09	2	5.7E+08	.146	.866	
Error	4.3E+10	11	3.9E+09			
Time	1.4E+11	6	2.3E+10	23.4	.000	*
Time*Morph	1.7E+10	12	1.4E+09	1.4	.170	
Error	6.5E+10	66	9.8E+08			

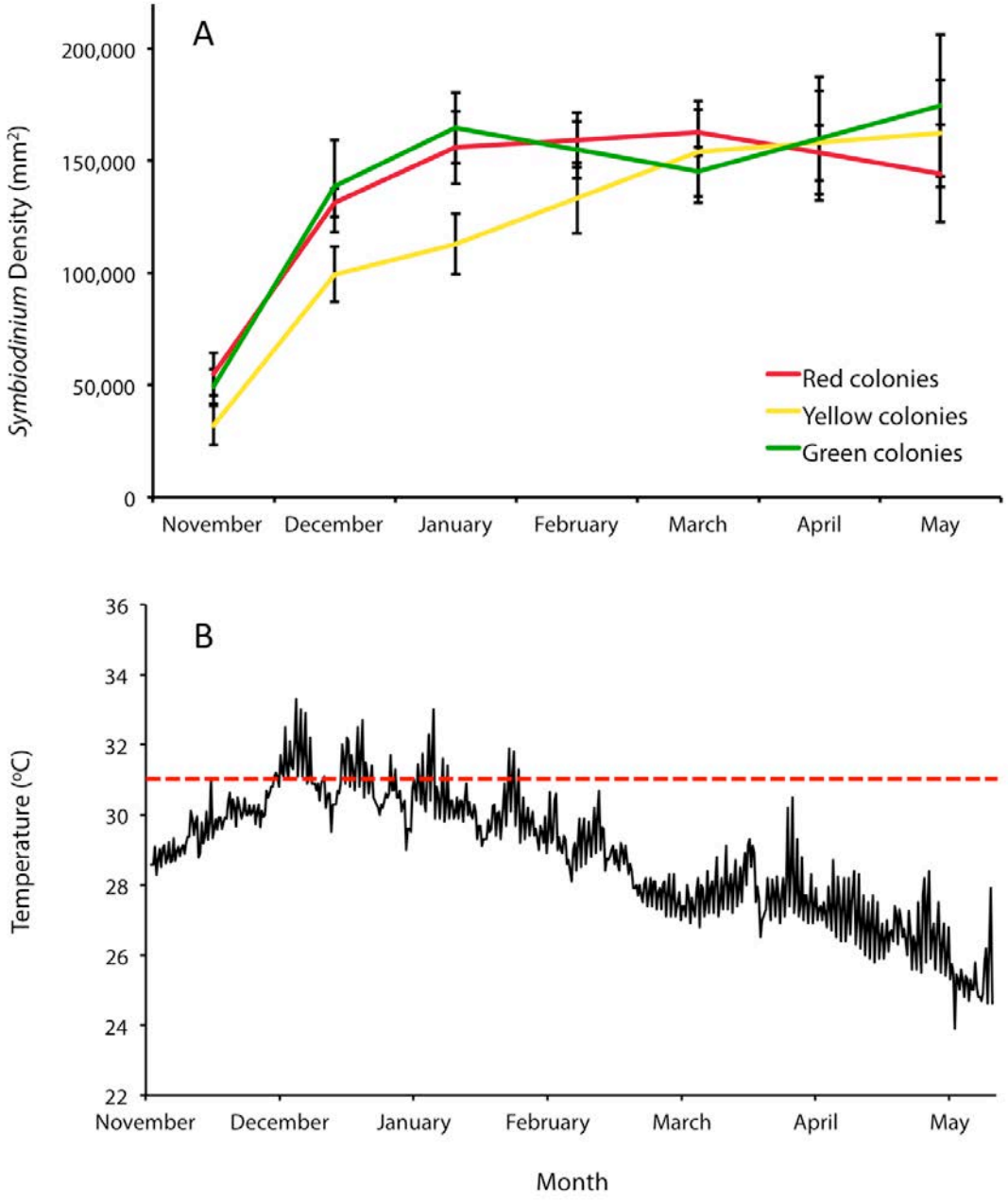

Fig. 3.6 Temporal patterns in A) *Symbiodinium* densities compared among three colour morphs of *Acropora millepora*, and B) seawater temperature in Pioneer Bay throughout the sampling period (November 2007 to May 2008). Temperature data represent daily midday average temperature readings acquired from two temperature loggers placed adjacent to tagged colonies. Red dashed line represents the 31°C 5-day local bleaching threshold for congeneric species identified by Berkelmans and Willis (1999).

3.3.3 Temporal variation in fluorescent and non-fluorescent protein content

Comparisons of temporal patterns in concentrations of the three FP types (Fig. 3.7) revealed that only patterns in CFP concentrations differed significantly among the three colour morphs ($p = 0.016$; Table 3.4A). CFP concentrations were significantly greater in yellow colonies sampled in March than in red colonies sampled at all time points and green colonies sampled in November (Table 3.5; Figure 3.7). Conversely, CFP concentrations were significantly lower in March samples of yellow colonies than in April samples of green colonies (Table 3.5). In contrast, concentrations of GFP and RFP did not differ significantly among colour morphs throughout the study period ($p_{(GFP)}=0.262$, Table 3.4B; $p_{(RFP)}=0.179$, Table 3.4C).

In yellow colonies, the relative proportions of the three FPs were constant throughout the study period ($X^2_{12} = 12.7$; $p = 0.394$), in contrast to temporally changing proportions of the three FPs in green ($X^2_{12}= 47.1$; $p = 0.000$) and red ($X^2_{12}= 21.2$; $p = 0.047$) colonies (Table 3.6). In red colonies, overall increases in FP concentrations, of between 9 and ~30-fold between the February and March 2008 time points, reflected differing patterns of increase for each FP type (Fig 3.7). In particular, mean GFP concentrations increased from levels that were approximately 50% those of CFP, the most abundant FP in the first 4 months, to levels that were more than 1.5-fold greater than CFP concentrations in March, thereby changing the relative proportions of FPs within red colonies through time. In green colonies, although CFP consistently had the highest mean concentration, levels of CFP only quadrupled (3.8-fold increase) between the February and March 2008 time points, whereas concentrations of GFP and RFP increased between 9 and nearly 22-fold, thereby changing the relative proportions of FP types within green colonies (Fig 3.7).

These changes in relative FP concentrations within colour morphs did not result in observable changes in colony colour or other visual aspects of colony appearance.

CP levels declined significantly with time (Fig. 3.8; p = 0.000; Table 3.7), a pattern that was consistent among colour morphs throughout the study period (p = 0.124; Table 3.7; Fig. 3.8). Mean CP levels declined in all morphs by ~35%, from a mean absorbance per mg of 3.7 to 1.3 over the 7 month study period (Fig. 3.8).

Table 3.4 Multivariate repeated measures ANOVA comparing levels of (A) Cyan, (B) Green, and (C) red FPs from green, yellow and red colour morphs of *A. millepora* from November 2007 to May 2008. *: denotes significance at α= 0.05.

A

Source of Variation	df	Pillai's Test Value	F	p	
Time	6	.919	11.4	.005	*
Error	6				
Time*Morph	12	1.5	3.4	.016	*
Error	14				

B

Source of Variation	df	Pillai's Test Value	F	p
Time	6	.518	1.1	.467
Error	6			
Time*Morph	12	1.1	1.4	.262
Error	14			

C

Source of Variation	df	Pillai's Test Value	F	p
Time	6	.711	2.5	.149
Error	6			
Time*Morph	12	1.2	1.7	.179
Error	14			

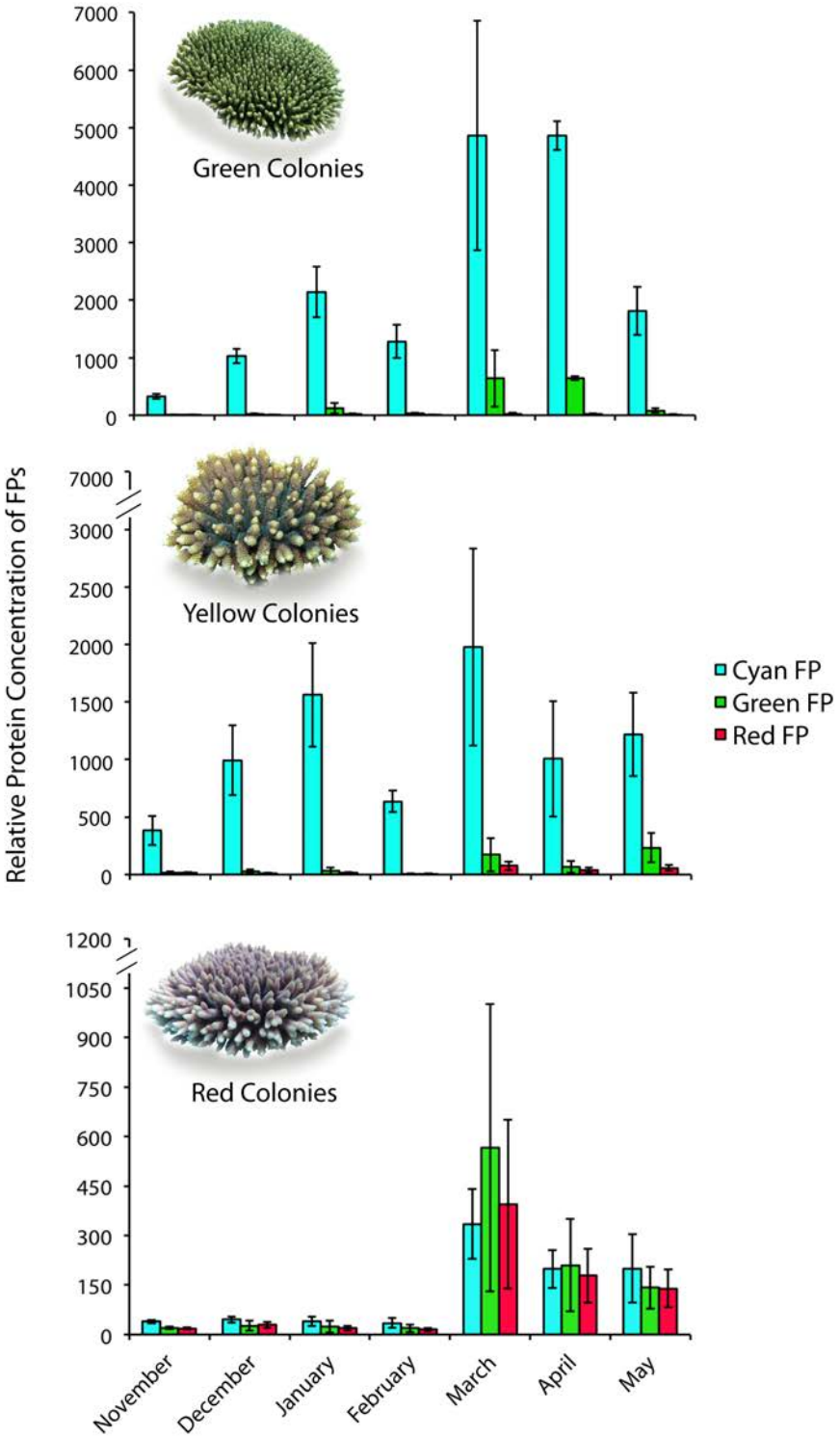

Fig. 3.7 Fluorescent protein concentrations compared among coral colour morphs through the study period. Mean relative concentration of fluorescent proteins (± SE) for green, yellow and red colonies from November 2007 through May 2008. n = 15 colonies (5 of each colour).

Table 3.5 Tukey's HSD for Cyan FP abundance compared among colour morphs of *A. millepora*, November 2007 to May 2008. *: denotes significance at α = .05.

Morph		Red							Yellow							Green						
Morph	Time	Nov	Dec	Jan	Feb	Mar	Apr	May	Nov	Dec	Jan	Feb	Mar	Apr	May	Nov	Dec	Jan	Feb	Mar	Apr	May
Red	Nov																					
	Dec	1.00																				
	Jan	1.00	1.00																			
	Feb	1.00	1.00	1.00																		
	Mar	1.00	1.00	1.00	1.00																	
	Apr	1.00	1.00	1.00	1.00	1.00																
	May	1.00	1.00	1.00	1.00	1.00	1.00															
Yellow	Nov	1.00	1.00	1.00	1.00	1.00	1.00	1.00														
	Dec	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00													
	Jan	.710	.715	.710	.706	.909	.832	.833	.857	1.00												
	Feb	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.0											
	Mar	**.001***	**.001***	**.001***	**.001***	**.004***	**.002***	**.002***	**.002***	**.020***	.455	**.005***										
	Apr	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	**.024***									
	May	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	**.035***	1.00								
Green	Nov	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	.953	1.00	**.010***	1.00	1.00							
	Dec	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	.127	1.00	1.00	1.00						
	Jan	.852	.855	.852	.849	0.97	.928	.929	0.97	1.00	1.00	1.00	.610	1.00	1.00	0.97	1.00					
	Feb	0.98	0.98	0.98	0.98	1.00	0.99	0.99	1.00	1.00	1.00	1.00	.319	1.00	1.00	1.00	1.00	1.00				
	Mar	.064	.066	.064	.063	.155	.105	.106	.161	.565	1.00	.309	1.00	.608	.686	.154	.695	.994	.923			
	Apr	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	**.049***	1.00	1.00	1.00	1.00	1.00	1.00	.434		
	May	.625	.630	.625	.621	.847	.754	.755	.854	.997	1.00	.961	.841	1.00	1.00	.849	1.00	1.00	1.00	1.00	0.99	

Table 3.6 Contingency table Chi^2 test comparing relative concentrations of the three fluorescent proteins among green, yellow and red colour morphs from November 2007 through May 2008. *: denotes significance at α= 0.05.

Colour Morph	X^2	df	p	
Green	21.2	12	.047	
Yellow	12.7	12	.394	
Red	47.1	12	.000	*

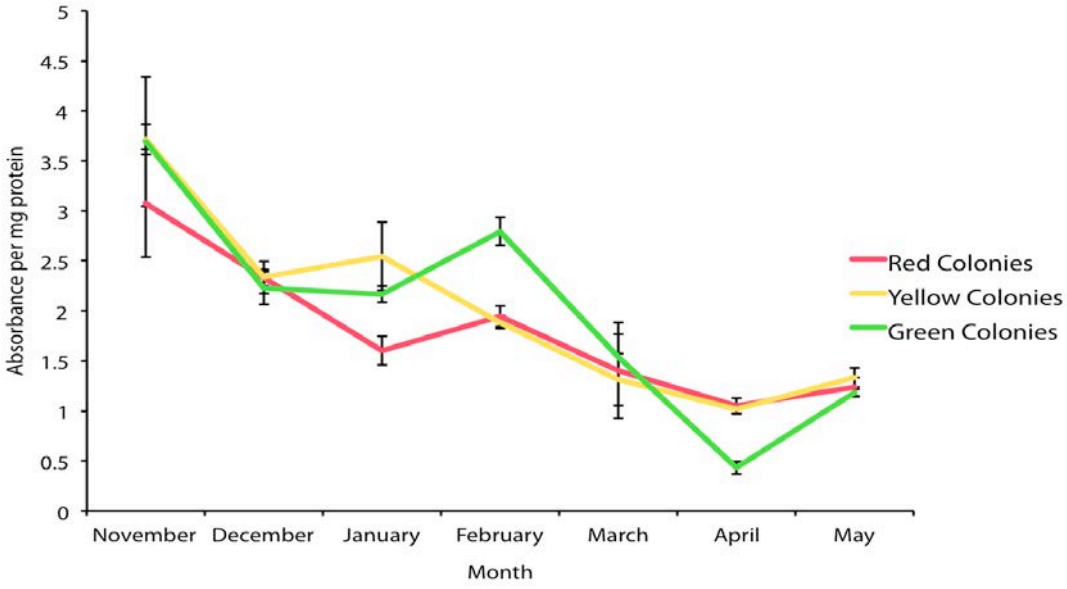

Fig 3.8 Mean (± SE) non-fluorescent chromoprotein concentrations compared among three colour morphs of *A. millepora* (November 2007 to May 2008). n = 15 colonies

Table 3.7 Univariate repeated measures ANOVA comparing levels of non-fluorescent protein among three colour morphs (green, yellow, red) of *A. millepora* through the study period (November 2007 through May 2008).

Source of Variation	SS	df	MS	F	p	
Morph	1.1	2	.569	1.7	.221	
Error	4.0	12	.331			
Time	68.4	6	11.4	35.5	.000	*
Time*Morph	6.0	12	.500	1.6	.124	
Error	23.1	72	.321			

3.3.4 Colony colour change and variation in FP content

Temporal patterns in the levels of CFP, GFP and RFP (Fig. 3.9) did not differ significantly between red colonies that remained red throughout the study and red colonies that changed to green in appearance, hereafter termed red-green (Tables 3.8, 3.9 A-C). Although mean CFP concentrations increased by more than 3-fold in red-green colonies, resulting in levels that were nearly 4.5-fold those of consistently red colonies (Fig. 3.9), these differences were not statistically significant. Despite these increases in CFP, levels in red-green colonies remained significantly lower than those measured in consistently green colonies in three of the four months (December, January and February; Fig. 3.9; Table 3.9A). Some temporal variation in FP concentrations appears normal (as observed in Pioneer Bay colonies in section 3.3.2 above), however the increase in CFP levels in colonies changing from red to green is noteworthy, as the intensity of FPs in red colonies typically remains low, with much narrower margins of monthly variability than observed in green colonies (Fig. 3.8). Health condition data for red-green colonies indicates that they all remained healthy (i.e. remained darkly pigmented) throughout the study (data not presented).

For colonies that changed colour from green to red, hereafter termed green-red, CFP levels were initially similar to those in consistently green colonies, but were more than 7.5-fold lower by the end of the study (Fig. 3.9; Table 3.9A). By February, there was no measurable difference in CFP levels between green-red colonies and consistently red colonies ($p = 1.00$; Table 3.9A). Condition data for green-red colonies revealed declines in health; one colony developed signs characteristic of white syndromes (WS) and the second became fragmented following disturbance and was found inverted with significant pigmentation loss. At the last sampling time point, these colonies displayed ~50% partial mortality, with continuing signs of WS and loss of pigmentation. There was no measurable difference in the levels of GFP or RFP between green-red, green or red colonies throughout the study period (Table 3.9B-C).

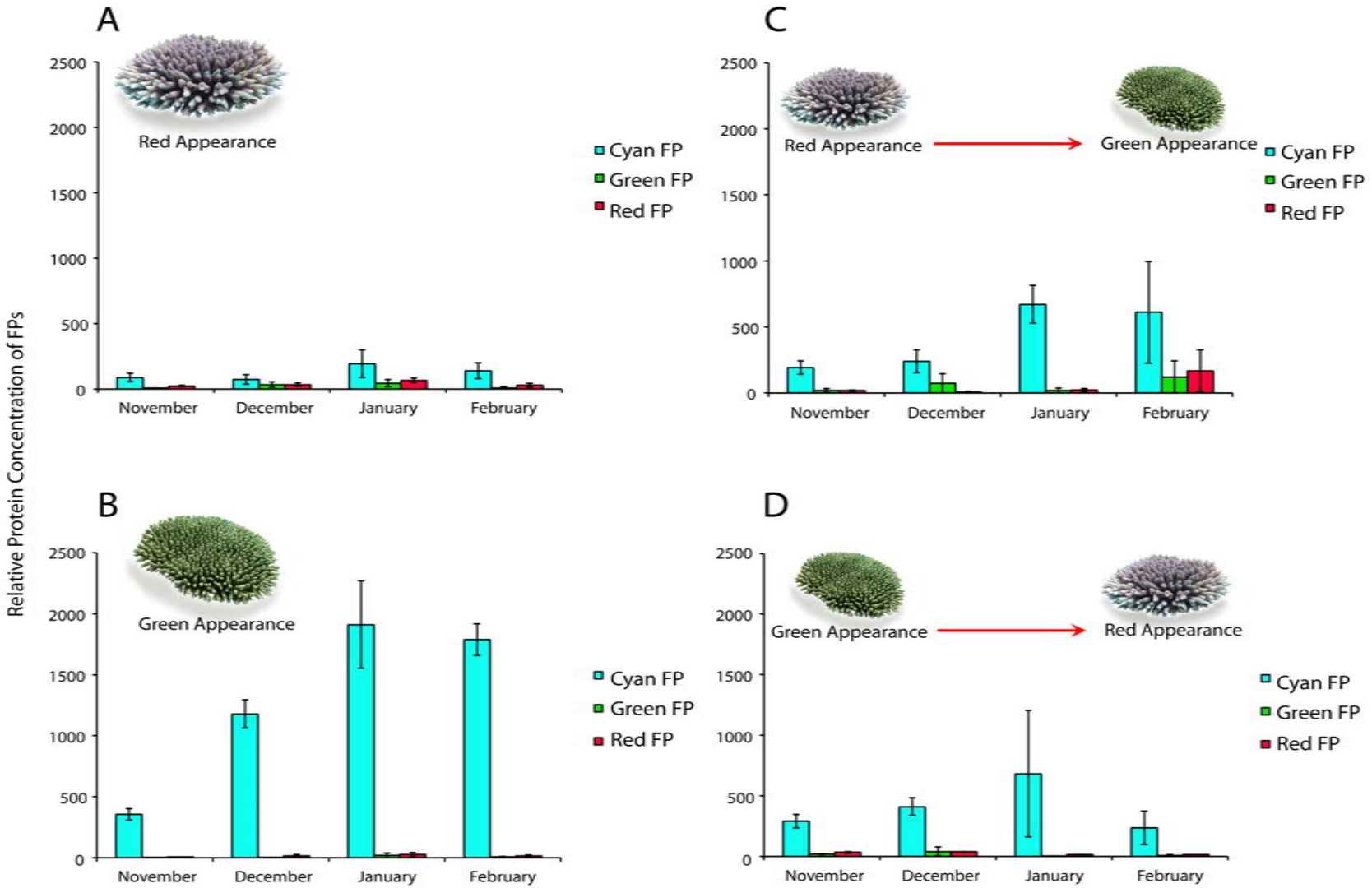

Fig. 3.9 Mean (±SE) relative fluorescent protein concentrations for red (A) and green (B) colonies compared with those of colonies that changed from red to green (C) and green to red (D) during the sampling period. n = 13 (4 red, 4 green, 2 red-green, 3 green-red) colonies.

Table 3.8 Multivariate repeated measures ANOVA comparing levels of (A) Cyan, (B) Green, and (C) red FPs between colonies of *A. millepora* that changed colour versus colonies that remained the same colour from November 2007 through May 2008. *: denotes significance at α= 0.05.

A

Source of Variation	df	Pillai's Test Value	F	p
Time	3	.846	12.8	.003 *
Error	7			
Time*Morph	9	1.1	3.6	.132
Error	27			

B

Source of Variation	df	Pillai's Test Value	F	p
Time	3	.285	.932	.474
Error	7			
Time*Morph	9	.808	1.1	.392
Error	27			

C

Source of Variation	df	Pillai's Test Value	F	p
Time	3	.257	.805	.149
Error	7			
Time*Morph	9	.854	1.2	.179
Error	27			

Table 3.9 Tukey's HSD tests comparing concentrations of (A) Cyan, (B) Green, and (C) Red FP among colonies of *A. millepora* that changed versus did not change colour from November 2007 through May 2008. *: denotes significance at $\alpha = .05$.

A

	Morph	Red				Green				Red to Green				Green to Red			
Morph	Time	Nov	Dec	Jan	Feb	Nov	Dec	Jan	Feb	Nov	Dec	Jan	Feb	Nov	Dec	Jan	Feb
Red	Nov																
	Dec	1.00															
	Jan	1.00	1.00														
	Feb	1.00	1.00	1.00													
Green	Nov	.998	.996	1.00	.999												
	Dec	**.003***	**.002***	**.010***	**.004***	.073											
	Jan	**.000***	**.000***	**.000***	**.000***	**.000***	.162										
	Feb	**.000***	**.000***	**.000***	**.000***	**.000***	.401	1.00									
Red to Green	Nov	1.00	1.00	1.00	1.00	1.00	**.024***	**.000***	**.000***								
	Dec	1.00	1.00	1.00	1.00	1.00	**.039***	**.000***	**.000***	1.00							
	Jan	.596	.555	.847	.668	.995	.785	**.001***	**.006***	.895	.949						
	Feb	.750	.712	.937	.811	.999	.636	**.001***	**.003***	.959	.985	1.00					
Green to Red	Nov	1.00	1.00	1.00	1.00	1.00	.162	**.000***	**.001***	1.00	1.00	.994	.999				
	Dec	.998	.997	1.00	.999	1.00	.354	**.001***	**.002***	1.00	1.00	1.00	1.00	1.00			
	Jan	.747	.714	.921	.802	.998	.914	**.008***	**.027***	.947	.977	1.00	1.00	.996	1.00		
	Feb	1.00	1.00	1.00	1.00	1.00	.107	**.000***	**.000***	1.00	1.00	.980	.995	1.00	1.00	.987	

B

	Morph	Red				Green				Red to Green				Green to Red			
Morph	Time	Nov	Dec	Jan	Feb	Nov	Dec	Jan	Feb	Nov	Dec	Jan	Feb	Nov	Dec	Jan	Feb
Red	Nov																
	Dec	1.00															
	Jan	1.00	1.00														
	Feb	1.00	1.00	1.00													
Green	Nov	1.00	1.00	1.00	1.00												
	Dec	1.00	1.00	1.00	1.00	1.00											
	Jan	1.00	1.00	1.00	1.00	1.00	1.00										
	Feb	1.00	1.00	1.00	1.00	1.00	1.00	1.00									
Red to Green	Nov	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00								
	Dec	.988	1.00	1.00	1.00	.975	.981	1.00	0.99	1.00							
	Jan	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	.996						
	Feb	.580	.894	.963	.712	.504	.533	.739	.580	.585	.998	.593					
Green to Red	Nov	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	.902				
	Dec	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	.986	1.00			
	Jan	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	.996	1.00	0.77	1.00	1.00		
	Feb	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	.825	1.00	1.00	1.00	

C

	Morph	Red				Green				Red to Green				Green to Red			
Morph	Time	Nov	Dec	Jan	Feb	Nov	Dec	Jan	Feb	Nov	Dec	Jan	Feb	Nov	Dec	Jan	Feb
Red	Nov																
	Dec	1.00															
	Jan	1.00	1.00														
	Feb	1.00	1.00	1.00													
Green	Nov	1.00	1.00	1.00	1.00												
	Dec	1.00	1.00	1.00	1.00	1.00											
	Jan	1.00	1.00	1.00	1.00	1.00	1.00										
	Feb	1.00	1.00	.999	1.00	1.00	1.00	1.00									
Red to Green	Nov	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00								
	Dec	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00							
	Jan	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00						
	Feb	.310	.424	.810	.522	.162	.248	.326	.221	.296	.226	0.33					
Green to Red	Nov	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	.699				
	Dec	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	.736	1.00			
	Jan	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	.503	1.00	1.00		
	Feb	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	.504	1.00	1.00	1.000	

3.4 Discussion

3.4.1 Temporal differences in FP and CP content within colour morphs

The mild temperature profile of the 2007-08 summer, relative to summers when seawater temperatures exceeded the local 5-day bleaching threshold of Palm Island corals (Berkelmans and Willis 1999), afforded an opportunity to identify colour-morph specific baselines for temporal variation in the FP/CP content of healthy colonies of *Acropora millepora*. This information is vital for understanding typical FP/CP maintenance in coral tissues and for interpretations of FP/CP levels under temperature stress.

Throughout a 7-month sampling program over the warmer spring-summer months, only concentrations of CFP differed among the green, yellow and red colour morphs of *A. millepora,* suggesting that production of CFP, which typically comprises the greatest percentage of the FP pool within coral tissues (except in the March and April samples of the red morph), is the primary fluorescent response of *A. millepora* to seasonally varying environmental conditions. The high concentrations of CFP in the green morph, which were more than two-fold greater than those in the yellow morph and more than 10-fold greater than those in the red morph, indicate that the green morph consistently devotes a greater percentage of its energy budget to CFP production in comparison to either of the other two morphs. Approximately four-fold increases in mean concentrations of CFP between the February and March samples in all three colour morphs, suggests a population-wide response to an environmental parameter, most likely either temperature or light or their co-correlation (D'Angelo et a. 2008). Although temporal differences in mean FP concentrations were only

statistically significant for CFP in the yellow morph, changes in mean concentrations of the other two FPs meant that the proportional concentrations of the three FPs remained constant over the seven months in this morph. In contrast, the proportional abundance of FPs in the red morph changed between the February and March sampling times, reflecting a substantial rise in investment in GFP and RFP, and leading to an unusual pattern in which CFP concentrations were the lowest of the three FPs. In contrast, comparatively stable patterns in GFP and RFP concentrations over the seven months for all three colour morphs suggest that the green, yellow and red morphs have a relatively consistent strategy for investing in these two FPs.

It is possible that seasonal seawater temperature patterns contributed, at least in some minor way, to temporal changes in FP concentrations within colour morphs, although the three morphs appeared to differ in their responses to the mild summer temperatures experienced in this study. In general, mean FP concentrations were substantially lower in red colonies in the warmer summer months (November – February) than in the cooler months of March to May, a pattern in accord with molecular studies of GFP-homolog genes (GFP, RFP and CP), which have shown that FPs are typically down-regulated in thermally stressed corals (Dove et al. 2006; Smith-Keune and Dove 2008). Interestingly, *Symbiodinium* densities more than doubled between November and January in the red morph (and also in the other two colour morphs), suggesting that FP concentrations would be a more sensitive method of detecting sub-lethal temperature stress than bleaching for the red morph.

Stability in the proportional concentrations of FPs in the yellow morph, combined with a lack of consistent pattern in overall FP concentrations through time, highlights that the yellow morph may have a higher temperature stress threshold than the red morph. The much greater investment in FPs by the green morph, particularly

in CFP across most months compared to the red and yellow morphs, may have implications for further increased thermal tolerance of this colour morph, but further studies are needed to evaluate this hypothesis. As mature GFP-homologs have slow decay rates (~20 days for red pigments; Leutenegger et al. 2007), significant reductions in GFP-homolog transcription may not impair biological function in the short term, as this is not likely to manifest into lower FP abundance for some time (Bay et al. 2009a, b). Accordingly, I suggest that it is unlikely that FP down-regulation occurred in response to thermal stress in green and yellow colonies, and that relatively minor fluctuations in relative FP abundance are likely due to cycles in up- and down-regulation required to maintain appropriate levels of these proteins within coral tissues. However, as light levels were not measured throughout the observation period, the influence of seasonal variability in light levels cannot be eliminated as a potential influence on FP concentrations like the fluctuating CFP levels documented here.

