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FACULTY OF NATURAL & ENVIRONMENTAL SCIENCES

Ocean & Earth Sciences

Exploring the biological function of green fluorescent protein (GFP)-like pigments in corals

by

Edward G. Smith

Thesis for the degree of Doctor of Philosophy

September 2012

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

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Doctor of Philosophy

EXPLORING THE BIOLOGICAL FUNCTION OF GREEN FLUORESCENT PROTEIN (GFP)-LIKE PIGMENTS IN CORALS

By Edward Geoffrey Smith

Green fluorescent protein (GFP)-like pigments are widespread among shallow water corals and are responsible for their cyan, green, red and purple pigmentation. Assigning a biological function to these proteins has proved challenging and proposed theories do not appear to be consistent for all groups of the GFP family. This thesis set out to explore the biological function of GFP-like proteins in corals by treating biochemically and photophysically distinct groups of GFP-like proteins separately. A suite of molecular, spectroscopic and biochemical analyses were used to assess three different groups: metal binding fluorescent proteins, non-fluorescent chromoproteins and photoconvertible red fluorescent proteins. The main findings of this research can be summarised as follows. Firstly, a metal binding domain was identified for the first time in a naturally occurring GFP-like protein although sequence and structural analyses suggest that this domain is not associated with a widespread function in corals. Secondly, coral chromoproteins are shown reduce the excitation of chlorophyll through screening, supporting a photoprotective role for these proteins in shallow water reefs. Lastly, a novel role for photoconvertible red fluorescent proteins in corals is proposed based on their ecological distribution, their unique spectral properties and analyses of light penetration in coral tissues. The studies presented in this thesis highlight the importance of the host in regulating the light environment experienced by the symbionts and in the susceptibility of the symbiosis to stress. These findings provide new insights into the photobiology of corals and help identify the corals most likely to tolerate future changes to environmental conditions on coral reefs.

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DECLARATION OF AUTHORSHIP

I, Edward G. Smith

declare that the thesis entitled

Exploring the biological function of green fluorescent protein (GFP)-like pigments in corals

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- Parts of this work have been published as: [please list references]

Smith EG, D'Angelo C, Oswald F, Nienhaus GU, Wiedenmann J (2012) Fluorescent proteins from the oceans: Marine macromolecules as advanced imaging tools for biomedical research. In: Fattorusso EG, W.H.; Taglialatela-Scafati, O. (ed) Handbook of Marine Natural Products. Springer, pp1231-1258

Smith, E.G., D'Angelo, C., Salih, A. & Wiedenmann, J. Screening by coral GFP-like chromoproteins supports a role in photoprotection of the zooxanthellae. DOI: 10.1007/s00338-012-0994-9

Signed: ……..

Date:……

Acknowledgements

I would like to say a big thank you to Joerg for being my supervisor and mentor for the past few years. I am incredibly grateful that you gave me the freedom to explore my ideas and that you involved me in the other projects within the lab. I feel I have learnt an incredible amount from you and that your training will help me become the scientist I aspire to be.

I would also like to thank Cecilia for training me in the lab. I came into the lab as a molecular biology novice but I leave with a skill set that I am incredibly proud of and I am incredibly grateful for that. You are an amazing role model and I have benefitted enormously from your expert guidance.

I'd like to express my gratitude to John, Martin and Andy for all of their support and help during the course of my PhD.

I don't quite know how to express my thanks to my parents for all of the love and support they have given me. I could never have done this without you! You always encouraged me to follow my dreams and taught me the value of hard work. You are there for me whenever I need you and I couldn't be more proud to have you as my parents.

Dave, Maddy and Edie, thank you all for always making me smile, particularly in the last couple of months as "thesis fever" took hold!

Everyone from 92 Belmont Road, thank you all for being there through the highs and lows of PhD life. Ben, thanks for your endless singing in the lab that kept me sane during long lab days sat in the dark. Team Surrey, thanks for always being interested in my work and celebrating my geekiness.

I know there are plenty of people I have missed out but please know that I am grateful to everyone who has helped me through this incredible journey.

Contributions

CHAPTER 1.

Edward Smith: Wrote chapter

CHAPTER 2.

Section 2.1. is part of a published book chapter.

Smith EG, D'Angelo C, Oswald F, Nienhaus GU, Wiedenmann J (2012) Fluorescent proteins from the oceans: Marine macromolecules as advanced imaging tools for biomedical research. In: Fattorusso EG, W.H.; Taglialatela-Scafati, O. (ed) Handbook of Marine Natural Products. Springer, pp1231-1258

Edward Smith wrote this section of the book chapter. The section was reviewed and edited by all of the other authors.
Joerg Wiedenmann supplied the figures (Figures 2.1 and 2.2 in this thesis).

CHAPTER 3.

Edward Smith wrote the manuscript and performed the experiments.
Joerg Wiedenmann commented on the manuscript.

CHAPTER 4.

Chapter 4 is published in Coral Reefs in a modified form.

Smith, E.G., D'Angelo, C., Salih, A. & Wiedenmann, J. Screening by coral GFP-like chromoproteins supports a role in photoprotection of the zooxanthellae. DOI: 10.1007/s00338-012-0994-9

Edward Smith wrote the manuscript and performed the majority of the experiments.
Joerg Wiedenmann edited the manuscript. Performed the experiments for Figure S4.1.
Provided photographs for Figures 4.2 and S4.6.
Cecilia D'Angelo cloned the three chromoproteins from *Acropora millepora* and commented on the manuscript.
Anya Salih performed the experiments with JW for Figure S4.1 and commented on the manuscript.

CHAPTER 5.

Edward Smith wrote the manuscript and performed the majority of the experiments.
Yoni Sharon collected the data for Figure 5.1.
Joerg Wiedenmann commented on the manuscript.

CHAPTER 6.

Edward Smith wrote the chapter.
Joerg Wiedenmann commented on the chapter.

CHAPTER 7.

Edward Smith wrote the text and performed the experiments.

CHAPTER 1. Introduction

Coral reefs are one of the most diverse and productive ecosystems on the planet. Approximately 500 million people rely directly on coral reefs (Wilkinson, 2008); they provide income through tourism and fisheries, coastal protection through dissipation of wave and storm energy and are a key source of protein for dependent populations in less economically developed countries (Moberg and Folke 1999). Nevertheless, the corals that are the fundamental building blocks of the reefs are under threat from both natural and anthropogenic stressors.

Coral reefs have undergone a dramatic decline over the past three decades. It is estimated that almost one fifth of the world's reefs have already been lost and that a further 15% are under the imminent threat of being lost within the next 10-20 years (Wilkinson 2008). The vulnerability of the corals that build the reefs can be attributed, to some extent, to the sensitivity of the symbiosis between the coral host and its algal symbionts. Considering that excess light plays a fundamental role in driving coral thermal bleaching (Iglesias-Prieto et al. 1992; Fitt and Warner 1995; Brown 1997; Jones et al. 1998; Warner et al. 1999; Brown et al. 2000; Coles and Brown 2003), a significant cause of coral reef decline, fully understanding the photobiology of the symbiosis is of paramount importance as factors that can reduce light stress may alleviate the impacts of elevated temperature (Coles and Brown 2003). Green fluorescent protein (GFP)-like proteins are widespread throughout shallow water corals (Salih et al. 2000) and have been proposed to modify the internal light field of corals for the benefit of their algal symbionts (syn. zooxanthellae) (Kawaguti 1944; Schlichter et al. 1986; Salih et al. 2000; Dove et al. 2001). GFP-like proteins are largely responsible for the colour diversity of reef anthozoans (Dove et al. 2001; Oswald et al. 2007) and represent a substantial proportion of their total soluble protein content, up to 14% in some scleractinians (Leutenegger et al. 2007b). Despite their prevalence and the potential energetic investment in accumulating their high tissue concentrations, the role of these pigment proteins in coral photobiology is poorly understood and remains controversial (Mazel et al. 2003; D'Angelo et al. 2008).

One of the main reasons that these proteins continue to be an enigma in coral ecology, is that they do not appear to have a function that is consistent for all types of GFP-like proteins. Recent studies have shown that this family of proteins is diverse and that the structural and biochemical differences between the different groups of GFP-like proteins may be associated with distinct functions (Alieva et al. 2008; D'Angelo et al. 2008). Indeed, evolutionary analyses on the fluorescent proteins (FPs) have identified multiple convergent evolution of certain fluorescent protein colours, indicative of

different functional roles (Ugalde et al. 2004; Field et al. 2006; Alieva et al. 2008). These analyses also indicate that there are conserved regions within the protein structure that may be involved in the interactions with other molecules (Field et al. 2006). Nevertheless, the functional roles that these proteins may play in coral biology are largely unknown although there have been suggestions that they may act as photoprotectants, photoenhancers or antioxidants (Kawaguti et al. 1944; Salih et al. 2000; Salih et al. 2006; Bou-Abdullah et al. 2006).

This study set out to use the biochemistry of these proteins to guide exploration of GFP-like protein function and help to address the wider hypothesis that **different GFP-like protein groups have different biological functions in corals**. As assigning biological functions to these proteins has proved remarkably challenging and that the GFP-family of proteins can be considered to contain numerous distinct groups of proteins, this thesis does not experimentally test this hypothesis *per se*, rather, it aims to further our knowledge of three biochemically distinct groups of GFP-like proteins. Understanding the biological importance of these proteins is of the upmost importance. Critical evaluation of proposed roles in the photobiology of corals and identification of the mechanisms behind these roles is urgently needed to understand their role in coral bleaching and to forecast how corals will respond to future environmental conditions such as increased temperatures and changing water quality on reefs. A greater understanding will also advance their use as coral health biomarkers. In addition to the ecological implications of these proteins, the pigmentation of corals is highly prized by the tourism and coral aquarist industries and therefore, understanding the expression and function of the proteins responsible for coral colouration has economic value. Finally, these proteins have revolutionised cell biology through their applications as molecular markers and have become one of the most widely used tools in biomedical science. However, without knowledge of their true biological function, the extent to which they could bias/influence the cell processes they are employed to monitor is unknown.

Studies into the biological function of GFP-like proteins in corals have largely focused on the spectral properties of these proteins rather than a function unrelated to the chromophore of the protein. This thesis aims to combine studies looking both at the biochemistry of the proteins in addition to roles in the photobiology of corals. The first study aims to identify whether metal binding by coral pigment proteins may serve a biological function in corals. The biological significance of the interaction between transition metals and naturally occurring GFP-like proteins has not previously been considered. However, interactions with metals are crucial to the roles of many proteins and in some cases, are essential for protein functionality (see Appendix Table 1 for

summary of the roles of metal binding in proteins). The study explores the metal-protein interactions in greater detail than could be considered necessary for assessing whether they serve a biological function in corals, however, these analyses are included in the thesis as they support the categorisation of actinarian FPs as a distinct group of GFP-like proteins, highlight potential metal binding functionality of certain proteins outside the scleractinian GFP homologs and demonstrate metal-induced quenching of fluorescence that could hinder the use of fluorescence intensity as a marker of coral reef health.

The second and third chapters of this thesis will focus on exploring the biological function of two distinct members of the GFP family: the chromoproteins and the photoconvertible red fluorescent proteins (PCRFPs). While the two protein groups absorb predominantly in the same region of the spectrum, they are contrasting in many aspects of their biology. The CPs are found predominantly in shallow waters (Salih et al., 2006) and are upregulated in response to light (D'Angelo et al. 2008). In contrast, the PCRFPs are typically found in species associated with low light environments (Oswald et al. 2007) and are constitutively expressed (Leutenegger et al. 2007b). By exploring the function of these two proteins, the thesis aims to understand the relevance of the different spectral properties and environmental conditions to the biological function of the proteins.

The specific questions to be answered in each chapter are outlined below:

Chapter 3: Identification and analysis of a metal binding site in the red fluorescent protein asFP595/1

- How prevalent is metal binding amongst GFP-like proteins?
- How do interactions with transition metals impact on the proteins properties?

Chapter 4: Screening by coral GFP-like chromoproteins supports a role in photoprotection of the zooxanthellae

- Do CPs provide screening for corals' algal symbionts?
- Are the wavelengths absorbed by the CPs damaging?

Chapter 5: Internal light modification by photoconvertible red fluorescent proteins as a potential mechanism for depth acclimation in corals.

- What is the ecological distribution of red fluorescence morphs?
- How do the optical properties of the photoconvertible red fluorescent proteins relate to their environment? Do these provide clues to their biological function?

CHAPTER 2. Background

This thesis sets out to explore the biological function of green fluorescent protein (GFP)-like proteins in corals. The purpose of this chapter is to provide additional background information to the studies presented in this thesis that is beyond the scope of the introductory sections of chapters presented in journal article format. The introduction chapter is divided into two main parts: Part A will provide an overview of GFP-like proteins while Part B will focus on the biological function of GFP-like proteins in the context of coral photobiology.

2.1. PART A: The GFP family of pigment proteins

2.1.1. History

Fluorescent proteins have become an indispensable component of the biomedical imaging toolbox (Mocz 2007; Day and Davidson 2009; Wiedenmann et al. 2009). The first representative of this protein family, the green fluorescent protein (GFP), emerged from a study of bioluminescence in the hydrozoan *Aequorea victoria* by Osamu Shimomura and colleagues (Shimomura et al. 1962). Further studies of bioluminescent cnidarians identified more GFPs from marine bioluminescent organisms such as the seapen *Renilla* and hydrozoan polyp *Obelia* (Morin and Hastings 1971b; Morin 1974). In bioluminescent cnidarians, GFP acts as secondary emitter in the bioluminescence reaction during which the chromophore of GFP is excited as result of a radiationless energy transfer from the photoprotein aequorin (Morin and Hastings 1971a; Morin 1974).

It took about 30 years from the discovery of GFP until the potential of this protein as a genetically encoded fluorescent marker was fully realised. The first important step towards its application as an imaging tool was the cloning of GFP encoding DNA by Prasher and colleagues (1992). Subsequently, Chalfie and co-workers succeeded in expressing the protein functionally in the nematode worm *Caenorhabditis elegans* (Chalfie et al. 1994). These groundbreaking studies transformed GFP from a scientifically interesting marine pigment to a fluorescent marker with enormous application and commercial potential. Studies soon began to focus on engineering of the GFP to provide new colours and to render the protein more suitable for cellular imaging applications, for instance, by enhancing its brightness or by producing blue, cyan, and yellow variants (Tsien 1998). The tremendous impact of GFP technology on

life science research was recognized by the award of the 2008 Nobel Prize for Chemistry to Osamu Shimomura, Martin Chalfie, and Roger Tsien (Nienhaus 2008; Day and Davidson 2009).

Despite extensive efforts to create novel colour variants of the GFP, red fluorescent proteins (RFPs) appeared elusive during the early years of GFP research (Mishin et al. 2008). These longer wavelength markers are particularly desirable for imaging applications because of reduced cellular autofluorescence in this spectral region, reduced phototoxicity of the excitation light, and the better penetration of long wavelength light in tissues (Wiedenmann et al. 2009). Furthermore, RFPs were in demand for novel fluorescence resonance energy transfer (FRET) applications (Erickson et al. 2003). Ultimately, it was nature that provided the first sources of RFPs. The search for natural FPs had originally focused on bioluminescent organisms as the discovery of GFPs in these animals suggested a strict coupling of FPs to bioluminescence reactions. Contrary to this common notion, Wiedenmann (1997) proposed the existence of red-emitting GFP-like proteins and non-fluorescent homologues in the sea anemone *Anemonia sulcata* and other cnidarians (Wiedenmann 1997). Subsequent studies confirmed the presence of GFP-like proteins in numerous non-bioluminescent anthozoans (Matz et al. 1999; Fradkov et al. 2000; Wiedenmann et al. 2000; Wiedenmann et al. 2002) while uncovering the RFPs DsRed and eqFP611; the latter being the natural RFP with the most red-shifted emission (Matz et al. 1999; Wiedenmann et al. 2002). These studies revealed non-bioluminescent anthozoans as a rich source of natural FPs, giving access to a broad palette of emission colours (Nienhaus and Wiedenmann 2009). In particular, the diversity of RFPs among anthozoans was exploited to create advanced marker proteins with optimized biochemical and spectral properties (Campbell et al. 2002; Karasawa et al. 2004; Shaner et al. 2004; Kredel et al. 2009). Interestingly, anthozoans, especially reef corals, harbour a variety of photoactivatable FPs (PAFPs) (Lukyanov et al. 2000; Ando et al. 2002; Ando et al. 2004; Wiedenmann et al. 2004b; Leutenegger et al. 2007b; Oswald et al. 2007). While the basic chromophore forms in an autocatalytic reaction, as in GFP from *Aequorea victoria*, the final emission colour is determined by the exposure to distinct light quantities and qualities (Nienhaus and Wiedenmann 2009). The chromoprotein asulCP (synonym asFP595), for instance, combines reversible “on”/“off” switching of red fluorescence in response to alternating irradiation with low intensities of green and blue light with an irreversible “on” switching under high levels of green light (Andresen et al. 2005). In contrast, photoconvertible red FPs (PCRFPs) such as Kaede and EosFP first develop a green fluorescent chromophore that is irreversibly converted into a red fluorescent state under illumination with violet light (Nienhaus et al. 2005). PAFPs have a high application potential as markers in pulse-chase

experiments, whereby the fate of a certain subset of labelled cells or cellular components can be monitored independently by selective excitation after targeted photoactivation (Wiedenmann and Nienhaus 2006). Moreover, photoactivatable proteins were key tools in the realisation of imaging techniques that enable light microscopy with a resolution beyond the diffraction barrier (Hofmann et al. 2005; Betzig et al. 2006).