In contrast to temporal patterns in FP abundance, CP abundance declined steadily throughout the study in all three colour morphs, with no differences in CP concentrations detected among colour morphs at any of the sampling times. Although biochemically related to GFPs and their homologs, CPs absorb substantial amounts of light but do not re-emit light (Dove et al. 2001; Smith et al. 2013). Assuming a photoprotective role (e.g. Wiedenmann et al. 1999; Salih et al. 2000; Dove et al. 2001; Smith et al. 2013) and with mean irradiance levels peaking annually during summer months on the GBR (Bureau of Meteorology), it is not surprising that CP levels declined along with easing water temperatures. However, it is interesting to note that concentrations of CPs show an inverse pattern when compared with symbiont densities. It has been hypothesised that expression of CPs in corals is linked to

densities of *Symbiodinium* in newly establishing symbiont populations. Because internal light fluxes decline with increasing symbiont density, the photoprotection offered by CPs is no longer required once healthy population densities are established, and consequently, costly CP production is shut-down (Smith et al. 2013). This mechanism may explain the decline in CPs observed under the otherwise benign temperature changes recorded between April and May. Although Smith et al. (2013) suggested CPs were found in greater concentrations at colony margins to promote colonisation of zooxanthellae populations in regions of new growth, the up-regulation of CPs throughout a colony could also aid in the re-population of zooxanthellae densities due to seasonal changes that are typical of corals (Fitt et al. 2000) and may explain the inverse relationship between zooxanthellae densities and CP concentrations measured in this study.

3.4.2 Temporal changes in colour within colonies

Only five colonies visibly changed colour during the four month study (n = 150 colonies), indicating that colour change in corals is rare, but possible (Todd et al. 2002a, b; Takabayashi and Hoegh-Guldberg 1997). Increases in FP concentrations in colonies that changed from red to green reached levels that are uncharacteristically high for red colonies (either in this comparison based on Pioneer Bay colonies, or as identified in Chapter 2, section 2.3.2), coupled with maintenance of an overall healthy appearance following the colour change, suggest that upregulation of FPs is associated with the maintenance of coral health. Despite the 3-fold increase in Cyan FP concentrations in red-green colonies, levels of fluorescence intensity did not reach those of green colonies. Nevertheless, changes in the proportions of pigments resulted

in phenotypic colour change in these colonies, highlighting the role of proportional FP concentrations in the manifestation of colour in corals.

The nearly 7.5-fold decrease in CFP levels in colonies that changed from green to red resulted in these colonies having CFP concentrations similar to those of red colonies by the end of the study. The occurrence of decreases in FP content over time, as colonies transitioned from a highly fluorescent state (green morph) to a weakly fluorescent state (red morph), following the appearance of disease signs or injury associated with detachment from the substratum and inversion, suggests that a change in colour in this direction is a useful indicator of compromised health.

3.4.3 Colour morph responses to seasonal temperature variation

General seasonal variation can affect corals negatively by creating oxidative stress during periods of high temperature and/or irradiance (Fitt and Warner 1995; Warner et al. 1996; Lesser and Farrell 2004; Lesser 2006) resulting in loss of photosynthetic pigments and/or algal cells, leading to pale or white appearance (Jokiel and Coles 1990; Brown 1997; Brown et al. 2000) and/or reduced carbon fixation by the symbiotic dinoflagellates (Brown et al. 1999). However, despite sea surface temperatures greater than 30^{o}C for much of December and January of 2007-08, *A. millepora* colonies examined here did not show signs of stress, as indicated by either visual loss of pigmentation or declines in *Symbiodinium* density. Although there are no light data available for the Palm Islands, the Bureau of Meteorology (BOM) reports higher than average cloud cover and therefore potentially decreased temperature/light stress throughout the 2007-08 summer for much of tropical North and Far North Queensland (BOM, 2009). This weather pattern likely led to lower than

usual levels of stress encountered by corals in Pioneer Bay (Orpheus Island Research Station Manager, pers. comm). In fact, *Symbiodinium* density increased from November to May, likely the result of seasonal changes in irradiance and temperature. The decline in CFP expression of green colonies between April and May could be a consequence of declining internal light fluxes with increasing *Symbiodinium* density (Smith et al. 2013). However, the changes in CFP concentration were non-significant and only occurred in one colour morph despite increases in *Symbiodinium* density across all colours.

The similarity in seasonal patterns in mean *Symbiodinium* density among the three colour morphs indicates that variation in FP concentrations among morphs is independent of *Symbiodinium* density in *A. millepora*. This finding contrasts with those of Dove (2004) and Klueter et al. (2006), who both found that, while colour morphs of *A. aspera* and *Montipora digitata* had similar symbiont densities, the more highly fluorescent morphs (the green morphs) often had higher densities of algal symbionts. The pattern of increasing *Symbiodinium* densities as seasonal temperatures declined by more than 9°C found here, is corroborated by an earlier study of *A. millepora* at two nearby sites in the Palm Islands (Moothien Pillay et al. 2005), which reported a similar inverse correlation between temperature and *Symbiodinium* density. Fitt et al. (2000) also found that *Symbiodinium* densities were highest in five species of coral from the Florida Keys and Caribbean during the coldest parts of the year, and this pattern was true for every coral species investigated from a range of depths. Similarly, several studies have demonstrated that symbiotic dinoflagellates grow faster in their hosts in the fall/wintertime when water temperatures are cooler than in the summer (Muller-Parker 1987; Fitt et al. 1993; Stimson 1997) in a wide range of

anthozoan taxa, including the reef corals *Pocillopora damicornis* and *Montastraea annularis*, and the anemone *Aiptasia pulchella*.

3.4.4 Conclusions

This is the first study to document and compare temporal changes in FPs and CP among intraspecific colour morphs in-situ. Fluctuations in FP content found in three colour morphs of *Acropora millepora* in Pioneer Bay likely reflect cyclical maintenance of optimal tissue concentrations of FPs, rather than a response to heat stress, as corroborated by the mild temperature profile, the maintenance of high mean *Symbiodinium* densities and the healthy appearance of colonies throughout the study. My finding that FP concentrations within coral tissues are temporally variable highlights the need for baselines against which FP concentrations during periods of thermal stress can be compared. Similarity in the pattern of declining mean chromoprotein abundance with declining temperatures among colour morphs indicates that highest investment in CP at highest summer temperatures fulfils a similar role in all three colour morphs. Colour change in *A. millepora* is correlated with changing concentrations of FP over time. Changes from the highly fluorescent green morph to the weakly fluorescent red morph signified declining health, whereas a colour change in the opposite direction did not correspond with any other visually perceivable change in coral health/condition.

Chapter 4

Bleaching condition varies among *Acropora millepora* colour morphs in the northern Great Barrier Reef

This chapter forms the basis for the publication:

Paley AS and Bay LK (2012) Bleaching condition varies among *Acropora millepora* color morphs. Proceedings of the International Coral Reef Symposium. 9A Coral bleaching and climate change.

Clockwise from Top: Blue, green, and red A. millepora morphs, Great Keppel Island, GBR

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4.0 Abstract

Understanding the mechanisms that underpin variation in bleaching susceptibility in corals is central to the conservation of reefs in the face of climate change. Fluorescent proteins (FPs) are pigments associated with the coral animal that are expressed by many coral species and govern colour polymorphisms in the wild. FPs can play photoprotective and anti-oxidant roles in corals; however, it is unclear whether the abundance of FPs affects bleaching susceptibility in the wild. Here, we analyse the bleaching condition of three colour morphs with different FP levels in *Acropora millepora* from the northern Great Barrier Reef (GBR). Colonies were surveyed at four sites during a cold winter and a warm summer. In winter, following unusually cold temperatures, proportionally fewer green (high FP) morphs paled (48%) than yellow (intermediate FP) and red (low FP) colour morphs (66 and 60%, respectively). In the warm summer, proportionally fewer green colonies bleached (20%) than the other two colour morphs (34 and 33% for yellow and red, respectively). This is the first demonstration of differential susceptibility to natural temperature stress among fluorescent colour morphs from similar habitats and highlights the functional importance of subtle intraspecific differences in FP content.

4.1 Introduction

Coral reefs are under threat from climate change that is causing chronic and acute stress to coral photosymbiosis and health, primarily through bleaching (Hoegh-Guldberg 1999). Coral bleaching, i.e. the loss or expulsion of endosymbiotic zooxanthellae (Donner et al. 2005), occurs most often in response to increased sea water temperature (Berkelmans 2009). Bleaching susceptibility is not equal among and within coral species (Buddemeier and Fautin 1993, Marshall and Baird 2000, Jones et al. 2004). Within populations, bleaching susceptibility often correlates with the algal symbiont type hosted by corals (Rowan 2004, Ulstrup et al. 2006, Abrego et al. 2008,), and the ability to swap algal partners for more tolerant strains can be important for coping with acute temperature stress (e.g., Baker 2003, Berkelmans and van Oppen 2006, Jones et al. 2008). However, differences in bleaching susceptibility still occur among populations without variation in symbiont strain, highlighting the importance of coral-host mediated factors that can influence colony level susceptibility to thermal stress (Baird et al. 2009, Barshis et al. 2010).

Fluorescent proteins (FPs) are found in many reef-building corals (Matz et al. 1999) and are an abundant component of the protein complement in coral tissues (Oswald et al. 2007). FPs contribute to the visual colour appearance of coral (Kawaguti 1944, Matz et al. 2002, Dove 2004) and underpin intraspecific colour polymorphisms (Mazel and Fuchs 2003, Alieva et al. 2008, Paley et al. in revision), which are common in many reef building coral species (Takabayashi and Hoegh-Guldberg 1995, Veron 2000). The functional role for FPs in coral tissues is not yet clear (see Leutenegger et al. 2007, Palmer et al. 2009 for review). A prominent hypothesis suggests FPs act as an ultraviolet-A (UVA)-screening shield to the algal-

symbionts, whereby FPs convert damaging excess UVA energy into longer, non-photosythentically active wavelengths or by filtering excessive photosynthetically active radiation (PAR) (Salih et al. 2000). Conversely, FPs may also have anti-oxidant properties (Bou-Abdallah 2006) and can help the host cope with radical oxygen species associated with bleaching stress (Lesser 2006). Despite their potential important role in coral physiology and stress tolerance, it is poorly understood how FPs affect bleaching susceptibility in corals (but see Salih et al. 2000, Dove 2004).

Here we compare the bleaching condition of three *Acropora millepora* colour morphs with different absolute FP levels among four sites in the Lizard Island Group on the northern Great Barrier Reef (GBR). Bleaching condition was compared among colour morphs during a colder and warmer than average winter and summer.

4.2 Material and Methods

4.2.1 Study Species and Sites

A. millepora is found on reefs throughout the Indo-Pacific and is common on inshore and mid-shelf reefs on the GBR. At least four colour morphs have been described in this species (Veron 2000, Cox et al. 2007, Alieva et al. 2008) and they commonly co-occur in shallow reef habitats. The visual colour appearance is explained by the concentration of three FPs (cyan, green and red, Paley et al. in revision). Green, yellow and red morphs contain the highest, intermediate, and lowest concentration of all three FPs corresponding to a 200% and 70% greater total FP

concentration in green morphs compared with red and yellow morphs, respectively (Paley et al. in revision).

Coral colony colour and bleaching surveys were conducted on four fringing reef flat sites (~3m depth) on the leeward side of Lizard, Palfrey and South Islands in the Lizard Island Group (14°40'S 145°28'E) (Fig 4.1). Lizard Island is located in the middle of the continental shelf, approximately 30 km off the far north Queensland coast in the northern region of the GBR. Southeasterly trade winds predominate for most of the year producing leeward and windward reef zonation. Here, three fluorescent colour morphs occupy the same habitats (Paley et al. in revision), thereby allowing us to study bleaching responses independent of habitat effects. At Lizard Island, *A. millepora* are dominated by symbiont *Symbiodinium* ITS-1 rDNA type C2, but up to 30% of corals also associate with type C2* in this region (Cooper et al. 2011). It is not yet clear whether C2 and C2* *Symbiodinium* have different levels of heat tolerance (Berkelmans and van Oppen 2006). We did not genotype the symbionts in the corals surveyed here and therefore assume that the distribution of dominant and background algal types was independent of coral colour as was the case in *A. millepora* at Orpheus Island (Cooper et al. 2011).

4.2.2 Temperature Data

Water temperature data for the Lizard Island region was acquired from the Australian Institute of Marine Science (http://data.aims.gov.au/aimsrtds) from data loggers deployed at 2.7m at Lizard Island. Daily maximum temperatures were averaged over a period of a year, from April to March, for all available data (~7 years)

spanning 2002-2010 and compared with daily maximum temperatures during sampling years (2008-2009).

4.2.3 Colour morph surveys

To examine their bleaching condition we surveyed 434 and 494 colonies of *A. millepora* in winter and summer, respectively, among 3 replicate 50 m x 10 m

Figure 4.1 Colour morph survey locations in the Lizard Island Group, northern Great Barrier Reef. Mermaid Cover, Research Point and Little Vicki's sites represent sheltered locations whereas Picnic Reef is more exposed to seasonal southeasterly trade winds.

haphazard belt transects at each site. Colony colour and bleaching condition was recorded for all *A. millepora* colonies, lying wholly or partially, within transect dimensions. Red, yellow, and green morphs were recognized based on the colour categories described in Paley et al. (in revision). Colony bleaching condition was determined using the CoralWatch Coral Health Chart (University of Queensland, see Chapter 3, Section 3.2.1). We used a scale of 1 - 6 where 1 = completely bleached and 6 = healthy/darkly pigmented using the chart's colour fields as a reference. For analysis, number categories were pooled into three bleaching categories (1 - 2 = Bleached, 3 - 4 = Pale and, 5 - 6 = Healthy (darkly pigmented)).

4.2.4 Data Analysis

To test whether bleaching condition differed between color morphs and seasons we used a 3-way ANOVA implemented in STATISTICA V12. We observed large differences in color morph abundances, so we first tested whether morph distribution and abundance was equal among sites using a 2-way ANOVA. To identify variation, among color morphs independent of their relative abundance we undertook our 3-way ANOVA analysis on proportional data and transects were pooled among sites as the replicate unit. A Kolmogorov-Smirnov test for normality ($p < 0.1$) and a Cochran's test ($p = 0.7$) for equality of variances confirmed conformation to ANOVA assumptions when data were squareroot transformed. Planned, post-hoc multiple comparisons of bleaching condition among color morphs and sampling times were interpreted with a Benjamini and Hochberg (1995) type I error correction at the false discovery rate of 0.0245.

4.3 Results

4.3.1. Colour morph abundance

The abundance of the three colour morphs was unequal ($F_{2,60} = 7.5$, $p = 0.001$) but the distribution of colour morphs was consistent among sites ($F_{6,60} = 0.1$, $p = 0.999$) where red colonies were always in highest abundance and green in lowest abundance (Table 4.1). The mean abundance among sites of red, yellow and green morphs was 130.3 (± 27), 64.3 (± 14) and 6.3 (± 3.2), respectively. Of total colonies surveyed 521 were red, 257 yellow and 48 green.

4.3.2. Mean summer and winter temperatures

Temperature regimes preceding winter and summer surveys exceeded the 7-year long-term average in this region (Fig. 4.2). During 2008/2009 daily maximum temperatures were below 23^{o}C for 11% of non-consecutive days compared with the long-term average where temperatures never reached below 23^{o}C (Fig 4.2). Similarly, daily maximum temperatures were above 32^{o}C for ~1.4% of non-consecutive days compared with the long-term average where temperatures never exceeded this temperature (Fig. 4.2).

4.3.3 Colony condition

The level of bleaching was ~40% greater in summer than winter and was dependent on colony colour (3-way interaction, p = 0.033; Table 4.2). Green colonies were more likely to pale than bleach during seasonal temperature changes when compared with red and/or yellow colonies, which more commonly bleached in summer. In winter, when temperatures were below average, between 34 and 54% of colonies were pale and only 0.5% of colonies were bleached (Fig. 4.3B-C). The proportion of pale red and yellow colonies was similar in winter (Fig 4.3B), but there was a trend of fewer pale green colonies compared to yellow (p = 0.048) but not red colonies (p = 0.236). In summer, between 22 and 50% of colonies bleached resulting

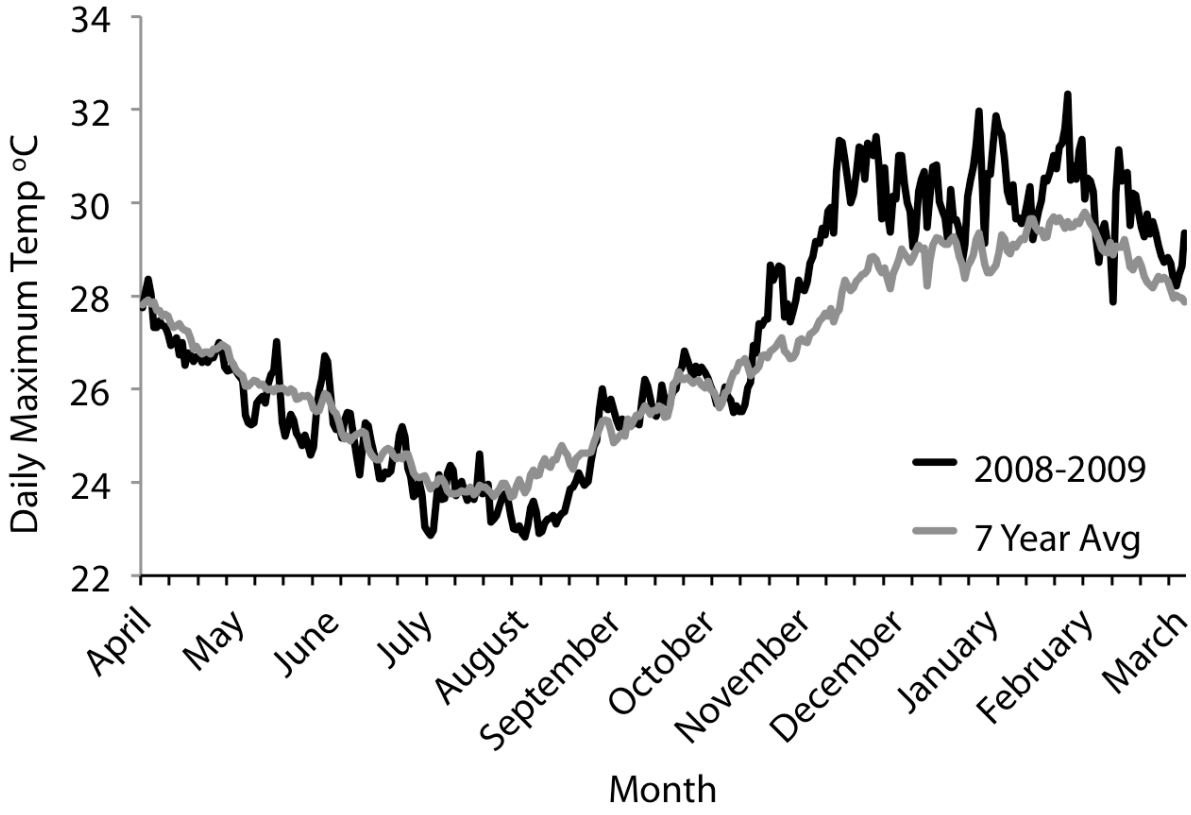

Figure 4.2 Daily maximum temperatures at Lizard Island from April to March. Temperature trends calculated from daily maximum values from 2002-2010.

in a further decline in healthy colonies of all colours from winter to summer (Fig. 4.3A). The proportion of pale red and yellow colonies was similar between winter and summer, but more green colonies became pale in summer compared to red ($p = 0.024$) but not yellow colonies ($p = 0.14$; Fig. 4.3B-C). A similar proportion of red and yellow colonies bleached in summer, however between 13.2 and 14.3% fewer green colonies bleached at this time compared with red and yellow colonies, respectively ($p = 0.01$ and $p = 0.007$, respectively; Fig. 4.3C).

4.4 Discussion

4.3.1 The influence of coral colour

Our results confirm that colony colour can influence the bleaching condition of corals (Gleason 1993, Salih et al. 2000, Dove 2004, Dove et al. 2006). Depth and light environment can affect FP abundance, and as a consequence coral colour is typically brighter (highly fluorescent) in shallow, high-light habitats and more drab (non-fluorescent) in deeper, low-light habitats (Gleason 1993, Takabayashi and Hoegh-Guldberg 1995). In our study, colours with high FP levels bleached less in shallow water than corals with low to intermediate FP levels at the same depth. Our results demonstrate for the first time that bleaching condition differs amongst fluorescent colour morphs within the same habitat. This finding highlights the value of considering fluorescent colour variation in future investigations into host-mediated stress tolerance and adaptive responses to climate change.

4.4.2 Local temperature regimes and bleaching condition

Corals live close to their thermal thresholds and commonly bleach when temperatures exceed long-term averages by only a few degrees (Jokiel and Coles 1990, Berkelmans 2002). Temperatures deviated substantially from those recorded between 2002 and 2010 in both our summer and winter sample and we recorded moderate levels of coral paling and bleaching in *A. millepora* (Fig. 3) and other branching pocilloporid and acroporid species (personal observations). These genera and growth forms are widely recognised for their bleaching sensitivity (e.g., Marshall and Baird 2000). The timing of bleaching was consistent with a temperature-driven response although other environmental parameters presumably varied between sampling times due to the field setting of our study.

Coral bleaching is a general stress response and can occur in response to colder, as well as warmer, than normal conditions (Coles and Jokiel 1997, Hoegh-Guldberg et al. 2005). For example, Saxby et al. (2003) observed similar physiological symptoms in cold and heat stressed coral including reduction of algal cell densities and loss of efficiency at photosystem II. Temperatures in August were lower than the long term average by 0.8°C, therefore, cold stress may explain why a substantial proportion of colonies (46 to 66%) were pale during the winter survey period. Then, from November to February temperatures routinely exceeded the long term average by 2-3^{o}C. Thermal history can be an important predictor of the health of corals, including the temperature regimes during winter months (e.g. Heron et al. 2010, Bruno et al. 2007). A colder-than-average winter may have pre-stressed corals and increased their susceptibility to bleaching during the warmer than average summer.

Table 4.1 Raw colour morph abundance data by site and season in the northern GBR.

Site	Trsct	Count Abundance (Winter)			Count Abundance (Summer)		
		Green	Yellow	Red	Green	Yellow	Red
Mermaid Cove	1	0	4	19	1	17	45
	2	2	8	18	3	15	33
	3	3	24	36	1	18	32
	4	0	1	1			
Research Point	1	4	7	23	4	17	36
	2	4	6	12	1	22	29
	3	2	7	11	1	8	23
Little Vicki's	1	3	19	31	4	15	30
	2	4	16	29	4	24	49
	3	3	6	6			
Picnic Reef	1	1	1	8	0	6	20
	2	1	3	2	0	7	8
	3	1	7	4	1	3	14

Table 4.2 Three-way factorial ANOVA of bleaching condition between seasons and among colour morphs of *A. millepora* at Lizard Island. *Denotes significance at $\alpha = 0.05$.

Source of Variation	SS	df	F	p	
Season	.005	1	.6	.457	
Morph	.014	2	.7	.498	
Condition	3.78	2	195.8	.000	*
Season*Morph	.004	2	.2	.814	
Season*Condition	4.14	2	214.5	.000	*
Morph*Condition	.071	4	1.8	.137	
Season*Morph*Condition	.110	4	2.8	.033	*
Error	.521	54	.1		

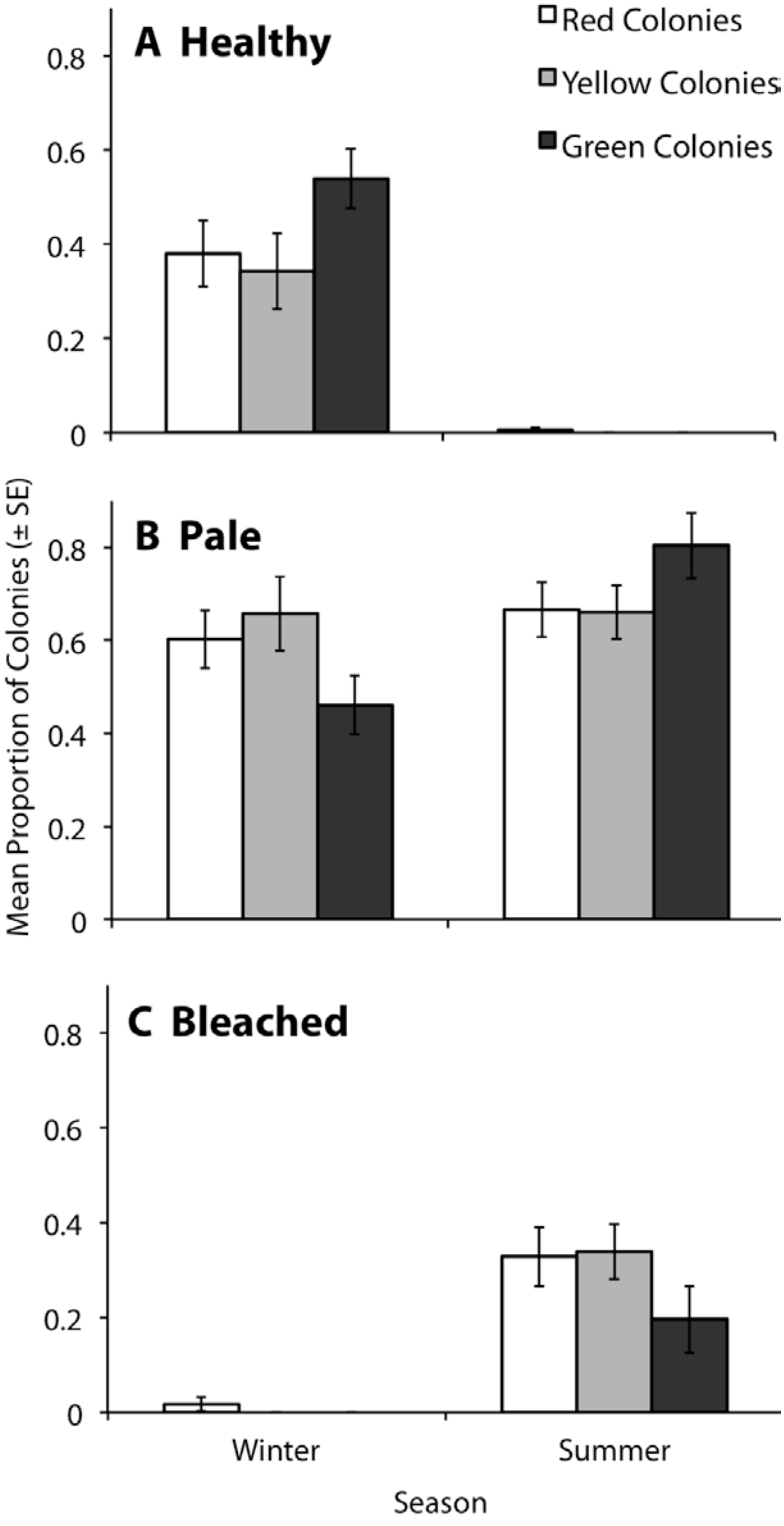

Figure 4.3 Mean proportion (±SE) of healthy, pale and bleached *A. millepora* colonies, by colour, in August 2008 (winter) and March 2009 (summer) in the Lizard Island Group.

4.4.3 Different bleaching susceptibility among colour morphs

Red and yellow colonies displayed similar bleaching condition in summer and winter despite having low and intermediate FP levels (Paley et al. in revision). In contrast, green morphs, with high FP levels had a lower proportion of bleached colonies in summer and lower incidence of paling in winter. This demonstrates different bleaching susceptibilities among fluorescent coral colour morphs that occupy similar habitats in the field. Our results therefore extend the laboratory findings of Salih et al. (2000) that coral morphs with high FP levels have higher bleaching tolerance compared to weakly or non-fluorescent morphs. This contrasts with experimental evidence that high FP levels confer a hypersensitivity to thermal stress (Dove 2004). Our field surveys suggest that green colonies are less likely to bleach during thermal stress possibly due to enhanced photoprotection (Salih et al. 2000) or antioxidant properties (Bou-Abdallah 2000) resulting from a greater abundance of FPs. The symbiont type and/or background symbiont levels were not quantified here (nor in Salih et al. 2000, Dove 2004) and remain potentially important sources of variation in bleaching condition (e.g., Berkelmans and van Oppen 2006). At present no information is available on associations between symbiont type and host colour and FP content, however, this must be considered in future studies.

4.4.4 Testing the importance of FP content in response to climate change

Our results suggest that the lower bleaching susceptibility of green colonies was a result of their higher FP concentration. Understanding of the costs and benefits

of FPs in coral-host tissues will be strengthened by long term studies of temporal FP variation in tagged colonies combined with the response of coral colour morphs under experimentally controlled heat stress. The ability to express FPs in high levels is likely to be important in determining the response of corals to environmental stress. An improved understanding of coral-host mediated processes in bleaching tolerance will enhance our knowledge of potential mechanisms of adaptation to climate change.

Chapter 5

Bleaching susceptibility differs among *Acropora millepora* colour morphs exposed to experimental heat stress

Table of Contents

5.0 Abstract

Coral fluorescent proteins (FPs) are hypothesised to influence stress tolerance in a number of ways, however, the potential for the thermal stress response to vary among sympatric colour morphs, as well as the role of colour polymorphisms in coral populations are not well understood. Here, a laboratory-based heat stress experiment was conducted to determine if susceptibility to, and recovery from mild (30°C) and severe (32°C) thermal stress differed among colour morphs of the coral *Acropora millepora*. Branches of the highly fluorescent green morph had greater bleaching resistance to severe thermal stress (64% bleached) compared with those of weakly and intermediately fluorescent red and yellow morphs (100% and 85% bleached, respectively), and also had greater recovery from exposure to severe thermal stress (54% compared with 0% and 15% healthy after recovery, respectively). Although *Symbiodinium* densities declined similarly among branches of the three colour morphs in all temperature treatments, densities only increased during the recovery phase in branches of the green morph, indicating its greater potential to recover from thermal stress within the timeframes observed. Three symbiont communities were identified among *A. millepora* colour morphs (C1, C2, C2+D), however, no symbiont type × colour morph associations were detected, and bleaching susceptibility was not affected by symbiont type. Overall, FP concentrations declined in all branches in comparison to initial field levels, indicating a response to aquarium conditions, however, patterns in FP concentrations differed among the temperature treatments. FP concentrations initially increased in all colour morphs in response to mild (30°C)

temperature stress, but then decreased during the recovery period. In contrast, FP concentrations decreased in all colour morphs in response to severe (32°C) thermal stress, but then increased by 2.5- to 15-fold during the recovery period. This study confirmed that high tissue concentrations of FPs correlate well with thermal stress tolerance, and highlights the contribution of host-mediated mechanisms in governing bleaching susceptibility.

5.1 Introduction

Global warming and ocean acidification are radically changing marine environments (Munday et al. 2013), with great biological consequence for ecosystems like coral reefs (Hoegh-Guldberg and Bruno 2010; Doney et al. 2012). Increased sea temperatures have contributed to coral loss globally, and are likely to lead to further net declines in coral cover, increased disease prevalence and changes in community structure (e.g. De'ath et al. 2012, Burge et al. 2014). The capacity of reef corals to physiologically acclimatize and/or rapidly adapt to changes in environmental conditions is paramount, but remains the subject of much debate (Fabricius et al. 2011; Logan et al. 2013; Palumbi et al. 2014).

Many examples of local acclimatisation to environmental extremes illustrate the potential for marine invertebrates to cope under a changing climate (e.g. amphipods, grastropods and acsidians: Sanford and Kelly 2011; corals: Coles and Riegl 2013). Reef-building corals have a suite of mechanisms that may aid their capacity to acclimatise to predicted increases in ocean temperatures, including adjusting the type and number of *Symbiodinium* cells harboured (Baker et al. 2001; Mieog et al. 2008) or by adjusting the complement of UV-screening mycosporin-like

amino acids (MAAs), antioxidant molecules (Falkowski and Dubinsky 1981, Hoegh-Guldberg and Smith 1989; Raina et al. 2013), photoprotective fluorescent proteins (FPs), and non-fluorescent chromoproteins (CPs) (Kawaguti 1969; Wiedenmann et al. 1999; Salih et al. 2000; Dove et al. 2001; Khang and Salih 2005; Cox et al. 2007; Smith et al. 2013). A central goal of research focused on corals under a changing climate is to determine the extent to which these mechanisms enhance the scope for coral populations and communities to persist under altered climate scenarios. Critical to this will be our ability to identify and manage their capacity for resilience, i.e., the ability of an organism to resist and recover from disturbances (Mumby et al. 2014; Hughes et al. 2013; McClanahan et al. 2002; 2014).