2.1.2. GFP-like proteins in marine organisms

The first GFP was found in the bioluminescent hydromedusa *A. victoria* (Shimomura et al. 1962); subsequent studies discovered GFPs also in other bioluminescent organisms including *Renilla* and *Obelia* (Morin and Hastings 1971b; Morin 1974). The studies of FPs in anthozoans showed that FPs are expressed throughout all major taxa of the anthozoans including sea anemones, corals, ceriantharians, octocorals, and corallimorpharians. The GFP-like pigments in these organisms show emission colours ranging from cyan to green over yellow to red (Nienhaus and Wiedenmann 2009). Some individuals also contain chromoproteins (CPs), another subpopulation within the family of GFP-like proteins which are intensely coloured but essentially nonfluorescent (Lukyanov et al. 2000; Prescott et al. 2003). In fact, several anthozoans, particularly corals, express more than five different GFP-like proteins within a single individual (Wiedenmann et al. 2000; D'Angelo et al. 2008). Different expression levels of both FPs and CPs are primarily responsible for the colour polymorphism that can be frequently observed among reef-building corals (Scleractinia) and sea anemones (Actiniaria) (Leutenegger et al. 2007a; Oswald et al. 2007).

GFP-like proteins are widespread throughout shallow water corals, with up to 97% of shallow water corals exhibiting fluorescent pigmentation (Salih et al. 2000). Indeed, of the 18 coral families identified by Veron (2000), individuals expressing GFP-like proteins are found in 17 of these families (Table 2.1). Corals accumulate high tissue concentrations of these proteins (>7%) (Leutenegger et al. 2007b) and evolutionary analyses demonstrate positive natural selection for the diverse colours, indicative that the distinct colours must serve an essential function (Field et al. 2006). Nevertheless, attempts to identify the biological significance of these proteins have proved inconclusive (see section 2.2.7.3.).

Coral Family	Reference
Acroporidae	1,2,3,4,6,7,9
Astrocoeniidae	5,9
Pocilloporidae	1,2,3,6,9
Euphyllidae	7
Oculinidae	9
Meandrinidae	5,9
Siderastreidae	9
Agariciidae	1,2,5,9
Fungiidae	2
Rhizangiidae	9
Pectiniidae	1,2,9
Merulinidae	7
Dendrophylliidae	
Caryophylliidae	10
Mussidae	1,2,5,9,10
Faviidae	1,2,5,9,10
Trachyphylliidae	8
Poritidae	1,2,5,9

Table 2.1. Coral families (as per Veron, 2000) expressing fluorescent pigments and chromoproteins. References: (1) Salih et al. 1998 (2) Salih et al. 2006 (3) Dove et al. 1995 (4) Dove et al. 2009 (5) Mazel et al. 2003b (6) Cox et al. 2007 (7) D'Angelo et al. 2008 (8) Ando et al. 2008 (9) Alieva et al. 2008 (10) Oswald et al. 2007.

GFP-like proteins are not only confined to the taxon Cnidaria, but have also been identified in other phyla such as Ctenophora, Crustacea, and even Chordata (Shagin et al. 2004; Deheyn et al. 2007; Haddock et al. 2010). Interestingly, a non-fluorescent, colourless structural homologue of GFP, the G2 domain of the extracellular matrix protein nidogen, is present even in mammals (Hopf et al. 2001).

2.1.3. Structural aspects of GFP-like proteins

2.1.3.1.The three-dimensional structure

GFP-like proteins have a distinct structure comprising of an 11-stranded β-barrel with a helix running along the central axis of the protein (Yang et al. 1996; Nienhaus and Wiedenmann 2009). Close to the geometric centre of the molecule, the central helix is interrupted by the chromophore (Figure 2.1). The chromophore is formed from a tripeptide sequence in an autocatalytic reaction in the presence of molecular oxygen (Heim et al. 1994). The chromophore is primarily responsible for the spectral characteristics of the GFP-like proteins, with the wavelength of absorption and emission increasing with the number of the conjugated π bonds (Remington 2006). Interactions with adjacent amino acids, however, can shift the absorbance and emission spectra by up to 20nm (Remington 2006). GFP exists in a predominantly monomeric form at concentrations below 1mg/mL (Ward 2005). In contrast, the GFP from *Renilla* exists as a stable dimer (Ward 2005). Most anthozoan FPs form tight tetrameric assemblages, although a number of dimeric variants have also been described in corals and sea anemones (Nienhaus et al. 2005; Nienhaus et al. 2006a,b) Figure 2.1).

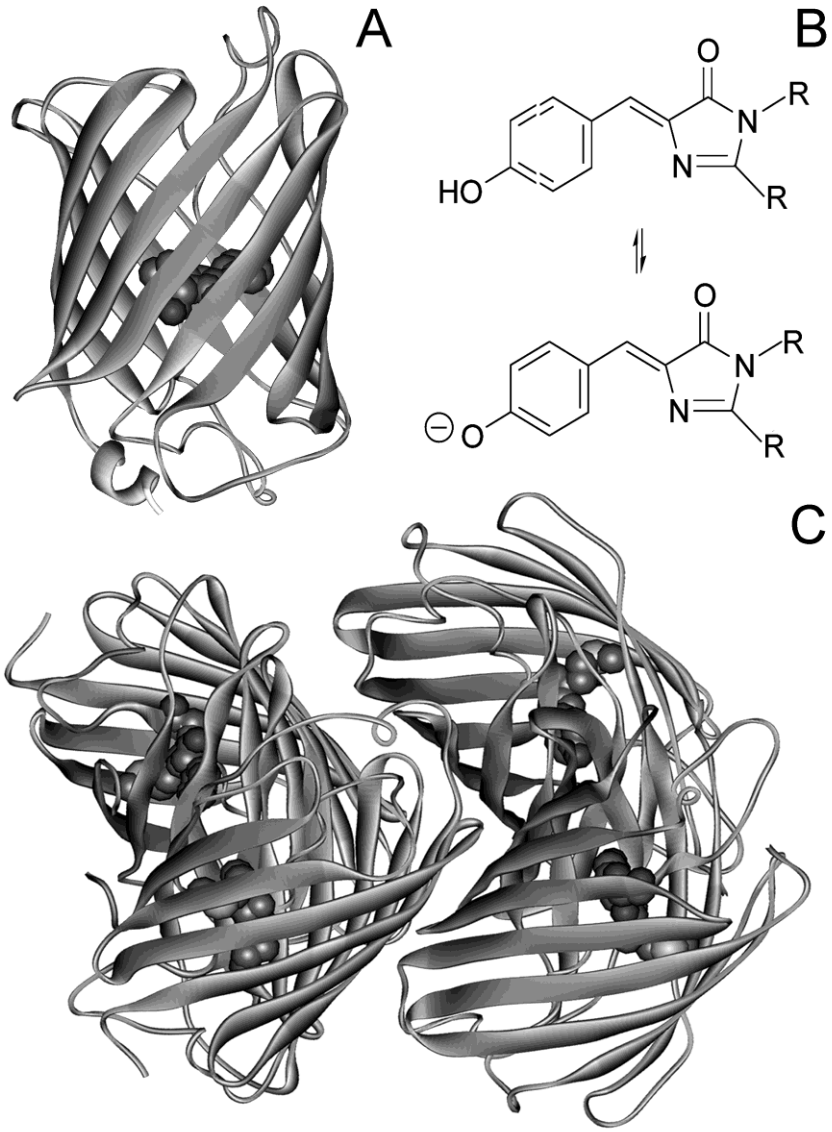

Figure 2.1. Structural aspects of GFP-like proteins. (a) Ribbon diagram of GFP exhibiting the arrangement of the 11 β-sheets around the central helix. The position of the chromophore in the center of the molecule is represented by Van der Waals spheres. (b) The p-HBI chromophore of GFP in its neutral (maximal absorption at 395nm; upper structure) and deprotonated state (maximal absorption at 475 nm; lower structure). (c) Typical assembly of protomers in a tetramer of an anthozoan FP as "dimers of dimers." The β-can fold of GFP is highly conserved.

2.1.3.2.Chromophore structures

The amino acid triad that forms the GFP chromophore, Ser65-Tyr66-Gly67, undergoes a three-step process of cyclization, dehydration, and oxidation to form the functional chromophore (Sniegowski et al. 2005). Firstly, the amide of Gly67 attacks the carbonyl of Ser65 to form imidazole. Dehydration converts the imidazole to imidazolinone and

subsequent oxidation of the Cα–Cβ bond of Tyr66 extends the conjugated p electron system from the imidazolinone to the aromatic group of Tyr66 to yield the final 4-(p-hydroxybenzylidene)-5-imidazolinone (p-HBI) chromophore (Sniegowski et al. 2005). The GFP chromophore features two states depending on the protonation of the hydroxybenzyl group (Figure 2.1). The neutral (protonated) chromophore absorbs maximally at 395 nm, whereas the anionic (deprotonated) form is optimally excited by blue light at 475 nm (Tsien 1998). The interesting phenomenon that both states emit mostly green fluorescence can be explained by an excited-state proton transfer that occurs upon excitation of the neutral chromophore (Chattoraj et al. 1996).

The colour diversity of natural FPs is derived from modifications of the green fluorescent p-HBI chromophore, which is considered to be the ancestral form (Alieva et al. 2008). In the following paragraphs, an overview of the chromophore types responsible for the different photophysical properties of GFP-like proteins will be presented.

Cyan/Green emission

Cyan/green emission of GFP-like proteins results from p-HBI chromophores (Nienhaus et al. 2006b,a). Emission maxima of cyan/green FPs have been recorded in the range from 470 to 520 nm (Alieva et al. 2008). The cyan FPs (CFPs) are characterized by wider excitation/emission bands and larger Stokes shifts compared to the green FPs (Alieva et al. 2008). The amino acid residues surrounding the chromophore play an important role in the spectral properties of these proteins. For example, it is responsible for the blue shift in the CFP emission relative to GFP. Fluorescence of both the anionic and the neutral forms of the chromophore are observed in some members of this group, for instance, in asFP499 (Nienhaus et al. 2006a) (Figure 2.2a).

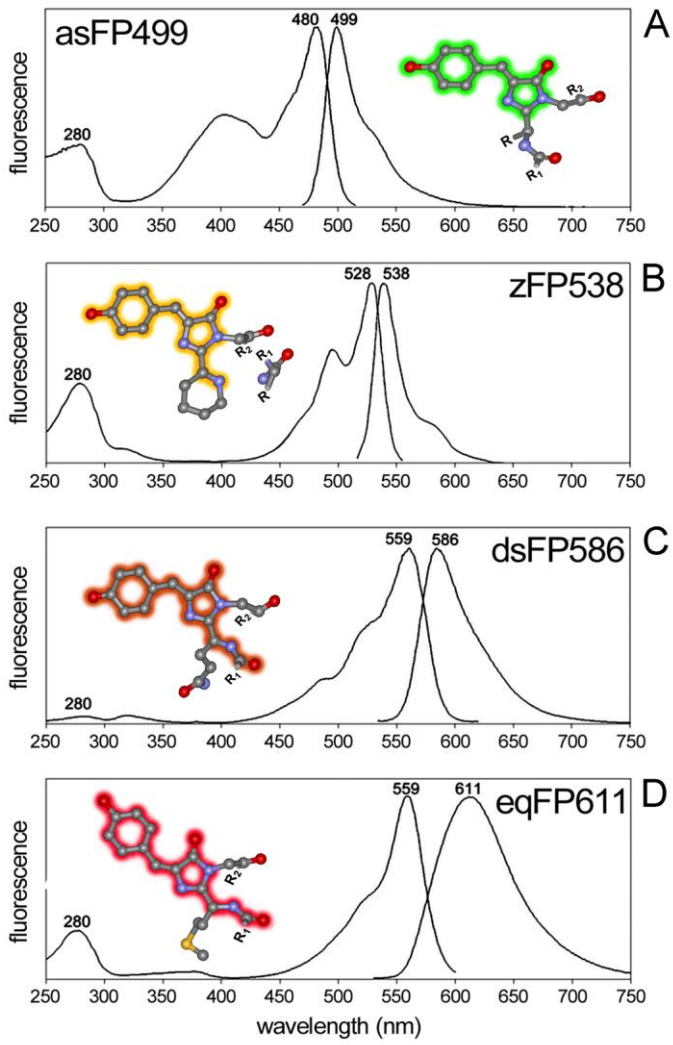

Figure 2.2. Chromophore structures of major FP colour classes. Excitation and emission spectra are shown with the position of the peak maxima in (nm) indicated above the peaks. The excitation spectrum in each panel is the spectrum to the left (blue shifted) due to the Stoke's shift. Structural models of the different chromophore types were constructed using representative GFP-like proteins (protein data bank code A: 1W7S, B: 2OGR, C: 1GGX, D: 3E5T). Carbon atoms are shown in grey, nitrogen atoms in blue, oxygen atoms in red and sulphur in yellow. The conjugated π-bond system is highlighted with shading around the bond by colours symbolizing the emission colour. (a) asFP499: The secondary excitation maximum at ~400 nm of the cyan-green FP results from the neutral p-HBI chromophore undergoing excited-state proton transfer. (b) The chromophore of the yellow fluorescent zFP538 features a 3-ring structure. (c) The DsRed-type chromophore of dsFP586 is extended along the peptide backbone. (d) The red-shifted emission of eqFP611 is associated with a DsRed-type chromophore adopting a cis conformation.

Yellow emission

To date, only two naturally occurring yellow FPs have been discovered, phiYFP (Shagin et al. 2004) and zFP538 (Matz et al. 1999) (Figure 2.2b). These two proteins have an emission maxima of 537 and 538nm and excitation maxima of 525 and 528nm, respectively. They achieve yellow fluorescence through different modifications of the p-HBI chromophore. The yellow fluorescence of zFP538 results from a three-ringed chromophore, whereas phiYFP's fluorescence results from π-stacking interactions of the tyrosyl moiety of p-HBI with the chromophore environment (Pletneva et al. 2007).

Red emission

Numerous natural orange/red FPs have been identified, and many more engineered, with emission wavelengths up to 611 nm (Wiedenmann et al. 2002; Nienhaus and Wiedenmann 2009). There are two classes of red fluorescent proteins, distinguished by the pathway in which the red fluorescent chromophore is achieved. Each class is named after the protein for which the type of chromophore was described: DsRed-type chromophores and Kaede/EosFP-type chromophores. Maturation of the DsRed-type chromophore begins with the formation of the neutral chromophore. At this point, the chromophore can follow one of two possible pathways. There have been different pathways proposed for the formation of the mature red chromophore but both acknowledge that it is a result of the formation of a double bond between the C(α) and the amide N of Gln66 (Verkhusha et al. 2004; Strack et al. 2010). DsRed-type chromophores usually adopt a cis conformation (Yarbrough et al. 2001) (Figure 2.2c). An exception is eqFP611 in which the red-shifted natural emission is produced by a DsRed-type chromophore in trans conformation (Petersen et al. 2003) (Figure 2.2d).

Once expressed in cells, both Kaede, EosFP, and related proteins form a green fluorescent p-HBI chromophore that exists predominantly in the deprotonated state (Wiedenmann et al. 2004b; Nienhaus et al. 2005). However, illumination of the neutral p-HBI chromophore with violet light (~390 nm) causes cleavage of the peptide backbone at the Nα–Cα bond of the first chromophore-forming residue, which is always a histidine in these photoconvertible proteins (Ando et al. 2002; Wiedenmann et al. 2004b; Oswald et al. 2007). The red emission arises from the extension of the conjugated π-electron system into the imidazole group of the histidine (Mizuno et al. 2003).

Chromoproteins

Non-fluorescent CPs absorb between ~550 and ~600nm and are characterized by purple, lilac, and blue hues (D'Angelo et al. 2008). Emission is virtually absent from the CPs. However, a CP from the sea anemone *Anemonia sulcata* becomes brightly fluorescent upon intense green-light irradiation (Lukyanov et al. 2000). The absence of fluorescence in chromoproteins is attributed to the nonplanarity and trans conformation of the DsRed-type chromophore (Prescott et al. 2003; Quillin et al. 2005).

2.1.3.3.Applications of fluorescent proteins from marine invertebrates

GFP-like proteins have revolutionized the field of cellular imaging over the past two decades (Day and Davidson 2009; Wiedenmann et al. 2009). The autocatalytic formation of the functional chromophore without the requirement for cofactors, their diverse spectral characteristics, and the *in vivo* stability of the chromophore over a range of physiological conditions has resulted in their widespread popularity as live-cell markers. As the chromophore can be expressed, matured, and maintained *in vivo*, FPs represent unrivaled tools with which, for instance, complex biochemical pathways can be studied within the living cell. Fluorescent proteins belonging to the GFP-like family have been used for various studies including gene expression, protein localization, protein–protein interactions, and monitoring of intracellular biochemistry (Day and Davidson 2009; Wiedenmann et al. 2009). In addition, photoactivatable proteins have enabled super-resolution optical imaging beyond the diffraction limit through techniques such as photoactivated localization microscopy (PALM) or reversible saturable optical fluorescence transitions (RESOLFT) (Hofmann et al. 2005; Betzig et al. 2006).

One emerging application of FPs is the use of these proteins as biosensors for transition metals (Eli and Chakrabartty 2006; Sumner et al. 2006; Rahimi et al. 2007b). A naturally occurring GFP-like protein, DsRed, displays a remarkable reduction in fluorescence in the presence of copper (I) and (II) ions (Sumner et al. 2006). The quenching is selective for copper ions and concentration dependent and hence DsRed has been proposed as a potential *in vitro* and *in vivo* copper sensor (Eli and Chakrabartty 2006; Sumner et al. 2006). The development of an *in vivo* copper sensor is highly desirable as dysregulation of copper homeostasis is involved in several neurological conditions such Alzheimer's, Wilson and Menkes disease (Strausak et al. 2001). The use of DsRed *in vivo*, although widespread, is hampered by its tendency to aggregate (Mizuno et al. 2001; Yanushevich et al. 2002; Wiedenmann et al. 2009). To date, the domain responsible for the metal binding is not known, however, the

identification of the domain would greatly assist in identifying other proteins with similar functionality that could serve as alternatives for *in vivo* applications.

2.2. PART B: The photobiology of corals

2.2.1. Introduction

This part of the introduction will focus on the photobiology of corals in relation to GFP-like protein expression. The currently theories regarding GFP-like proteins' function in corals predominantly involve a role in modulating the light environment experienced by the symbionts. Nevertheless, these theories remain controversial and in order to appreciate the complexities involved in assigning a function to these proteins, one must understand the light interactions occurring upstream and downstream of GFP-like protein expressing tissues. Firstly, this section will address the interactions of light along its path from the sun to the antennae of the symbionts, highlighting the localisation of the GFP-like proteins and their role in attenuation of light. Secondly, an overview of the importance of light in the symbiosis will be provided. This is necessary to provide a basis for understanding the impacts of sub- and supra-optimal light on the photosynthetic machinery. Lastly, the acclimation and adaptation strategies used by the coral host and the symbionts to deal with light will be briefly discussed along with a more in-depth discussion of proposed GFP-like functions.

2.2.2. Light path to the zooxanthellae

The following section will outline the path of light from the sun the zooxanthellae. The journey is divided into seven sections corresponding to the numbered stages identified in figures 2.3 and 2.5. Each stage is discussed in relation to the different processes affecting the intensity and spectral distribution of light.

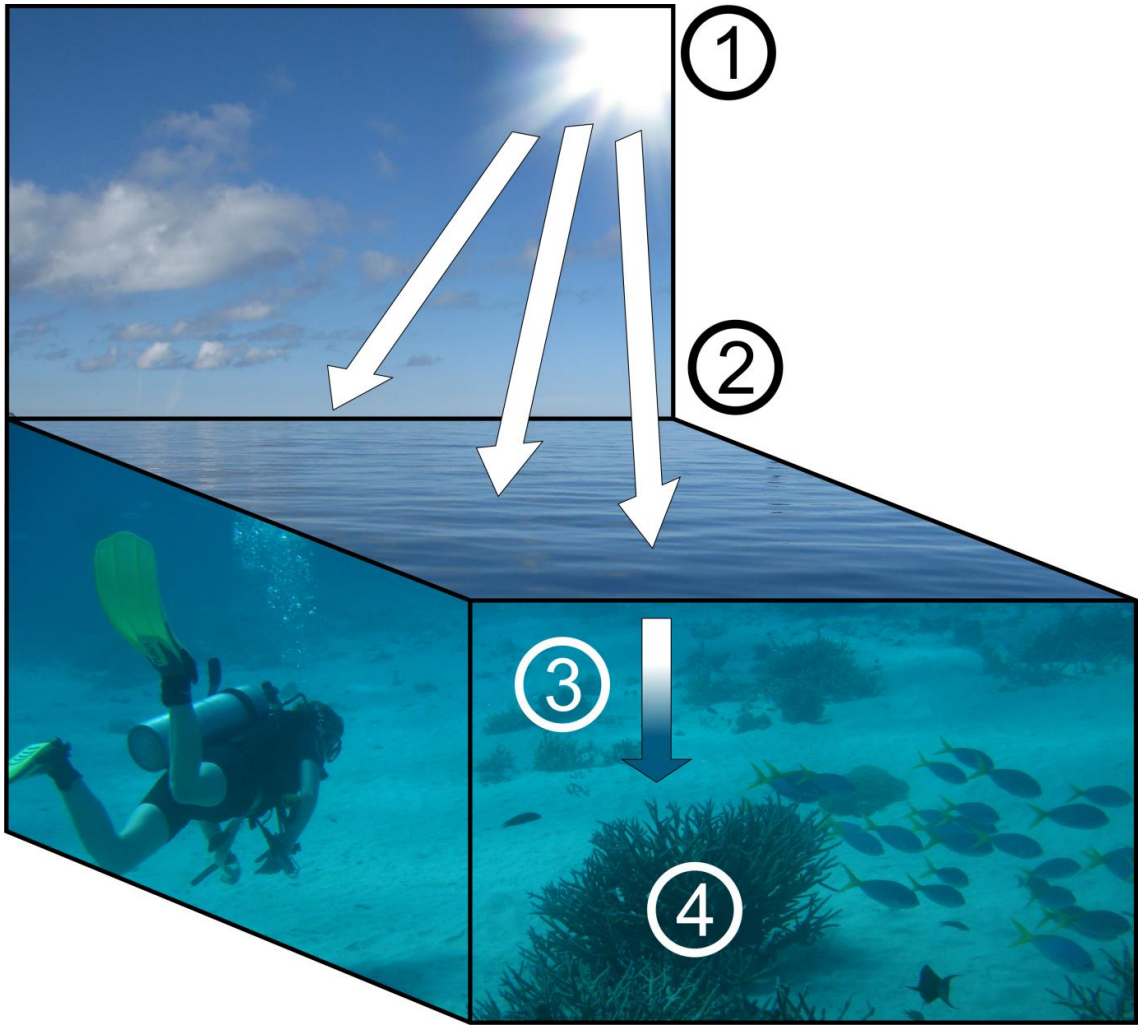

Figure 2.3. Pathway of light from the sun to the coral (Stages 1-4). The white arrows denote the path of light and the numbers correspond to the different stages referred to in the text.

1. Solar radiation

The nuclear fusion of hydrogen nuclei into helium in the sun results in a blackbody emission of radiation peaking at 500nm (Kirk 1994). Of primary interest in the photobiology of corals is the emission of light in the visible region of the spectrum ranging from 400nm to 700nm (also known as photosynthetically active radiation: PAR), representing 38% of the extraterrestrial solar irradiance (Kirk 1994), as this region of the spectrum is responsible for driving the photosynthesis of the zooxanthellae. Whilst this review will largely focus on the emission of light in the visible region of the spectrum, it should be noted that UV light is also important in the photobiology of corals (Lesser 1996; Banaszak and Lesser 2009) and it will be discussed briefly due to negative impacts on the symbiosis.

2. From the sun to the water's surface

The amount of light reaching a point on the Earth's surface at any particular moment is dependent on the orientation of the Earth in relation to the sun. This results in variation in solar irradiance on timescales from hours to seasons (Falkowski 1984). Of great importance to photosynthetic organisms is the diurnal variability in irradiance intensity (Falkowski 1984) which may change by 9 orders of magnitude (U.S. Department of the Navy, 1952; cited in Ryer and Olla 1999). For coral reefs, this relates to peak PAR irradiances peaking at around 1800μmol m^{-2} s^{-1} for shallow water corals (Levy et al. 2004).

While travelling through the Earth's atmosphere, light is attenuated due to absorption and scattering processes. Absorption of visible light in the Earth's atmosphere is due to oxygen and ozone at shorter wavelengths and water vapour at longer red wavelengths (Kirk 1994). Two types of scattering occur in the atmosphere, Rayleigh and Mie scattering and are dependent on the size of the molecule/particle in relation to the wavelength of the radiation (Kirk 1994). Absorption and scattering are responsible for about a 14-40% loss of radiation, depending on atmospheric constituents, for a solar zenith angle of 0° and will increase with increasing zenith angle due to the longer pathlength (Kirk 1994).

The presence of clouds will also affect the solar radiation reaching the water's surface due to scattering/reflection on the upper surface of the cloud and absorption within the cloud (Falkowski and Raven 2007). Sparse clouds that are not blocking the direct sunlight can increase the contribution of diffuse light and consequently increase the total irradiance by up to 10% (Kirk 1994). Nevertheless, a study of the impact of clouds on Great Barrier Reef reefs showed that cloud cover can episodically reduce irradiance to 20-40% of the rolling maximum during the summer and reduce the annual irradiance by 14-17% (Anthony et al. 2004). Furthermore, it has been proposed that the spatial distribution of cloud cover (and subsequent reduction in irradiance) can help explain records of regional patchiness of coral bleaching (Mumby et al. 2001).

3. Surface of the water to the coral colony

At the air-water interface, some of the incident irradiance is reflected from the water's surface. The amount of reflection is dependent on the angle of the sun, with nearly 100% reflection as the solar zenith angle approaches 90° (Kirk 1994). The surface state of the water's surface, related to wind speed, will affect the magnitude of the reflection

at high zenith angles, with less reflection as the surface roughness increases due to the changes in the surface orientation in relation to the incident light (Kirk 1994). The surface state of the water also will impact the underwater light field due to wave focussing effects (Veal et al. 2010). Here, the curved surfaces of the waves act to focus and defocus light resulting in flashes of light across the benthic surface which can be nearly three times greater than the mean downwelling irradiance at that depth (Veal et al. 2010). The impact of wave focusing effects decreases with depth so is most relevant to shallow water corals (Falkowski et al. 1990).

The intensity and spectral composition of the irradiance that a coral colony experiences depends on the depth at which it resides due to attenuation of light by the water column (Dustan 1982; Mass et al. 2010). This results in decreasing irradiance and chromaticity with increasing depth. Indeed, corals inhabit environments spanning over two orders of magnitude in intensity (Falkowski and Dubinsky 1981) from the high light exposed colonies in shallow waters to the shaded and mesophotic corals (Figure 2.4). In the tropical waters of coral reefs, the attenuation in the water column is largely due to the water molecules themselves although terrestrial sources can increase the contributions of chromophoric dissolved organic matter and chlorophyll (Falkowski et al. 1990; Shick et al. 1996; Banaszak and Lesser 2009; Mass et al. 2010). Absorption by water increases almost exponentially with increasing wavelength of visible light from an absorption coefficient of $0.017m^{-1}$ at 400nm to $0.650m^{-1}$ at 700nm (Smith and Baker 1981). As a result, corals residing in depths greater than 10m are largely deprived of red light. In addition to absorption, water molecules and particles in the water column scatter the light. The diversion of photons from their original path increases the pathlength through which they travel and therefore the probability with which they are absorbed is increased, acting to decrease the attenuation coefficient (Kirk 1994).

Although the light conditions on coral reefs are considered to be generally dominated by the optical properties of the water column, human impacts in coastal regions are changing the light regimes (Hennige et al. 2010) and this phenomena is expected to increase in the future (Fabricius 2005). Human impacts can increase both the organic and inorganic components in the water column. While the inorganic constituents will decrease largely decrease the overall light intensity through scattering, the alteration of the organic constituents (potentially coupled with phytoplankton blooms) will additionally alter the light quality.

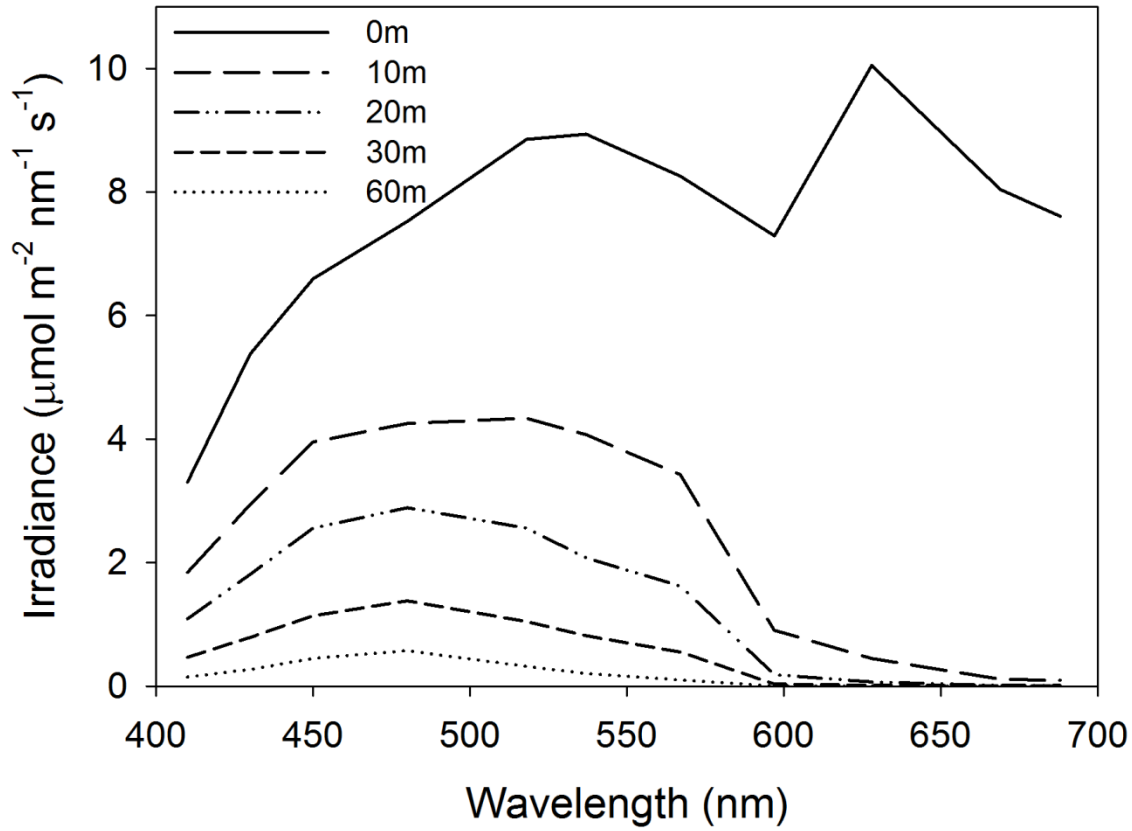

Figure 2.4. Spectral irradiance at different depths for an "average sunny day" on a Jamaican coral reef. Redrawn from Dustan (1982).

4. Colony to tissue scale

The light flux reaching the coral tissue depends on two key parameters, the exposure of the colony and the morphology of the colony. Coral colonies growing at the same depth can experience very different light regimes depending on the exposure of the colony. For example, a colony located in a cave, crevice or under another colony will experience much less light than a colony that is directly exposed to the downwelling irradiance. Falkowski and Dubinsky (1981) found shaded colonies "under ledges, in grottos, or near the reef floor (~15m)" received less than 1% of the surface irradiance compared to exposed colonies in lagoons or upper reef areas which received 50-90%. The differences in intensity observed between these shallow water individuals is on the same order of magnitude as differences between colonies at ~5m and ~90m (Lesser et al. 2010).

The exposure of tissue also depends on the morphology of the coral. The radiance distribution, the orientation of the tissue on the coral's surface relative to the solar zenith angle and the degree of self shading will dictate the photon flux available for interaction with the coral tissue (Anthony et al. 2005). Different colony morphologies provide different distributions of tissue surface orientations and different degrees of self shading (Stambler and Dubinsky 2005; Kaniewska et al. 2011). Measurements on branching colonies of *Stylophora pistillata* have shown that irradiances at the tips of branches are 10 times greater than at 2cm from the tip (Kaniewska et al. 2011). In contrast, measurements on the massive coral *Lobophyllia corymbosa* found that the irradiance was more evenly distributed over the surface of the colony (Kaniewska et al. 2011). As a general rule, the larger the colony size in terms of its footprint on the reef, the greater the light absorption, however, integrating sphere approaches have demonstrated that while in spherical and planer colonies this increase in absorption is approximately linear with size, light collection by branching corals was less efficient at larger sizes due to the greater tissue surface area of the colonies (Stambler and Dubinsky 2005).

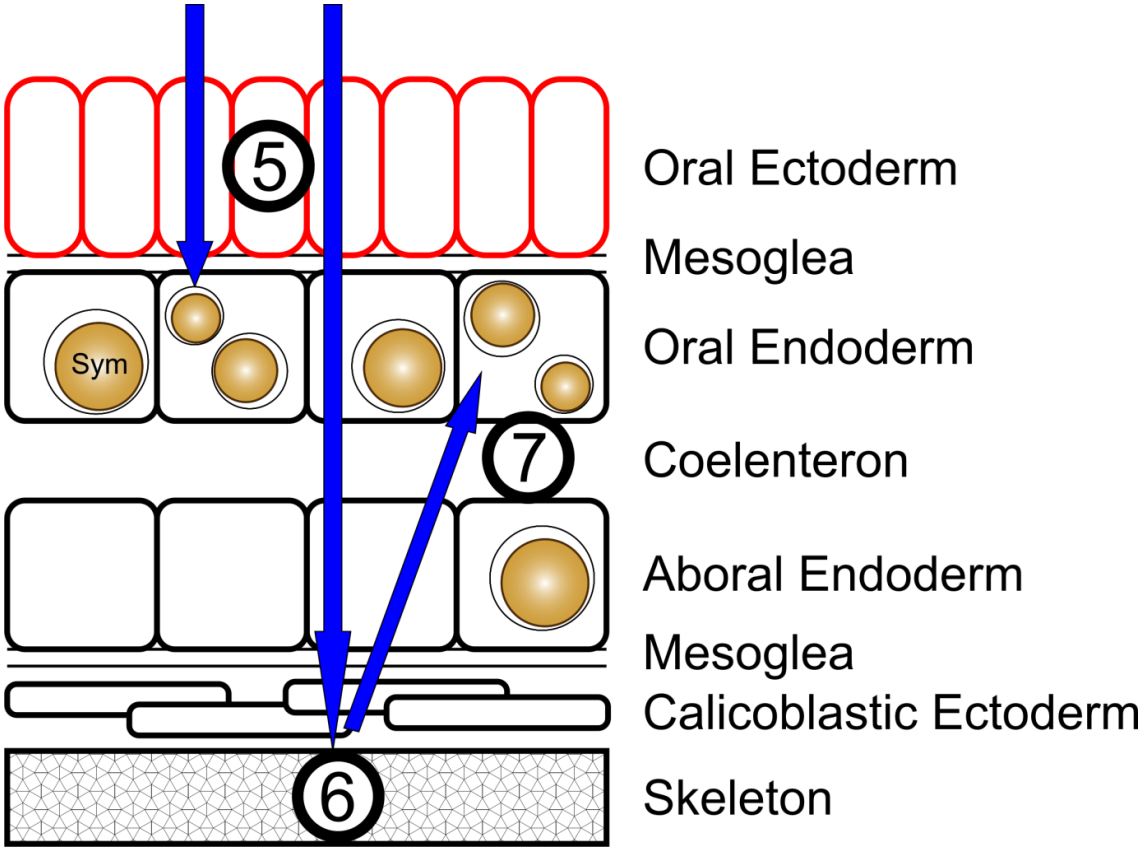

Figure 2.5. Pathway of light from the surface of the coral tissue to the algal symbionts (Stages 5-7). The path of light is shown by the blue arrows. Each tissue layer is labelled on the right of the schematic. Sym = Symbiodinium. Synthesised and adapted from Kvennefors et al. (2010). Not to scale.