It has long been established that variability in bleaching susceptibility in response to thermal stress exists among coral taxa (Marshall and Baird 2000; Loya et al. 2001; van Hooidonk et al. 2013). Coral bleaching, or the whitening of corals, is caused by disruptions to the coral-*Symbiodinium* association, resulting from oxidative stress (Lesser 2006) and damage to the photosynthetic apparatus of the symbiont and ultimately to the coral host (Jones et al. 1998; Tchernov et al. 2004). The consequences of coral bleaching are manyfold; bleached corals suffer from reduced tissue regeneration (Meesters and Bak 1993), growth rates (Goreau and Macfarlane 1990), reproductive success (Jokiel and Guinther 1978; Ward and Hoegh-Guldberg 2000), and increased susceptibility to disease (Muller et al. 2008; Burge et al. 2014). While differences in bleaching susceptibility among corals are partly explained by growth form (Marshall and Baird 2000; Loya et al. 2001) and symbiont type (Baker 2001; Rowan et al. 2004), and may contribute to their capacity to recover (e.g. Jones et al. 2008), host factors play a large but comparatively unexplored role in governing recovery capacity (e.g. Fitt et al. 2000; Grottoli et al. 2004, 2006). Further research

on the role of the coral host in bleaching recovery may provide important insights into mechanisms of resilience in reef corals.

There is considerable evidence demonstrating that FPs located in coral tissues confer increased thermal tolerance on the coral host (Salih et al. 2000, Paley and Bay 2012 but see Dove 2004) and may be used as a proxy for health (D'Angelo et al. 2008; Roth and Deheyn 2013) or as biomarkers (D'Angelo et al 2012) in some coral species. Colour polymorphisms exhibited by many coral species are determined by variation in the relative contributions of different FP types in their tissues (Mazel and Fuchs 2003, Alieva et al. 2008, Chapter 2), but the extent to which such variation might enable a coral species to respond to variation in thermal stress is unknown. Previous research on the role of coral FPs in thermal stress has focused on either a single colour variant within a species (e.g. Smith-Keune and Dove 2008), a comparison between fluorescent and non-fluorescent (not containing FPs/CPs) morphs (e.g. Dove 2004), or on a single GFP-like protein chromophore (Smith et al. 2013; Roth and Deheyn 2013). Understanding the role of coral FPs in the thermal stress response requires inclusion of the full range of intraspecific colour variation and FPs present. Such studies would greatly advance our understanding of the function of FPs in mitigating thermal stress in corals.

Although the role of *Symbiodinium* type has received much attention in investigations of intraspecific variation in bleaching susceptibility (e.g., Berkelmans and van Oppen 2006; Jones et al. 2008), the role of intraspecific colour polymorphisms in the thermal stress response of a coral population has largely been overlooked (Baird et al. 2009). To advance current understanding of intraspecific variation in thermal tolerance among closely related coral colour morphs (Salih et al. 2000; Dove 2004; Paley and Bay 2012), there is need for targeted studies of variation

in thermal bleaching resistance within coral populations characterised by distinctive colour polymorphisms.

My objectives in this chapter are to determine if bleaching resilience differs among intraspecific colour morphs of the coral *Acropora millepora*, and if any variation in the bleaching response detected is influenced by the associated *Symbiodinium* assemblage. Populations of *A. millepora* exhibit distinctive colour polymorphism, and the FPs and algal symbiont types the species associates with are well documented. Colonies of *A. millepora* on the GBR contain the full complement of the three most common FPs (cyan, green, and red; Wiedenmann 1999; Matz et al. 1999; Oswald 2007; Alieva et al. 2008)), and associate with four types of dinoflagellate endosymbionts in the genus *Symbiodinium* (ITS-1 types C1, C2, C2* and D1; Cooper et al. 2011), making this species an ideal model for studies of the role of colour polymorphism in the thermal stress response of corals.

5.2 Materials and Methods

5.2.1 Laboratory set-up and coral collection

Three colour morphs of *A. millepora* were exposed to thermal stress in a temperature- and photoperiod-controlled experimental facility at Orpheus Island Research Station (OIRS). Seawater was filtered to 1 μm and delivered to experimental tanks after two UV sterilization treatments, at a rate of ~20 litres/hour in a flow-through system (Fig. 5.1A). Two air stones enhanced circulation and oxygenation of each tank. The photoperiod regime was 14 hour light: 10 hour dark commencing at 06:30, with light levels maintained at 150-250 μmol quanta $m^{-2}s^{-1}$

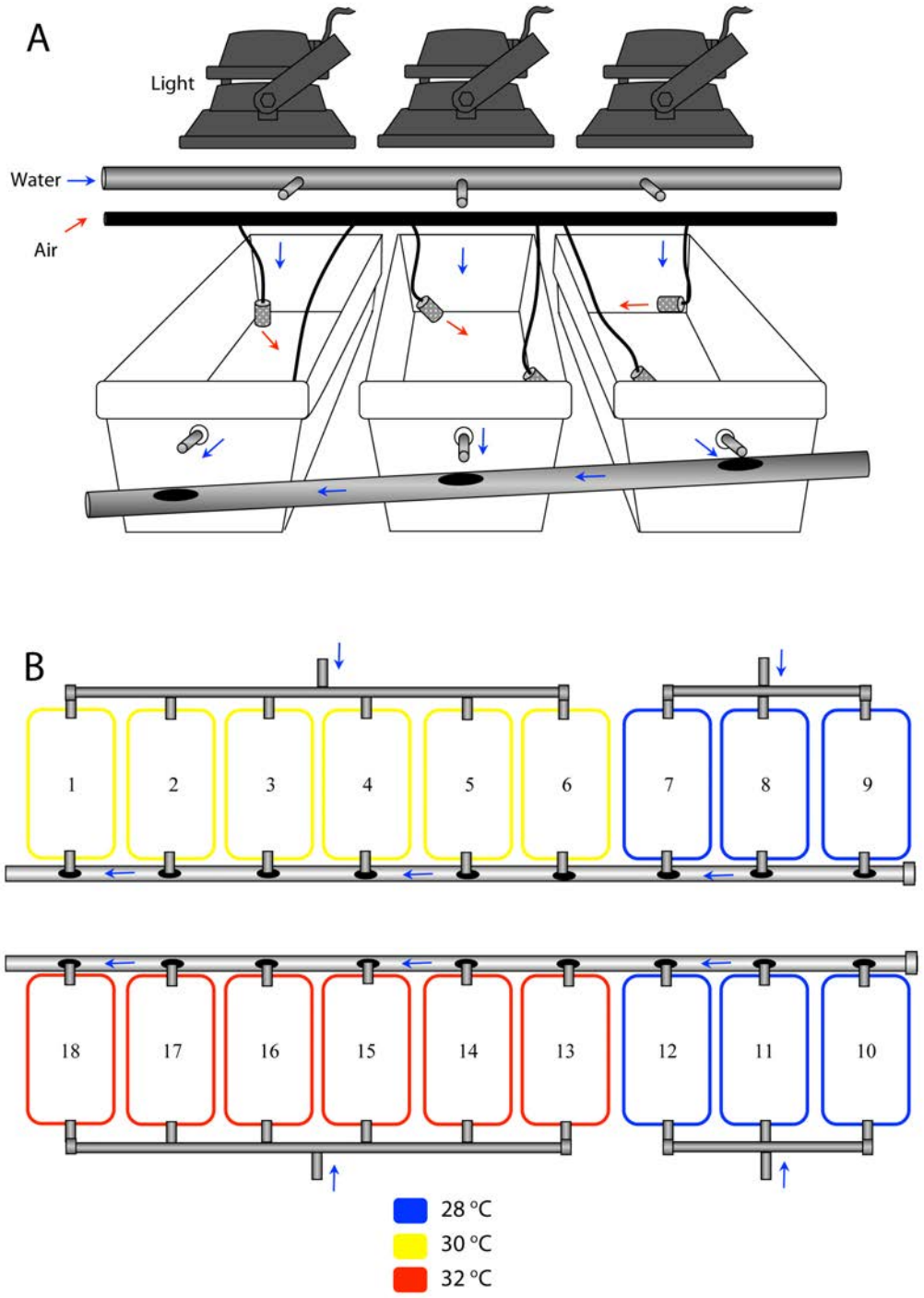

Figure 5.1 Schematic of flow-through experimental set-up: (A) tank set-up; and (B) experiment design showing seawater temperature treatments, where blue represents tanks in the 28°C treatment, yellow represents tanks in the 30°C treatment, and red represents tanks in the 32°C treatment. Thirty-six branches (two of each coral genotype; n = 18 colonies) were placed within each tank.

using one 150 watt MH 20k halide coral lamp (Sylvania Oracle Wide Beam) per tank. Experimental light levels were similar to those measured in the field under cloud cover (between 200-450 μmol quanta $m^{-2}s^{-1}$) at the time of collection.

Three temperature treatments were established: a control, which remained at ambient temperature (tanks 1-6), a mild (30°C) thermal stress treatment (tanks 7-12), and a severe (32°C) thermal stress treatment (tanks 13-18) (Fig 5.1B). Control tanks were heated with 240 volt and 2000 Watt semi-submersible heaters (Control Distributions Pty. Ltd.), controlled by a Carel remote keypad. Seawater for the 30°C

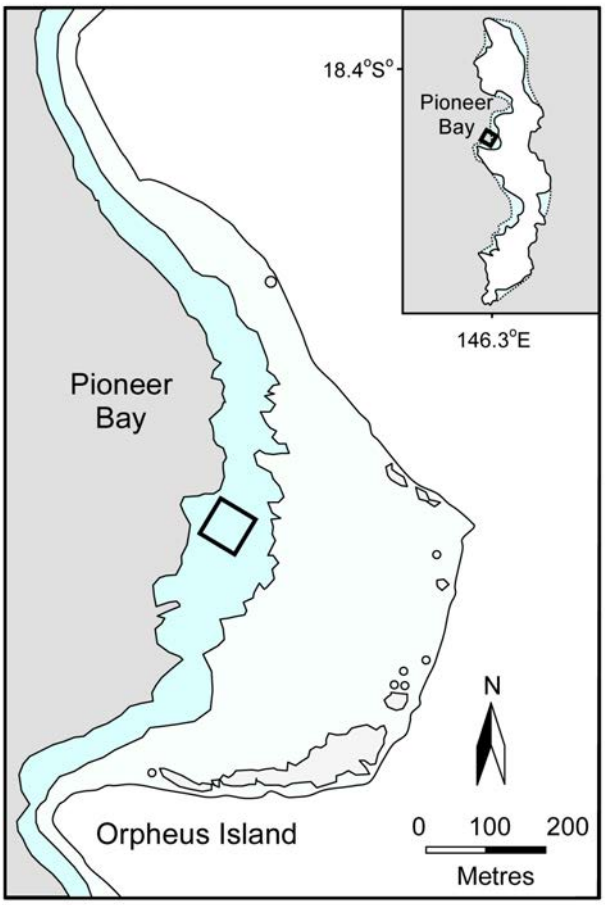

Figure 5.2 Schematic showing the fringing reef (in blue) in Pioneer Bay, Orpheus Island. Rectangle represents approximate sampling area along the reef crest.

Figure 5.3 Profile (A) and aerial (B) views of experimental branches of *Acropora millepora* affixed onto perspex racks.

and 32^{o}C treatments flowed through Carrier (30RH Series Air to Water) heater/chiller units with 210 kwr capacity; treatment temperatures were digitally set and maintained with 0.5^{o}C accuracy. Tank temperatures were monitored thrice daily using a Digi-Key digital thermometer.

Replicate fragments (~12 cm diameter) were collected from 18 genetically distinct colonies of *Acropora millepora*, which included six colonies from each of the three colour morphs (red, yellow and green), from an area 75 m^{2} on the outer reef flat in Pioneer Bay (Fig. 5.2). Donor coral colonies occurred sub-tidally, at 2.5m to 4m depth below tidal datum, and were exposed to a 3.5 tidal range and a mid-day light intensity range of 300 to 890 μmol quanta m^{-2}s^{-1} at the time of collection. Colony fragments were further divided into small branches (nubbins) in the laboratory to produce 42 nubbins per colony, and affixed onto clear perspex racks using plastic clamps (Fig. 5.3).

One rack was placed in each of the 18 experimental tanks. Of the 42 branches collected from each colony, six were immediately snap frozen in liquid nitrogen to serve as a baseline sample of colony condition in the field, hereafter referred to as the

"Field" sample. One frozen branch from each colony was transferred to 100% EtOH for down-stream symbiont genetic analysis (see section 5.2.4 below). Thirty-six branches were then haphazardly positioned on each rack, such that two replicates of each coral genotype were represented in each tank (n = 648 nubbins in total).

5.2.2 Sampling regime

Coral health was scored daily throughout the experiment using the CoralWatch Coral Health Chart (see description in Chapter 2, Section 2.2.2). Additionally, sampling occurred at three times throughout the experiment: (1) at the end of the acclimation period and prior to temperature increase on day 1 of the experiment, which will be referred to as the "Acclimation" sample; (2) at the end of the heating period and prior to temperature reduction on day 16, referred to as the "Heating" sample; and (3) at the end of the recovery period at ambient temperature on day 44, referred to as the "Recovery" sample. Acclimated samples provided a baseline for branch condition prior to the start of the experiment, Heating samples served as indicators of coral condition under varying regimes of thermal stress, and Recovery samples provided indicators of branch condition following nearly four weeks at ambient temperatures. At each sampling time point, four replicate branches from each coral genotype were randomly sampled (using a random number chart) from across the six tanks within each temperature treatment. The random sampling resulted in an uneven distribution of replicate genotypes among tanks within a temperature treatment, but the total number of branches for each genotype remained equal among temperature treatments. Following collection, the branches were immediately snap-frozen in liquid nitrogen and stored at -80^{o}C for down-stream laboratory analysis.

5.2.3 Acclimation and experimental design

Branches were allowed to acclimate in experimental tanks for a period of 7 days. During acclimation, any branches showing signs of poor health (defined as paling using the coral health chart as a guide) were replaced (~80 branches, of which 84% were red branches) with healthier counterparts of the same genotype, which had been kept as supplementary material in an adjacent laboratory room with the identical tank set-up. During acclimation, photochemical efficiency of the coral symbiont *Symbiodinium* was measured by variable chlorophyll *a* fluorescence analysis using a Diving-PAM (pulse-amplitude-modulation) chlorophyll fluorometer. Maximum (Fv/Fm) and effective ($\Delta F/Fm^1$) quantum yields of photosystem II (PSII) were recorded. The ratio of these measurements provides an estimation of the excitation pressure over PSII (Q_m), calculated as:

Equation 5.2.1

$$Q_m = 1 - \left(\frac{\Delta F/Fm'}{Fv/Fm} \right)$$

where ΔF/Fm', or the light-adapted maximum fluorescence, is a reading taken while the PSII reaction centres are open and Fv/Fm, or the dark-adapted maximum fluorescence, is the proportion of the maximum possible fluorescence used for photosynthesis (Iglesias-Prieto et al. 2004; Ulstrup et al. 2007). Q_m was used as a proxy for the physiological performance of the coral symbionts (Ulstrup et al. 2007). Q_m values close to 0 indicated that photosynthesis was light limited, even at high irradiance, and values close to 1.0 indicated closure of PSII at high irradiance (e.g. due to photoinhibition). Daily mean Q_m was calculated from two repeated

measurements per branch of (1) effective and (2) maximum quantum yield taken two hours after lights were switched on and off (08:30 and 20:30 hrs), from 50 randomly selected experimental coral branches. These Q_m values were used as indicators of the coral holobiont's overall condition during the acclimation period. Measurements were taken using a Diving-PAM with Diving-F fiberoptics (Heinz Walz Pty. Ltd.) The measuring light intensity level was raised to and maintained at '3' in order to give optimal fluorescence signals for all coral nubbins. The fiberoptic probe was oriented so all readings were taken at a 90 degree angle to the coral surface. Q_m measurements acquired during the acclimation period were compared among days in a one-way ANOVA (Levene's test for homogeneity of variances: $p = 0.736$; Test for normality: $p < 0.1$) and significance of main effects tested with Tukey's HSD post-hoc tests. The ratio of maximum to effective photosynthetic yields (Q_m) varied significantly throughout the 7 day acclimation period ($F_{6,321} = 3.2$; $p = 0.004$), reflecting an adjustment to experimental light conditions (Table 5.1 Fig. 5.4). Although Q_m initially varied 22-fold between days 1 and 3 ($p = 0.029$) of the acclimation period, Q_m did not vary after day 4 (Table 5.2; Fig. 5.4). These data, combined with observations of new growth around plastic clamps, the absence of mucus or tissue necrosis, and lack of bleaching in visual surveys of branch condition indicated that experimental branches were in a stable condition within tanks. By the end of acclimation, all coral branches in experimental tanks received a visual health score of 5 or 6 (out of a total possible score of 6).

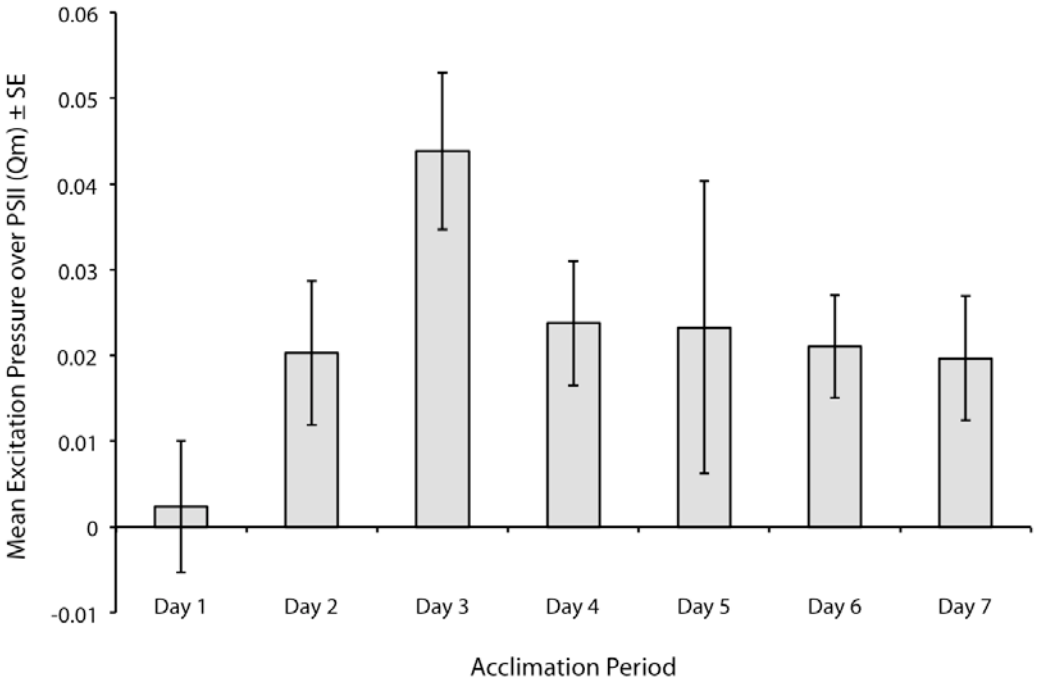

Figure 5.4 Daily measurements of *Symbiodinium* photochemical efficiency throughout the acclimation period, based on the daily ratio of mean maximum (Fv/Fm) and effective ($\Delta F/Fm^1$) quantum yields of photosystem II (Q_m).

Table 5.1 One-way ANOVA comparing the ratio of maximum to effective photosynthetic yields (Q_m) among days during the acclimation period. *: denotes significance at $\alpha = .05$.

Effect	SS	df	MS	F	p	
Day	0.084909	6	.014	3.22	.004	*
Error	1.412148	321	.004			

Table 5.2 Tukey's HSD tests comparing the ratio of maximum to effective photosynthetic yields (Q_m) among days during the acclimation period. *: denotes significance at $\alpha = .05$.

Day	1	2	3	4	5	6	7
1							
2	.980						
3	**.029***	**.002***					
4	.960	.550	.317				
5	.604	.153	.821	.988			
6	1.00	.879	.124	.999	.876		
7	.870	.410	.706	1.00	1.00	.981	

On day 8, seawater temperatures in Treatments 1 and 2 were increased at a rate of $\leq 1^{o}$C per day (0.1^{o}C per hour from 08:00 to 16:00 hrs) over a period of 4.5 days, until reaching final treatment temperatures of 30^{o}C and 32^{o}C, respectively. Experimental temperatures were maintained for a further 10 days, after which they were lowered at a rate of $\leq 2^{o}$C per day (0.2^{o}C per hour from 08:00 to 16:00 hrs) over 2.5 days or until seawater in experimental tanks reached ambient temperatures (28^{o}C). Branches were maintained in ambient seawater for a further 24 days.

5.2.4 *Symbiodinium* identification

DNA Extraction: DNA was extracted from samples of coral fragments approximately 1 cm^3 in size. Coral fragments were blotted dry to remove ethanol, and subsequently added to 750 µl of grinding buffer (final concentrations: 100 mM Tris pH 9.0, 100 mM EDTA, 1% SDS, 100 mM NaCl, Milli-Q water) containing 20 µ1 of proteinase-K ($20mg.ml^{-1}$), vortexed and incubated at 65^{o}C for three hours. After incubation, samples were vortexed, put on ice, and 187.5 µl of 5 M KOAc was added to a final concentration of 1 M. Samples were mixed and left on ice for 10 min, then centrifuged for 20 min at 16,100 rcf at room temperature. The supernatant was transferred to a new tube with 600 µl Isopropanol added and gently mixed. After five min at room temperature, samples were centrifuged at 18,000 rcf for 15 min and the supernatant carefully removed. 150 µl of 70% EtOH was then added, mixed gently and centrifuged for five minutes at 18,000 rcf. The supernatant was again removed, and the resulting pellet left to dry for five minutes and then resuspended in 100 µl of 10 mM Tris (pH 9). DNA was quantified using a NanoDrop 1000 Spectophotometer

(Thermo Scientific). DNA quality was checked by running 2 µL on a 1% Agarose gel with TAE buffer (90 V, 25 min) against a 100 bp ladder.

PCR: Polymerase chain reaction (PCR) amplification of the LSU rDNA region was carried out using the following primer pairs and conditions. *LSU rDNA:* One microlitre of DNA was added to 14 µl of a PCR cocktail mix, consisting of 0.3 µM of each primer (forward and reverse) at a concentration of 0.3 µM, 1 × reaction buffer (MyTaq, Bioline), 0.03 U *Taq* DNA polymerase (MyTaq, Bioline). The PCR profile consisted of an initial denaturation step of 5 min at 95^{o}C, followed by 35 cycles of 20 sec at 95^{o}C; 30 sec at 60^{o}C; and 90 sec at 72^{o}C. Products were spun in a table top centrifuge and the quality of PCR products was examined by running 3 µl on a 0.8% high resolution TAE-agarose gel (100 V, 60 min).

Restriction digestion and gel electrophoresis: 10 µl of PCR product were combined with 10 µl of restriction enzyme mix (0.3 µl Taq1 Restriction Enzyme (10 units/µl), 2 µl 10x restriction buffer (New England Biolab) and 7.7 µl dH2O). Samples were digested at 65^{o}C for 2 hours after van Oppen et al. (2001). Ten µl of digest was run on a 2% high resolution TAE-agarose gel (100 V, 60 min) (Astral). LSU of known *Symbiodinium* samples (ITS-1 types C1, C2, and D1) were amplified, restriction digested and electrophoresed as above and used to designate symbiont types in our samples.

5.2.5 Fluorescent pigment protein and fluorescence analysis

Protein concentrations and relative FP abundance measurements were acquired for 216 branches from the four sampling times following the methods outlined in detail in Chapter 2 (see Sections 2.2.3. and 2.2.4.).

5.2.6 *Symbiodinium* density

The densities of *Symbiodinium* cells within coral tissues were quantified from 216 nubbins sampled throughout the experiment. Methods of extraction and quantification of *Symbiodinium* and coral surface area followed the protocols and procedures outlined in Chapter 3 (see Section 3.2.3).

5.2.7 Statistical analyses

Bleaching Condition: The proportions of healthy, pale and bleached branches in the control (28^{o}C), mild stress (30^{o}C), and severe stress (32^{o}C) temperature treatments were compared among colour morphs at 2 time points: at the height of bleaching (following 16 days of heating and 6 days of recovery), and after 23 days at ambient temperatures. Due to random sampling within each temperature treatment, replicate branches of each genotype became unevenly distributed across tanks. Therefore, the total numbers of healthy, pale and bleached branches by colour across replicate tanks were tested in a Chi^2 test of homogeneity and independence in contingency tables. Note that ANOVA comparisons of the mean proportion of healthy, pale and bleached branches per temperature treatment for each colour morph, were not considered appropriate, given the uneven distribution of colour morphs across tanks within each temperature treatment. Mean daily temperatures were averaged across tanks within treatments and plotted against daily condition data throughout the experiment.

Symbiodinium cell Density and FP Concentration: Changes in *Symbiodinium* density and FP (CFP, GFP and RFP) content were compared among corals that differed in symbiont complement (C1, C2 and C2+D), colour and temperature treatment using a series of 2-factor ANOVAs. The proportional change in *Symbiodinium* density or FP content was calculated as the change in values over the time period of interest, divided by the starting value for that period. Mean proportional changes were compared between the following samples: (1) Acclimation and Heating, (2) Heating and Recovery, and (3) Field and Recovery sampling times (implemented in R (Team RDC 2013)). Raw data conformed to the assumptions of homogeneity of variances and normality. Significance of fixed factors was estimated using Tukey's HSD tests, also implemented in R.

Identity of Symbiodinium community: Multiple symbiont types were identified in the coral samples used in the experiment. To compare associations between *A. millepora* colour morphs and *Symbiodinium* complements, the proportions of samples having C1, C2, or C2+D *Symbiodinium* assemblages in each colour morph (green, yellow, red) were compared in a Chi^2 test of homogeneity and independence in a contingency table. To determine the effect of *Symbiodinium* assemblage on bleaching condition in the 32^oC treatment, two 1-factor ANOVAs were used to compare the proportion of fragments in each tank that were either (1) healthy, or (2) bleached among the three *Symbiodinium* assemblage types. Additionally, to determine if *Symbiodinium* assemblage influenced susceptibility to or recovery from bleaching, the health status of branches (healthy, pale, bleached) was compared with *Symbiodinium* community (C1, C2, C2+D) during the acclimation, heating and recovery phases of

the experiment, using Chi^2 tests of homogeneity and independence in contingency tables.

5.3. Results

5.3.1 Branch condition

The proportion of healthy, pale, and bleached branches in the severe (32°C) temperature stress treatment differed among colour morphs at the end of both the heating ($X^2_4 = 31.7$; $p = 0.000$) and recovery ($X^2_4 = 26.9$; $p = 0.000$) phases (Table 5.3). Changes in the proportions of healthy, pale, and bleached branches occurred two days after seawater temperatures reached 32°C, when branches of all colours began to pale in appearance (Fig. 5.5). The red morph was the first to bleach, with bleaching apparent after 9 days of severe temperature stress, followed by the green (after 10 days of heating) and yellow morphs (after 11 days of heating; Fig. 5.5A-C). After 14 days of heating at 32°C, 86% of red, 79% of yellow and 58% of green branches were scored as "bleached" (Fig. 5.5). The peak of bleaching for red and yellow branches (100% and 87.5%, respectively) was recorded two days after water temperatures were lowered to ambient, and one day after water temperature was lowered for green branches (when 66.7% were scored as "bleached"; Fig. 5.5A-C). After a 20-day recovery period at ambient temperature, 75% of red and yellow branches were scored as "bleached", compared with 54% of green branches (Fig. 5.5). The remaining 46% of green branches were scored as "healthy", compared with 17% of yellow and 0% of red branches (Fig 5.5A-C).

In the mild (30 ^{o}C) temperature stress treatment, the proportion of healthy and pale branches did not differ among colour morphs at the end of either the heating or recovery phases (Table 5.3). Overall, 26% of branches (40% of red, 23% of yellow and 14.5% of green branches) were scored as "pale" after 14 days of heating (Fig. 5.6). No branches in the 30^{o}C treatment were scored as "bleached" at any point throughout the experiment (Fig. 5.6).

Similarly, at ambient (control) temperatures (28 ^{o}C), the proportion of healthy and pale branches did not differ among colour morphs (Table 5.3). Overall, nearly 96% of branches remained "healthy" throughout the heating phase (92% of red, 98% of yellow, and 98% of green) (Fig. 5.7A-C). Although 20% of yellow branches in the ambient treatment appeared pale during the recovery phase (compared to 12.5% of red and 6.3% of green branches), no branches of any colour appeared bleached at any time in this control treatment (Fig. 5.7A-C).

In summary, paling and bleaching was observed among branches of all colour morphs in the elevated temperature treatments (between 66-100% bleached at 32^{o}C, and between 5-45% were pale at 30^{o}C), but green branches suffered the least bleaching and showed strongest recovery within tanks heated to 32^{o}C (Fig. 5.5C). Branches of the red morph were the most sensitive to experimental heating, bleaching 1 and 2 days before branches of other colour morphs, and attaining the greatest levels of paling (40%) and bleaching (100%) within both elevated temperature treatments. Moreover, branches of the red morph showed the weakest signs of recovery after exposure to temperatures of 32^{o}C (Figs. 5.5 and 5.6).