5. Transmission through the coral tissue

The coral ectoderm acts as the interface between the external environment and the endoderm below which houses the zooxanthellae. As such, any modulation of light in the ectoderm will impact the light field experienced by the zooxanthellae. The ectoderm of corals often contains green fluorescent protein (GFP)-like pigments (Kawaguti 1944; Salih et al. 2000; Mazel et al. 2003b; Oswald et al. 2007). Fluorescent proteins are most frequently encountered in shallow water corals with up to 97% of shallow water corals possessing fluorescent pigments (Salih et al. 2000). These proteins have the potential to modify the light field experienced by the zooxanthellae due to their absorption, fluorescence and scattering properties. All GFP-like proteins in corals absorb light, with the degree depending on the tissue concentration, spatial distribution across the colony and spectral properties of the protein. While in the CPs, the absorbed light is almost entirely lost as heat, in the FPs, the light is re-emitted at a longer wavelength (Salih et al. 2000; Dove et al. 2001; Alieva et al. 2008). The efficiency of the fluorescence is governed by the quantum yield, i.e. the number of photons emitted per photon absorbed, which ranges from 0.43-0.96 in FPs (Alieva et al. 2008). Some FPs can be found in aggregates (Kawaguti 1944; Schlichter et al. 1986; Salih et al. 2000; Mazel et al. 2003b), also known as fluorescent pigment granules and it has been proposed that these aggregates can scatter the light either increasing reflectance or increasing the pathlength of light in the tissue and hence absorption (Salih et al. 2000).

The concentrations of GFP-like proteins across a coral colony can be spatially variable (Figure 2.6; (Mazel et al. 2003b; Gruber et al. 2008; D'Angelo et al. 2012). In some colonies, the FPs and CPs are uniformly distributed whereas in other colonies distinct localisation in regions, such as growth zones, is evident (D'Angelo et al. 2008; D'Angelo et al. 2012). Furthermore, while frequently localised in the epidermis, some GFP-like proteins are located in the endoderm (Kawaguti 1944; Salih et al. 2000; Mazel et al. 2003b; Salih et al. 2006; Oswald et al. 2007).

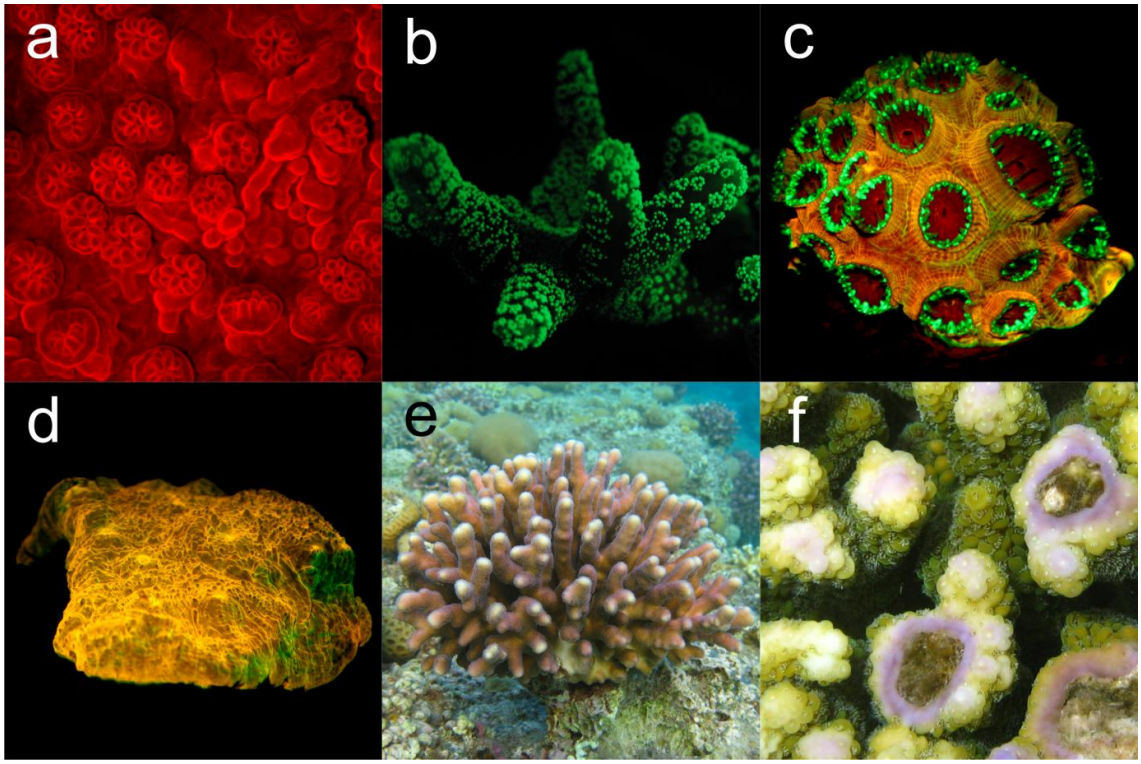

Figure 2.6. Localisation of fluorescence in coral colonies. (a) Uniform distribution of red fluorescence across a *Montipora* sp. colony. (b) Green fluorescence localised in the tentacles of *Stylophora* sp. (c) *Montastrea cavernosa* colony showing different localisation of distinct FPs with red fluorescence uniformly distributed across the coenosarc and green fluorescence in the tentacles. (d) *Oxypora* sp. colony with a uniformly distributed photoconvertible red fluorescent protein. The green fluorescence on the right of the colony relates to an entirely unconverted population (green form of the protein) due to shading. (e) A pink chromoprotein uniformly distributed across the light exposed tissues of a *Stylophora pistillata* colony. (f) Localisation of chromoproteins in the growth margins of an acroporid coral.

6. Interaction with the coral skeleton

The skeleton of corals plays a fundamental role in light collection (Kuhl et al. 1995; Enriquez et al. 2005; Terán et al. 2010). Observations of enhanced light fluxes within coral tissues were built upon by theoretical examinations and experimental measurements of multiple scattering within the coral skeleton (Kuhl et al. 1995; Enriquez et al. 2005; Terán et al. 2010). The skeleton is highly reflective (R_{400nm} = ~60% to R_{700nm} = ~95%) and so light that has initially passed through the tissue without being absorbed, is reflected back towards the zooxanthellae in the endoderm increasing the pathlength of the light (Enriquez et al. 2005). The structure of the skeleton, particularly the presence of cup-like structures and channels, traps light enabling

potentially multiple interactions with the skeleton and increasing the overall absorption efficiency of the coral (Enriquez et al. 2005; Terán et al. 2010; Kahng et al. 2012). Consequently, corals can absorb similar amounts of incident light compared with higher plants but with an order of magnitude less chlorophyll (Enriquez et al. 2005).

7. Absorption by the zooxanthellae

Absorption of light by the coral's algal symbionts is governed by the spectral properties of their photosynthetic pigments. The major light harvesting pigments in corals are chlorophyll a, chlorophyll c_2 and peridinin (Iglesias-Prieto and Trench 1997). Chlorophylls a and c_2 absorb strongly in the blue and red regions of the spectrum but absorption is virtually absent at green wavelengths. In contrast, peridinin absorbs strongly in the green region of the spectrum. This combination of pigments gives the dinoflagellates their characteristic brown appearance and is a key determinant in the colouration of corals (Hochberg et al. 2004). These pigments are bound in pigment-protein complexes that slightly modify the spectral properties of the pigments in isolation. The photosynthetic apparatus of symbiotic dinoflagellates in corals comprises of the soluble peridinin-chlorophyll protein complex (sPCP) and the membrane bound chlorophyll a – chlorophyll c_2 – peridinin protein complex (acPCP) coupled to the reaction centre complexes of photosystems I and II (Figure 2.7) (Iglesias-Prieto et al. 1991,1993; Iglesias-Prieto and Trench 1997; Hennige et al. 2009).

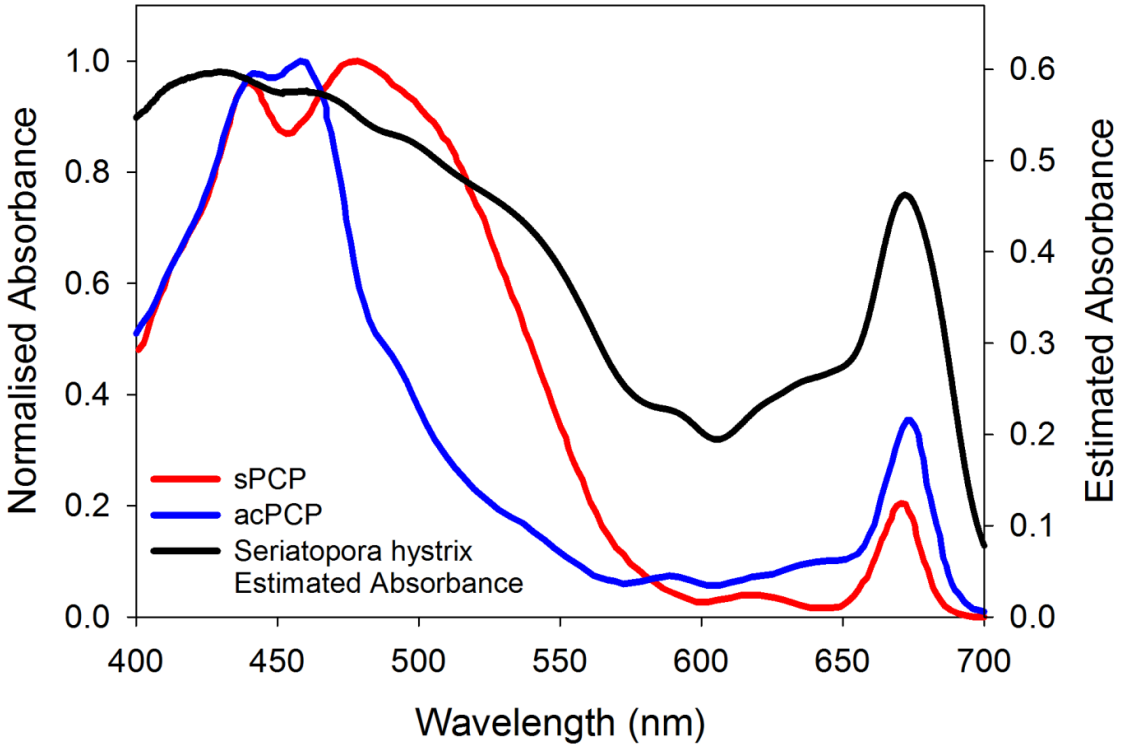

Figure 2.7. Absorption by the light harvesting complexes sPCP and acPCP from *Symbiodium microadriaticum* in comparison to the absorption of a coral tissue. The light harvesting complex pigment spectra are normalised to a maximum value of 1.0 and are redrawn from Iglesias Prieto et al. (1991, 1993). The absorption spectrum for the coral *Seriatopora hystrix* was estimated using measurements of shaded tissue reflectance spectra (as per Enriquez et al. 2005).

The degree of absorption by the pigments is dependent on the concentration of the pigments in the dinoflagellate cells and the number of cells (Stambler and Dubinsky 2005). The increase in absorption with increasing concentration is non-linear, however, due to the package effect (Duysens 1956; Kirk 1994; Stambler and Dubinsky 2005; Hennige et al. 2009). The package effect, also known as the flattening effect, describes the decrease in efficiency with which light is absorbed with increasing concentration due to self shading. The package effect is greatest at wavelengths of strongest absorption consequently increasing the relative absorption of wavelengths with weaker absorption coefficients and thus causing a 'flattening' of the spectra (Duysens 1956; Kirk 1994). As a result of the package effect, the absorption spectrum of coral tissues shows a greater contribution of yellow-orange light in the absorption spectrum than would be predicted using isolated pigment spectra.

2.2.3. The role of light in the symbiosis

Light plays a fundamental role in the success of the coral-dinoflagellate symbiosis due to the photosynthetic fixation of carbon by the zooxanthellae and its subsequent translocation to the host. It has been demonstrated that the supply of these energy rich compounds can exceed the host's daily requirement for respiration (Edmunds and Davies 1986; Muscatine 1990). It is, therefore, in the coral's interests to ensure that the symbionts are provided with optimal conditions for photosynthesis. The symbiont is isolated from the external seawater by host tissue layers and a membrane complex called the symbiosome (Davy et al. 2012). Consequently, the host needs to ensure that the symbiont is supplied with various resources required for photosynthesis; this includes inorganic carbon, nutrients and light. Inorganic carbon (Weis et al. 1989) and nutrients (D'Elia 1977) are actively transported across the symbiosome and therefore are regulated by the host. The regulation of nutrients, for example, is thought to play a key role in how the host maintains the symbiont population (Falkowski et al. 1993). By restricting the growth of the symbionts through nutrient limitation, the population can be kept under control as photosynthate transfer declines as the population size increases due to the symbionts conserving carbon supplies (Dubinsky et al. 1990). Like nutrients, the supply of light is essential for symbiont photosynthesis and the host plays a part in providing an optimal light environment for its zooxanthellae.

The challenge for the symbiosis is that corals inhabit a range of light environments and as sessile organisms, can be exposed to fluctuations of irradiance over timescales ranging from microseconds to seasons (Falkowski 1984). The important question to address is what constitutes the optimal amount of light and what is the impact of sub/supra-optimal light on the symbiosis?

2.2.4. Optimal light requirements

For a given coral tissue under set environmental conditions, the symbiont population will have a relationship between the rate of photosynthesis at different irradiances (Photosynthesis vs Irradiance - P vs E curve – Figure 2.8). This relationship can be considered to have 3 distinct regions: Light limited, light saturated and photoinhibited region (Falkowski and Raven 2007). The light limited phase describes the linear increase in the rate of photosynthesis with increasing light at low irradiances. The slope of this initial phase, α, is related to the maximum quantum yield. As irradiance increases, the relationship between photosynthetic rate and irradiance becomes non-linear and reaches an irradiance, Pmax, above which the photosynthetic rate is light saturated (rate of photon absorption>rate of electron transport) (Falkowski and Raven

2007). The irradiance at which extrapolations from α and Pmax intersect indicates the optimal irradiance for the photosynthesis, E_k, reflecting the trade-off between the efficiency of photon utilisation and overall photosynthetic production (Talling 1957; Falkowski and Raven 2007). Values for E_k in coral zooxanthellae *in vivo* range from 10μmol m^{-2} s^{-1} in deep (110-120m) mesophotic reefs (Fricke et al. 1987) to >750μmol m^{-2} s^{-1} in shallow waters (Hennige et al. 2008). Supraoptimal irradiances can bring about a decrease in the zooxanthellae's photosynthetic rate below Pmax (e.g. Shick et al. 1995; Lesser 1996), referred to as the photoinhibited region of the P vs E curve. Photoinhibtion is defined as "the light dependent and slowly reversible retardation of photosynthesis, independent of any developmental change" (Long et al. 1994) and therefore is not restricted to only a decline in Pmax but can be evident in other parts of the P vs E curve. The reduction in photosynthetic rate occurs due to a reduction in the turnover time of the electron transfer chain and/or a reduction in the number of functional PSII reaction centres (Falkowski and Raven 2007). It is important to note, however, that external conditions can act to alter the impact of a given irradiance on populations of the same species and hence alter the P vs E relationship. Any factor that slows the rate of utilisation of absorbed light will act to increase the excitation pressure (Long et al. 1994; Maxwell et al. 1995). A decrease in the zooxanthellae's ability to use the reducing power generated by photochemistry, known as sink limitation, has been implicated as a cause of photoinhibition in corals (Jones et al. 1998; Rodríguez-Román and Iglesias-Prieto 2005).

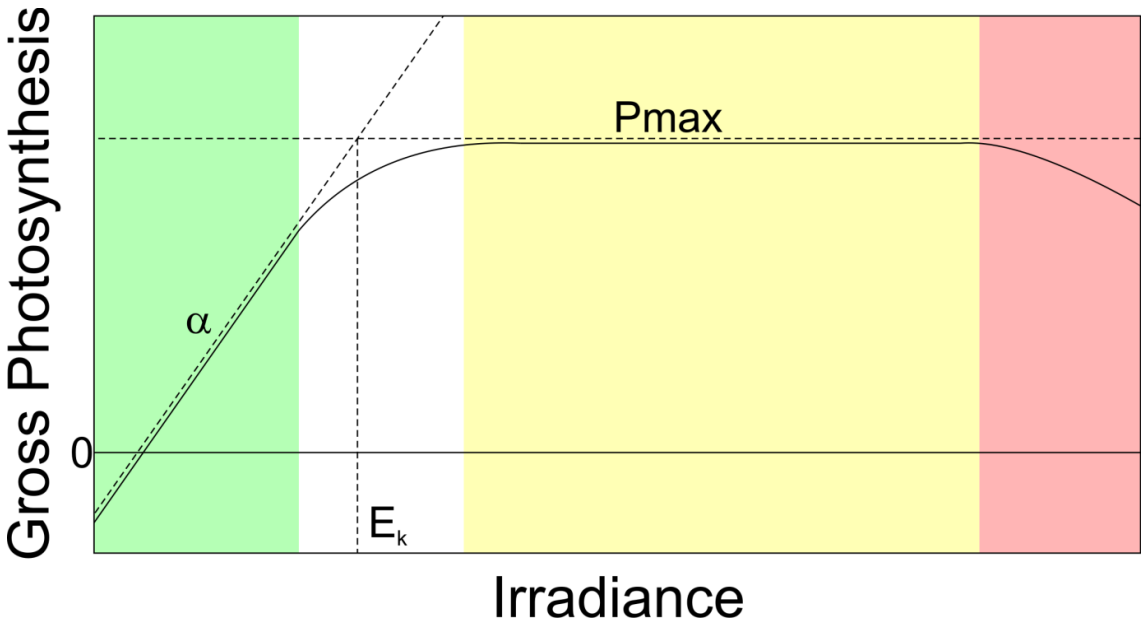

Figure 2.8. Hypothetical photosynthesis vs irradiance curve. The solid line shows the photosynthesis vs irradiance relationship and the parameters α, E_k and Pmax are described in the text. The green, yellow and red regions of the graph relate to the light limited, light saturated and photoinhibited regions, respectively.

Two types of photoinhibition have been identified in corals, dynamic and chronic (Gorbunov et al. 2001), and their occurrence depends on the equilibrium between damage and repair of the photosystem II reaction centre protein D1 (Warner et al. 1999). Damage to D1 occurs even at low light intensities, however, the capacity for repair and replacement of the protein greatly exceeds the rate of damage (Smith et al. 2005). Under higher light levels, the rate of damage increases, particularly if the capacity to utilise the energy for photosynthesis and thermally dissipate excess excitation energy is exceeded (Niyogi 1999; Smith et al. 2005). Here, temporary damage to the D1 protein leads to dynamic photoinhibition. In shallow water corals, about 10-30% of the reaction centres are transiently damaged under irradiances of 1500-1800µmol m^{-2} s^{-1}, however, they are repaired over relatively short timescales (tens of minutes to several hours) (Gorbunov et al. 2001). The efficacy of the repair mechanisms appears to be greater in more thermally tolerant zooxanthellae/corals (Takahashi et al. 2004). If further excitation pressure is applied or is sustained for long periods of time, the photoinhibition can become chronic. Chronic photoinhibition describes the complete degradation of the D1 protein at a rate greater than can be repaired (Warner et al. 1999) and the restoration of PSII photochemical activity takes >20 hours (Gorbunov et al. 2001).

D1 is damaged by reactive oxygen species generated by the photosynthetic machinery (Richter et al. 1990). When the plastoquinone pool is fully reduced, P680* is unable to transfer excitation energy due to the loss of an available electron acceptor resulting in the formation of a triplet state (Smith et al. 2005). The triplet state readily reacts with O_2 to form oxygen radicals, which damage the D1 protein (Niyogi 1999; Smith et al. 2005). The formation of reactive oxygen species can also occur in the antenna as well as through the operation of the Mehler reaction (Smith et al. 2005) and exposure to ultraviolet radiation (Banaszak and Lesser 2009). The generation of oxygen radicals can cause damage to cell components such as DNA, proteins, pigments and lipids (Niyogi 1999; Banaszak and Lesser 2009). This photooxidative damage has been widely implicated in mass coral bleaching, the widespread explusion/loss of zooxanthellae from the coral host.

Mass coral bleaching occurs predominantly in temperatures that exceed the thermal threshold of the corals in the region, usually 1˚C above the long-term average of the warmest month (Goreau and Hayes 1994). Light plays an important role in this thermal bleaching as the direct consequence of thermal stress is to alter how the coral can deal with light (Iglesias-Prieto et al. 1992; Fitt and Warner 1995; Brown 1997; Jones et al. 1998; Brown et al. 1999; Hoegh-Guldberg 1999; Warner et al. 1999; Coles and Brown

2003). This is supported by field observations of thermal bleaching specifically localised to the light exposed regions of colonies (Coles and Brown 2003; Salih et al. 2006). The primary mechanism of photoinhibition under thermal stress has been linked to PSII (Warner et al. 1999), the stability of the thylakoid membranes (Tchernov et al. 2004; Wiedenmann et al. 2012b), the dark reactions of photosynthesis (Jones et al. 1998), photosynthetic machinery's repair mechanisms (Takahashi et al. 2004) and the oxygen evolving complex (Baird et al. 2009).

To minimise their susceptibility to photooxidative stress, the algal symbionts and the coral host possess mechanisms to effectively reduce the excess light. These mechanisms and the approximate timescales over which they operate are summarised in Figure 2.9.The next section will briefly discuss mechanisms employed by the symbionts and the host to deal with different irradiances with a particular focus on reducing excess excitation energy in high light environments (shallow water corals) and maximising productivity under low light environments (shaded/mesophotic corals).

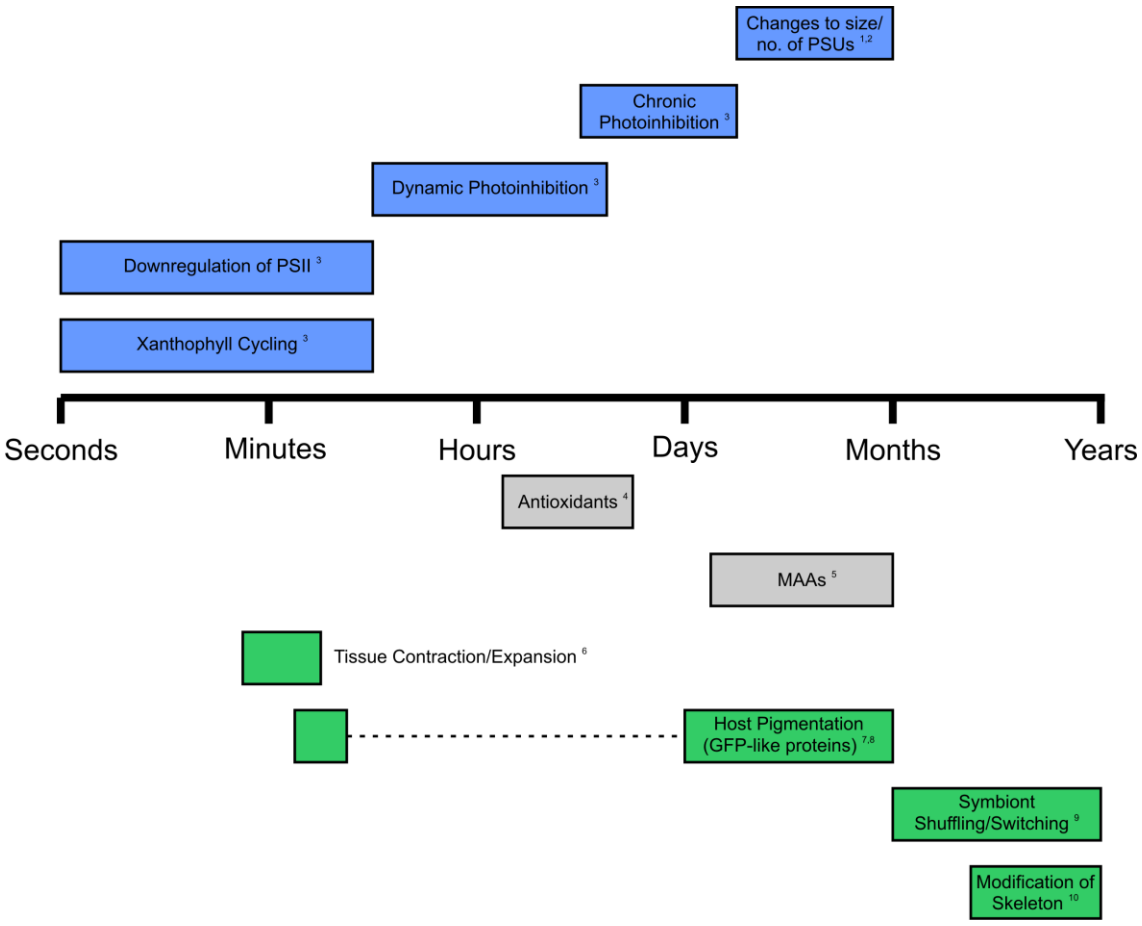

Figure 2.9. Approximate timescales of host and symbiont responses to light. Timescales are estimated based on published data but should be considered as approximate as they are inherently limited by the resolution of the respective studies.

References: (1) Falkowski 1984 (2) Winters et al. 2009 (3) Gorbunov et al. 2001 (4) Levy et al. 2006 (5) Shick 2004 (6) Levy et al. 2003 (7) D'Angelo et al. 2008 (8) Wiedenmann Unpublished data (9) Chen et al. 2005 (10) Muko et al. 2000. * Dashed line on host pigmentation box represents the time between the upregulation at the transcript level on the scale of minutes and the appearance of the functional protein.

2.2.5. Symbiont mechanisms:

2.2.5.1.Symbiont genetic diversity

The endosymbiotic dinoflagellates found in corals belong to the genus *Symbiodinium*. It is a remarkably diverse group that is believed to compromise of different species (LaJeunesse 2001). Early work identified different clades of the symbionts (Rowan and Powers 1991), however, further analyses of internal transcribed regions (ITS) regions of rDNA have shown that there is substantial intracladal diversity (LaJeunesse 2001; LaJeunesse 2002). Understanding genetic variation within the genus *Symbiodinium* is important because variants have different physiological tolerances (Iglesias-Prieto and Trench 1994; Iglesias-Prieto and Trench 1997; Tchernov et al. 2004; Frade et al. 2008) with some clades/sublades exhibiting exclusive photoprotective mechanisms (McCabe Reynolds et al. 2008) and distinct ecological distributions (Iglesias-Prieto et al. 2004; Frade et al. 2008). Nevertheless, some generalist symbionts have been identified in deep mesophotic reefs and this discovery, in combination with analyses of host diversity, suggest that symbiont diversity alone is not the only factor involved in the depth distribution of corals (Chan et al. 2009).

It is also important to consider that the extent of the genetic diversity of the symbionts is not fully understood as the current ITS2 site may not be the most suitable marker (Stat et al. 2011). Microsatellite analyses have revealed that there is further diversity within subclades found in different populations (Santos et al. 2003; Howells et al. 2009). Furthermore, analyses of C1 symbionts in culture from two different populations found that they exhibited different thermal tolerances based on the thermal regime of their normal environment (Howells et al. 2011).

2.2.5.2.Symbiont pigments

The efficacy with which light is collected at low irradiances depends, to a large extent, on the photosynthetic pigment concentrations in the tissue (Dubinsky et al. 1984). This can be altered by keeping the pigment concentrations per cell the same but increasing

the number of cells or by increasing the size or number of photosynthetic units (PSUs). Increased pigmentation of corals with depth in the field has been demonstrated to occur through increases in the size of the PSUs (Falkowski and Dubinsky 1981) and changes in the zooxanthellae numbers (Titlyanov et al. 2001). Analyses on zooxanthellae cultures indicate that changes to the number of PSUs is also a strategy employed in response to low light (Iglesias-Prieto and Trench 1994) and potentially dependent on symbiont subclade (Hennige et al. 2009).

2.2.5.3.Non-photochemical quenching

When chlorophyll is excited by a photon, the excitation energy is destined for either photochemistry, non-photochemical quenching (NPQ), chlorophyll fluorescence or decay via the triplet state (Müller et al. 2001). NPQ is the dissipation of excess excitation energy as heat (Müller et al. 2001). Under low light regimes, the majority of photons are used for photochemistry, however, under supraoptimal irradiances, the zooxanthellae must balance the relative use of photochemistry and NPQ to prevent damage to the photosynthetic machinery but still maintain high rates of photosynthesis (Falkowski and Raven 2007).

There are different mechanisms through which NPQ can occur. Xanthophyll de-epoxidation is one of the main mechanisms for NPQ in higher plants and is also present in symbiotic dinoflagellates as the diadinoxanthin cycle (Demmig-Adams and Adams III 1996; Brown et al. 1999; Müller et al. 2001). The diadinoxanthin cycle dissipates excitation energy through reversible de-epoxidation (loss of the epoxy group) of diadinoxanthin to form diatoxanthin. Although the exact mechanism/s of the xanthophyll cycle's role in NPQ remains inconclusive, the evidence supports a role in the direct de-excitation of singlet chlorophyll (Demmig-Adams and Adams III 1996; Goss and Jakob 2010). In corals, Brown and co-workers (1999) have shown that the proportion of diatoxanthin within the xanthophylls pool varies with the diurnal light cycle and that it is correlated with the decline in effective quantum yield, supporting a role in photoprotection of shallow water corals. Nevertheless, there does not appear to be a clear link between the size of the xanthophylls pool and bleaching resistance (Venn et al. 2006).

Gorbunov and co-workers (2001) found that downregulation of the PSII reaction centres thermally dissipates up to 80% of the excess excitation energy experienced by shallow water colonies. Here, the temporary suspension of the reaction centre, as opposed to permanent damage, enables rapid restoration of photochemical activity

when required (Weis and Berry 1987; Gorbunov et al. 2001) and appears to be more important in corals than antenna based quenching (Gorbunov et al. 2001).

Other symbiont NPQ/photoprotective mechanisms include some pathways that are specific to certain symbiont clades such as the observation of cyclic electron transport and sPCP dissociation from the light harvesting complex in clade A Symbiodinium but not in B-D or F (McCabe Reynolds et al. 2008). Interestingly, clade A symbionts are frequently encountered in high light environments (LaJeunesse 2002) and these additional photoprotective mechanisms may provide their enhanced fitness (McCabe Reynolds et al. 2008). Furthermore, a mechanism found in algae which involves migration of the LHCs between PSII and PSI to balance excitation energy between photosystems has been proposed to occur in zooxanthellae (Jones and Hoegh-Guldberg 2001). Nevertheless, these state transitions have not been conclusively demonstrated for *Symbiodinium*.

2.2.6. Shared mechanisms between host and symbiont

There are certain photoprotective mechanisms shared by the coral host and its symbionts such as the production of mycosporine-like amino acids (MAAs) and antioxidant enzymes. MAAs, abundant in shallow water corals, exhibit characteristics consistent with a role as UV photoprotectants (Shick and Dunlap 2002). They absorb strongly (molar extinction coefficient = 28000-50000M^{-1} cm^{-1}) in the UV and are more abundant in shallow than deeper waters, in agreement with the higher flux of UV light (Gleason and Wellington 1993; Shick et al. 1995; Shick et al. 1996). Although the host MAAs were originally thought to originate from the symbiont partner (Shick and Dunlap 2002), analysis of a coral genome has identified that corals have the ability to perform *de novo* synthesis of MAAs (Shinzato et al. 2011).

Antioxidants, such as superoxide dismutase (SOD) and catalase (CAT), are also present in both the coral and symbionts (Levy et al. 2006). These enzymes deal with the reactive oxygen species that are the products of photooxidative stress and their tissue activity is greater under blue irradiance (compared to green, red and white light) (Levy et al. 2006) and at shallower depths (Shick et al. 1995).

2.2.7. Host

2.2.7.1.Morphology

As previously mentioned, the shape of a coral colony has an impact on the light absorption by the coral's tissues (Stambler and Dubinsky 2005). Branching morphologies generally provide substantial self shading and therefore are thought to benefit shallow water environments, however, changes in branching morphology have been observed with depth as *Stylophora pistillata* branches are more planar and fan-like in low irradiance environments (Dubinsky and Jokiel 1994; Mass et al. 2007; Einbinder et al. 2009). As irradiance decreases, there tends to be a higher abundance of massive, planar and encrusting forms (Falkowski et al. 1990) in order to intercept as much light as possible and analyses using an integrating sphere has shown that these growth forms are particularly efficient at light collection (Stambler and Dubinsky 2005).

2.2.7.2.Skeleton

Modelling by Teran and co-workers (2010) identified that the complexity and architecture of the skeleton determines the degree of amplification of the internal light field. Measurements from the field appear to support these findings showing differences in the skeletal optical properties between mesophotic *Leptoseris* spp. and shallow water *Porites* spp. (Kahng et al. 2012). As the *Leptoseris* colonies exhibit greater absorption with lower areal chlorophyll concentrations, the authors hypothesise that the differences observed are due to the architecture of the coral skeleton, reflecting an adaptive response to the irradiance conditions (Kahng et al. 2012). Further work is required to assess the optical properties of the skeletal matter from the species in this study in an unstructured form. In addition, an assessment of the modification of the skeleton on the same species at different irradiances would greatly support their findings.