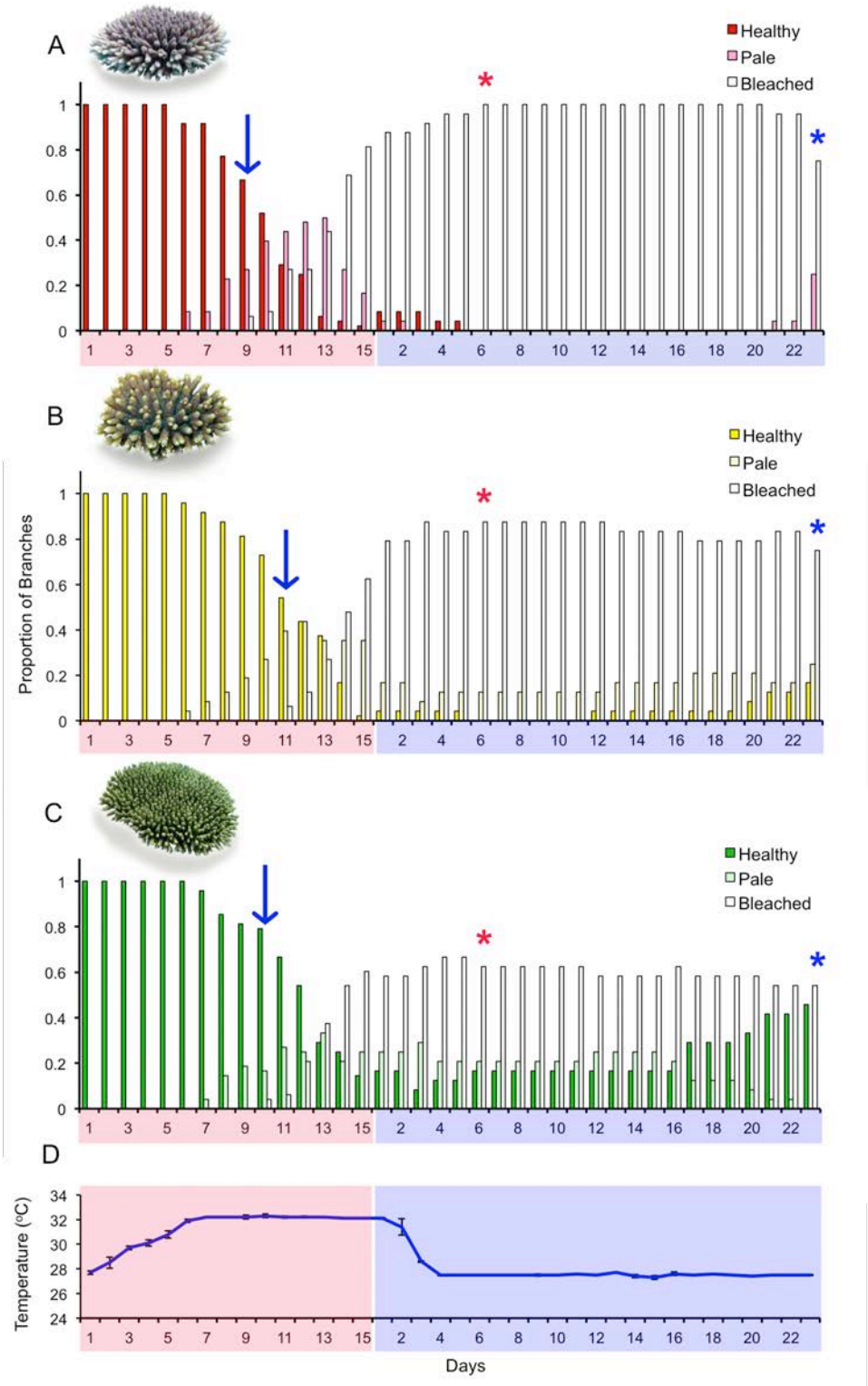

Figure 5.5 Health status of experimental branches in the severe (32°C) stress treatment during the heating (pink box) and recovery (blue box) phases. The proportions of branches in healthy, pale and bleached categories are shown for the (A) red, (B) yellow, and (C) green morphs, as well as (D) mean daily seawater temperatures (±SE). Blue arrows indicate onset of bleaching. Red and blue asterisks indicate the peak of bleaching and end of recovery phase, respectively.

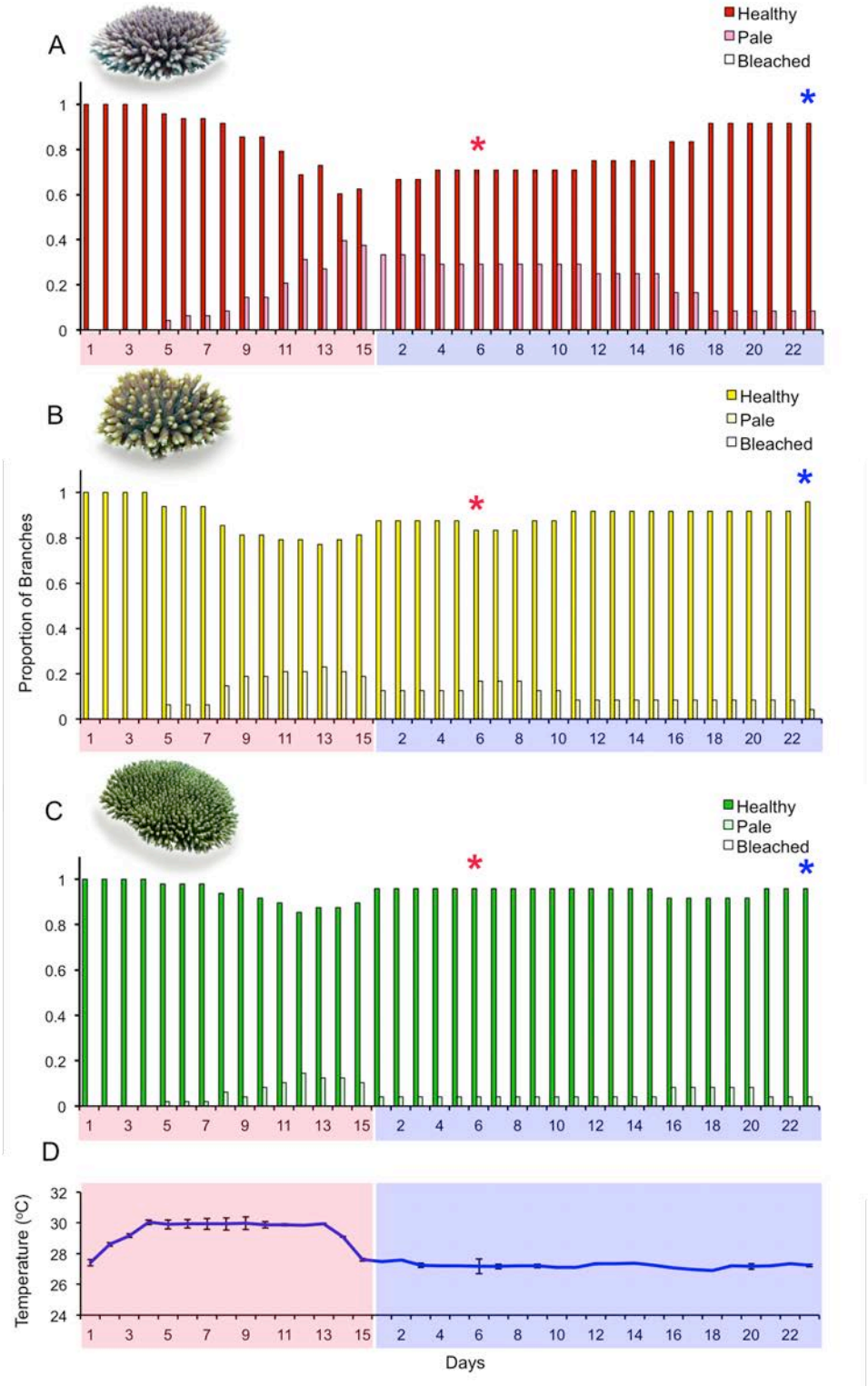

Figure 5.6 Health status of experimental branches in the mild (30°C) temperature treatment during the heating (pink box) and recovery (blue box) phases. The proportions of branches in healthy, pale and bleached categories are shown for the (A) red, (B) yellow, and (C) green morphs, as well as (D) mean daily seawater temperatures (±SE). The peak of bleaching in 32°C tanks and end of recovery phase are indicated by red and blue asterisks, respectively.

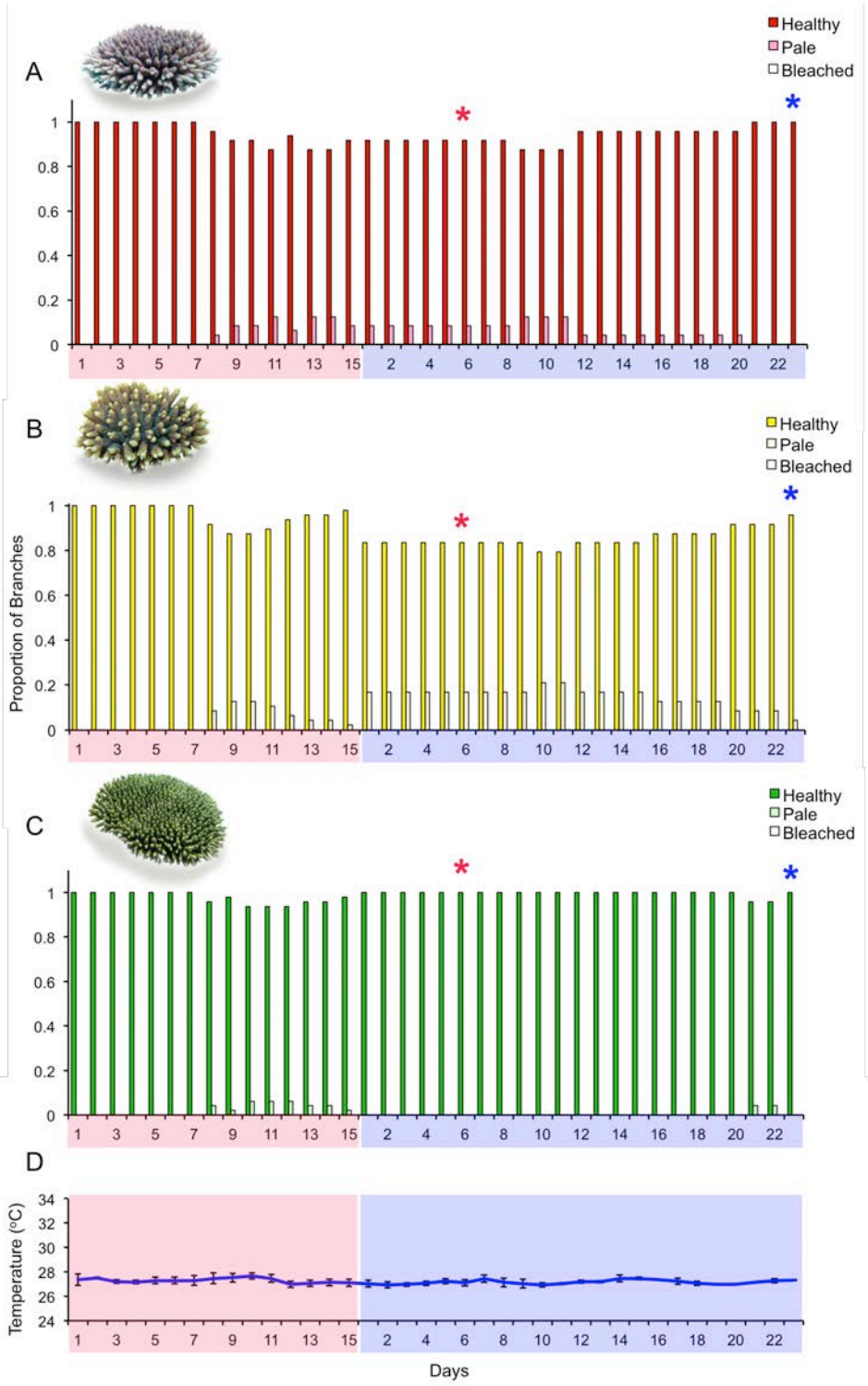

Figure 5.7 Health status of experimental branches in the control (28°C) temperature treatment during the heating (pink box) and recovery (blue box) phases. The proportions of branches in healthy, pale and bleached categories are shown for the (A) red, (B) yellow, and (C) green morphs, as well as (D) mean daily seawater temperatures (±SE). The peak of bleaching in 32°C tanks and end of recovery phase are indicated by red and blue asterisks, respectively.

Table 5.3 Chi^2 contingency tables comparing the proportions of branches per colour morph in each of three health categories (healthy, pale, bleached) for the severe (32^{o}C) stress treatment, and in each of two health categories (healthy, pale) for the mild (30^{o}C) stress and control (28^{o}C) treatments, after each of the heating and recovery phases.

32°C

	X^2	df	p	
Heated	31.7	4	.000	*
Recovering	26.9	4	.000	*

30°C

	X^2	df	p
Heated	4.8	3	.187
Recovering	0.1	3	.992

28°C

	X^2	df	p
Heated	0.3	3	.960
Recovering	0.1	3	.992

5.3.2 Host-*Symbiodinium* Associations

Despite colour morphs of *A. millepora* associating with three types of *Symbiodinium* assemblages (C1, C2 or mixed C2 + D), the proportion of colonies associating with each of the three assemblage types did not differ among colour morphs ($X^2_4 = 1.6$; p = 0.809). Of the 17 colonies sampled to characterise *Symbiodinium* assemblages, ten (59%) contained *Symbiodinium* type C2 assemblages, and five (29%) contained *Symbiodinium* type C1 assemblages (Fig. 5.8). Only two yellow colonies contained mixed type C2 and D assemblages, representing a low (17%) percentage of the colonies sampled. Overall, *Symbiodinium* assemblage type did not influence the health status of branches (i.e. the percentage of colonies in each health category; Fig. 5.9), during either the acclimation, heating or recovery phases of the experiment (Table 5.4). An ANOVA provided further support for this conclusion, as the proportion of branches that bleached ($F_{2,14} = 0.868$; p = 0.441) or were healthy ($F_{2,14}$ =

0.063; p = 0.940) was similar among branches that were associated with each of the three *Symbiodinium* assemblage types. Likewise, the percentages of branches that were healthy, pale, or bleached were similar among branches harbouring different symbiont assemblages, during each of the acclimation, heating and recovery phases (Fig. 5.9). During the acclimation phase, 99-100% of coral branches that were associated with each of the three *Symbiodinium* assemblage types were healthy (Fig. 5.9A). After heating to 32°C and recovery at ambient temperature, between 44 and 65% of branches appeared bleached (Fig. 5.9B-C).

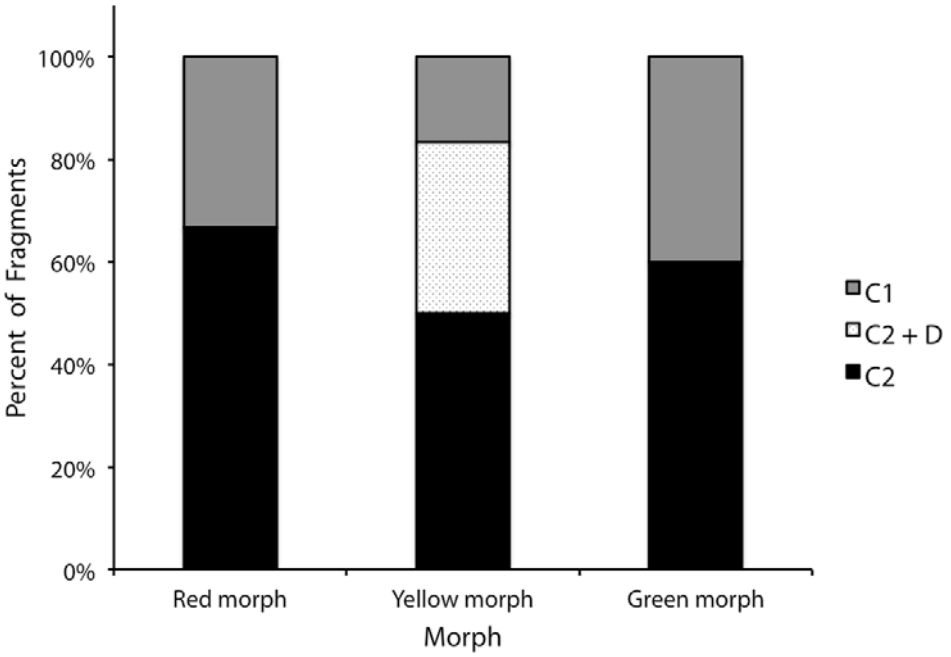

Figure 5.8 Comparison of the percent of experimental branches within each colour morph harbouring assemblages comprised of C1, C2 or mixed C2 and D *Symbiodinium* types. (Red: n = 6 branches; Yellow: n = 6 branches; Green: n = 5 branches).

Table 5.4 Chi^2 contingency tables comparing the proportion of branches within each health status category (healthy, pale, bleached) in the severe (32°C) stress treatment among branches associating with three *Symbiodinium* assemblage types (C1, C2, mixed C2 + D), as characterised after each of the acclimation, heating and recovery phases.

	X^2	df	p
Acclimated	0	4	1.00
Heated	0.4	4	.983
Recovering	2.7	4	.609

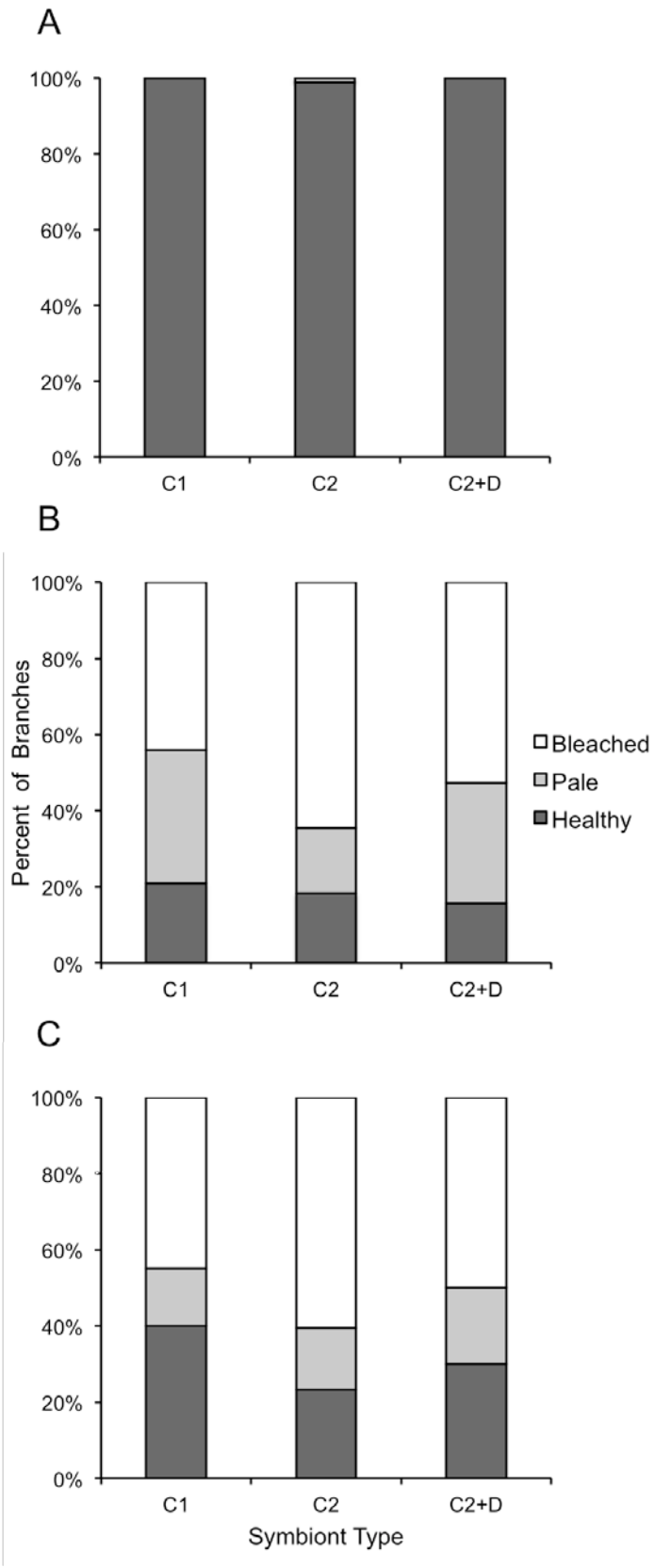

Figure 5.9 Comparison of the percent of coral branches in the severe (32°C) stress treatment that were healthy, pale or bleached among branches that were associated with each of three *Symbiodinium* assemblage types (C1, C2, mixed C2 + D) during (A) acclimation, (B) heating, and (C) recovery. (C1: n = 5 branches, C2: n = 10 branches, C2+D: n = 2 branches).

5.3.3. Changes in *Symbiodinium* densities during three experimental phases

Proportional changes in *Symbiodinium* cell densities in experimental branches from the beginning to the end of the heating phase (i.e. between acclimated and heated samples; Fig. 5.10A) differed significantly among temperature treatments (Table 5.5A), in particular, between the control and 32^{o}C treatments ($p = 0.012$; Table 5.6). *Symbiodinium* cell densities in the 32^{o}C treatment declined by 9 to 64% for all colour morphs, whereas proportional changes in *Symbiodinium* cell densities in the control (28^{o}C) treatment varied from 15% declines to increases of more than 120% (Fig. 5.10A). Statistically, overall patterns in the proportional changes of *Symbiodinium* cell densities in the three treatments were similar among colour morphs over the heating phase (Table 5.5A), although mean changes were negative in all treatments for green colonies, but positive for yellow and red colonies in the control and mild stress treatments (Fig 5.10A).

In the recovery phase, *Symbiodinium* cell densities did not differ among colour morphs nor among experimental treatments (i.e. between the heated and recovered samples; Table 5.5B). Interestingly, the green morph was the only colour morph to show an increase in *Symbiodinium* density during recovery, with cell densities increasing by nearly 33% in the 32^{o}C treatment, compared with declines of 14% and 36% for yellow and red branches, respectively (Fig. 5.10B). Over the timeframe of the whole study (i.e. between field and recovered samples), changes in *Symbiodinium* densities were similar among colour morphs and among experimental temperature treatments (Table 5.5C). Over all treatments, *Symbiodinium* densities between these two sampling times declined 3% to 68% for all colour morphs, with the exception of cell densities of yellow branches in the 30^{o}C tanks, which increased by 62%, and of green branches in 28^{o}C tanks, which increased by 27% (Fig. 5.10C).

Table. 5.5 Two-factor ANOVAs comparing proportional changes in *Symbiodinium* cell densities among colour morphs and temperature treatments between (A) acclimated and heated samples, (B) heated and recovered samples, and (C) field and recovered samples. *: denotes significance at α = .05.

A

Source of Variation	df	SS	F	p	
Morph	2	2.50E+09	.423	.658	
Temperature	2	2.50E+10	4.2	.021	*
Morph x Temperature	4	1.80E+09	.152	.961	
Error	45	1.30E+11			

B

Source of Variation	df	SS	F	p
Morph	2	0.8	1.100	.353
Temperature	2	.504	.696	.506
Morph x Temperature	4	1.1	.727	.578
Error	45	16.4		

C

Source of Variation	df	SS	F	p
Morph	2	1.5	1.500	.232
Temperature	2	2.8	2.9	.067
Morph x Temperature	4	2.7	1.400	.248
Error	45	21.9		

Table 5.6 Tukey's HSD tests comparing proportional changes in *Symbiodinium* cell densities among temperature treatments over the timeframe of experimental heating (i.e. between acclimated and heated samples). *: denotes significance at α = .05.

Treatment	28°C	30°C	32°C
28°C			
30°C	.537		
32°C	**.012***	.142	

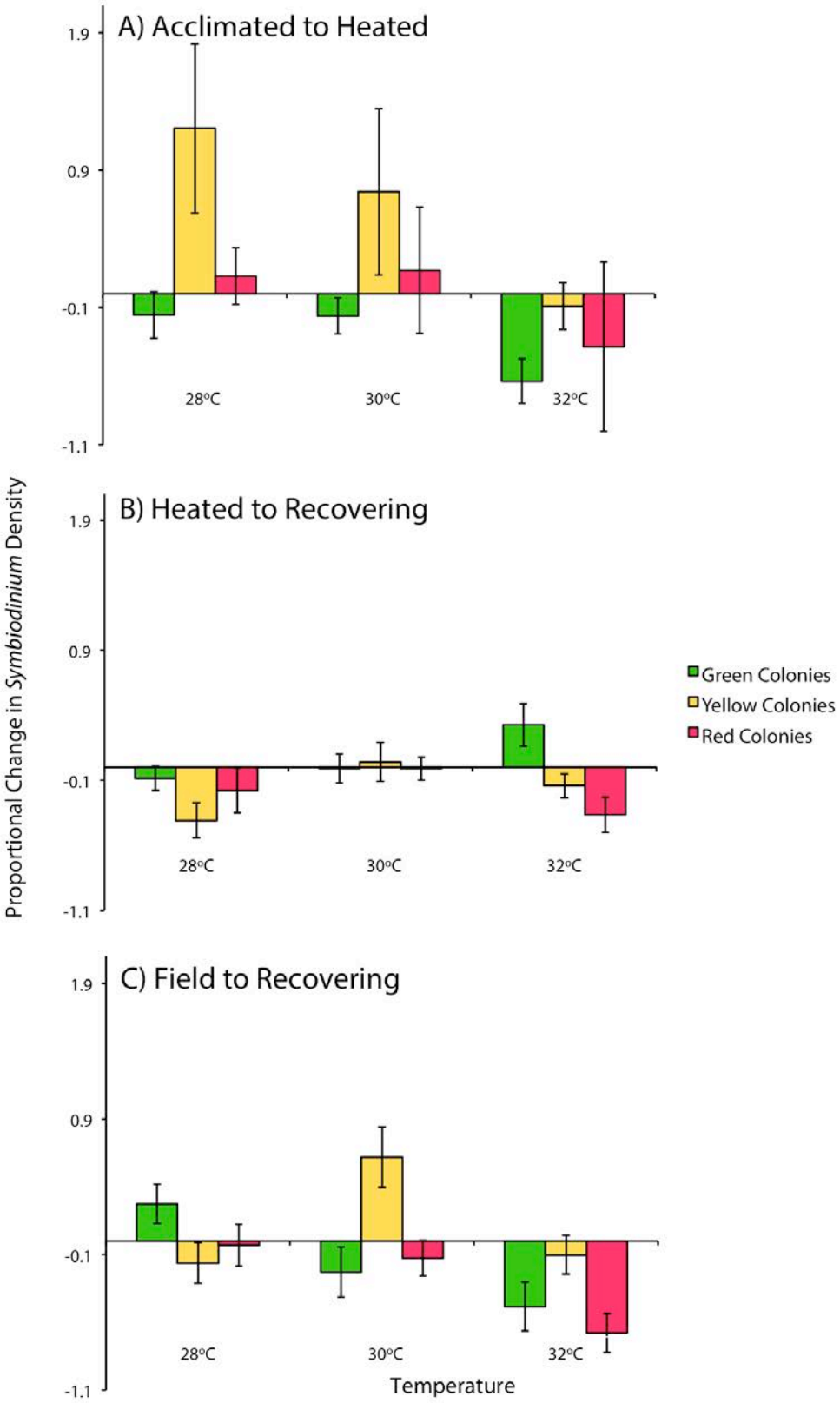

Figure 5.10 Comparisons of proportional changes in *Symbiodinium* cell densities among three colour morphs (green, yellow, red) of *Acropora millepora*, and among three experimental treatments (28°C, 30°C, 32°C), in each of three experimental phases, i.e. between: A) acclimated and heated samples, B) heated and recovered samples, and C) field and recovered samples.

5.3.4 Fluorescent protein levels

Over the period of experimental heating (i.e. between acclimated and heated samples), FP levels in experimental branches typically increased in the control and 30^{o}C treatments, but declined in the 32^{o}C treatment (Fig. 5.11A-C), a pattern that was consistent among colour morphs for all three FP types (CFP, GFP, RFP; Table 5.7A-C). In the control treatment, increases in concentrations of the three FPs of 16% to 180% were observed across all three colour morphs, with the exception of GFP levels in the green morph, which declined by 11% (Fig. 5.11A). Similarly in the 30^{o}C treatment, increases in FP concentrations of 48% to 150% were observed for all colour morphs, with the exception of CFP and GFP in red branches, which declined by 8 and 19%, respectively (Fig 5.11B). In the 32^{o}C treatment, the proportional change in FP concentration was much lower, ranging from -5% to -32% for all colour morphs (Fig. 5.11C). Differences in proportional changes in FP concentrations among experimental temperature treatments for RFP (Table 5.7C) reflected significant declines in RFP in the 32^{o}C treatment compared to both the control ($p = 0.016$) and 30^{o}C ($p = 0.023$) treatments (Table 5.8; Fig 5.11C).

Proportional changes in the concentrations of all three FPs over the recovery period (i.e. between heated and recovered samples; Fig 5.12) differed significantly among temperature treatments (pCFP = 8.9e-05; pGFP =8.7e-05; pRFP =0.000; Table 5.9A-C), and these patterns were consistent among colour morphs (Table 5.9A-C). Specifically, concentrations of CFP, GFP and RFP differed between the 32^{o}C treatment and both the control and 30^{o}C treatments (Table 5.10A-C). In the control treatment, concentrations of the three FPs declined by 5% to 33% in all three colour morphs (Fig. 5.12A-C). Similarly, in the 30oC treatment, changes in FP

concentrations were comparatively small, ranging from 18% declines to 15% increases. In contrast, concentrations of the three FPs increased by 98% to 260% in the 32^{o}C treatment (Fig. 5.12A-C).

Proportional changes in the concentrations of all three FPs from the beginning to the end of the study (i.e. between field and recovered samples; Fig 5.13) differed among temperature treatments, and patterns of proportional change were again similar among the three colour morphs (Table 5.11A-C). FP concentrations in recovered samples from the control and 30^{o}C treatments declined by a similar proportion (by 46% to 83%) in comparison to concentrations measured in field samples (Fig. 5.13 A-B). In contrast, proportional declines in FP concentrations in the 32^{o}C treatment (-67% to +5%) were significantly less than those in both the control and 30^{o}C treatments. This pattern was consistent across the three FPs, with the exception of RFP concentrations in the 32°C treatment, which differed only with concentrations in the control treatment (Table 5.12; Fig. 5.13C). Despite typically lower FP concentrations in recovered branches than in the initial field samples, FP concentrations in five green branches (i.e. in ~27% of branches) in the 32^{o}C treatment increased by 2% to 200% compared to those of initial field samples, and constituted the only cases where FP concentrations increased following exposure to thermal stress (Fig. 5.13C)**.**

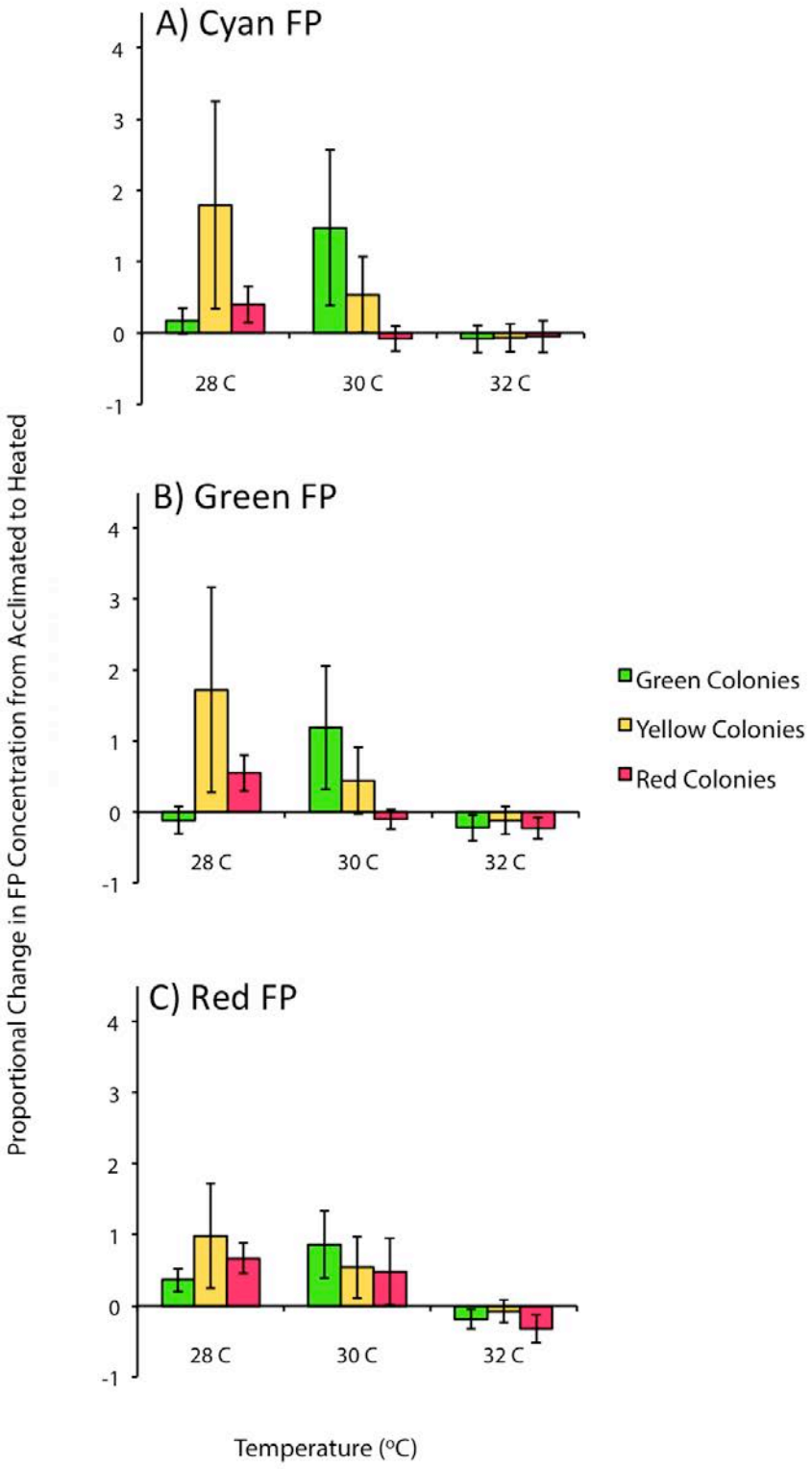

Figure 5.11 Comparisons of mean proportional changes (± SE) in FP concentrations over the period of experimental heating (i.e. between acclimated and heated samples) among control, 30°C, and 32°C temperature treatments and among three colour morphs of *A. millepora* for A) CFP, B) GFP, and C) RFP.