2.2.7.3.Host pigments

<u>Fluorescent Proteins</u>

High light

Fluorescent pigments of the GFP family have been proposed to be involved in photoacclimation to high and low light environments, however, these roles remain

controversial. In high light, fluorescent proteins are thought to perform a photoprotective function in corals. This was first proposed by Kawaguti (1944) and his pioneering work was subsequently built upon by Salih and co-workers (2000). It is postulated that the FPs provide photoprotection by two methods. Firstly, the shorter wavelength FPs (cyan and green) absorb wavelengths of light that are strongly absorbed by the photosynthetic pigments of the symbionts and through fluorescence resonance energy transfer (FRET) and radiative energy transfer, pass the excitation energy onto longer wavelength FPs that would emit in a region of low photosynthetic pigment absorption (Salih et al. 2000; Gilmore et al. 2003b; Cox et al. 2007). Thus, the concept is that excitation pressure on the photosynthetic machinery is reduced by reducing the efficiency with which light is absorbed by the symbionts. Secondly, scattering by fluorescent pigment granules is proposed to increase broadband reflectance (Salih et al. 2000).

There are observations and characteristics of fluorescent proteins in corals that are consistent with a photoprotective function. The high abundance of fluorescence in shallow water corals (>97%) (Salih et al. 2000) is supported by the demonstration of light regulation of fluorescent proteins (D'Angelo et al. 2008; Roth et al. 2010; Aranda et al. 2011). D'Angelo and colleagues (2008) showed that while almost absent in low light, green and red fluorescent proteins are upregulated in response to increasing light intensities. Such a regulation pattern would ensure that investment in photoprotection is only made where required. Furthermore, the slow turnover and stability of these proteins implies that the expression of these proteins is relatively inexpensive for the corals as once the tissue concentration is established, it will require less energy to maintain it at that level. The low maintenance costs are thought to enable corals to accumulate tissue concentrations up to 14% of the total soluble protein content (Leutenegger et al. 2007b). Observations of the symbiont community within fluorescent colonies suggests that they can house greater symbiont densities (Kawaguti 1944), host symbionts typical of deeper waters (Frade et al. 2008) and that under bleaching conditions, tissue FP content correlates positively with zooxanthellae density (Salih et al. 2000). Kawaguti (1944) also observed that declines in oxygen evolution associated with midday irradiances were greater for brown morphs of *Acropora hyacynthus* and *Montipora ramosa* compared to green morphs.

The main argument against the function of FPs in photoprotection centres on the findings of Mazel and co-workers (2003b). They argued that if GFPs were serving a photoprotective function, then GFP absorption should be evident as a wavelength specific reduction in the action spectrum of photosynthesis. Using the chlorophyll excitation spectrum as a proxy for the photosynthetic action spectrum, they found that

the reduction in excitation associated with GFP absorption was minimal and therefore argued that GFPs could not be serving a photoprotective function (Mazel et al. 2003b). In addition, they observed that there was no depth dependent decrease in green corals with depth which contrasts to the depth dependent decrease in other photoprotectants and photoprotective mechanisms such as MAAs and NPQ, respectively (Mazel et al. 2003b). It has also been identified that the FPs most capable of providing photoprotection, the cyan FPs whose absorption has greatest overlap with the photosynthetic pigments, belong to the low threshold group of light regulation. The low threshold group are present at low light intensities up to ~400µmol m^{-2} s^{-1} before decreasing at higher intensities which is opposite to the expected pattern for a photoprotectant. Examples of FP expression inconsistent with a photoprotective have also been observed in organisms outside of the scleractinians (Leutenegger et al. 2007a; Vogt et al. 2008).

Another function that has been proposed for GFP-like proteins under high light conditions is an antioxidant function (Bou-Abdallah et al. 2006; Palmer et al. 2009). Nevertheless, GFP-like protein tissue concentrations do not explain much of the variation in tissue homogenate scavenging of H_2O_2 (r^2 = 0.08-0.34) (Palmer et al. 2009) and other studies do not support an antioxidant function (Leutenegger et al. 2007a; Aranda et al. 2011).

Low light

In low light conditions, fluorescent pigments have been proposed to perform a photoenhancing role (Schlichter et al. 1986; Salih et al. 2000; Dove et al. 2001). The photoenhancing role has been assigned to FPs that are located in the endoderm of the coral where it is proposed that they absorb UV light at depth and transfer it to cyan wavelengths suitable for photosynthesis thus broadening the spectral availability for photosynthesis into the UV (Schlichter et al. 1986; Dove et al. 2001). In addition, scattering by these pigment granules is expected to increase scattering within the tissue and therefore enhance the absorption of visible wavelengths (Salih et al. 2000). The photoenhancement effect may not be functionally significant due to the low UV irradiances at the depths at which the corals studied by Schlichter and coworkers (1986) reside and the theory has largely been discredited due to studies showing the absence of energy transfer between fluorescent pigments and chlorophyll, using chlorophyll fluorescent excitation spectra (Mazel et al. 2003b) and confocal microscope based lifetime spectroscopy (Gilmore et al. 2003b). It is important to consider that these studies were performed on shallow water individuals and therefore may not be the most appropriate models. Nevertheless, direct energy transfer via FRET

is highly unlikely due to the reduction in FRET efficiency by a factor of r^6 where r is the distance between the donor and acceptor (Gilmore et al. 2003b).

Chromoproteins

Chromoproteins, photophysically distinct members of the GFP-like protein family, have also been proposed to serve as photoprotectants for shallow water corals (Dove et al. 2001; Salih et al. 2006). The CPs are upregulated in response to higher light intensities (Takabayashi and Hoegh-Guldberg 1995; D'Angelo et al. 2008) and a depth dependent decrease in the proportion of CP pigmented colonies has been observed (Salih et al. 2006). In common with their fluorescent counterparts, the photoprotective function has been challenged largely due to a lack of evidence for the mechanism and because the absorption occurs in a region of the spectrum that is weakly absorbed by the photosynthetic pigments (Dove et al. 1995; D'Angelo et al. 2008). However, there have been reports of a superior competitive ability and reduced light stress at high irradiances in corals expressing CPs compared to their brown counterparts (Rinkevich and Loya 1983; Dove 2004). In addition, it has recently has been postulated that the development of an expanded CP repertoire in *Acropora digitifera* is related to its occurrence in high light environments (Shinzato et al. 2012).

One factor that may affect the CP's potential to provide protection under thermal stress is the downregulation of transcripts in response to elevated temperatures (Smith-Keune and Dove 2008). The high susceptibility of CP pigmented morphs to thermal bleaching is thought to result from the loss of the CP pigment shield that subsequently leaves the previously shaded zooxanthellae exposed to higher light intensities (Dove 2004). In contrast to the work of Dove (2004), other studies have found that CP pigmented morphs suffered less mortality in a natural thermal bleaching event (Salih et al. 2006) and upregulation of CP transcripts during bleaching events (Seneca et al. 2010). It is clear that without full understanding the mechanism behind the proposed photoprotective mechanism, interpreting the causes for contrasting findings amongst different studies is challenging.

CHAPTER 3. Identification and analysis of a metal binding site in the red fluorescent protein asFP595/1

3.1. Abstract

Fluorescent proteins from the green fluorescent protein (GFP) family have become indispensible tools for cell biologists. Evolutionary analyses of these proteins suggest that there may be binding sites present on the proteins surface and therefore a greater understanding of their biological function should enable identification of interactions with other molecules. These binding domains could then be exploited for marker applications. One emerging application of fluorescent proteins is the use of these proteins to monitor *in vivo*/*in vitro* concentrations of transition metal ions through metal-induced quenching of the proteins' fluorescence. While some fluorescent proteins have an inherent metal affinity, attempts to engineer binding sites onto GFP mutants have been hindered by our limited understanding of the motifs involved. In order to successfully develop transition metal ion biosensors, identification and characterisation of naturally occurring metal binding domains is needed. Furthermore, characterisation of naturally occurring metal binding motifs will enable elucidation of the prevalence of metal binding amongst GFP-like proteins and could provide clues as to a biological function. To this end, GFP-like proteins from the reef building corals *Acropora millepora* and *Lobophyllia hemprichii* and the sea anemone *Anemonia sulcata* were screened for the presence of metal binding domains using immobilised metal affinity chromatography. Metal binding was observed in a red fluorescent protein from *A. sulcata*, asFP595/1. *In silico* structural analysis and site directed mutagenesis identified a two histidine motif in the C-terminus as the binding domain. The screening of coral GFP-like proteins and *in silico* analysis suggest that the domain is not present in scleractinian GFP homologs and therefore does not perform a biological function in these organisms, Overall, the protein is rare within the GFP family and requires a C terminus structure that is unique to the Actinarian GFP-like proteins.

Fluorescence quenching analyses on the asFP595/1 mutants showed that the metal affinity and transition metal induced fluorescence quenching are likely to relate to different motifs. Furthermore, it was identified that the C terminus also plays an important role in protein aggregation in the presence of transition metals. This study provides the first in-depth analysis of a naturally occurring metal binding site of a GFP-like protein and demonstrates an unreported link between aggregation and metal

binding. Development of mutants using the site directed mutagenesis approach employed in this study could improve the aggregation tendencies of fluorescent proteins with similar properties and therefore enhance their suitability for cell imaging.

3.2. Introduction

Green fluorescent proteins (GFP)-like proteins have revolutionised the field of cell imaging over the past two decades (Verkhusha and Lukyanov 2004; Chudakov et al. 2005; Wiedenmann and Nienhaus 2006; Wiedenmann et al. 2009). The autocatalytic formation of the functional chromophore without the requirement for cofactors, their diverse spectral characteristics and the *in vivo* stability of the chromophore over a range of physiological conditions has resulted in their widespread popularity. Fluorescent proteins (FPs) belonging to the GFP family have been used for various studies including gene expression, protein localisation, protein-protein interactions and monitoring aspects of intracellular biochemistry (see reviews; Verkhusha and Lukyanov 2004; Chudakov et al. 2005; Wiedenmann and Nienhaus 2006; Wiedenmann et al. 2009). The use of FPs as molecular markers, however, hinges on the assumption that they behave in an inert fashion within a cellular environment (Wiedenmann et al. 2009). This is not always the case, particularly with anthozoan FPs, where toxicity, aggregation and interactions with other cellular components are frequently encountered (Mizuno et al. 2001; Yanushevich et al. 2002; Wiedenmann et al. 2009). Nevertheless, if these behaviours and the mechanisms controlling them are understood, mutation strategies can be targeted to eliminate them or enhance them to develop new applications (Tsien 1998; Jayaraman et al. 2000; Yanushevich et al. 2002). Indeed, fluorescent protein sensors have been created based on observations of intracellular behaviour of certain GFP-like proteins and detailed structural analyses of these proteins. FP-based sensors are now available for pH (Kneen et al. 1998; Llopis et al. 1998; Arosio et al. 2010), halides (Jayaraman et al. 2000; Wachter et al. 2000) and metal ions (Miyawaki et al. 1997; Baird et al. 1999; Nakai et al. 2001; Zou et al. 2007).

A greater understanding of the biological function of GFP-like proteins may help to identify and develop novel markers. Previous studies into the evolutionary history of GFP-like proteins have noted the conservation of a putative binding domain (Field et al. 2006). This domain and other unexplored domains may be responsible for interactions with other molecules in the cell and could be exploited for marker purposes. This is exemplified by the observation of metal binding in the RFP, DsRed (Sumner et al. 2006; Eli and Chakrabarrty, 2006). Development of transition metal ion sensors based on engineering mutants of the GFP has been met with limited success (Tansila et al. 2008). Meanwhile, the studies of metal binding in the naturally occurring DsRed have

shown it to be sensitive and specific for copper ions (Sumner et al. 2006)highlighting their potential as *in vitro*/*in vivo* copper sensors (Sumner et al. 2006). Nevertheless, the effect of these interactions between FPs and metal ions *in vivo* is currently unknown. FPs use as metal sensors or other *in vivo* applications could be limited by the potential for these FPs to scavenge metal ions from the cell, altering its physiology.

Despite the attention metal binding in anthozoan FPs has received, the binding domain and mechanism of quenching remain inconclusive. There are two mechanisms whereby metal ions cause static fluorescence quenching, energy transfer and electron transfer (Bergonzi et al. 1998). The quenching of the fluorescence in metal binding FPs has been attributed to both energy transfer (Richmond et al. 2000; Sumner et al. 2006) and electron transfer (Barondeau et al. 2002; Rahimi et al. 2007b). Meanwhile, the location and amino acids residues involved in natural binding sites also remain controversial. Whilst several sites have been proposed, it remains unresolved whether cysteine (Eli and Chakrabartty 2006; Rahimi et al. 2008) and/or histidine (Rahimi et al. 2007b; Rahimi et al. 2008) residues are involved. Locating the site/motifs responsible for these FP-metal interactions would enable identification of other proteins with similar properties.

Considering estimations that about one third of all purified proteins and enzymes require metal cofactors to perform their biological function (Holm et al. 1996), it is somewhat surprising that the biological significance of metal binding by naturally occurring GFP-like proteins has not been previously explored. Aside from binding cofactors, metal binding in proteins may also be involved in other functions such as storage of metals and transfer of electrons (Holm et al. 1996). To date, it is not known how prevalent metal binding is amongst GFP-like proteins although it occurs in at least one naturally occurring GFP-like protein (DsRed). Understanding the prevalence and characteristics of binding will enable assessment of whether metal binding by GFP-like proteins is functionally significant for the organisms that express these proteins. To this end, this study set out to test the hypotheses that metal affinity is an inherent property of all GFP-like proteins and that metal binding alters the protein's properties as this may be indicative of activation of the molecule.

3.3. Methods

3.3.1. Protein expression and purification

To enable screening of different GFP-like proteins, fluorescent proteins and non-fluorescent chromoproteins (CPs) cloned from the sea anemone *Anemonia sulcata* (Wiedenmann et al. 2000), the corallimorpharian *Discosoma* sp. (Wiedenmann et al. 2012a) and the hard corals *Acropora millepora* (D'Angelo et al. 2008) and *Lobophyllia hemprichii* (Wiedenmann et al. 2004b) were subcloned into the pET17b vector. The proteins were selected based on their distribution amongst the different clades from previous phylogenetic analyses of GFP-like proteins (Alieva et al. 2008) and included cyan, green and red (DsRed and Kaede-type chromophores) FPs and two CPs. The focus of the study was on the diversity of the proteins rather than the host species although the organisms selected included representatives from the Orders Scleractinia, Corallimorpharia and Actinaria. Specifically, *A. millepora* was selected due to its potential as a model for GFPs in corals (Alieva et al. 2008) and *L. hemprichii* was selected as it is the source of the characterised PCRFP, EosFP. *A. sulcata* and *Discosoma sp.* were selected as sources of well studied GFP-like proteins from organisms from other Orders of the Class Anthozoa. The constructs were transformed into competent *Escherichia coli* BL21 cells and grown on Luria-Bertani agar plates. Three colonies were used to inoculate 500ml of 2YT medium and cell cultures were grown overnight at 37°C. The cultures were then transferred to 4°C for expression in the absence of an inducer (Wiedenmann et al. 2002). The cells were harvested by centrifugation and resuspended in phosphate buffered saline (PBS) pH7.2. Proteins were extracted by sonicating the cells on ice for 3 minutes (15 seconds on/off cycle) and removing the cellular debris by centrifugation (20,000xg, 45 minutes).

3.3.2. Immobilised metal affinity chromatography assay

Immobilised metal affinity chromatography (IMAC), using a cobalt matrix, was performed on the protein extracts to determine the strength of metal binding. 300µl of the protein extracts were incubated with 250µl Talon Metal Affinity Resin (Clontech Laboratories Inc., Mountain View, CA) in a spin column for 40 minutes at room temperature. Unbound protein was subsequently removed by centrifugation (4,000xg, 1 minute). The matrix was washed with 300µl of extraction buffer (50mM PBS, 300mM NaCl) six times before three elution stages with 300µl of elution buffer (50mM PBS, 300mM NaCl, 300mM Imidazole). The fluorescence of the flow-through fraction from the initial protein extract and the subsequent wash and elution stages was recorded on a fluorescence spectrophotometer (Cary Eclipse, Varian Inc., Palo Alto, CA). The

fluorescence at the wavelength of maximum emission for each FP was used as a proxy for FP concentration during the different IMAC stages. The IMAC assays were repeated with diluted samples (0.5x) and the binding strength compared to the original experiments to ensure the IMAC resin was not overloaded. A full protocol for this assay is included in the Appendix (section 7.3.1.).

3.3.3. Site-directed mutagenesis

In silico analyses of the protein sequences and structure were performed using Lasergene Core Suite (DNASTAR) and Deep View (Guex and Peitsch 1997). In order to constrain the search for metal binding motifs, sequence comparisons between binding and non-binding FPs identified in the random trials, were performed. Potential amino acids involved in the binding site were selected according to:

- their presence in the binding proteins (according to the IMAC binding assay)
- amino acids that are known to be involved in cobalt coordination in other proteins (Hsin et al. 2008)
- the exposure of the residues in the 3D structure of a homologous CP from *A. sulcata*, asuICP (PDB: 2A50; Andresen et al. 2005)

After identifying the C terminus as a potential site for metal binding, mutations were introduced to asFP595/1 using mismatching primers (Kadowaki et al. 1989). The PCR products were restricted and ligated into pET17b vectors; constructs were sequenced (Eurofins MWG Operon) to confirm the presence of the mutations. The impact of the mutations was subsequently assessed using the aforementioned IMAC binding assay.

3.3.4. Protein purification

The asFP595/1 wild type and mutant protein extracts were purified using IMAC. The proteins' natural metal affinity was used for purification and as previous observations had identified a higher affinity for nickel (Wiedenmann, pers. obs.), a nickel IMAC column was used. Where natural metal affinity was low, a polyhistidine tag was used for purification. An enterokinase site was cloned between the N-terminus of the protein and the polyhistidine tag to enable cleavage of the polyhistidine tag using Tag Off Cleavage Capture Kit (Novagen) prior to analyses. After cleavage and capture of the enterokinase site, the purified proteins were passed through the IMAC column again to remove any uncleaved proteins and any remaining polyhistidine tags that could interfere with the fluorescence reduction analyses.