Table 5.7 Two-factor ANOVAs comparing mean proportional changes in FP concentrations over the period of experimental heating (i.e. between acclimated and heated samples) among colour morphs and temperature treatments for (A) CFP, (B) GFP and (C) RFP. *: denotes significance at α = .05.

A) CFP

Source of Variation	df	SS	F	p	
Morph	2	4.05	2.0	.461	
Temperature	2	7.6	3.8	.241	
Morph x Temperature	4	12.6	3.2	.314	
Error	45	115.7	2.6		

B) GFP

Source of Variation	df	SS	F	p	
Morph	2	3.3	.758	.474	
Temperature	2	9.2	2.1	.135	
Morph x Temperature	4	10.1	1.2	.343	
Error	45	98.4			

C) RFP

Source of Variation	df	SS	F	p	
Morph	2	0.4	.227	.798	
Temperature	2	8.6	4.9	.012	*
Morph x Temperature	4	1.4	.406	.803	
Error	45	39.9			

Table 5.8 Tukey's HSD tests comparing the proportional changes in FP concentrations over the period of experimental heating (i.e. between acclimated and heated samples) among temperature treatments for RFP. *: denotes significance at α = .05.

Treatment	28°C	30°C	32°C
28°C			
30°C	.989		
32°C	**.016***	**.023***	

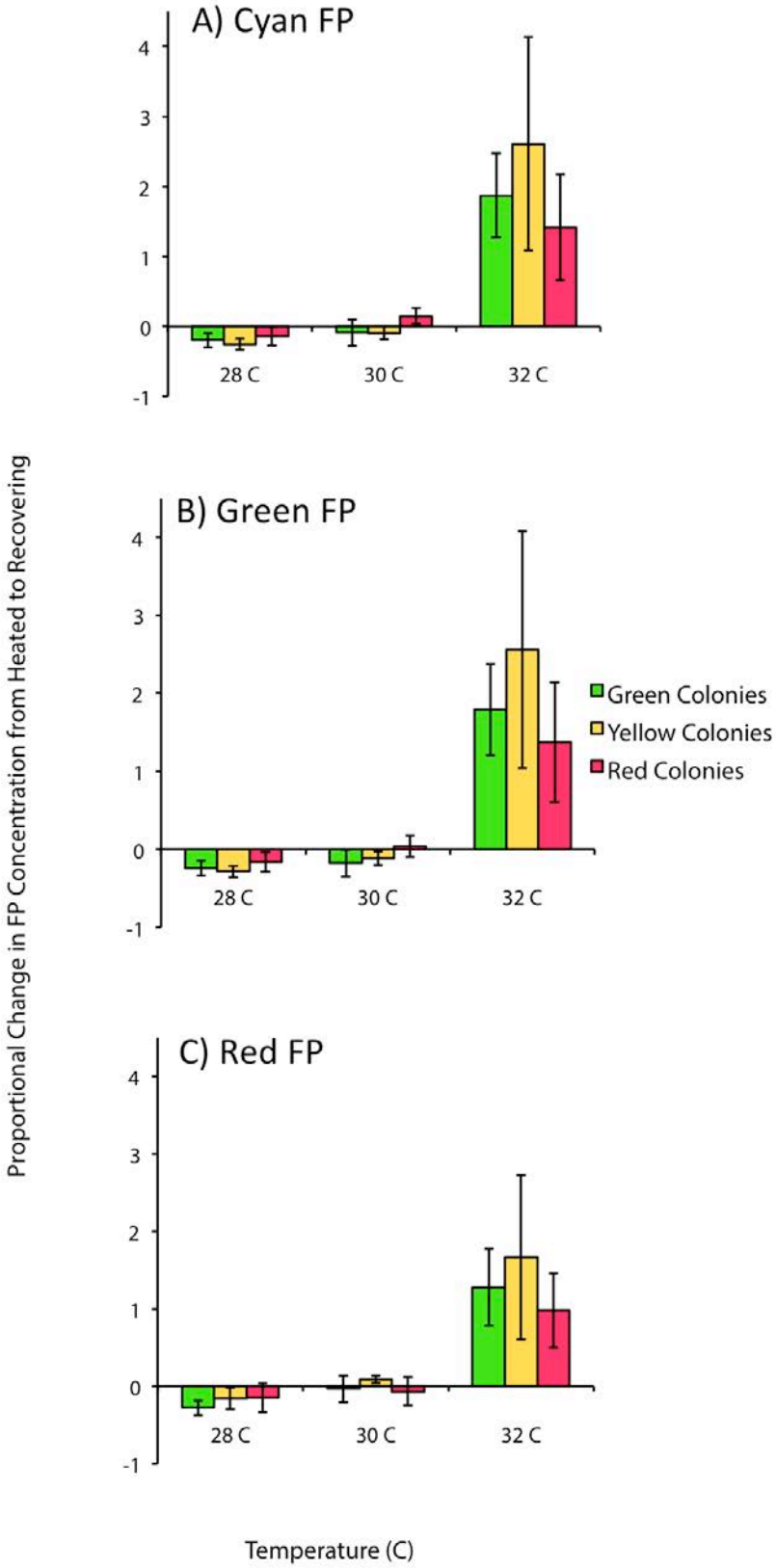

Figure 5.12 Comparisons of mean proportional changes (± SE) in FP concentrations over the period of experimental recovery (i.e. between heated and recovering samples) among control, 30°C, and 32°C temperature treatments and among three colour morphs of *A. millepora* for A) CFP, B) GFP, and C) RFP.

Table 5.9 Two-factor ANOVAs comparing mean proportional changes in FP concentrations over the period of experimental recovery (i.e. between heated and recovering samples) among colour morphs and temperature treatments for (A) CFP, (B) GFP and (C) RFP. *: denotes significance at α = .05.

A) CFP

Source of Variation	df	SS	F	p	
Morph	2	.800	.178	.837	
Temperature	2	51.7	11.6	8.90E-05	*
Morph x Temperature	4	3.9	.429	.787	
Error	45	100.5			

B) GFP

Source of Variation	df	SS	F	p	
Morph	2	1.0	.223	.801	
Temperature	2	51.3	11.6	8.70E-05	*
Morph x Temperature	4	3.6	.404	.805	
Error	45	99.6			

C) RFP

Source of Variation	df	SS	F	p	
Morph	2	.760	.328	.722	
Temperature	2	24	10.4	.000	*
Morph x Temperature	4	.810	.175	.950	
Error	45	52			

Table 5.10 Tukey's HSD tests comparing the proportional changes in FP concentrations over the period of experimental recovery (i.e. between heated and recovering samples) among temperature treatments for (A) CFP, (B) GFP and (C) RFP. *: denotes significance at α = .05.

A

Treatment	28°C	30°C	32°C
28°C			
30°C	.920		
32°C	**.000***	**.000***	

B

Treatment	28°C	30°C	32°C
28°C			
30°C	.950		
32°C	**.000***	**.000***	

C

Treatment	28°C	30°C	32°C
28°C			
30°C	.841		
32°C	**.000***	**.001***	

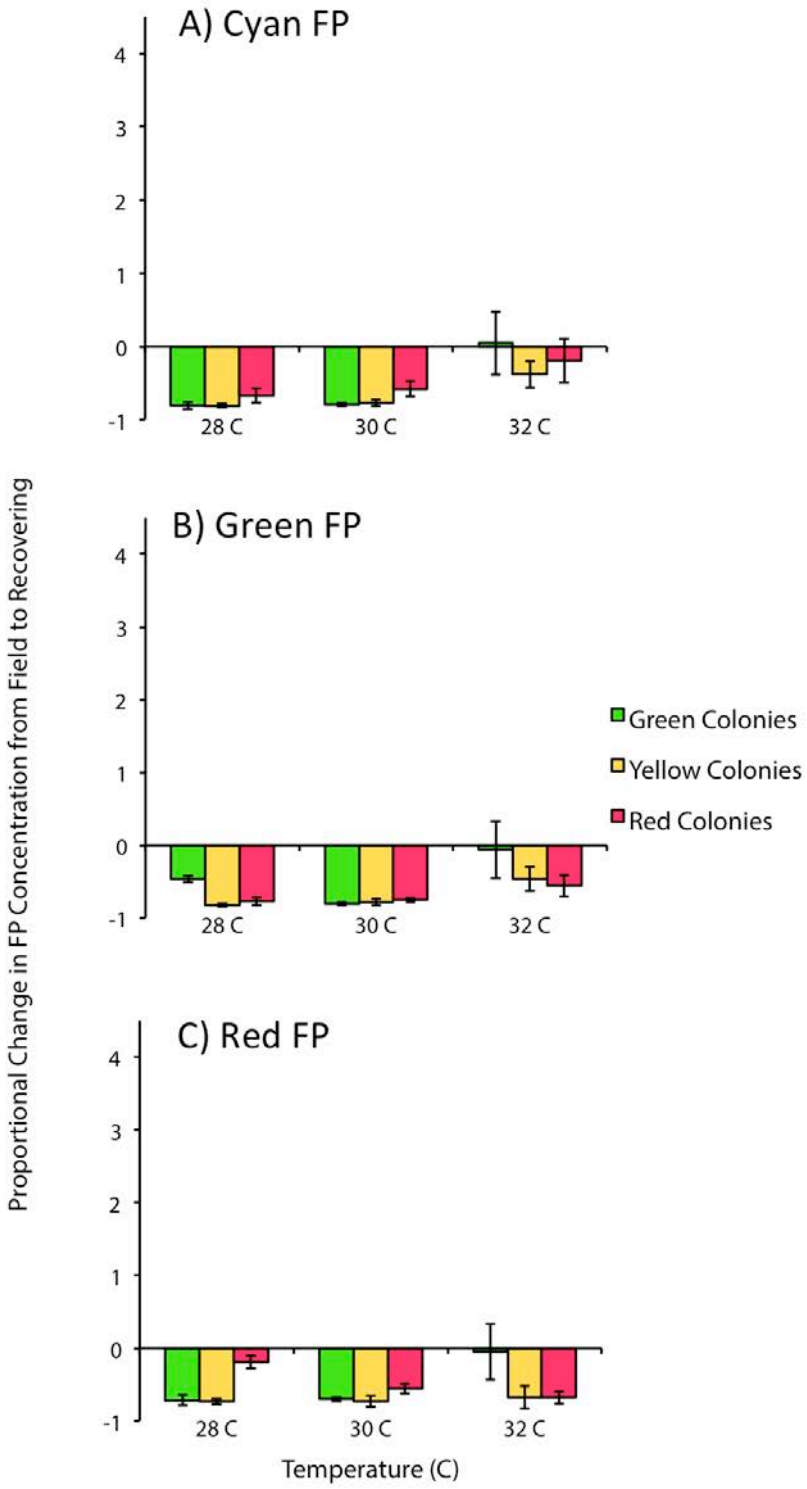

Figure 5.13 Comparisons of mean proportional changes (± SE) in FP concentrations from the beginning to the end of the experiment (i.e. between field and recovered samples) among control, 30°C, and 32°C temperature treatments and among three colour morphs of *A. millepora* for A) CFP, B) GFP, C) RFP.

Table 5.11 Two-factor ANOVAs comparing mean proportional changes in FP concentrations between field and recovering samples among colour morphs and temperature treatments for (A) CFP, (B) GFP and (C) RFP. *: denotes significance at α = .05.

A) CFP

Source of Variation	df	SS	F	p	
Morph	2	.290	.656	.524	
Temperature	2	3.8	8.6	.001	*
Morph x Temperature	4	.489	.553	.698	
Error	45	10			

B) GFP

Source of Variation	df	SS	F	p	
Morph	2	0.2	.730	.488	
Temperature	2	2.5	8.0	.001	*
Morph x Temperature	4	0.634	1.1	.361	
Error	45	6.4			

C) RFP

Source of Variation	df	SS	F	p	
Morph	2	0.5	1.7	.193	
Temperature	2	1.2	2.5	.017	*
Morph x Temperature	4	.773	1.4	.245	
Error	45	6.2			

Table 5.12 Tukey's HSD tests comparing the proportional changes in FP concentrations from the beginning to the end of the experiment (i.e. between field and recovering samples) among temperature treatments for (A) CFP, (B) GFP and (C) RFP. *: denotes significance at α = .05.

A

Treatment	28°C	30°C	32°C
28°C			
30°C	.943		
32°C	**.003***	**.001***	

C

Treatment	28°C	30°C	32°C
28°C			
30°C	.970		
32°C	**.030***	.053	

B

Treatment	28°C	30°C	32°C
28°C			
30°C	.982		
32°C	**.003***	**.004***	

5.4 Discussion

5.4.1 Colour morphs of *A. millepora* vary in bleaching susceptibility

Experimental manipulations of seawater temperatures within an environmentally controlled facility demonstrated that colour morphs of *A. millepora* vary in their susceptibility to elevated (+2^{o}C and +4^{o}C) temperatures. Of the three colour morphs examined, the weakly fluorescent red morph is the most sensitive to thermal stress, as corroborated by evidence that red branches bleached most severely following severe thermal stress and remained white bleached throughout 15 days of the recovery period. The strongly fluorescent green morph has comparatively greater capacity to resist thermal stress, as evidenced by the nearly 2-fold lower proportion of green branches that bleached compared to red branches. The poor recovery of red branches as a result of greater bleaching severity highlights the longer term consequences of variability in thermal tolerance among intra-specific colour morphs.

Correspondence between variation in bleaching resilience and variation in tissue concentrations of FPs among colour morphs suggests that FPs contribute to the coral thermal response. In particular, colonies of the thermally sensitive red morph have the lowest tissue concentrations of FPs (see Chapter 2 Section 2.3.1). Conversely, colonies of the more thermally tolerant green morph have the highest tissue concentrations of FPs and are characterized as being highly fluorescent (see Chapter 2, Section 2.4.2). The enhanced bleaching resilience of highly fluorescent morphs found here is in direct contrast to results of earlier studies, which reported that the most fluorescent morph of *A. aspera* was the least likely to survive experimental

heating (Dove 2004). Dove (2004) suggested that the photoprotective nature of coral FPs are critically damaged during periods of heat stress, in the same manner as GFP maturation has been shown to be hindered at high temperatures (Lim et al. 1995). Their findings suggest that even subtle changes in temperature can impact a photoprotective mechanism that relies on protein synthesis and maturation, but are in direct contrast to those of Roth and Deheyn (2013), who showed that heat-treated corals that bleached continued to show strong fluorescence despite reduced GFP concentrations. Additionally, mature GFPs are stable at high temperatures in vivo (Tsein 1998), suggesting that while heat stress may affect the production and maturation of new FPs, existing mature FPs are likely to remain intact and functioning within coral tissues.

The comparatively poor adjustment of fragments of the red morph to aquarium conditions during the acclimation period, as indicated by the 9.6- to 13.4-fold greater number of red fragments that were replaced based on their visual appearance than yellow or green fragments, provides further evidence that low levels of tissue FPs are associated with overall poor stress tolerance. Given evidence that sublethal stress may occur prior to any changes in visual condition in corals (Edge et al. 2013), I cannot discount the possibility that the condition of some red colonies may have been more compromised than those of the other two morphs at the time of collection. However, a number of lines of evidence suggest that the overall poor performance of the red morph represents a valid biological difference among the colour morphs of *A. millepora,* including the stability in mean photochemical efficiency of *Symbiodinium* endosymbionts for branches of all three colour morphs at the end of the acclimation

period, the observation that no fragments of any of the three morphs bleached in the control treatment, and analyses showing that the proportions of pale branches in the control treatment were low and similar across all three colour morphs.

5.4.2 Patterns in *Symbiodinium* density are consistent with thermal robustness of the green morph

Similarity in the overall proportional declines in *Symbiodinium* density among branches of the three colour morphs exposed to severe (32^{o}C) thermal stress suggests that tissue concentrations of FPs play a minor role in ameliorating loss of *Symbiodinium* cells during thermal stress. However, the proportional analyses presented in this chapter do not take into account differences in absolute cell densities among the three colour morphs. Bleaching of green branches was 1.5-fold lower than among red branches in the 32°C treatment, despite a similar proportional decline in *Symbiodinium* density, and similar initial densities of *Symbiodinium* cells to begin with (see Appendix 1, Fig. S1). *Symbiodinium* density samples were collected directly after 15 days of heating, although the proportion of visual bleaching continued to increase among branches of all colours through the first seven days of the recovery period. This resulted in similar *Symbiodinium* densities among all colour morphs after heating, but yielded observations of 1.5-fold greater bleaching of red branches than green branches following heating during the recovery period. Changes in the absolute cell densities indicate that red branches continued to decline by 1.5-fold between the heating and recovery samples (see Appendix 1, Fig. S1). Moreover, although overall proportional changes in *Symbiodinium* density during the recovery

phase did not differ among the three colour morphs in statistical analyses, the green morph was the only morph that increased *Symbiodinium* cell densities during the recovery phase, suggesting a stronger recovery potential compared with the red and yellow morphs of *A. millepora* (see Appendix 1, Fig. S1). This trend further supports the notion that the most highly fluorescent colour morph (the green morph) is the most robust to thermal stress.

5.4.3 *Symbiodinium* assemblage did not affect thermal stress responses of colour morphs

Despite diversity among the assemblages of symbiont types harboured, the lack of a colour morph × *Symbiodinium* association detected in this study indicates that *Symbiodinium* type did not bias conclusions about variation in the thermal stress response among the three colour morphs. Great genetic diversity exists among *Symbiodinium* types associating with corals (Rowan and Knowlton 1995; LaJeunesse 2001; van Oppen et al. 2001; Baker 2003), and the nature of *Symbiodinium* assemblages established also varies, including the ability to harbour from one to multiple *Symbiodinium* types simultaneously (Rowan and Knowlton 1995; Ulstrup and van Oppen 2003; Baker 2003; Mieog et al. 2007). Different combinations of host and *Symbiodinium* types may provide ecological advantages under different environmental scenarios, including under elevated water temperatures (e.g. Baker 2001; Berkelmans and van Oppen 2006). Although thermal tolerance has been linked to *Symbiodinium* type D in *A. millepora* (Berkelmans and van Oppen 2006; Mieog et al. 2009), my study shows that fragments containing *Symbiodinium* type D were equally as likely to bleach as those associating solely with C1 or C2. Application of

higher resolution molecular methodology, such as NGS (Quigley et al. 2014), would be useful to detect potential background symbiont types in the fragments sampled, and to measure potential changes in dominant symbiont types following bleaching.

5.4.4 FP concentrations decline following temperature stress but increase following recovery

Following heating to 32^{o}C, significant proportional declines in the abundance of all FPs measured in the three coral colour morphs suggest that FPs are either metabolised or degraded during thermal stress and also that corals lack sufficient energy stores to maintain optimal FP concentrations under thermal stress. Down-regulation of FP production in *A. millepora* has been observed following experimental heating in a previous study (Smith-Keune and Dove 2008), however, only the weakly fluorescent 'pink' colour morph (termed 'red' in this thesis) was examined, thus the potential impact of thermal stress on the regulation of FP concentrations in highly fluorescent morphs was not assessed. Also, changes in FP expression were only documented over a ~6 day period of thermal stress and did not include observations through recovery of experimental branches (Smith-Keune and Dove 2008). Following recovery at ambient temperatures, FP concentrations in experimental branches in my study increased within ~3 weeks to levels comparable with those first observed in the field. Although the synthesis of new FPs likely ceased during heat stress, as suggested by Dove (2004) and observed by Smith-Keune and Dove (2008), residual pigments may have continued to protect *Symbiodinium* symbionts from the likely photoinhibition under thermal stress during the recovery period (Salih et al. 2000).

Hence the symbiosis is more stable in corals with typically high concentrations of FPs under ambient temperatures (i.e. the green morph; Appendix 1, Fig. S2), because of higher residual levels of functional FPs remaining within their tissues after heat stress.

While heat may be damaging to FP synthesis and maturation (Lim et al. 1995; Dove 2004; Smith-Keune and Dove 2008), it does not affect mature GFPs (Tsein 1998). Accordingly, I suggest that corals containing high concentrations of FPs are better equipped to respond to heat stress because of high initial concentrations of mature photoprotective pigments that remain functionally intact within their tissues. Although this does not explain why Dove (2004) observed such high mortality among fluorescent blue morphs of *A. aspera* during thermal stress, (66% of blue branches died compared with 0 and 11% of light blue and cream branches), it does align with the findings of Salih et al. (2000), who found a high positive correlation ($R^2 = .09471$; $p < 0.0001$) between bleaching resistance of *A. palifera* and concentrations of FPs within coral tissues. Salih et al. (2000) also found that non-fluorescent morphs of *A. palifera* were significantly more photoinhibited and recovered to pre-inhibition rates more slowly compared to fluorescent morphs. Correlations between FPs and reduced photoinhibition were also recorded for other coral species including *A. nobilis* (Salih et al. 2000). Greater residual concentrations of mature, functional FPs under heat stress as an artefact of retaining high concentrations of FPs under ambient temperatures may therefore be advantageous to corals under threat of ocean warming.

Following bleaching, highly fluorescent morphs are also more likely to rapidly up-regulate production of FPs to maintain optimal concentrations once environmental conditions have returned to normal than weakly fluorescent morphs, which typically

function with comparatively lower tissue concentrations of FPs. Although proportional increases in FP concentrations during recovery for branches exposed to the severe (32°C) stress treatment were similar among all three colour morphs, mean tissue FP concentrations for the green morph were 2.4- to 3.6-fold higher than those of the red and yellow morphs (see Appendix 1, Fig. S2). Thus up-regulation of FPs occurred much more rapidly in the green morph than other morphs during recovery from severe (32^{o}C) thermal stress. Maintainer lower tissue concentrations of FPs in weakly fluorescent morphs may be advantageous in terms of energy budgets under normal conditions, however may become a liability under thermal stress.

The history of severe thermal stress at the study site, including severe thermal stress events in 1998 and 2002 which resulted in mass bleaching and up to 70% coral mortality (Marshall and Baird 2000; Berkelmans et al. 2004), combined with the greater thermal tolerance of highly fluorescent corals, implies that the highly fluorescent morph should be more abundant than the weakly fluorescent morph in contemporary populations. However, the present rarity of highly fluorescent colonies (see Chapter 2, Section 2.4.1) suggests that greater thermal tolerance is not the only life history trait that has given rise to current patterns of abundance and distribution of colour morphs. The low abundance of the highly fluorescent green morph raises the possibility that maintenance of high concentrations of photoprotective FPs comes at the expense of other metabolic processes.

5.4.5 Conclusions

Highly fluorescent colour morphs of *A. millepora* have greater bleaching resilience than weakly fluorescent counterparts, as corroborated by visual census of bleaching status and *Symbiodinium* density measurements throughout a laboratory-based heat stress experiment. Colour morphs of *A. millepora* in populations at Orpheus Island associated with three *Symbiodinium* communities (C1, C2, and C2+D), but bleaching susceptibility was not influenced by *Symbiodinium* type in this study. FP levels were rapidly up-regulated in heat-treated coral fragments during the recovery phase, even in weakly fluorescent colour morphs, indicating their importance in maintaining optimal fitness following disturbance events. Molecular studies comparing down regulation and degradation of FPs between strongly and weakly fluorescent morphs are required to further understand the causes of declines in FP concentrations during thermal stress and the impacts these declines have on this species.

Currently, the weakly fluorescent (red) morph is the most abundant on the Great Barrier Reef, comprising up to 85% of the populations surveyed (see Chapter 2 section 2.4.1), but given its high susceptibility to thermal stress (this Chapter) and current predictions for oceanic warming (IPCC 2001), its persistence as the dominant phenotype in this species appears tenuous. If conditions conducive to bleaching occur yearly within the next several decades (Hoegh-Guldberg 1999), the high bleaching susceptibility of the red morph suggests that, although more highly fluorescent colour morphs will become proportionally more abundant within populations, the overall abundance of *A. millepora* will decline on coral reefs.

Chapter 6

The consequences of coral colour: high FP concentration versus reproductive fitness

Juvenile yellow A. millepora morph, Miall Island, Keppel Islands, GBR

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6.0 Abstract

When resources are finite, trade-offs in the allocation of energy towards key life-history processes are required, but the possibility that resource allocation strategies might vary within a species, for example in association with colour polymorphism, has not been explored previously in a coral species. The striking colour morphs of the coral *Acropora millepora* vary in their response to thermal stress as a consequence of differential constituent levels of FPs, yet the more thermally tolerant green morph is rare across a range of environmental gradients, including locations with a history of significant thermal stress. To investigate patterns of resource allocation to FPs versus other key physiological processes, I compared concentrations of FPs, storage lipids (representative of oocyte development), and *Symbiodinium* density among three colour morphs of *A. millepora* that were exposed to four nutritional treatments in the three months leading up to their annual reproductive event. In the unrestricted nutritional regime (natural light, added *Artemia*), total lipid content increased between 1.7- and 3-fold in yellow and red colonies in the 20 days preceding spawning, but did not change for green colonies. Three-fold greater concentrations of wax esters in red and yellow colonies compared with green colonies the day before spawning, suggest that colour morphs of *A. millepora* vary in their energetic allotment towards reproduction. Increases of 3.7-fold in total FP concentrations in green colonies during the 20 days preceding spawning highlight that investment in high FP concentrations comes at the cost of resources available to invest in reproduction. These findings are the first to document intraspecific variation in the allocation of resources to reproduction, and suggest that maintaining high FP concentrations characteristic of the green morph of *A. millepora*

is energetically costly and results in comparatively lower investment into reproduction.

6.1 Introduction

Functional interactions (i.e. trade-offs) between life-history traits that enhance survival occur at two levels: evolutionary trade-offs that shape life history strategies at a species level, and physiological trade-offs that determine energy allocation under resource limitation at the organismal level (Stearns 1976, 1989). While many investigations have explored aspects of coral life-history strategies, such studies typically focus on specific life history traits, like growth, juvenile development, reproduction, and recruitment (Meesters et al. 2001; Garrabou and Harmelin 2002; Negri et al. 2005; Vermeij 2006), but few studies focus on physiological or evolutionary trade-offs involving life history traits that mitigate stress (but see Bak and Engel 1979; Hoeksema 1991). Under stress, organisms may reallocate energy away from core processes towards defense or repair mechanisms in order to survive (Petes et al. 2008). Energetic requirements associated with reproduction are one of the most costly, thus many trade-offs involve reproduction to ensure survival and future reproduction (Stearns 1992). Examples of reproductive failure of corals following bleaching and reduced reproduction for up to two years following disturbance (Szmant and Gassman 1989; Michalek-Wagner and Willis 2001) highlight trade-offs between current and future reproduction. Such evidence of trade-offs between reproduction and survival also suggests that coral species with colour polymorphisms

linked to intraspecific variation in thermal tolerance are also likely to vary in their investment into reproduction following disturbance.

A surprisingly large fraction (14%) of the protein complement of corals is composed of fluorescent proteins (FPs) (Oswald et al. 2007), which have long been thought to play a role in coral stress tolerance (Kawaguti 1944; Salih et al. 2000; Bou-Abdallah et al. 2006; Palmer et al. 2009). Intraspecific colour morphs of the coral *Acropora millepora* contain different concentrations of FPs (Chapter 2), which have been linked to their variation in thermal tolerance (Chapter 5). The production and maintenance of high concentrations of FPs, like those measured in colonies of the green morph of *A. millepora*, are hypothesized to be energetically inexpensive because of their relatively slow decay rates (~20 days for red pigments) (Leutenegger et al. 2007). However down-regulation of FP transcription has been observed over timeframes much shorter than 20 days (Smith-Keune and Dove 2006; Bay et al. 2009a; Roth and Deheyn 2013), indicating that FPs are rapidly regulated in response to environmental cues, potentially as an energy saving mechanism. If FP synthesis is energetically more costly than previously assumed, then one consequence of variation in FP concentrations and thermal tolerance among colour morphs of *A. millepora* could be that allocation of energy towards reproduction varies among morphs, with resultant variation in the trade-off between thermal stress tolerance and reproductive investment.

Comparisons of colony condition during periods of resource deprivation or during periods of enhanced allocation into energetically costly processes, like reproduction, provide insights into the strategies corals have evolved to maximize investment returns when allocating limited energy reserves into key physiological processes. Lipids are known to play an important role in the energy budgets of corals

(Crossland 1987; Harriott 1991), and total lipid content is widely acknowledged as a useful indicator of coral condition (Anthony 2006). In particular, the proportion of polar to non-polar lipids describes the ratio of lipid allocation into structural and storage components, providing further insights into the proportion of lipids available to invest in reproduction (i.e. storage lipids). For example, Saunders et al. (2005) found that variation in lipid ratios within tissues of the coral *Acropora nobilis* was correlated with local light and sediment regimes; declining light regimes and increasing sediment deposition rates were linked to declining lipid ratios in coral tissue samples. Cooper et al. (2011) found a similar reduction in storage lipids with increasing depth (declining light) in the coral *Pachyseris speciosa*. Sediment deposition onto coral surfaces results in increasing energy expenditure through elevated respiration and the need to allocate energy to sediment removal mechanisms (Riegl and Branch 1995; Philipp and Fabricus 2003), and low light decreases the amount of energy produced through photosynthesis, ultimately resulting in less energy available for storage (Patton et al. 1977). Therefore, environmental parameters can negatively affect the energetic budgets of coral colonies, and the relative investment in structural versus storage lipids can be used as a bio-indicator of coral condition (Glynn et al. 1985; Saunders et al. 2005).

Wax esters are a primary component of coral storage lipids, and comprise between 52 and more than 86% of coral propagules (Arai et al. 1993; Harii et al. 2007; Figueiredo et al. 2012). Wax esters contribute considerably to coral larval buoyancy and energy reserves, making them a substantial component of the storage lipids typically involved in coral reproduction (Arai et al. 1992; Wellington and Fitt 2003; Figueiredo et al. 2012), and accounting for up to 80% of the total lipid content of corals (Benson and Muscatine 1974; Harland et al. 1993). Seasonal variation in

total lipid content has been attributed to changes in wax ester content (Oku et al. 2003). Although Oku et al. (2003) attributed fluctuating wax ester content to variation in sea surface temperatures, sharp declines in both total lipid and wax ester content have also been recorded directly following of coral spawning (Oku et al. 2003; Leuzinger et al. 2003). Therefore comparisons of wax ester content during the final months of oocyte maturation and preparation for spawning can provide an estimate of resources (storage lipids) directed towards reproduction.