3.3.5. Fluorescence reduction analysis

Fluorescent protein concentrations were determined spectrophotometrically using absorbance at 575nm and an extinction coefficient of 50,000 M^{-1} cm^{-1} .The purified FPs were diluted with PBS to a concentration of 2μM. Each FP was subsequently mixed with an equal volume of a $CoCl_2$ solution to final $CoCl_2$ concentrations ranging from 10μM-10mM and a final FP concentration of 1μM. Each sample was divided between two microcentrifuge tubes and incubated for seven hours at room temperature. After incubation, the first subsample, the total protein extract (TPE), was transferred directly to a well plate for fluorescence measurements. The second subsample was centrifuged (12,000xg for 10 minutes) to remove aggregates and the supernatant was loaded into the well plate; the precipitate was retained for quantification of the FP concentration using SDS-PAGE. The centrifuge samples are herein referred to as the soluble protein fraction (SPF). The fluorescence intensity of the TPE and SPF for each mutant across the cobalt chloride concentration series was recorded in a fluorescence spectrophotometer. Ethylenediaminetetraacetic acid (EDTA; 0.5M), a chelating agent, was then added to each well and the fluorescence was recorded once more after overnight incubation. The protocol used for these analyses are provided in the Appendix (section 7.3.2.).

3.3.6. SDS-PAGE analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Summers et al. 1965) was performed to assess the FP content in the precipitate removed by centrifugation during the separation of the second subsample into its soluble (SPF) and insoluble (precipitate) protein fractions. Briefly, the precipitates, where present, were dissolved in SDS loading buffer (10% SDS, glycerol, bromophenol blue, β-mercaptoethanol) using a pipette. The samples were denatured at 95°C for five minutes and duplicate samples were loaded onto polyacrylamide gels. The polyacrylamide gels were stained with Coomassie Blue and imaged using a gel documentation imaging system (U:Genius, Syngene, Synoptics Ltd., Cambridge, UK). FP concentrations were estimated by digital image analysis of the relevant bands (Matlab® 2009b, MathWorks Inc., Natick, MA) and compared to quantifications based on fluorescence intensity of EDTA-treated samples using Pearson's correlation coefficient (Minitab 15, Minitab Inc., State College, PA).

3.3.7. Statistics

Statistical analyses on the IMAC assays were performed using SigmaPlot. Comparisons between the IMAC retention of the mutant compared to the wild-type proteins were made using a t-test. To meet the assumption of normality, the non-binomial proportion data in the IMAC assay were logit transformed according to the recommendations of Warton and Hui (2011). All samples passed the Shapiro-Wilk normality test after transformation.

3.4. Results

3.4.1. Binding site identification

3.4.1.1.Random Trial

The identification of the binding site began with a randomised trial of FPs for metal affinity. To test for metal binding, an IMAC binding assay was performed to determine the relative metal affinity of a protein, based on its retention to a cobalt IMAC resin. From the trial of 11 proteins, different IMAC elution patterns were evident and enabled categorisation of the proteins into three groups based on the degree of retention: <10% Non-binding, 10-30% weak binding, +30% binding (Figure 3.1). Three proteins with high retention in the IMAC assay were identified; all three proteins were from *A. sulcata* (asFP595/1, asFP595/2, asCP562).

Binding			
asFP595/1	214	**E** A A V A R **Y C D** A A P **S** K L G **H H**	231
asFP595/2	214	**E** A A V A R **Y C D** A A P **S** K L G **H H**	231
asCP562	215	**E** A A V A R **Y C D** A A P **S** K L G **H H**	232
Weak binding			
asulCP	215	**E** A A V G R **Y C D** A A P **S** K L G **H** N	232
asFP499	212	**E S** V V A **S Y S** Q - V P **S** K L G **H** N	228
dsFP586	215	**E** R **T E** G R **H H** L F L	225
Non-binding			
amilFP484	219	**E H** A A A **H** V N P - L K V K	231
amilFP497	219	**E H** A A A **H** V N P - L K V K	231
amilFP512	219	A **H** A A A **H** V N P - L K V K	231
amilFP597	221	**E H** A V A **S** R **S** A - L P G	232
EosFP	212	**E H** A V A - **H S** G - L P **D** N A R R	226
Other Studies			
DsRed	215	**E** R **T E** G R **H H** L F L	225

Figure 3.1 – Sequence alignments of the C termini from selected coral and anemone GFP-like proteins. Binding categories defined based on IMAC assays. Amino acid

residues in bold are amino acids found in cobalt binding sites (Hsin et al., 2008) and the boxes highlight the proposed binding motif. The C terminus of DsRed is shown for reference as copper IMAC affinity was identified in a previous study (Rahimi *et al.*, 2007a)

3.4.1.2.Sequence and structural analyses

Based on these criteria outlined in the Methods section and giving preference to potential sites that were exclusive to the binding proteins, the C terminus of the protein was identified as the potential site for metal binding (Figure 3.1; Figure S3.1). The C terminus of the asFP595/1 is positioned at the bottom of the β-barrel and provides an accessible site for binding to the immobilised cobalt ions. A two histidine motif found was found in all binding proteins in this study and even in DsRed (Figure S3.1; Figure S3.2). The *A. sulcata* RFPs and CPs were selected for further study based on the availability of homologous proteins with different C termini and binding performances. The histidines, located at positions 230 and 231 in asFP595/1, were selected as candidates for site directed mutagenesis. The absence of one of these histidines in asulCP enabled the impact of reverse mutation to be analysed.

3.4.1.3.Site directed mutagenesis of binding motif

The histidines at positions 230 and 231 in asFP595/1 were substituted with the corresponding amino acid found in FPs with similar C termini, arginine (homologous position in eqFP611) and asparagine (homologous position in asulCP) respectively. These mutants will herein be referred to as H230R and H231N. After mutagenesis of the C terminus, the IMAC binding assay was performed with each of the mutants and their binding strength compared to the wild type. The binding in the mutants was signifcantly lower compared to the wild type (H230R vs Wild Type, t-test, $t = -4.480$, $DOF = 4$, $p<0.05$; H231N vs Wild Type, t-test, $t = -7.923$, $DOF = 4$, $p<0.05$), with a more than fourfold reduction in IMAC retention (Figure 3.2a).

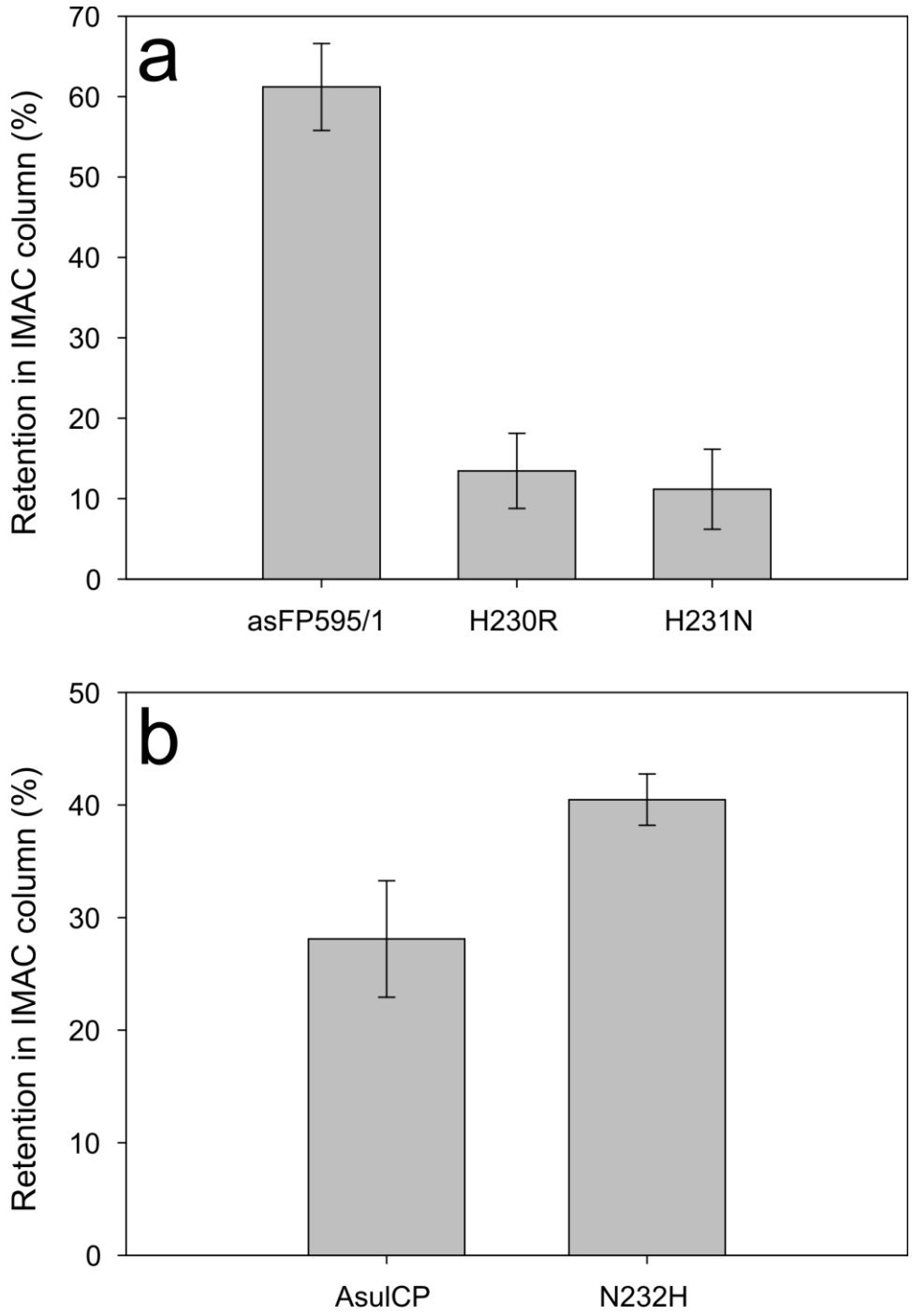

Figure 3.2 – Percentage retention of *Anemonia sulcata* wild type and mutant proteins in IMAC binding assays. The results for asFP595/1 and asulCP proteins are shown in panels (a) and (b), respectively. Percentage retention is the total eluted protein expressed as a fraction of the total protein content where fluorescence is used as a proxy for protein concentration. Error bars indicate the standard deviation of triplicate measurements.

To provide additional confirmation of the role of the C termini histidines in metal binding, the mutation of H232N was introduced in the weakly binding asulCP. The

substitution of the asparagine for histidine resulted in a significant increase the binding of the asulCP mutant, by 30%, compared to the wild type protein (t-test, t = 3.472, DOF = 4, $p<0.05$; Figure 3.2b).

3.4.2. Metal ion mediated reduction of fluorescence

The wild type and mutant asFP595/1 proteins provide a unique opportunity to assess the link between IMAC retention and metal induced loss of fluorescence in anthozoan FPs. The three proteins (wild type, H230R, H231N) were incubated with different $CoCl_2$ concentrations. Cobalt was chosen to enable comparison with the IMAC assays. The fluorescence of the asFP595/1 wild type decreases with increasing cobalt chloride concentration, however, the "non-binding" mutants, H230R and H231N, also show a similar decrease in fluorescence over the cobalt concentration range (Figure 3.3). The gradient of decrease in fluorescence is slightly different in the mutants with a 50% reduction in fluorescence achieved at cobalt chloride concentrations of approximately 1mM, 7mM and 8mM for the wild type, H230R and H231N mutants respectively. Some of the samples, however, contained aggregates/precipitates consistent with previous observations of DsRed incubations with copper (Eli and Chakrabartty 2006).

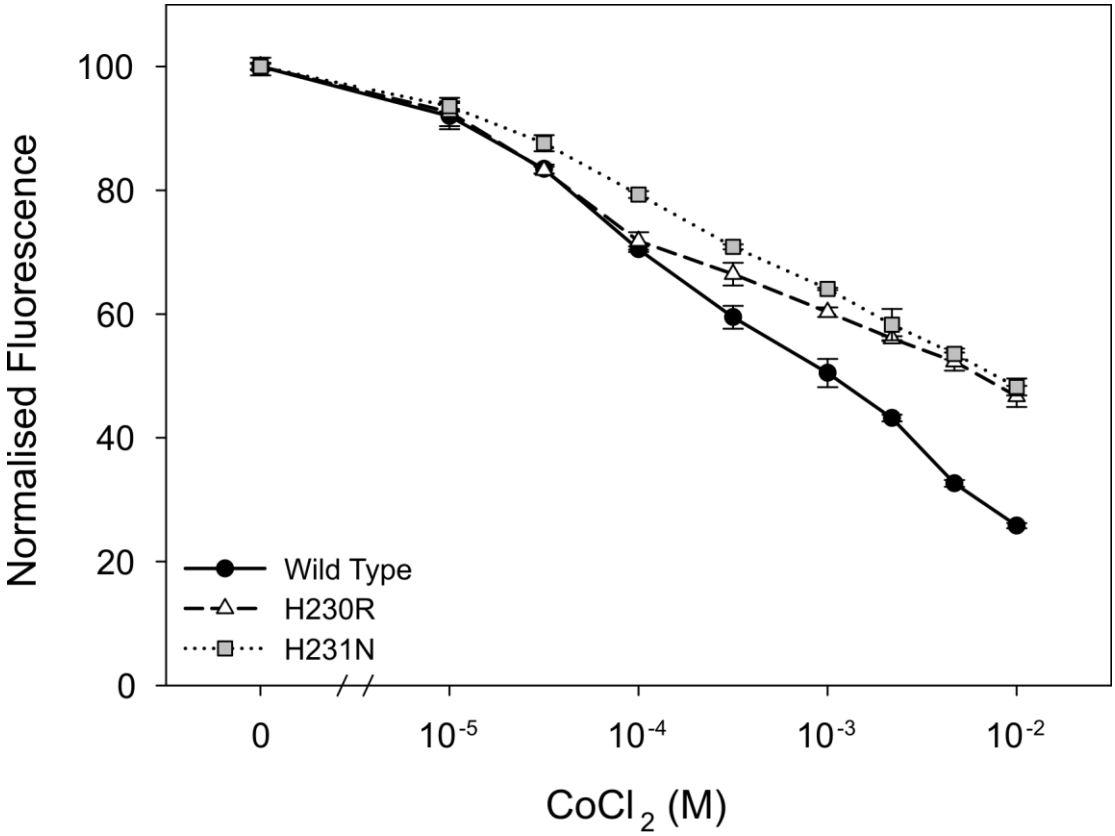

Figure 3.3 – Fluorescence intensity of asFP595/1 proteins in the presence of $CoCl_2$. Fluorescence is normalised to the intensity of the untreated control. Error bars show the standard deviation of triplicate measurements.

3.4.3. Aggregation

Aggregation of the fluorescent proteins may serve to reduce the fluorescence intensity, whereby functional fluorescent chromophores may be shielded from the excitation source by the other FP molecules in the aggregate. To assess the extent of the aggregation amongst the asFP595/1 proteins, the total protein extract (TPE) was divided into a soluble protein fraction (SPF) and an insoluble precipitate by centrifugation. The degree of aggregation was analysed spectroscopically and using SDS-PAGE. The spectroscopic measurements involved treating the samples with EDTA, firstly to assess whether the chromophore fluorescence could be restored and whether the removal of insoluble/aggregated material would affect the fluorescent protein concentration. Figure 3.4 shows the fluorescence recovery in all three proteins upon the addition of EDTA. The fluorescence of the SPF behaves differently amongst the three proteins with different aggregation tendencies observed. The fluorescence in the SPF is reduced by up to 45% (H230R mutant) when compared to the corresponding

EDTA treated TPE whereas the corresponding decrease in fluorescence was 12% in the H231N mutant.