My overarching objective in this study is to compare resource allocation strategies among colour morphs of *A. millepora* under conditions of resource limitation to determine if the greater FP concentrations that characterize the green morph come at the cost of reduced resources for investment in reproduction. Specifically, I compare colony condition indicators among three colour morphs in response to four nutritional treatments spanning a range of autotrophic and heterotrophic regimes: 1) natural light plus heterotrophic input (high nutrition); 2) natural light without heterotrophic input (autotrophic only); 3) low light with heterotrophic input (heterotrophy only); 4) low light without heterotrophic input (low nutrition). Running the experiment in the three months leading up to the annual mass spawning enabled me to assess the impact of resource limitation on the allocation of resources to FP production versus reproduction. Indicators of coral condition include total FP concentration and lipid content as indicators of the health of the coral host, and *Symbiodinium* density as an indicator of the intactness of the coral-*Symbiodinium* symbiosis. Allocation of resources into reproduction is examined by comparing changes in the concentrations of structural and storage lipid classes, as well as wax esters, the storage lipids that make up to 82% of *A. millepora* eggs (Arai et al. 1992; other refs), among colour morphs. Comparisons among colour morphs will reveal

whether energetic investment into reproduction varies among colonies with characteristically different concentrations of FPs and thermal tolerances.

6.2 Materials and Methods

6.2.1 Coral collection and experimental design

Fifteen colonies, comprised of five discrete genotypes from each of the green, yellow, and red morphs, were collected from the eastern side of Orpheus Island in August 2011. After collection, each colony was fragmented into four approximately equal-sized, 10 x 10 cm^2 fragments, which were randomly allocated to four experimental treatments. Four experimental tanks (one per treatment) each contained 15 fragments, such that all genotypes were represented in every tank (Fig. 6.1A). Four round polyethylene water tanks, each 1090 x 350 mm^2, with a 320 litre capacity were located outdoors under natural light but sheltered from rain by a transparent roof (laserlite; transmits 92% visible light; blocks 99.9% UV light). Experimental tanks received 0.5 μm filtered seawater at a rate of approximately 75 litres/hour in a flow-through system. Water was filtered using a combination of X100 propylene bag filters (1 μm) and 10-inch in-line wide-spun sediment cartridge filters (0.5 μm) (Fig. 6.1B). A minimum of six air stones per tank aided circulation and oxygenation. Sunny, midday light levels ranged from 590 to 1140 μmol quanta $m^{-2}s^{-1}$, which were similar to midday light levels measured in the field at the time of collection (680 – 999 μmol quanta $m^{-2}s^{-1}$) at ~ 4 m depth. Colony fragments were placed on inverted plastic dish racks, elevating them up off the bottom of the tank by ~ 5 cm. During the 15-day acclimation period, experimental tanks received supplemental feedings of *Artemia* sp.

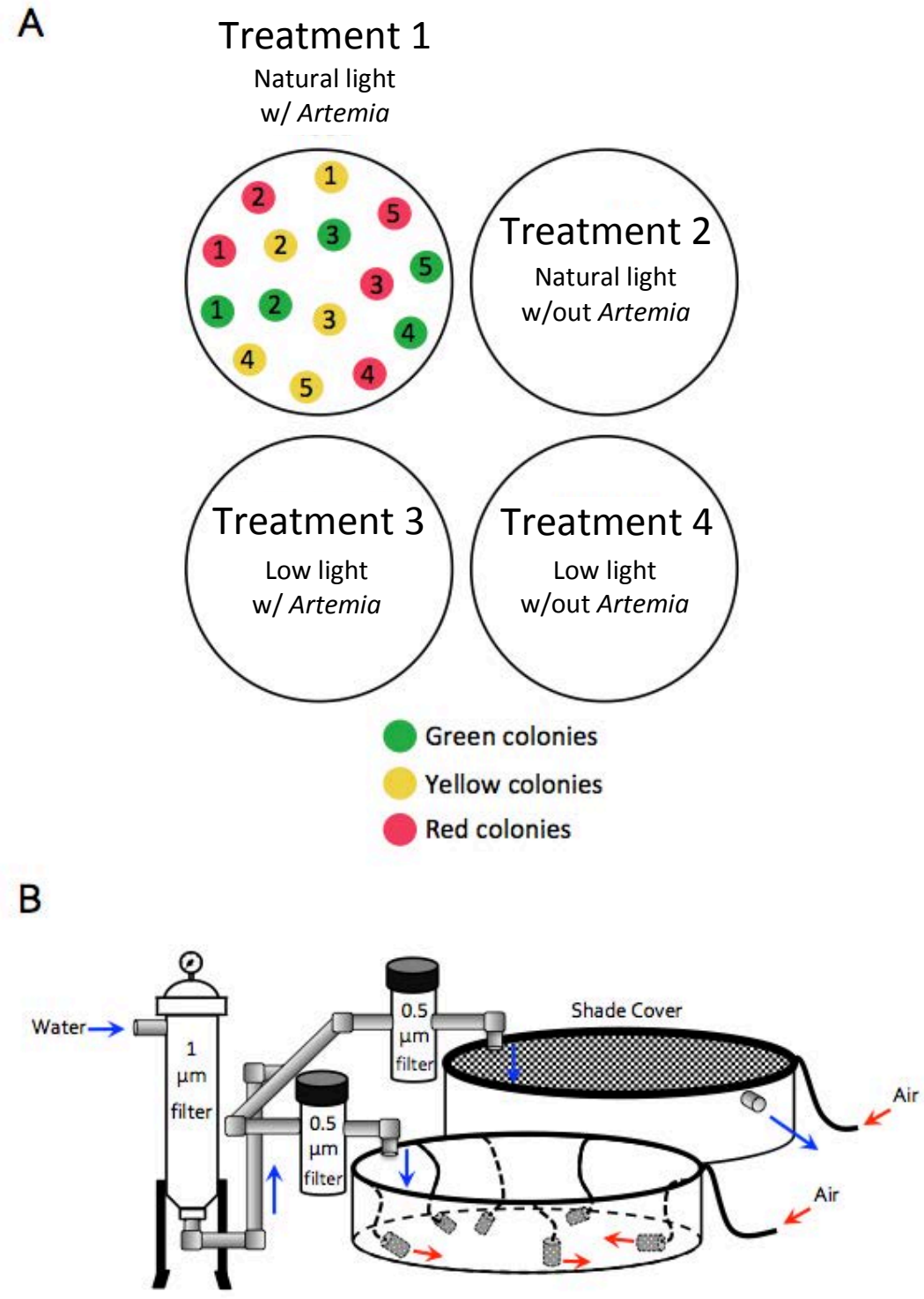

Figure 6.1 Schematic of flow-through laboratory set-up: (A) experimental design showing light and feeding treatments, and fragment replication for fragments of the green, yellow and red morphs of *A. millepora*, and (B) tank set-up showing shading and filtration. Fifteen genetically distinct coral fragments (5 of each colour morph) were represented within each tank.

every second night (see Section 6.2.2 below for details). On these nights, flow rates of seawater and air were halved to increase *Artemia* retention and accommodate heterotrophic feeding.

The following four nutritional treatments commenced on day 16: 1) high nutritional regime, i.e., natural light with *Artemia*; 2) autotrophic regime, i.e., natural light without *Artemia*; 3) primarily heterotrophic regime, i.e., low light with *Artemia*; and 4) restricted nutritional regime, i.e., low light without *Artemia* (Fig. 6.1A). Light levels were reduced by ~85% in low light treatments 3 and 4 using heavyweight UV-stable shade cloth (90% blockout rate), which reduced light levels to between 84 and 124 μmol quanta $m^{-2}s^{-1}$. *Artemia* feeding occurred every second day.

Sampling occurred four times throughout the experiment, i.e. (1) after 15 days of acclimation to tank conditions, and after (2) one, (3) two, and (4) nearly three months of experimental conditions. At each sampling, three vertically-oriented branches from each coral fragment were collected, immediately snap frozen in liquid nitrogen, and maintained at -80^{o}C. All coral samples were air-blasted on ice following the protocols outlined in Chapter 2 (see Section 2.2.3), but without centrifugation, and the tissue homogenates from the three replicate branches per fragment were combined in one sample. The resulting tissue slurry was divided into three sub-samples as follows: 1.0 ml for protein analyses, 0.9 ml for *Symbiodinium* density measurements (to which 0.1 ml of 10% formaldehyde was added), and ~ 13 ml for lipid extraction. Protein and lipid sub-samples were re-frozen at -80^{o}C until analysis. Bare coral skeletons were immersed in a 20% bleach solution to remove residual tissues and dried to estimate surface area (see Section 6.2.4 below).

6.2.2 Feeding

Two fresh *Artemia* cultures were established every second day throughout the duration of the experiment. For each culture, 4g of *Artemia* cysts (Gulf Breeze Premium *Artemia*) were submerged in approximately 2 litres of a filtered solution of freshwater and seawater (1 : 2 by volume), aerated with two air stones, and maintained under natural light. Fifteen minutes prior to feeding, aeration was turned off, allowing un-hatched cysts to settle to the bottom, hatched shells to float to the surface and living nauplii larvae to swim mid-water. Living *Artemia* nauplii larvae were harvested 24 hours after eggs were submerged by siphoning the mid-water layer through 106 µm plankton mesh and diluted with fresh filtered seawater to obtain a two litre solution containing between 30-50 nauplii ml^{-1}. The contents from each culture were released into experimental tanks 3 hours after dusk, when polyps typically feed actively.

6.2.3 Fluorescent protein and spectral analyses

Protein concentrations and relative FP abundances were measured for each fragment in each of the four tanks at each sampling time (n = 60 measurements/sampling time) using the first of the three tissue slurry subsamples produced per fragment (see Section 6.2.1) and the methods outlined in Chapter 2 (see Sections 2.2.3. and 2.2.4).

6.2.4 Estimating *Symbiodinium* density and branch surface area

Symbiodinium density was determined from the second of the three tissue slurry subsamples produced per fragment (see Section 6.2.1) in each of the four tanks at each sampling time (i.e. 6 replicate measurements for each of the 15 fragments in each of the 4 treatments; i.e. n = 360 measurements per sampling time) using the methods outlined in Chapter 3 (Section 3.2.3). Branch surface area was calculated as the total surface area of the three branches collected to produce each tissue slurry for each fragment using the methods outlined in Section 3.2.3.

6.2.5 Total lipid extraction

The third of the three tissue slurry subsamples produced per fragment was freeze-dried for 48 hours, and stored at -20^{o}C until analysed. During processing, each sample was crushed with a mortar and pestle, weighed, and extracted in 6 ml of dichloromethane : methanol (2 : 1 by volume) using sonication following a modified version of the technique described by Folch et al. (1957). Following filtration, samples were washed in 3.5 ml of potassium chloride : water : methanol (0.44% : 3 : 1 by volume) and allowed to separate overnight. The lipid portion of the sample was recovered and concentrated under nitrogen evaporation, and then reconstituted with dichloromethane: methanol (2:1) to a known volume (1000 µl). Dry weights were recorded from the average of three, 25 µl subsamples using a microbalance (Perkin Elmer A4 microbalance), and lipid per sample (mg) was calculated based on a concentrated sample volume as follows:

Equation 6.1 lipid weight (mg/ml) = avg. extract dry weight / concentrated vol. of sample

Total lipid content (mg) per cm^2 was back calculated for each sample using initial freeze-dried sample weights and percent lipid content, and divided by branch surface area as follows:

Equation 6.2 lipid (mg) cm^{-2} = (freeze-dried sample wt * lipid weight) / branch SA

Extracted lipids were preserved under nitrogen and stored at -20^{o}C until analysed for lipid class analysis.

6.2.6 Lipid class separation

Lipid class composition was analysed from total lipid fractions using an Iatroscan MK-6s thin layer chromatography – flame ionization detector (Mitsubishi Chemical Medience, Tokyo Japan) according to the methods outlined in Nichols et al. (2001). Briefly, each lipid extract was completely dried under nitrogen evaporation and re-suspended in 100% dichloromethane for a final concentration of ~ 10 mg ml^{-1}. Total lipids were separated into lipid classes by spotting 1 µl of each sample, in duplicate, onto silica gel S4-Chromarods® (5 µm particle size) and developing in a glass tank containing filter paper. Lipid separation was achieved through a two-step elution sequence: 1) elution of monoacylglycerol (MG), phosphatidylcholine (PC), phosphatidylserine and phosphatidylinositol (PS-PI), and phosphatidylethanolamine

(PE) was performed in a hexane / diethyl ether / formic acid (60 : 15 : 1.5, by volume) solvent system run to half height (~15 min); and 2) after air drying, elution of wax esters (WEs), triacylglycerol (TG), free fatty acid (FFA) 1,3-diacylglycerol (1,3-DG), sterol (Chol), and 1,2-diaylglycerol (1,2-DG) was performed in a dichloromethane / methanol / water (50 : 20 : 2, by volume) solvent system run to full height (~ 30 min) before rods were placed in an oven at 100°C for 10 min. Chromatograms were acquired by scanning with the Iatroscan MK-6s. The amount of each lipid class from each sample was determined by calibration from known compound classes with concentrations ranging from 0.625, 1.25, 2.5, 3.5, and 5.0 mg ml^{-1}. The concentration of lipid classes in each sample was determined by calculating peak area versus standard curves of concentration using PowerChrom version 2.6.15 (eDAQ Pty Ltd). Lipid class concentration was normalized to total lipid initially extracted for each sample.

6.2.7 Data analyses

Total Lipid Content: To evaluate if colony condition changed during the experiment, a three-factor ANOVA was used to compare total lipid content among colour morphs (green, yellow, red), sampling times (days 1, 29, 61, and 81), and treatments (1, 2, 3, 4); both colour morph and nutritional treatment were fixed factors. Log_{10} transformed data conformed to the assumption of normality (Kolmogorov-Smirnov test for normality: $p < 0.1$) but violated the assumption of homogeneity of variances (Cochran's test: $p = 0.00$). Since ANOVA is somewhat robust to variance heterogeneity when samples sizes are equal (Glass and Stanley 1970), the log_{10} transformed data were analysed and a significance level of $\alpha = 0.01$ was used.

Storage and structural lipids and wax ester concentrations: To explore a 3-fold increase in total lipid content in red colonies from treatment 1 between the first and last samples (see Fig. 6.2 below), the concentrations of 3 lipid classes (storage lipids, structural lipids, wax esters) were analysed. Concentrations of each of the three lipid classes were compared among morphs between the most nutritionally restricted (4: low light without *Artemia*) and unrestricted (1: natural light with *Artemia*) treatments for the first (day 1) and last sampling time (day 81), in a separate 3-factor (sampling time, treatment, morph) ANOVA. Log_{10} transformed data conformed to the assumptions of homogeneity of variances (Cochran's test) and normality (Kolmogorov-Smirnov test) for each lipid class (storage lipids: Cochran's test, $p = 0.541$,: $d = 0.138$; $p > 0.2$; structural lipids: Cochran's test, $p = 0.207$, $d = 0.081$, $p > 0.2$; wax esters Cochran's test, $p = 0.067$, $d = 0.136$, $p > 0.2$). For storage lipids and wax ester concentrations, post-hoc, pairwise comparisons between morph and sample time were interpreted with Tukey's HSD tests.

Symbiodinium density: To determine if the impact of the nutritional treatments on the *Symbiodinium*-coral symbiosis differed among colour morphs throughout the experiment, mean *Symbiodinium* densities were compared in a 3-factor ANOVA, where the factors colour morph (green, yellow, red), sampling time (days 1, 29, 61, and 81), and nutritional treatment (1, 2, 3, 4) were all fixed factors. Log_{10} transformed *Symbiodinium* densities conformed to the assumptions of homogeneity of variances (Levene's test: $p = 0.156$) and normality (Kolmogorov-Smirnov test for normality: $p > 0.1$). Post-hoc comparisons of the effect of treatment on *Symbiodinium* density among colour morphs were interpreted with Tukey's HSD tests.

Total FP concentration: As differences in the patterns of CFP, GFP, and RFP concentrations among colour morphs of *A. millepora* are well established (see

Chapters 2, 3, and 5), FP concentrations in this study were estimated as the total FP concentrations (the sum of CFP, GFP and RFP) for each sample. Total FP concentrations differ by up to 7.4-fold among the three colour morphs (see Chapters 2, 3 & 5), therefore mean total FP concentrations were compared among sampling times and treatments using a separate ANOVA for each colour morph. In all cases, Log_{10} transformed total FP concentrations conformed to the assumptions of homogeneity of variances (Cochran's tests: $p_{Green} = 0.724$; $p_{Yellow} = 0.344$; $p_{Red} = 0.115$) and normality (Kolmogorov-Smirnov test for normality: $p_{Green} > 0.2$; $p_{Yellow} > 0.2$; $p_{Red} > 0.1$). Post-hoc pairwise comparisons were interpreted with Tukey's HSD tests. All analyses were performed in STATISTICA version 12 (StatSoft).

6.3 Results

6.3.1 Total lipid content

Mean total lipid concentration increased more than 3-fold (from ~ 2.0 to more than 6.0 mg cm-^{2}) in red colonies in the high nutrition treatment (treatment 1) between days 61 and 81, and was 3-fold higher than the mean lipid concentration in green colonies at the end of the study (Fig. 6.2). Mean lipid concentration also increased approximately 1.7-fold in yellow colonies, from 2.4 to nearly 4.0 mg cm^{-2} in treatment 1 over the same time period (Fig 6.2). In contrast, mean lipid concentration in green colonies in the high nutrition treatment was comparatively constant throughout the study (Fig 6.2). Statistically, however, mean total lipid concentration did not differ significantly among colour morphs ($p = 0.781$; Table 6.1), nor among

nutritional treatments (p = 0.58; Table 6.1) or sampling times (p = 0.303; Table 6.1). Although mean lipid concentrations at the end of the study were consistently two-fold lower in the low light treatments (3 and 4) compared to the high nutrition treatment (1) in all three colour morphs (Fig. 6.2), statistically, these differences were not significant (p = 0.058; Table 6.1). This lack of statistical difference among morphs and nutrition treatments was consistent throughout the experiment (p = 0.554; Table 6.1).

6.3.2 Storage and structural lipids and wax esters

Patterns in the mean concentration of storage lipids from the beginning to the end of the study differed significantly among colour morphs (p = 0.038; Table 6.2). Mean concentrations of storage lipids were similar in all three morphs on day 1 of the study, in both the high and low nutrition treatments (1, 4) (Fig. 6.3). By day 81, however, mean concentrations of storage lipids in red and yellow colonies were 2- and 3-fold greater than the mean concentration found for green colonies in the high and low nutritional treatments, respectively (Fig. 6.3). This pattern of increasing concentrations of storage lipids through time in the red and yellow morphs (p = 0.000 for both morphs; Table 6.3) but constant concentrations in the green morph (p = 0.504; Table 6.3) was consistent in both the high and low nutritional treatments (p = 0.565; Table 6.2). Overall, concentrations of storage lipids were significantly greater (by 2.4-fold in the red morph, and two-fold in yellow and green morphs) in the high nutrition treatment compared to the low nutrition treatment by the end of the study (p = 0.023; Table 6.2).

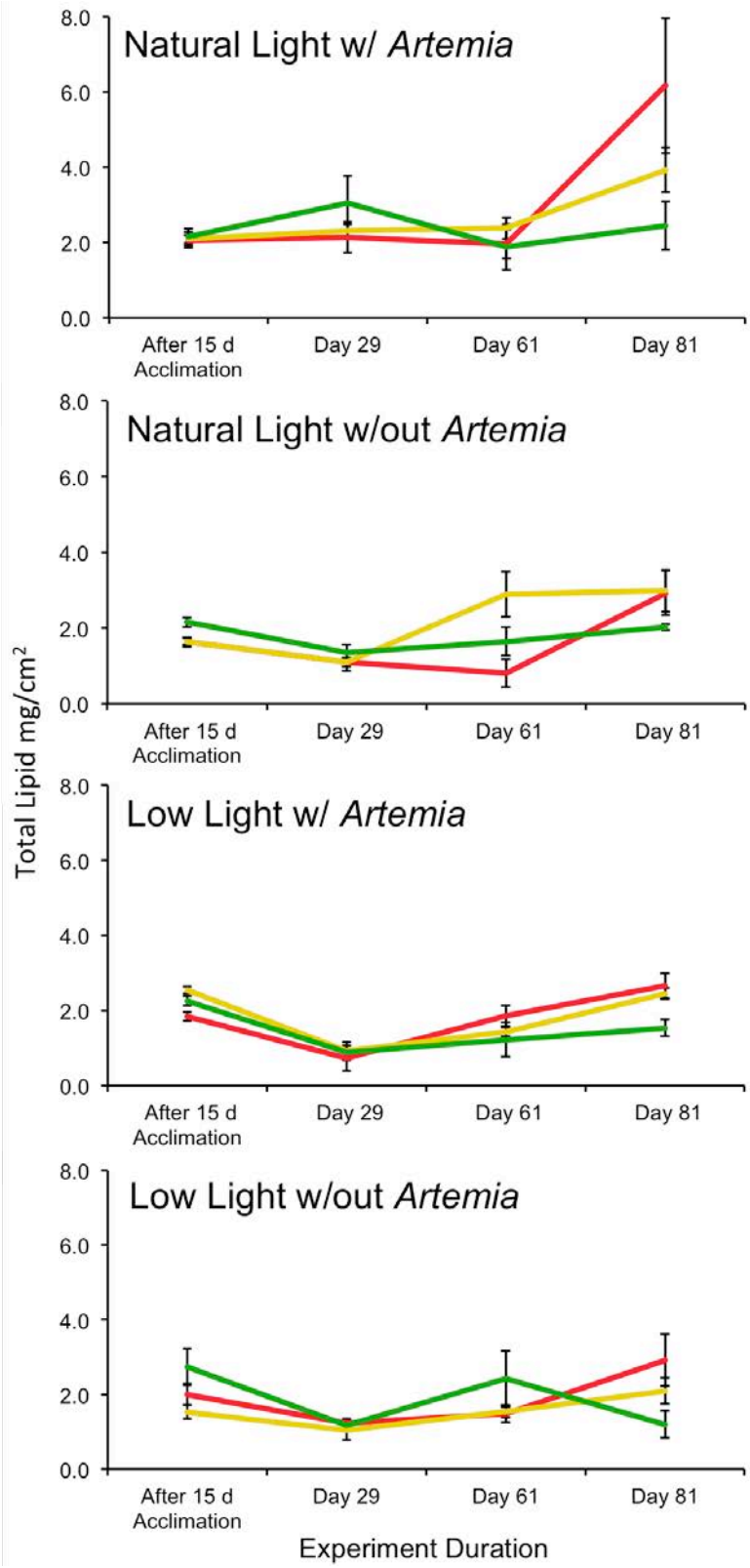

Figure 6.2 Mean total lipids (± SE) compared among three colour morphs (green, yellow, red) of *A. millepora* exposed to four nutritional treatments: 1) high nutritional regime (natural light with *Artemia*), 2) natural light without *Artemia*, 3) low light with *Artemia*, and 4) low nutritional regime (low light without *Artemia*) at four time points throughout the experiment. (Red: n = 5; Yellow: n = 5; Green: n = 5).

Table 6.1 Three-factor ANOVA comparing total lipid content among three colour morphs of *A. millepora,* four nutritional treatments, and four sampling times.

Source of Variation	SS	df	F	p
Sampling Period	.406	3	1.2	.303
Treatment	.843	3	2.5	.058
Morph	.055	2	.248	.781
Sampling Period*Treatme	.702	9	.705	.704
Sampling Period*Morph	.802	6	1.2	.305
Treatment*Morph	.546	6	.822	.554
Sampling Period*Treatme	1.8	18	.884	.599
Error	18.5	167		

In contrast to patterns in storage lipids, mean concentrations of structural lipids were constant throughout the study (Fig. 6.4). In summary, mean structural lipid concentrations did not differ significantly among the three colour morphs ($p = 0.435$; Table 6.4), or between the high and low nutritional treatments ($p = 0.181$; Table 6.4), or between samples collected on the first and last (day 81) day of the experiment ($p = 0.84$; Table 6.4).

Similar to patterns found for storage lipids, patterns in the mean concentration of wax esters between the first and last sampling times differed significantly among colour morphs ($p = 0.005$; Table 6.5). At the beginning of the experiment, mean wax ester concentrations were similar among colour morphs in both the high and low nutrition treatments (Fig. 6.5; Table 6.6). By the last day of the experiment, however, mean wax ester concentrations in red and yellow colonies had increased by more than 4-fold, and were between 3- and 4.5-fold greater than the mean concentration found for green colonies in the high and low nutrition treatments, respectively (Fig. 6.5; Table 6.6). The pattern of increasing concentrations of wax esters through time in the red and yellow morphs, but constant concentrations in the green morph was consistent in both the high and low nutritional treatments ($p = 0.977$; Table 6.5).

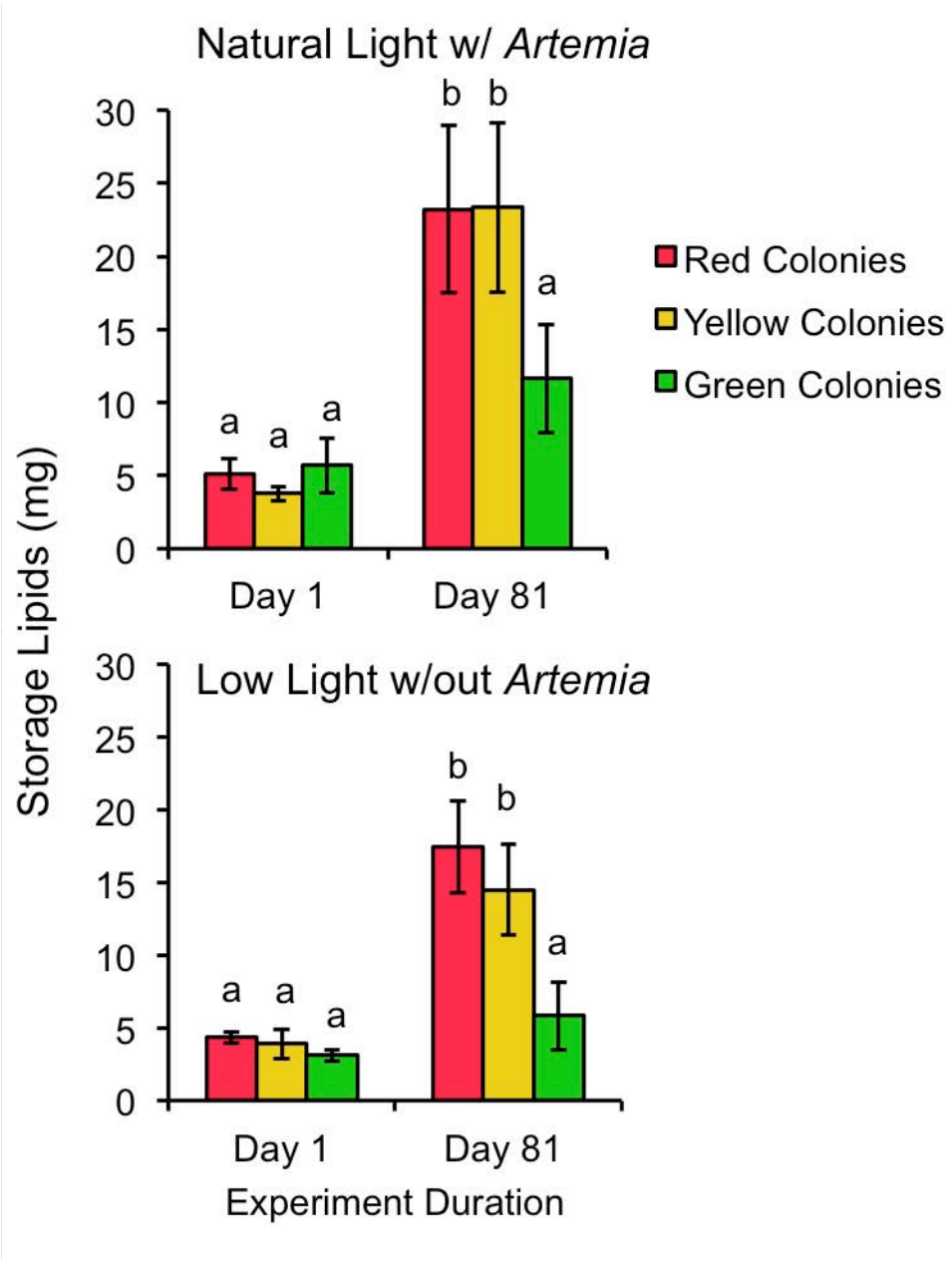

Figure 6.3 Mean concentrations of storage lipids (±SE) compared among three colour morphs (green, yellow, red) of *A. millepora* exposed to two nutritional treatments: 1) high nutritional regime (natural light with *Artemia*), and 2) low nutritional regime (low light without *Artemia*) at the first and last time points throughout the experiment. (Red: n = 5; Yellow: n = 5; Green: n = 5). Letter sets 'a' and 'b' signify similar concentrations, as determined by Tukey's HSD post-hoc analyses.

Table 6.2 Three-factor ANOVA comparing the mean concentrations of storage lipids among three colour morphs of *A. millepora*, two nutritional treatments and two sampling times. *: denotes significance at α = .05.

Source of Variation	SS	df	F	p	
Sampling Time	2.3	1	60.2	.000	*
Treatment	.2	1	5.6	.023	*
Morph	.3	2	4.3	.021	*
Sampling Time*Treatment	.043	1	1.100	.299	
Sampling Time*Morph	.3	2	3.6	.038	*
Treatment*Morph	.045	2	.579	.565	
Sampling Time*Treatment*Morph	.006	2	.081	.922	
Error	1.5	39			

Table 6.3 Tukey's HSD tests for pairwise comparisons of the concentrations of storage lipids among three colour morphs of *A. millepora* and two sampling times. *: denotes significance at α = .05.

Sampling Time		Day 1			Day 81		
	Morph	Red	Yellow	Green	Red	Yellow	Green
Day 1	Red						
	Yellow	.926					
	Green	1.00	0.99				
Day 81	Red	**.000***	**.000***	**.000***			
	Yellow	**.000***	**.000***	**.000***	1.00		
	Green	.499	.132	.504	**.005***	**.011***	

Table 6.4 Three-factor ANOVA comparing the mean concentrations of structural lipids among three colour morphs of *A. millepora*, two nutritional treatments and two sampling times. *: denotes significance at α = .05.

Source of Variation	SS	df	F	p
Sampling Time	.8	1	3.1	.084
Treatment	.5	1	1.9	.181
Morph	.5	2	.9	.435
Sampling Time*Treatment	.006	1	.022	.884
Sampling Time*Morph	.0	2	.0	.976
Treatment*Morph	.313	2	.590	.559
Sampling Time*Treatment*Morph	.230	2	.433	.651
Error	10.6	40		

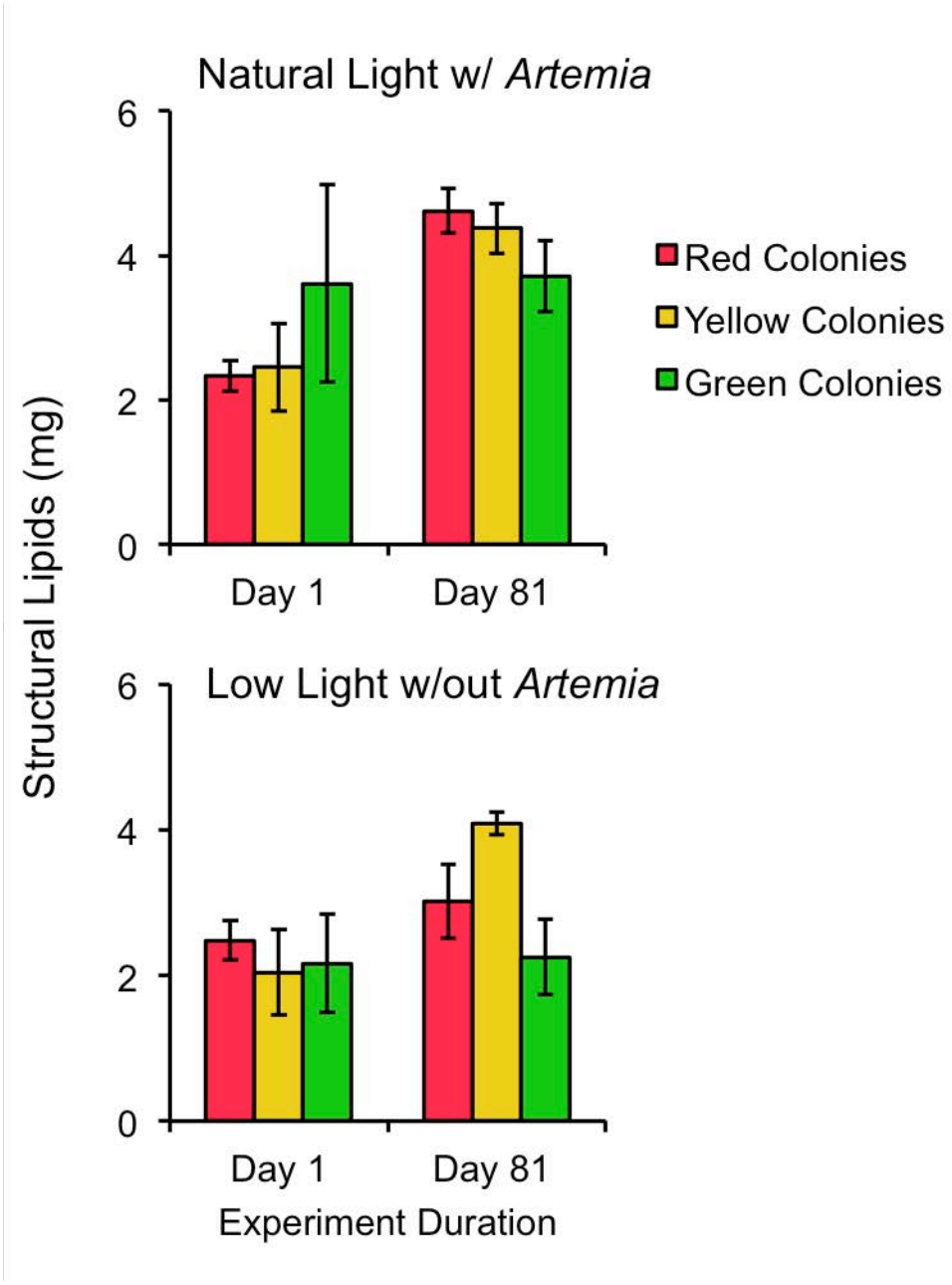

Fig. 6.4 Mean concentrations of structural lipids (±SE) compared among three colour morphs (green, yellow red) of *A. millepora* exposed to two nutritional treatments: 1) high nutritional regime (natural light with *Artemia*), and 2) low nutritional regime (low light without *Artemia*) at the first and last time points throughout the experiment. (Red: n = 5; Yellow: n = 5; Green: n = 5). Letter sets 'a' and 'b' signify similar concentrations, as determined by Tukey's HSD post-hoc analyses.

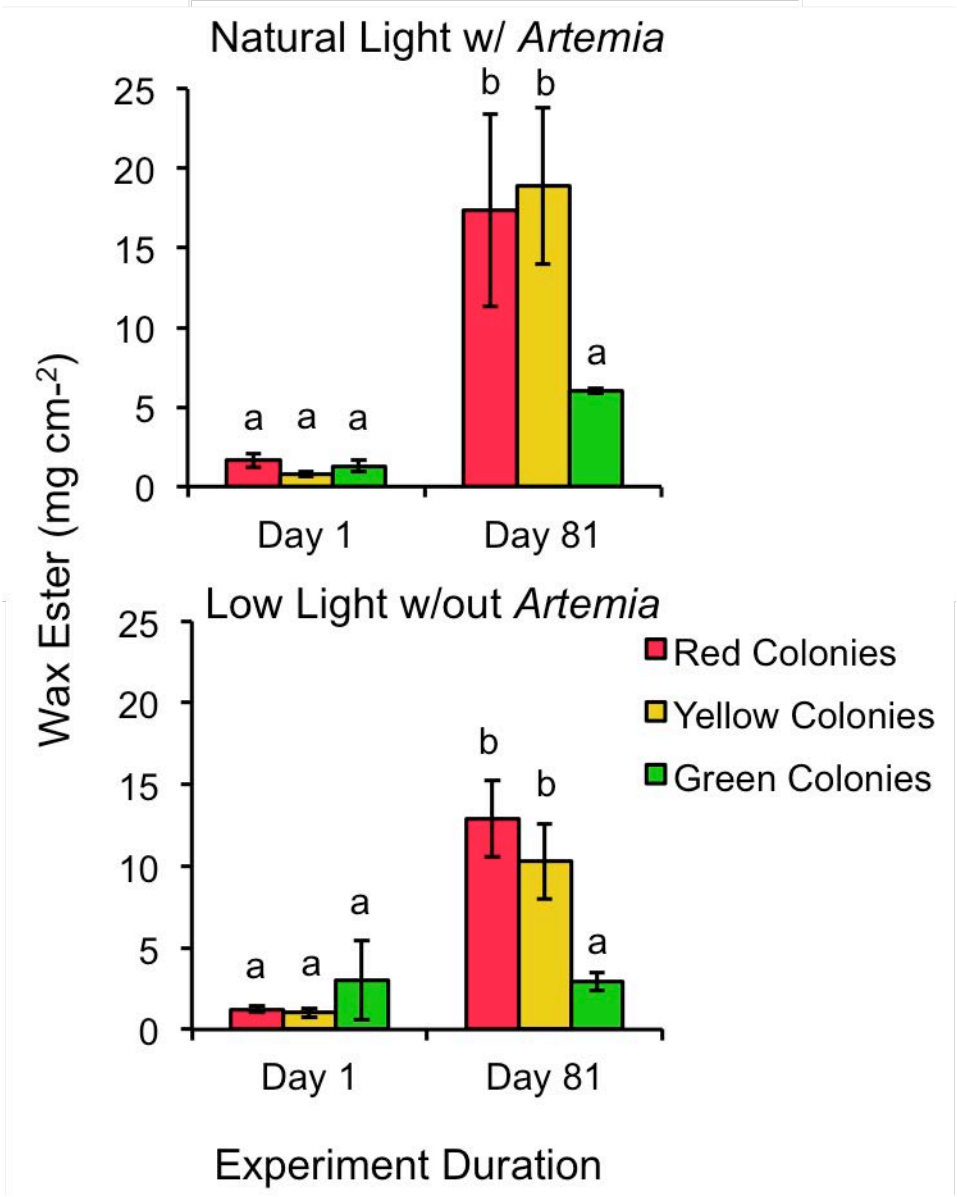

Figure 6.5 Mean concentrations of wax esters (±SE) compared among three colour morphs (green, yellow red) of *A. millepora* exposed to two nutritional treatments: 1) high nutritional regime (natural light with *Artemia*), and 2) low nutritional regime (low light without *Artemia*) at the first and last time points throughout the experiment. (Red: n = 5; Yellow: n = 5; Green: n = 5). Letter sets 'a' and 'b' signify similar concentrations, as determined by Tukey's HSD post-hoc analyses.

Table 6.5 Three-factor ANOVA comparing the mean concentrations of wax esters among three colour morphs of *A. millepora*, two nutritional treatments and two sampling times. *: denotes significance at α = .05.

Source of Variation	SS	df	F	p	
Sampling Time	3.6	1	40.2	.000	*
Treatment	.1	1	.633	.431	
Morph	.5	2	2.8	.076	
Sampling Time*Treatment	.2	1	1.900	.178	
Sampling Time*Colour	1.1	2	6.1	.005	*
Treatment*Colour	.0	2	.023	.977	
Sampling Time*Treatment*Colour	.087	2	.482	.621	
Error	3.5	39			

Table. 6.6 Tukey's HSD pairwise comparisons of wax ester concentrations among three morphs and two sampling times. *: denotes significance at α = .05.

Sampling Time		Day 1			Day 81		
	Morph	Red	Yellow	Green	Red	Yellow	Green
Day 1	Red						
	Yellow	.974					
	Green	.999	.959				
Day 81	Red	**.000***	**.000***	**.002***			
	Yellow	**.000***	**.000***	**.001***	.998		
	Green	.873	.452	.958	**.007***	**.003***	

6.3.3 Fluorescent proteins

Overall, green colonies had the highest total concentrations of FPs, followed by yellow and then red colonies (Fig. 6.6), consistent with results presented in previous chapters (Chapters 2, 3, 5). However, patterns in mean total FP concentrations through time differed among nutritional treatments for each of the green (p = 0.00; Table 6.7), yellow (p = 0.00; Table 6.7) and red morphs (p = 0.41; Table 6.7). In all three colour morphs, the general pattern was for total FP

concentrations to decline initially between days 1 and 29 in all nutritional treatments (except for FP concentrations in green colonies in the primarily heterotrophic treatment, which stayed low and constant throughout this period) (Fig. 6.6). However, whereas FP concentrations increased in the high nutrition treatment by day 81 (by 3.7-fold in green colonies: $p = 0.007$; 2.5-fold in yellow colonies: $p = 0.002$; and 1.3-fold in red colonies (non-significant): $p = 0.9990$; Table 6.8), they stayed low in the low nutrition treatment ($p = 1.00$ for green, yellow and red colonies; Table 6.8), patterns that were consistent for all three colour morphs (Fig. 6.6).

6.3.4 *Symbiodinium* density

Overall, mean *Symbiodinium* densities declined significantly throughout the experiment ($p = 0.000$; Table 6.9), by more than 2.5-fold between the first and final sampling times of the experiment across all nutritional treatments and morphs (Table 6.9; Fig. 6.7). Patterns in *Symbiodinium* densities in the four nutritional treatments differed among colour morphs ($p = 0.25$; Table 6.9). In the high nutritional treatment, mean *Symbiodinium* densities were greatest in the red morph, whereas, in the low nutrition treatment, mean *Symbiodinium* densities were greatest in the green morph. Overall, mean *Symbiodinium* densities were highest in the red morph when *Artemia* was added (treatments 1, 3), and highest in the green morph when *Artemia* was not added (treatments 2, 4).

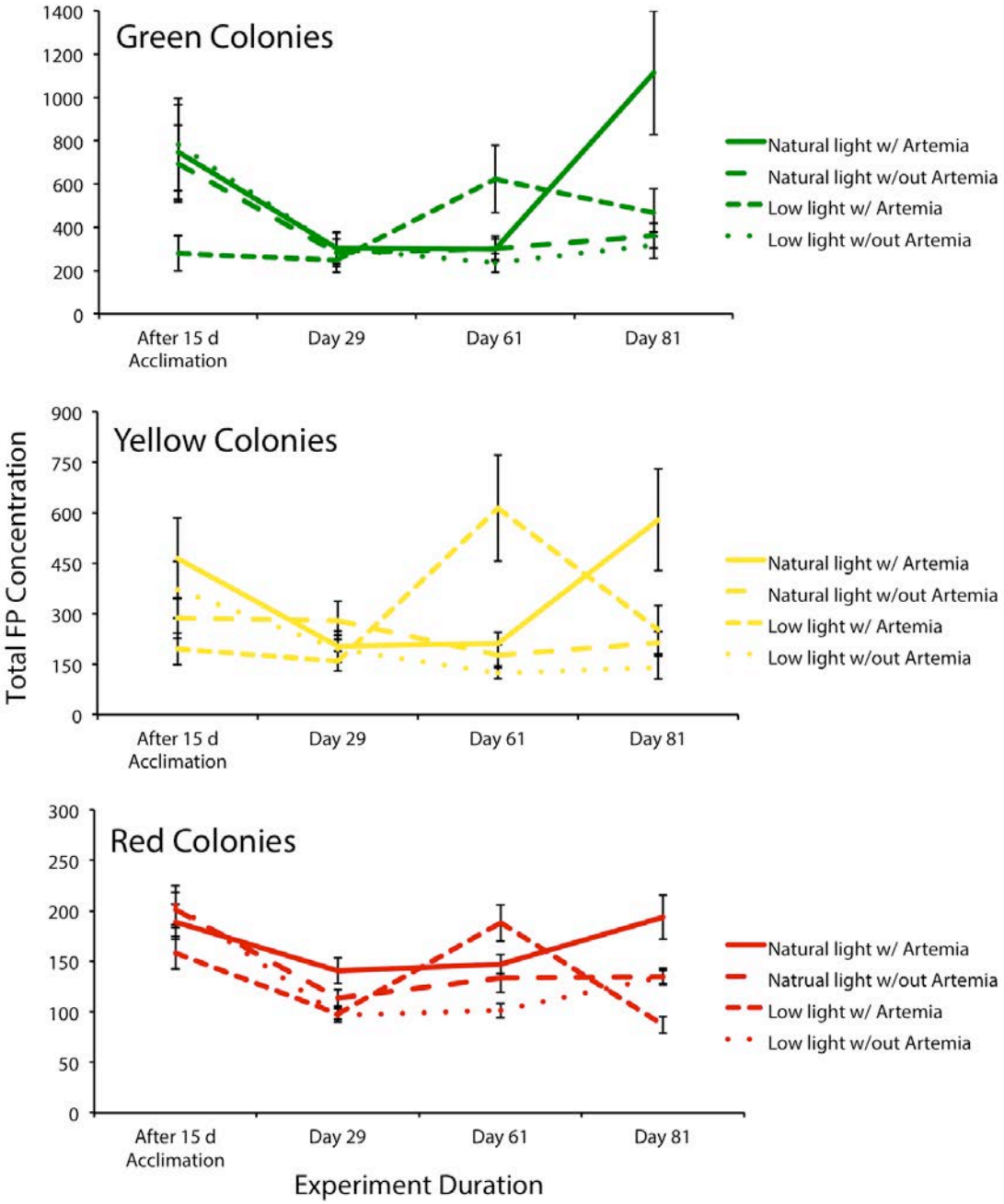

Figure. 6.6 Mean total FP concentrations (± SE) compared among three colour morphs (green, yellow, red) of *A. millepora* exposed to four nutritional treatments: 1) high nutritional regime (natural light with *Artemia*), 2) natural light without *Artemia*, 3) low light with *Artemia*, and 4) low nutritional regime (low light without *Artemia*) at four time points throughout the experiment. (Red: n = 5; Yellow: n = 5; Green: n = 5).

Table 6.7 Two-factor ANOVAs comparing mean total FP concentrations among three colour morphs of *A. millepora*, four nutritional treatments and four sampling times. *: denotes significance at α = .05.

Green Colonies

Source of Variation	SS	df	F	p	
Treatment	.308	3	3.7	.017	*
Sampling Time	1.0	3	12.5	.000	*
Treatment*Sampling Time	1.3	9	5.3	.000	*
Error	1.3	47			

Yellow Colonies

Source of Variation	SS	df	F	p	
Treatment	.352	3	7.2	.000	*
Sampling Time	.290	3	5.9	.002	*
Treatment*Sampling Time	1.6	9	12.0	.000	*
Error	.781	48			

Red Colonies

Source of Variation	SS	df	F	p	
Treatment	.190	3	2.5	.071	
Sampling Time	.375	3	4.9	.004	*
Treatment*Sampling Time	.493	9	2.1	.041	*
Error	1.7	64			

Table 6.8 Tukey's HSD pairwise comparisons of total FP concentrations among three colour morphs of *A. millepora*, four nutritional treatments and four sampling times. *: denotes significance at α = .05.

Green Colonies

Treatment		Natural light w/ Artemia				Natural light w/out Artemia				Low light w/ Artemia				Low light w/out Artemia			
	Sampling Time	After Acclim.	Day 29	Day 61	Day 81	After Acclim.	Day 29	Day 61	Day 81	After Acclim.	Day 29	Day 61	Day 81	After Acclim.	Day 29	Day 61	Day 81
Natural light w/ Artemia	After Acclim.																
	Day 29	.075															
	Day 61	.131	1.00														
	Day 81	.997	**.002***	**.007***													
Natural light w/out Artemia	After Acclim.	1.00	**.032***	.094	.223												
	Day 29	.266	1.00	1.00	1.00	.134											
	Day 61	.372	1.00	1.00	1.00	.313	1.00										
	Day 81	.907	**.000***	**.003***	**.011***	.567	1.00	1.00									
Low light w/ Artemia	After Acclim.	.048	**.009***	1.00	.890	.188	1.00	1.00	1.00								
	Day 29	1.00	1.00	.407	.957	**.043***	1.00	1.00	.995	1.00							
	Day 61	1.00	1.00	.521	.974	1.00	.225	.466	.733	.303	.081						
	Day 81	**.001***	**.000***	.790	.189	.996	.838	.973	.999	.906	.545	1.00					
Low light w/out Artemia	After Acclim.	1.00	.051	**.003***	.183	1.00	**.018***	.057	.147	.028	1.00	1.00	1.00				
	Day 29	**.046***	1.00	.999	1.00	.198	1.00	1.00	1.00	**.005***	1.00	1.00	.998	**.030***			
	Day 61	.085	1.00	1.00	1.00	.018	1.00	.997	.959	1.00	.317	**.036***	.670	**.002***	1.00		
	Day 81	1.00	.002	**.000***	**.008***	.500	1.00	1.00	1.00	.793	.915	.339	.997	.118	1.00	.976	

Yellow Colonies

Treatment		Natural light w/ Artemia				Natural light w/out Artemia				Low light w/ Artemia				Low light w/out Artemia			
	Sampling Time	After Acclim.	Day 29	Day 61	Day 81	After Acclim.	Day 29	Day 61	Day 81	After Acclim.	Day 29	Day 61	Day 81	After Acclim.	Day 29	Day 61	Day 81
Natural light w/ Artemia	After Acclim.																
	Day 29	**.024***															
	Day 61	**.023***	1.00														
	Day 81	1.00	**.002***	**.002***													
Natural light w/out Artemia	After Acclim.	.581	.644	**.002***	.053												
	Day 29	.975	.959	1.00	1.00	1.00											
	Day 61	.973	.956	1.00	1.00	.624	.560										
	Day 81	.152	.185	**.000***	**.006***	.997	.993	1.00									
Low light w/ Artemia	After Acclim.	**.007***	**.001***	.987	.301	.828	.777	1.00	1.00								
	Day 29	1.00	1.00	**.000***	.999	.373	.320	1.00	.983	1.00							
	Day 61	1.00	1.00	**.000***	.999	**.032***	**.042**	.000	**.001***	**.000***	**.000***						
	Day 81	**.001***	**.000***	1.00	.053	1.00	1.00	.879	1.00	.973	.667	**.009***					
Low light w/out Artemia	After Acclim.	1.00	**.011***	**.000***	**.004***	.986	.993	**.038***	.395	.087	1.00	.79	1.00				
	Day 29	.237	1.00	.484	1.00	.903	.865	1.00	1.00	**.013***	1.00	.99	1.00	.129			
	Day 61	.229	1.00	.496	1.00	.016	**.013***	.931	.306	.585	**.000***	**.000***	**.000***	**.000***	.682		
	Day 81	.927	**.001***	**.000***	**.000***	.710	.649	1.00	1.00	.882	.990	.054	.927	.053	1.00	.886	

Red Colonies

Treatment		Natural light w/ Artemia				Natural light w/out Artemia				Low light w/ Artemia				Low light w/out Artemia			
	Sampling Time	After	Day 29	Day 61	Day 81	After	Day 29	Day 61	Day 81	After	Day 29	Day 61	Day 81	After	Day 29	Day 61	Day 81
Natural light w/ Artemia	After Acclim.																
	Day 29	1.00															
	Day 61	1.00	1.00														
	Day 81	1.00	.995	.999													
Natural light w/out Artemia	After Acclim.	1.00	.871	.993	1.00												
	Day 29	.996	1.00	1.00	1.00	.662											
	Day 61	.999	1.00	1.00	1.00	.940	1.00										
	Day 81	1.00	.653	.937	.992	.992	1.00	1.00									
Low light w/ Artemia	After Acclim.	.997	.512	1.00	.170	.962	1.00	1.00	1.00								
	Day 29	1.00	.973	.997	.725	.286	1.00	1.00	.982	1.00							
	Day 61	1.00	.930	1.000	.596	1.00	.706	.956	.995	.973	.322						
	Day 81	.960	.279	1.000	.070	**.072***	.998	.930	.770	.897	1.000	.09					
Low light w/out Artemia	After Acclim.	1.00	.507	.661	.999	1.00	.424	.794	.942	.845	1.00	1.00	1.00				
	Day 29	.959	.972	.993	1.00	.283	1.00	1.00	.981	.139	1.00	1.00	.990	.137			
	Day 61	.986	.928	.976	1.00	.414	1.00	1.00	.996	1.00	.319	.457	.991	.221	1.00		
	Day 81	1.00	.276	.406	.984	.985	1.00	1.00	1.00	.028	1.00	1.00	.822	.914	.990	.998	

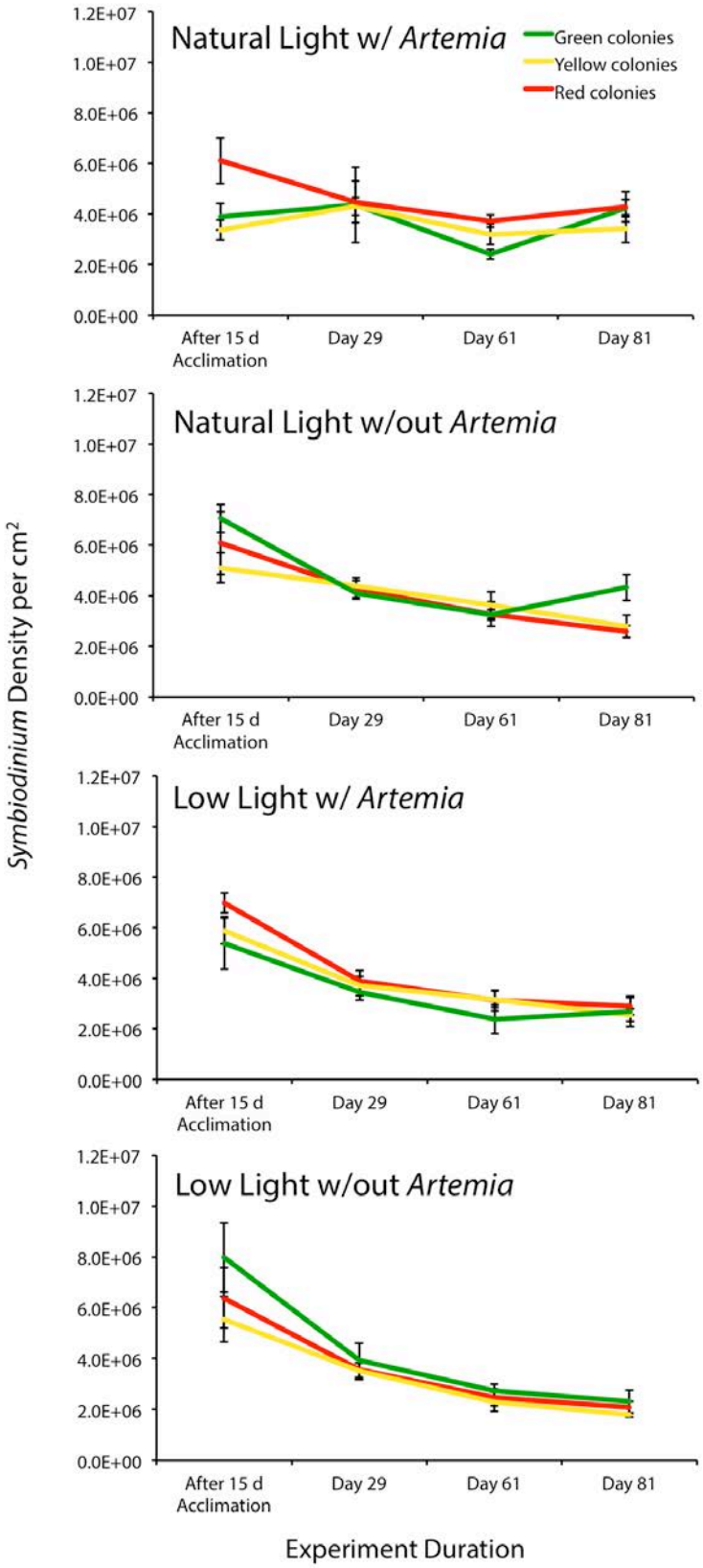

Figure. 6.7 Mean *Symbiodinium* densities (± SE) compared among three colour morphs (green, yellow, red) of *A. millepora* exposed to four nutritional treatments: 1) high nutritional regime (natural light with *Artemia*), 2) natural light without *Artemia*, 3) low light with *Artemia*, and 4) low nutritional regime (low light without *Artemia*) at four time points throughout the experiment. (Red: n = 5; Yellow: n = 5; Green: n = 5).

Table 6.9 Three-factor ANOVA comparing mean *Symbiodinium* densities among three colour morphs of *A. millepora*, four nutritional treatments and four sampling times. *: denotes significance at α = .05.

Source of Variation	SS	df	F	p	
Sampling Period	2.7	3.0	17.2	.000	*
Treatment	.091	3.0	.580	.629	
Morph	.131	2.0	1.3	.289	
Sampling Period*Treatment	.890	9.0	1.9	.056	
Sampling Period*Morph	.141	6.0	.450	.844	
Treatment*Morph	.777	6.0	2.5	.025	*
Sampling Period*Treatment*Morph	.936	18.0	.995	.468	
Error	9.0	173.0			

Table 6.10 Tukey's HSD pairwise comparisons of *Symbiodinium* densities among three colour morphs of *A. millepora* and four nutritional treatments. *: denotes significance at α = .05.

Treatment		1: Natural light w/ *Artemia*			2: Natural light w/out *Artemia*			3: Low light w/ *Artemia*			4: Low light w/out *Artemia*		
	Morph	Red	Yellow	Green	Red	Yellow	Green	Red	Yellow	Green	Red	Yellow	Green
1: Natural light w/ *Artemia*	Red												
	Yellow	.135											
	Green	.151	.980										
2: Natural light w/out *Artemia*	Red	.303	.341	.965									
	Yellow	.639	.585	.146	.939								
	Green	.640	.589	.160	.311	.346							
3: Low light w/ *Artemia*	Red	.435	.287	**.030***	.220	.124	.125						
	Yellow	.472	.694	.443	.374	.232	.075	.759					
	Green	.482	.692	.481	.774	.980	**.005***	.149	.263				
4: Low light w/out *Artemia*	Red	**.046***	**.022***	.423	.327	.291	.054	.220	.124	.125			
	Yellow	.610	.406	**.028***	.196	.172	**.027***	.374	.232	.075	.743		
	Green	.648	.447	**.035***	.078	.090	.473	.774	.980	**.005***	.056	.054	

6.4 Discussion

6.4.1 A trend for patterns in mean total lipid to differ among colour morphs

Despite prolonged deprivation of light and food resources, total lipid content in three colour morphs of *Acropora millepora* did not differ between the restricted nutritional treatment and treatments with higher light or supplied plankton, or between the initial and final samples throughout the 3-month resource limitation experiment. An increase in lipids associated with oocyte maturation had been expected, given that the experiment coincided with the final three months of oocyte maturation, with the final sample collected on the night before the annual mass spawning of this species at Orpheus Island (Willis et al. 1985; Babcock et al. 1986). Although not statistically significant, the 1.7 to more than 3-fold increase in mean lipid content in yellow and red colonies, respectively, within the unrestricted (high) nutrition treatment over the three months is consistent with increasing allocation of resources to reproduction leading up to spawning. In contrast, constancy in mean lipid content in green colonies in the high nutrition treatment throughout the 3 months leading up to spawning is consistent with reduced allocation to reproduction in comparison to the red and yellow morphs. It is possible that declining colony condition under experimental conditions offset potential increases in lipid content associated with oocyte maturation in all three colour morphs, explaining the lack of statistical significance associated with the trend for increasing concentrations of total lipids in red and yellow morphs, and the lack of any detectable increase in lipid content in the green morph. Although patterns did not differ statistically, these trends in mean total lipid content through time suggest intraspecific variation in resource allocation to reproduction among

colour morphs of *A. millepora*. The comparatively lower resource allocation to reproduction in the green morph suggests that it might preferentially allocate resources to other key physiological processes, for example to FP production.

6.4.2 Resource allocation to reproduction differs among colour morphs

In contrast to the lack of statistical difference in patterns of mean total lipid content among colour morphs, concentrations of storage lipids and wax esters did differ significantly, confirming that colour morphs of *A. millepora* differ in their patterns of resource allocation to reproduction. The 2- and 3-fold greater concentrations of storage lipids and wax esters in red and yellow colonies compared with green colonies, in all four nutritional treatments, provides strong support for my conclusion that the red and yellow morphs of *A. millepora* allocate more resources to reproduction than the green morph. Moreover, this pattern was consistent regardless of resource availability. Similarity in patterns of increasing concentrations of these two parameters in red and yellow colonies prior to the annual spawning event indicates investment of resources into the production of storage lipids for use in reproduction. Wax esters comprise up to 82% of oocytes in *Acropora* spp., and as much as 92% of oocytes in *A. millepora* (Arai et al. 1992). The magnitude of the contribution of wax esters to oocyte composition, coupled with evidence of significant declines in the concentration of storage lipids and wax esters directly following spawning (Oku et al. 2003; Leuzinger et al. 2003), indicate that patterns in wax ester concentrations can be used as a proxy for reproductive output. The 2- to 5-fold lower concentrations of storage lipids and wax esters in green colonies at the end of the study, in all nutritional treatments, indicates that the green morph had reduced

reproductive output in comparison with the yellow and especially the red morph. These findings, of lower allocation of resources to reproduction by the green morph in comparison to the red and yellow morphs, provide the first evidence of intraspecific variation in strategies for investing energetic resources into reproduction in a coral species.

Consistency in the concentration of structural lipids among colour morphs of *A. millepora* throughout the experiment indicates that a minimum allocation of resources is required to sustain cellular and tissue structures. Polar lipids provide the structural basis of cell membranes in corals and their symbionts (Imbs and Yakovleva 2012). Declines in structural lipid content are typically associated with severe stress, such as high temperatures leading to coral bleaching and the loss of energy-acquiring dinoflagellate symbionts (Grottoli et al. 2004; Rodrigues et al. 2008; Imbs and Yakovleva 2012). The lack of variation in structural lipid concentrations among colour morphs in my study, in comparison with the marked increased in storage lipids in the red and yellow morphs, could be interpreted as differences in either resource allocation or energy acquisition among colour morphs. For example, red and yellow colonies generated substantial reserves of storage lipids, which were then available for investment into reproduction. In comparison, green colonies contained much lower reserves of storage lipids, either because: (1) energy acquired was directed towards processes other than reproduction, or (2) energy acquisition was lower. Although *Symbiodinium* density declined in all colour morphs, regardless of nutritional treatment, the magnitude of declines (considerably less than 10-fold) and the lack of change in colony pigmentation in any colour morph (data not presented) are consistent with an adjustment to experimental light conditions rather than declining health. In the absence of any evidence of reduced health of green colonies in

comparison to red or yellow colonies, I conclude that resource allocation strategies differ among the three colour morphs, with the green morph preferentially allocating resources to key physiological processes other than reproduction.

6.4.3 A trade-off between reproduction and FP production

Comparisons of FP concentrations alongside changes in lipid content among the three colour morphs suggest that an energetic trade-off has evolved and underpins the maintenance of colour polymorphism in populations of *A. millepora*. Inverse patterns of energy investment, as confirmed by high tissue concentrations of FPs coupled with low concentrations of storage lipids in the green morph, in contrast to low tissue concentrations of FPs coupled with high concentrations of storage lipids in the yellow and especially the red morph, signify the presence of an evolutionary trade-off between the energetically costly processes of reproduction and the production of stress-mitigating FPs. The fact that in the final 20 days of the study, corresponding to the three weeks leading up to spawning, total FP content only increased within the unrestricted (high) nutritional treatment suggests that FP production is energetically costly. Thus the 1.8- and 5.5-fold greater total FP concentrations in green colonies compared to yellow and red colonies, respectively, represented a significant impost on the energy budget of green colonies. The inverse patterns of FP and storage lipid concentrations between green colonies versus red and yellow colonies are consistent with differential resource partitioning among intraspecific coral colour morphs, and provide evidence of an energetic trade-off between investment in reproduction versus FP production. These findings directly

contradict those of previous studies, which led to the conclusion that coral FP expression is energetically 'cheap' to maintain based on the slow decay rates of RFP (Leutenegger et al. 2007). As documented in chapters 2 and 3, RFP is typically the FP in lowest concentration in colour morphs of *A. millepora*. Comparatively less is known about decay rates of the most abundant coral FP, CFP, which might be much more energetically costly to maintain in the high concentrations recorded in green colonies (Chapters 2 and 3) than previously suggested. I therefore propose that it is energetically costly to produce and maintain the colour and vibrancy of the highly fluorescent green morph of *A. millepora,* and maintenance of high tissue concentrations of the CFP, in particular, may occur at the expense of reproductive fitness. Such a trade-off explains the lower levels of storage lipids and wax esters in the green morph, as resources required for the energetically costly process of reproduction were depleted to maintain high concentrations of FPs (Chapters 2 and 3). Conversely, red colonies, which invest little into the production and maintenance of coral FPs, have surplus energetic resources available to invest into reproduction.

Evidence of lower investment in reproduction by the green morph of *A. millepora* corroborates patterns in the abundance and distribution of the three colour morphs found across large spatial scales and environmental gradients on the Great Barrier Reef (Chapter 2). In particular, these results explain why green colonies are consistently rare in comparison with red and yellow colonies. A comparatively greater investment into reproduction by red and yellow colonies would explain their greater abundance within populations. Such intraspecific variation in resource allocation among colour morphs has far-reaching ecological implications, leading to differences in recruitment potential versus resilience to environmental disturbance among colour morphs of this species. In combination with results from previous chapters, my results

suggest that red colonies have stronger recruitment potential as a result of greater reproductive output, but have lower tolerance to thermal stress, whereas green colonies have the potential for greater thermal tolerance but at the cost of reduced recruitment (Chapters 4 and 5). Although greater reproductive output may be advantageous for population replenishment following disturbance (Hughes et al. 2000), the reduced resilience to environmental perturbations, particularly thermal stress, of the red morph is a significant disadvantage given predictions of ocean warming (IPCC 2013). Conversely, although the green morph has superior performance under thermal stress, its abundance within populations remains low as a consequence of lower investment in reproduction. These intraspecific differences in resource allocation have resulted in populations currently being dominated by colour morphs that are thermally less resilient. However, by maintaining colour polymorphisms corresponding to differing FP characteristics, *A. millepora* has increased the resilience of the species as a whole to predicted ocean warming.

6.4.4 Conclusions

Results presented in this chapter represent the first documentation of differential investment into reproduction among intraspecific colour morphs of a coral species. Importantly, they uncover an energetic trade-off between resource allocation to reproduction versus FP production, thereby providing significant insight into the underlying basis for the maintenance of colour polymorphism in populations of *A. millepora*. These data also substantiate patterns of abundance and distribution presented earlier in this thesis (see Chapter 2); the comparatively lower investment

into reproduction of green colonies explains their lower abundance in comparison with those of the red and yellow morphs, both of which invest comparatively greater resources into reproduction. These findings also explain the lack of correlation of colour morph abundance with environmental gradients, and instead offer strong support for the suggestion that coral colour is largely a heritable trait, with discrete colour morphs maintained by disruptive selection. In summary, selection for different physiological attributes under typical versus stressful thermal regimes maintains colour polymorphism with populations of *A. millepora*.

Chapter 7

General Discussion: Coral colour/FPs as useful bio-indicators

Understanding the suite of mechanisms corals employ to combat changes in environmental conditions underpins our capacity to predict and manage coral reefs under climate change. Research presented in this thesis significantly advances current understanding of the influences that FPs have on stress tolerance in corals. In this final chapter, I synthesise results presented in chapters 2 through 6 to offer an ecological perspective on the role of FPs in corals, and highlight the relevance of documenting coral colour polymorphism for both coral reef biologists and managers. In particular, my research establishes the importance of colour polymorphism as a mechanism for providing coral species with options for responding to thermal stress. In addition, I show that colour provides a useful indicator of susceptibility to thermal stress, as well as levels of energy investment into somatic growth. I conclude by identifying future research directions that will further resolve our understanding of the importance of colour diversity within coral populations.

7.1 The *A. millepora* FP complement and its relationship with colony colouration and the ecology of colour polymorphisms

Patterns in the distribution and abundance of colour morphs within the many marine invertebrate species that exhibit intraspecific colour polymorphisms are

governed by either environmental stimuli (Harley et al. 2006) or genetic factors (Kelmanson and Matz 2003). Accordingly, insights into the ecology or biology of a species can be gleaned from observations of colour variation patterns and their distribution. Despite coral colour polymorphisms featuring in studies of intraspecific thermal tolerance (e.g. Dove 2004), current understanding of how FPs contribute to coral colour is limited to two studies (e.g. Mazel and Fuchs 2003; Cox et al. 2007), neither of which documented the relative contributions of the multiple FPs that are present in coral tissues. Although several studies have compared colour morph abundance patterns between different habitats, none have investigated these patterns across large spatial scales or found consistency in patterns across varying habitats (e.g. Gleason 1993; 1998; Takabayashi and Hoegh-Guldberg 1995). The results presented in Chapter 2 are the first to characterize the FP complement of intraspecific coral colour morphs and to document their patterns of abundance and distribution across a large latitudinal gradient. FP concentrations were estimated from fluorescent spectrums by modelling empirical data from extracted, purified and cloned *A. millepora* FPs. This technique uses fluorescence spectrums to estimate FP concentration and is validated by actual FP concentrations extracted from this species and their corresponding fluorescent spectrums. FP emission spectra modelling allows for comparatively quick analysis of FP concentrations compared with Western-blot analysis, and may be adapted to suit living coral tissue, thus eliminating problematic use of spectrophotometers with fibre optics where fluorescent intensity varies greatly depending on the point of illumination.

I show that the four common colour morphs of *A. millepora* (green, blue, yellow and red) differ in the relative concentrations of three FPs and one CP, and colour morph-specific patterns were conserved both spatially across a large latitudinal

gradient (Chapter 2) and temporally throughout a mild summer-spring-fall season (Chapter 3). CFP is the most abundant FP in all four colour morphs, regardless of sampling time (Chapter 3) or location (Chapter 2), despite the visually distinctive colours of the four morphs. Although the visual impact of a FP differs depending on the wavelength of its emission (i.e. the longer the wavelength, the stronger the visual effect; Mazel and Fuchs 2003), it was previously thought that colour differences among morphs of *Acropora millepora* were determined by the FP in highest abundance (Cox et al. 2007). The results from Chapter 2 indicate that, while concentration is an important consideration in colour determination, the relative abundance of other, less abundant FP types and their type-specific visual impacts result in the extensive colour variation observed in this species, variation that spans the entire visible spectrum. These findings highlight that FP concentration corresponds with coral colouration and suggest that colour may serve as a gauge of fluorescent intensity to the naked eye, with the green morph being the most strongly fluorescent and the red morph being the most weakly fluorescent.

I found that patterns in the differential abundance of the four colour morphs are conserved across a variety of spatial and ecological scales on the Great Barrier Reef (Chapter 2). The most abundant (red) colour morph was also the least fluorescent, exhibiting comparatively weaker fluorescence for both CFP and GFP than other morphs. Conversely, the most fluorescent (green) morph was one of the rarest, comprising between 3 and 13% of the populations surveyed (Chapter 2). These abundance patterns were maintained across study sites of varying depth and wind exposure, and across a 4^{o} latitudinal gradient in seawater temperature (Chapter 2). Despite rarity of the green morph, and especially of the blue morph, these two morphs were present within all populations and across all environmental parameters

investigated. These patterns highlight the absence of environmental drivers of colour morph distribution patterns in *A. millepora*. Given the widely-held, yet largely unconfirmed, photoprotective role of coral FPs, the most surprising of the relationships revealed in Chapter 2 was the lack of correlation between colour morph abundance and depth. The consistent pattern of high abundance of the weakly fluorescent morph and low abundance of the highly fluorescent morph, at both shallow (<5m) and deep (>5m) reef sites, suggests the local light regime plays little role in controlling FP concentration in this species. This ecological evidence therefore suggests a hereditary basis for coral colouration, as we would have expected the proportion of phenotypes to differ among habitats if coral colour were the result of varying environmental conditions.

7.2 The role of FPs in the variable thermal tolerances of coral colour morphs

Uncertainties about what advantages FPs afford to corals continue in the literature largely as a consequence of conflicting results reported in the last two decades, with some studies demonstrating that possession of coral FPs confers greater bleaching resilience (e.g., Salih et al. 2000), while others report that FPs leave colonies exposed to high light levels during thermal stress (e.g., Dove 2004). The majority of studies examining intraspecific variation in thermal tolerance do not consider FPs, instead focusing on the influence of genetic diversity in *Symbiodinium* types on physiological properties of the coral host (e.g. Abrego et al. 2008; Meiog et al. 2009; Howells et al. 2012). Although several authors have acknowledged that the coral host may also possess a suite of mechanisms to mitigate stress (reviewed in

Baird et al. 2009), to date no studies have incorporated both *Symbiodinium* genetic diversity and FP content in assessments of variation in bleaching susceptibility among intraspecific colour morphs.

Observations of colony condition in the field prior to and during thermal stress in a moderate, natural bleaching event (Chapter 4) and under experimental conditions during a controlled thermal stress study (Chapter 5), suggest that higher concentrations of FPs confer greater bleaching resilience to *A. millepora*. Bleaching resistance was significantly higher for fragments of the highly fluorescent, green morph compared with fragments of the intermediately and weakly fluorescent yellow and red morphs, respectively. Similarly, recovery was greatest for the highly fluorescent green fragments following severe heat stress, as scored visually and through measurements of *Symbiodinium* density (Chapter 5). Coral health assessments following 3 weeks of recovery at ambient temperatures revealed that greater bleaching susceptibility of the yellow and red morphs corresponded with reduced recovery rates. Although few studies incorporate recovery into experimental designs, bleaching signs have been observed in the field for several months following disturbance (Thompson et al. 2011), therefore I cannot conclude absolutely that recovery levels differed among colour morphs. However, the greater sensitivity of red colony fragments during acclimation and following experimental heating indicates their resilience to changes in environmental conditions is poor and indicates that their recovery potential following disturbance is lower.

Although multiple *Symbiodinium* types were present within experimental colonies, no colour morph × *Symbiodinium* genotype associations were detected, nor did bleaching susceptibility correspond with *Symbiodinium* type alone (Chapter 5). These findings corroborate those of other studies, which have shown that

Symbiodinium type is not the sole determinant of coral thermal tolerance (Abrego et al. 2008; Baird et al. 2009; Kenkel et al. 2013; other refs), and they signify the importance of considering coral host-mediated processes in the regulation of thermal tolerance of the coral holobiont. The role of coral FPs in regulating coral thermal tolerance is routinely acknowledged in the literature (Salih et al. 2000; Abrego et al. 2008; Dove et al. 2008; Baird et al. 2009), although to my knowledge, no studies have combined comparisons of relative FP concentrations and *Symbiodinium* assemblages to explain differences in thermal tolerance among intraspecific colour morphs. Although the molecular techniques used in this thesis lacked the sensitivity required to detect background levels of rare *Symbiodinium* types, they add to our knowledge of the *Symbiodinium* diversity present within populations of *A. millepora* in the Palm Islands. Previously, *Symbiodinium* ITS-1 type C2 has been reported to be the dominant type present in populations of *A. millepora* from the Palm Islands, with background levels of D1 also typically present (Cooper et al. 2011; Howells et al. 2013). This is the first study to detect ITS-1 type C1 from colonies sampled in Pioneer Bay, Orpheus Island, implying a greater diversity than previously thought of Symbiodinium type associations than previously documented in this region.

Significant declines in FP concentrations when experimental fragments were translocated from the field to experimental tanks (Chapter 5) were consistent with similar patterns of reduced FP concentrations following transfer to lower-light experimental conditions, well documented in previous studies (Bay et al. 2009a, b), and suggest that FPs have a photoprotective role (i.e. high FP concentrations are unnecessary in lower light conditions and therefore expression declines). However, increases in FP expression during recovery of all colour morphs in tanks with elevated temperatures, despite unaltered experimental light levels (Chapter 5), indicate that FP

expression can also change in response to temperature stress. These results further substantiate the claim the coral FPs function in thermal resistance in addition to photoprotection.

7.3 The consequences of coral colour: fluorescence at the expense of fitness?

The rarity of the highly fluorescent (green) morph across multiple spatial scales, despite (1) the documented advantage of maintaining high FP concentrations under thermal stress, and (2) the occurrence of thermal stress in thermal histories of the populations surveyed, underscores the likelihood that there are disadvantages associated with maintaining high FP concentrations in coral tissues. The discovery that RFP has a half-life of approximately 20 days led Leutenegger et al. (2007) to propose that coral FPs are long-lived and energetically 'cheap' to maintain at high concentrations in coral tissues. RFP was the least abundant of all coral FPs detected in tissues of the four colour morphs of *A. millepora* (Chapter 2); moreover, its concentrations were less variable under thermal stress (Chapter 5) and resource deprivation (Chapter 6) than either CFP or GFP. We know much less about the stability of, or energetic investment required to produce CFPs, the most abundant FP in tissues of *A. millepora* colour morphs (Chapter 2), but if CFP is also relatively stable, then fluctuating tissue concentrations of this FP (Chapter 3) represent rapid biosynthesis and use of this protein, presumably as part of a biological response of the coral host. Changes in coral FP expression are among the most rapid tissue responses reported to changes in light and temperature conditions (Bay et al. 2009a; Desalvo et al. 2008), occurring within as little as 7 days. Such responses reflect changes in FP

concentration within time spans much shorter than the natural decay rates observed in Leutenegger et al. (2007). Although further studies of the stability of CFP and GFP are needed, rapid fluctuations in tissue concentrations of these two FPs under changing environmental conditions provides additional evidence that they are part of the coral stress response.

In light of evidence that the highly fluorescent green morph is the least abundant morph *of A. millepora* across large spatial scales on the GBR (Chapter 2), combined with evidence that it has the highest thermal tolerance (Chapters 4 & 5), I propose that the production of high concentrations of coral FPs, particularly CFP, comes at the expense of other physiological processes. This conclusion is supported by evidence from my starvation study (Chapter 6), which showed that under conditions of resource limitation, the weakly fluorescent red morph was able to channel more resources into lipid synthesis for egg production (i.e. non-polar lipids) than the highly fluorescent green morph. Results of this experiment suggest that colour in *A. millepora* represents a trade-off between energetic investment into reproduction (as indicated through measurements of non-polar lipid class data) versus other physiological functions, for both healthy colonies and those under starvation.

Change in *Symbiodinium* density is a commonly used indicator of coral holobiont health during disturbances such as bleaching, however, when stressors (e.g. high temperatures and irradiance; Berkelmans and Oliver 1999; Hoegh-Guldberg 1999) are below threshold levels that disrupt the coral-algal symbiont association, total lipid content is widely acknowledged as a more useful indicator of coral condition (Anthony 2006). In particular, changes in lipid fraction ratio of non-polar (storage lipids) to polar (structural) lipids has received recent attention as a more rigorous bio-indicator of coral condition (Saunders et al. 2005; Cooper et al. 2009;

2011). Three-fold increases in total lipid content in tissues of healthy red colonies, during the 22 days preceding spawning (Chapter 6) reflected increases in wax esters (indicated through lipid class measurements; Chapter 6) that were highest in red colonies. As wax esters are typically a main component of lipids in coral eggs (Arai et al. 1993; Harii et al. 2007), these findings suggest that increasing lipid content in healthy corals preceding spawning was the result of preparations for release of lipid-rich gametes during spawning, with such preparations being greatest in the red morph.

Comparisons of lipid content and FP concentration levels among healthy, highly fluorescent (green) and weakly fluorescent (red) colonies provide an interesting and unexpected insight into how resources are directed towards different functions within different colour morphs of this species. If investment of resources into reproduction is greatest among red colonies, and if coral colour is indeed inherited as proposed here and by several studies (Takabayashi and Heogh-Guldberg 1995; Veron 1995; Leutenegger et al. 2007), these findings provide corroborative evidence that the dominance of red colonies among the coral populations surveyed (Chapter 2) is a consequence of directing greater resources towards reproduction. Comparatively lower investment of resources into reproduction among green colonies may free-up resources available for the production and maintenance of high concentrations of coral FPs at the expense of reproduction (Chapter 6), which explains their low prevalence among populations of this species on the GBR. These findings suggest that low FP concentrations within red colonies may be the result of fewer resources available to direct towards their production because of energetic trade-offs required to maintain high investment of resources into reproduction.

My findings are the first to suggest that strategies for investing resources into reproduction differ among intraspecific colour morphs, and contribute to explaining

the occurrence of uneven abundances of this species across extensive geographic scales and environmental gradients. This work also suggests that investment in production and maintenance of high concentrations of some coral FPs (namely CFP) may be energetically more costly than previous proposed (e.g. Leutnegger et al. 2007).

7.4 Maintenance of colour polymorphism in populations of *A. millepora*

The maintenance of genetic polymorphisms within populations is generally the consequence of disruptive selection (Mather 1995; Rueffler et al. 2006). Populations of *A. millepora* have been exposed to periodic warm thermal anomalies, hence selection for the maintenance of the green morph in such years would maintain this morph within populations. In years without thermal anomalies, there would be selection for morphs that invest energetic resources in functions other than FP production. Hence disruptive selection would maintain colour polymorphism within populations of *A. millepora.* Predictions for ocean warming indicate that coral reefs may reach or exceed their bleaching thresholds annually within as little as several decades (Hoegh-Guldberg 1999; Hoegh-Guldberg et al. 2007). Increasing frequency of thermal anomalies may result in changes to the patterns of abundance of *A. millepora* colour morphs where the highly fluorescent green morph increases in prevalence. Increasing abundance of green *A. millepora* colonies would result in thermally hardier populations, however may come at the expense of the reproductive fitness if green colonies invest comparatively less resources into reproduction. Disruptive selection in this direction

7.5 Recommendations for future directions in the field of coral fluorescence

My conclusion that the dominance of red colonies within populations of *A. millepora* on the GBR is due to greater reproductive investment enabled by reduced investment in FPs hinges on coral colour having a hereditary basis. However, current understanding of colour inheritance remains speculative and unclear. Breeding experiments involving controlled crosses within and between colour morphs will help to reveal the genetic basis of colour inheritance.

Techniques used to genotype *Symbiodinium* in Chapter 5 were not sensitive enough to detect background levels of other genotypes simultaneously present. My research also did not investigate whether changes in *Symbiodinium* genotype occurred during recovery from bleaching. Therefore further examinations into the diversity of *Symbiodinium* – colour morph associations both pre- and post-thermal stress are warranted.

To elucidate the potential photoprotective roles of coral FPs, changes in FP expression could be monitored under different coloured light conditions. Aquarists have long practised altering the expression and intensity of fluorescent coral colours by exposing coral fragments to different wavelengths of light (e.g. altering the Kelvin rating of light sources) in aquaria (Borneman 2001). These techniques could be implemented in an experimental design to determine whether changes in light intensity and quality influence changes in the expression patterns of coral FPs. Although D'Angelo et al. (2008) has looked at FP expression in a single coral colour morph under experimentally manipulated light regimes, new insights would be

revealed by using multiple colour variants from a species with colour polymorphism, like *A. millepora.* Further, intra-colour morph variation in FP expression should be assessed *in-situ* and compared with environmental correlates such as light /depth gradients to further test my conclusion that fluorescence plays only a small role in governing the ecology of this species.

This thesis demonstrated that the possession of greater concentrations of CFPs resulted in increased thermal resilience of corals. Although it is possible that FPs possess some property other than the capacity to dissipate thermal stress that could have provided bleaching tolerance, the known stress-dissipating properties of FPs makes this the most parsimonious explanation. However, investigating the comparative properties of similar concentrations of CFPs versus RFPs or GFPs particularly in response to heat stress would be a useful area for future research.

The potential for coral colony colour to be used as an *in-situ* bio-indicator has been proposed by several authors (e.g., D'Angelo et al. 2008; 2012; Smith et al. 2013), although direction for how such measures could be practically applied are wanting. Given the propensity for highly fluorescent (typically green) coral colonies to possess greater thermal resilience (Chapters 4 & 5; Salih et al. 2000), and the high prevalence of acroporid coral species exhibiting multiple colour polymorphisms on the GBR (Veron 2000), recording the prevalence of common coral colony colours may aid already well-established reef surveys (e.g. BleachWatch) typically conducted by non-scientific reef users to provide meaningful data on the potential vulnerability of sites visited. High prevalence of fluorescent colonies may indicate a recently disturbed location or conversely, low prevalence of fluorescent colonies may indicate low levels of disturbance or successful proliferation and recruitment.

7.6 Concluding remarks

Ongoing degradation of coral reef habitats is a likely consequence of climate change (Hoegh-Guldberg 1999; Hughes et al. 2003; Pandolfi et al. 2011), and may create a scenario where some coral colour types prevail over others. The relative merits of thermal tolerance of the highly fluorescent green morph versus reproductive success of the weakly fluorescent red morph are likely to become increasingly important under future climate scenarios, although difficult to predict with the current state of knowledge of coral resource allocation. Perhaps those colour types that represent the intermediate between these extremes possess the greatest ability to persist under future climate scenarios.

While a comprehensive understanding of the many roles of FPs within the coral holobiont will undoubtedly continue to develop with further research, this thesis provides a novel ecological perspective on both the positive and negative effects of maintaining high versus low FP concentrations within tissues of intraspecific coral colour morphs. This research improves our understanding of the extensive intraspecific variability that exists among colour morphs in their responses to their environment. Including or, at the very least, acknowledging colour variation in experimental design and reef monitoring are practical applications that should arise from this research, as a step towards a more holistic understanding of variable responses among corals to varying environmental changes.

Appendix

Chapter 5 supplemental figures

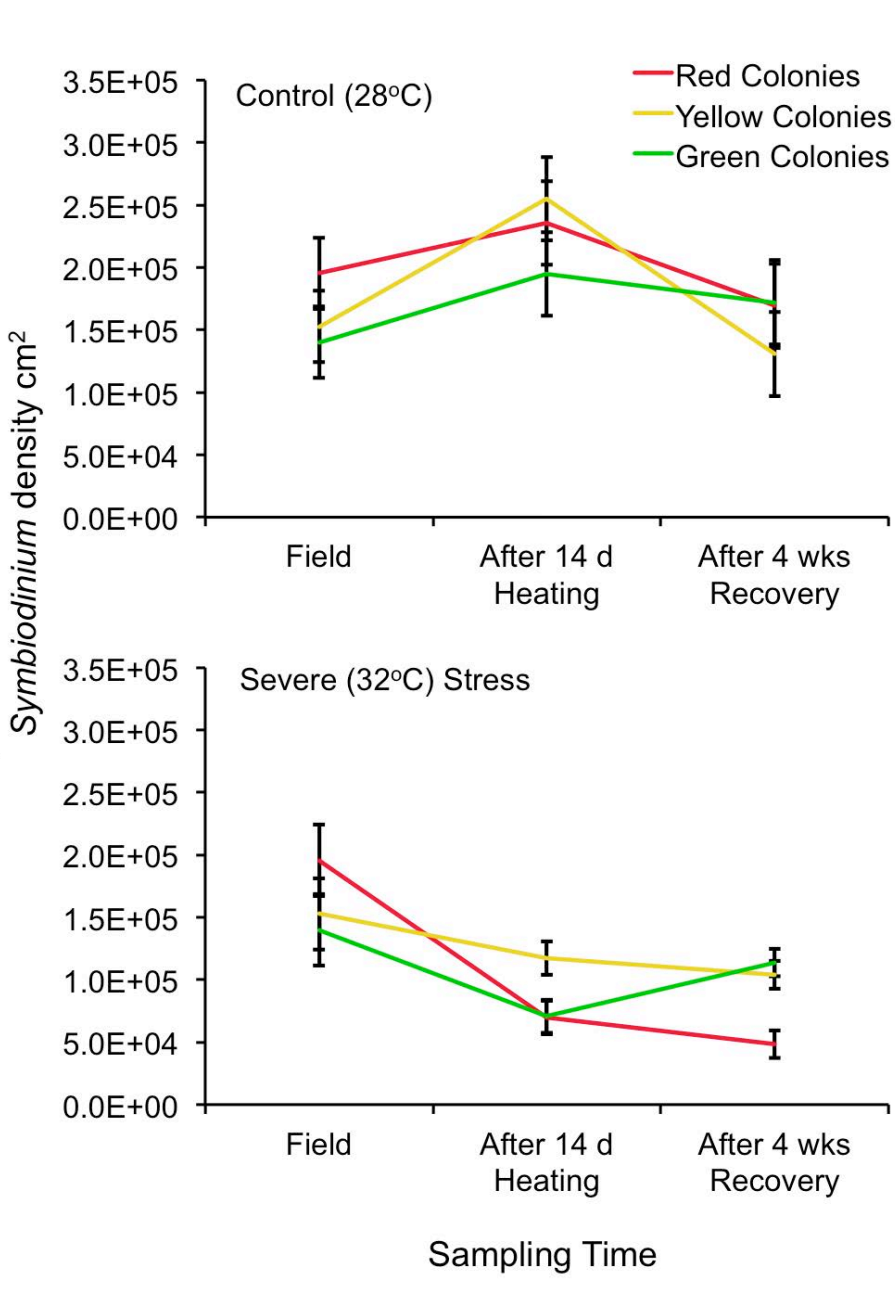

Figure S1. Mean *Symbiodinium* density (±SE) for red, yellow and green branches sampled in the field, after heating and at the end of recovery in the control (28°C) and severe (32°C) stress treatments. (Red: n = 6; Yellow: n = 6, Green: n = 6).

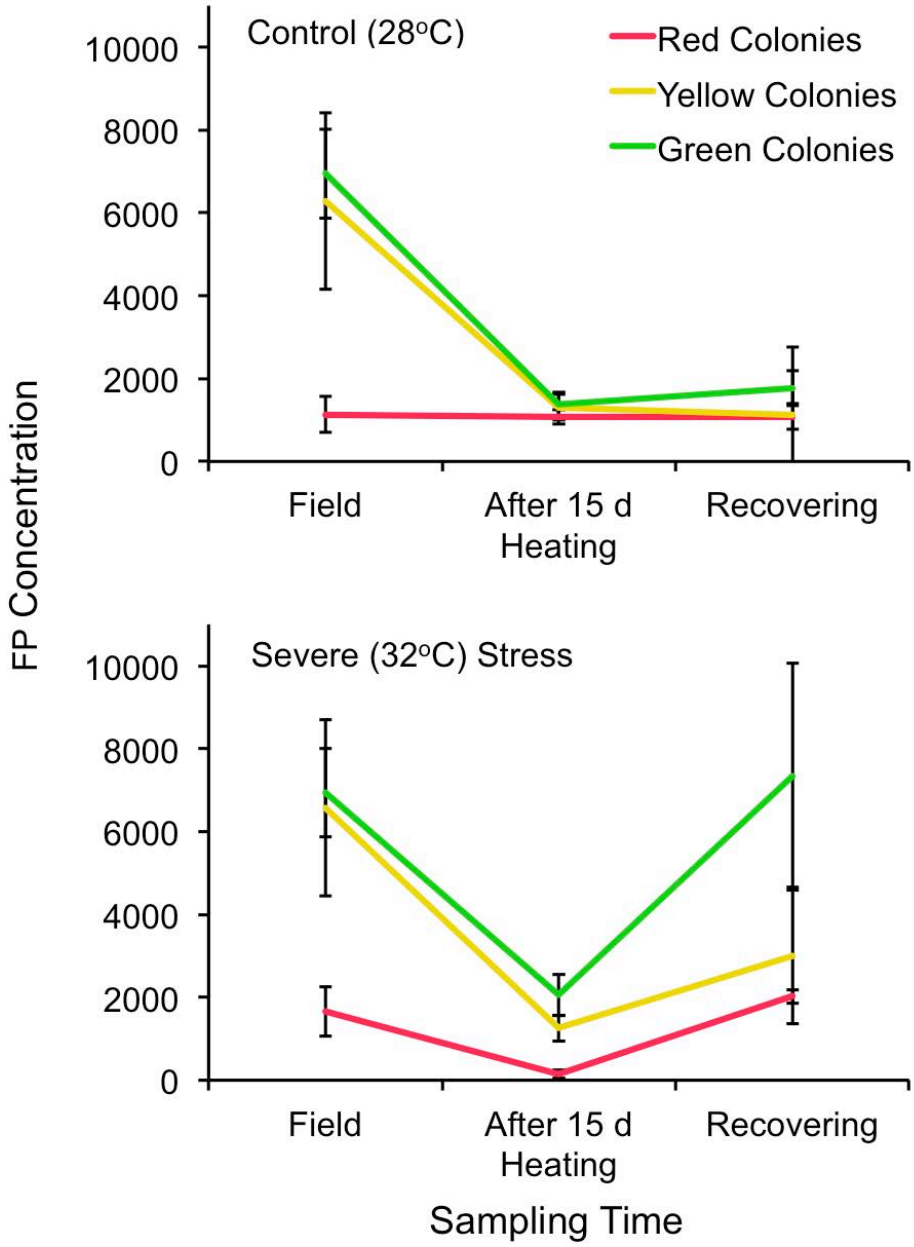

Figure S2. Mean total FP concentration (±SE) for red, yellow and green branches sampled in the field, after heating and at the end of recovery in the control (28°C) and severe (32°C) stress treatments. Mean values represent the sum of the mean values of the three coral FPs (CFP, GFP, RFP) for each colour morph at each sampling time. (Red: n = 6; Yellow: n = 6, Green: n = 6).

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