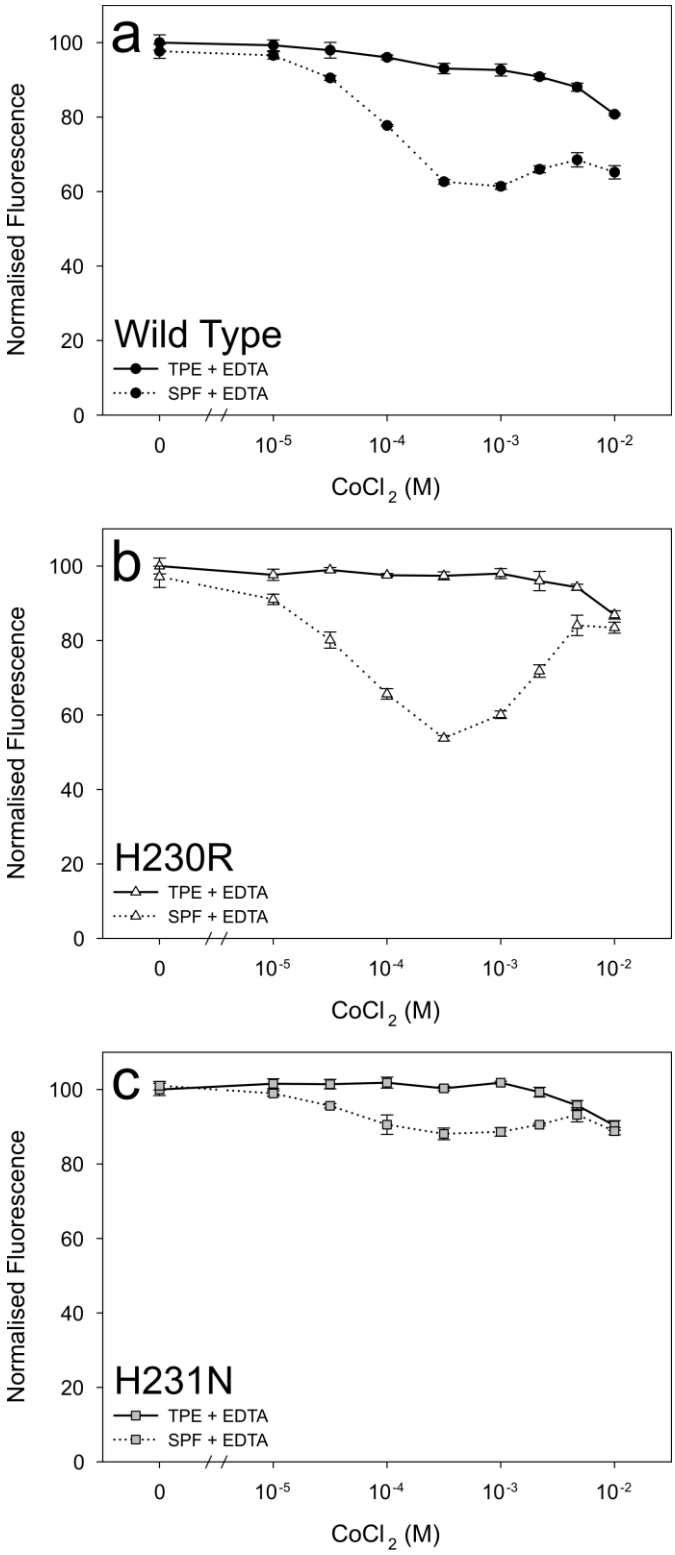

Figure 3.4 – Fluorescence intensity of $CoCl_2$ incubated asFP595/1 proteins upon the addition of EDTA. Solid line denotes the total protein extract (TPE); dashed line indicates the soluble protein fraction (SPF). The responses of the asFP595/1 wild type (A), H230R mutant (B) and H231N mutant (C) are shown respectively. Fluorescence is

normalised to the intensity of the untreated control. Error bars show the standard deviation of triplicate measurements.

The precipitates removed from the SPFs were analysed to confirm the extent to which precipitation was responsible for the differences between the EDTA treated TPEs and SPFs (Figure 3.5). The FP concentrations quantified using band optical densities from polyacrylamide gels were slightly underestimated compared to the quenching analysis. Nevertheless, there was good agreement between the two methods (Pearson's correlation, $r = 0.95$, $p < 0.05$).

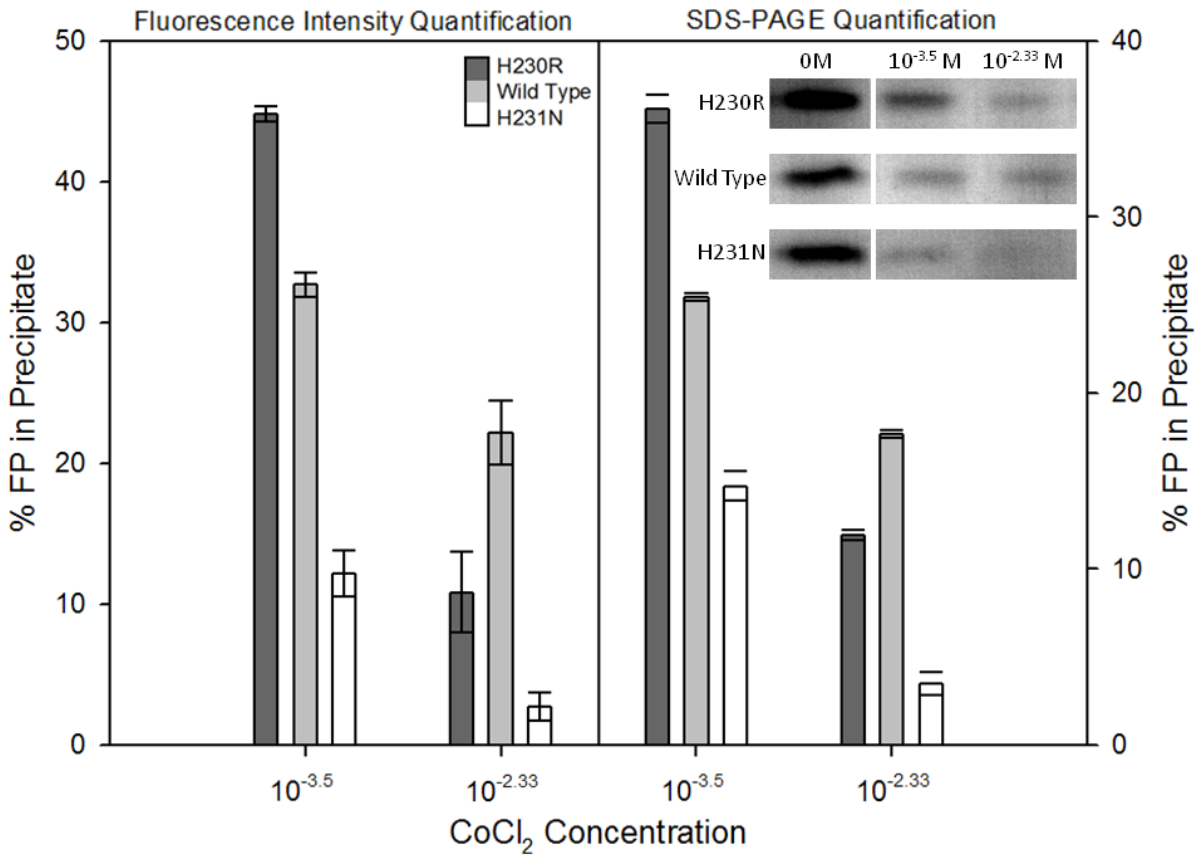

Figure 3.5 – Quantification of FP concentrations from precipitates formed during $CoCl_2$ incubation. Left Panel: Precipitate FP concentration determined from fluorescence measurements of the difference between the EDTA treated TPE and SPF. Error bars show the standard deviation of triplicate measurements. Right Panel: Quantification of precipitate FP concentration from SDS-PAGE analysis. Error bars show the individual duplicate values. Inset; Representative bands for each of the mutants from the polyacrylamide gels used for quantification.

3.5. Discussion

3.5.1. Location of the binding domain

This study set out to test the hypothesis that metal affinity is an inherent property of all GFP-like proteins. The results of the screening assay found that metal binding associated with cobalt ions was not ubiquitous amongst the GFP-like proteins studied and absent from the coral GFP homologs. Nevertheless, despite the screening covering a broad suite of GFP-like proteins from the different clades identified by Alieva and coworkers (2008), the proteins tested in this study were from only four species. In order to test the hypothesis, it was necessary to identify the motif responsible for binding, enabling *in silico* analyses.

In the RFP from *A. sulcata*, asFP595/1, the binding domain responsible for affinity to immobilised cobalt ions has been identified by this study. Screening of a suite of anthozoan FPs and *in silico* analysis of the 3D protein structures enabled a targeted site directed mutagenesis strategy that successfully reduced cobalt affinity by single point substitutions. The two histidines in the C terminal region (H230, H231) of asFP595/1 presumably cooperatively coordinate the cobalt ions resulting in retention of the protein in IMAC column.

The localisation of the cobalt affinity domain in the C terminus was confirmed by performing a reverse mutation on a homologous protein asulCP. This chromoprotein, one of a suite of GFP-like proteins expressed by *A. sulcata* (Wiedenmann et al. 1999; Wiedenmann et al. 2000), shares 88% sequence similarity with asFP595/1, however, it possesses a histidine-asparagine sequence at the end of the C terminus as opposed to the histidine-histidine sequence of asFP595/1. Mutation of the asparagine residue (N232H), increased metal affinity in this protein supporting the identification of the two histidine residue as the site of binding. Two histidine sites have been observed in other proteins binding cobalt (Hsin et al. 2008) and engineered into proteins to form chelating metal binding sites (McGrath et al. 1993). Nevertheless, the presence of other residues in the site cannot be ruled out.

The sequence alignments performed during this study identified a similar double histidine domain in positions 221 and 222 in the C terminus of DsRed, a FP with previously identified metal binding capabilities. The binding of the DsRed homolog, dsFP586, was considerably lower than in asFP595/1 and potentially non-specific. In order to confirm the nature of the binding, mutagenesis of one of the C terminal histidines was performed which did not result in a substantial reduction in binding as

observed for asFP595/1 (Figure S3.3). DsRed has been shown to have an affinity for copper IMAC resins (Rahimi et al. 2007a), however, copper IMAC requires only a single exposed histidine for binding (Sulkowski 1989; Chaga 2001). In contrast, cobalt IMAC requires the presence of at least two adjacent exposed histidines (Chaga 2001), suggesting that although the two histidine motif is present in DsRed, its accessibility limits the binding. Indeed, the C terminus of asFP595/1 is longer than the DsRed C terminus, extending out from the base of the β-can forming a hook like structure (Figure S3.1; Figure S3.2; Wall et al. 2000; Andresen et al. 2005). The asFP595/1 C terminus is typical of other actinarian GFP-like proteins (Nienhaus et al. 2006a; Nienhaus 2008) whereas the C terminus of DsRed is more similar to scleractinian coral termini (Nienhaus et al. 2005) and this probably reflects their different evolutionary histories (Alieva et al. 2008). Furthermore, the His-His motif is located at the end of the C terminus in asFP595/1, in contrast to DsRed, where the His-His site is closer to the β-can. This finding highlights the importance of the location of the two histidines in C terminus for efficient binding. The C terminus double histidine motif identified in this study is also found in other anthozoan FPs: a chromoprotein from the sea anemone *Actinia equina*, aeCP597 (Shkrob et al. 2005), and another protein with high sequence similarity to DsRed, dsFP483 (Matz et al. 1999). Considering structure and binding strengths of asFP595/1 and dsFP586, aeCP597 is more likely to possess cobalt binding capabilities and be suitable for the mutagenesis strategy applied in this study as its two histidines are located at the end of the C terminus. The absence of the metal binding domain from the sequences of the majority of GFP-like proteins available suggests that metal binding associated with this domain is not widespread throughout the GFP-like proteins.

3.5.2. Quenching of fluorescence

The affinity of the metal binding FP, DsRed, has been linked to its quenching behaviour in the presence of copper (Rahimi et al. 2007a). In the case of asFP595/1, quenching behaviour is observed in all three forms of this protein including the mutants with significantly reduced affinity in the IMAC assay. This indicates that quenching and affinity are most likely related to different domains and, in addition, this could hold true for DsRed. The quenching of DsRed is specific to copper with no discernable effect from nickel (Sumner et al. 2006). Nevertheless, when the affinity of DsRed to IMAC resins was tested, a weak affinity for nickel was observed (Rahimi et al. 2007a). The proposition that the fluorescence reduction and IMAC retention derive from different domains would explain this behaviour. It is worthwhile noting that although the quenching occurs in all three asFP595/1 proteins studied (wild-type, H230R, H231N), the concentrations required to achieve 50% quenching are slightly different amongst

the proteins; these differences may result from altered access to the quenching domain/chromophore associated with the mutations. These results are in good agreement with the findings of previous work assessing the relationship between affinity and quenching in introduced histidines on the green fluorescent protein mutant, GFPuv (Tansila et al. 2008). It was observed that affinity is dictated by the surface accessibility of the histidines whereas the distance from the chromophore is the determining factor in controlling the degree of quenching. It was concluded that although the N146H mutant was the most metal accessible mutant, its distance from the chromophore, 10.15Å, meant that the interacting metal ions would not be close enough for efficient quenching to occur (Tansila et al. 2008). Considering that the C terminus affinity site identified in the study is ~25Å from the chromophore, it is unlikely that loss of fluorescence is caused by a direct interaction between the metal ions at this site and the chromophore.

The use of metal induced quenching in GFP-like proteins as a biomarker requires specificity of the quenching to a particular metal ion. While other studies on metal binding in naturally occurring GFP-like proteins have focused on copper as the quencher, cobalt was used in this study to maintain consistency with the IMAC assay and enable inferences with regard to the binding site. Nevertheless, asFP595/1, like DsRed and HcRed (Eli and Chakrabartty 2006; Sumner et al. 2006; Rahimi et al. 2007b), is also quenched by copper (II) ions (Figure S3.4). The results presented here show that cobalt induces quenching at higher concentrations and over a larger concentration range than copper presumably due to a lower affinity for cobalt ions compared to copper ions. It is interesting to note that although regarded as specific to copper, quenching of DsRed fluorescence has only been tested at a concentrations of 1μM (Sumner et al. 2006) and 10μM (Eli and Chakrabartty 2006) where the low levels of quenching occurring at these concentrations in DsRed are similar to those found in asFP595/1. Depending on the application, the use of DsRed as a copper sensor may require further testing at different cobalt concentrations if the samples are likely to contain cobalt concentrations exceeding 10μM.

3.5.3. Significance of metal interactions to the use of RFPs as molecular markers and biosensors

3.5.3.1.Scavenging of metal ions

As the use of FPs as biomarkers relies on the assumption that they behave in an inert manner (Chalfie et al. 1994), the interaction of certain FPs and metals may have consequences for their use *in vivo*. The concentrations of cellular cobalt are

prohibitively low to have a substantial effect on asFP595/1 fluorescence intensity and therefore it could not be used as a cobalt sensor *in vivo*. In contrast, the higher affinity for copper of asFP595/1 and DsRed, and high local copper concentrations, for example, up to ~300μM in synaptic vesicles (Hopt et al. 2003), could result in metal mediated effects on fluorescence where sensor applications could be developed. Nevertheless, the impact of the relationship between metal ions and FPs must not only be considered from the point of view of the cellular environments effect on the FP but also on the impact of the FP on the cell's trace metal chemistry. An over expressed metal binding fluorescent protein such as asFP595/1 or DsRed could efficiently scavenge certain metal ions, inhibiting fundamental pathways and potentially compromising the cell's vitality. For this reason, it is not only important to identify proteins with similar functionality but elucidate a strategy to neutralise the metal binding. The site directed mutagenesis strategy used in this study may be transferable to other proteins to reduce metal binding, as shown for asFP595/1.

3.5.3.2.Aggregation

Aggregation may have an impact on the potential of RFPs to serve as *in vivo*/*vitro* biosensors and *in vivo* molecular markers due to their renowned aggregation tendencies. Indeed, there are numerous reports of aggregation of DsRed *in vivo* (Jakobs et al. 2000; Mizuno et al. 2001; Wiedenmann et al. 2009) and during quenching analyses (Eli and Chakrabartty 2006). This study found that at concentrations relevant for assessing quenching behaviour in asFP595/1, aggregation occurs heterogeneously across the concentration gradient. The cellular concentration of cobalt is unlikely to induce aggregation as it is prohibitively low, however, reported *in vivo* copper concentrations (Kardos et al. 1989; Hopt et al. 2003) are comparable to aggregation inducing concentrations determined *in vitro* (Figure S3.4). The aggregation would also be dependent on the FP concentration and this would depend on the concentration of the tagged protein but micromolar concentrations of tagged proteins have been recorded (Hack et al. 2000). Further work is needed to explore the metal induced aggregation of these proteins both *in vitro* and *in vivo*. Of particular interest is the non-linearity in the relationship with concentration. It does not appear to follow a pattern consistent with salting-out and therefore the reason for the relationship between aggregation and concentration is unclear.

This could have implications for biosensing as it may reduce ion accessibility to the quenching domain, reduce fluorescence due to the shielding of functional chromophores by other proteins in the aggregate or exert toxicity on the host cell in *in vivo* sensing (Yanushevich et al. 2002). In DsRed, a difference between the dissociation

constant (K_d) for copper of the tetrameric DsRed express (K_d = 5.4μM) and the monomeric DsRed monomer (K_d = 1.7μM) was observed (Rahimi et al. 2008); Higher order aggregates could act to increase the K_d. The aggregation tendency was not uniform amongst the generated mutants. In fact, the H231N mutant showed over 60% less aggregation compared to the wild type (according to spectroscopic measurements). This provides a promising mutagenesis strategy for metal binding FPs to reduce their aggregation tendencies. Previous mutagenesis strategies to reduce aggregation in FPs have focused on the N terminus (Yanushevich et al. 2002), however, the C terminus is also located on the exterior of the tetramer and could play a similar role in inter-tetrameric interactions.

3.5.4. Biological Significance

The screening of 11 GFP-like proteins showed that the metal binding observed in the *A. sulcata* proteins is absent in the coral GFP-like proteins. Furthermore, having characterised the binding motif responsible for the chelation to immobilised cobalt ions, *in silico* screening found that the C terminus histidine-histidine motif is not a common feature of GFP-like proteins. Based on these findings, it is concluded that metal binding by the site observed in this study is unlikely to represent a widespread biological function amongst GFP-like proteins, particularly coral GFP homologs.

The binding motif identified in this study does appear to have minor effects on the metal induced quenching of the protein's fluorescence but it does not appear to be responsible for this phenomenon. Therefore, other metal interactions may be occurring elsewhere in the protein. In terms of the biological function of these interactions, the role of metal binding by certain members of these proteins remains unclear. This study identifies changes in the properties of the protein associated with binding which could be indicative of changes in the protein structure associated with binding. Whether this reflects activation of a functional form of the protein remains to be tested.

3.5.5. Conclusions

3.5.5.1.Biological function

The findings of this study do not support a biological function of metal binding by GFP-like proteins in corals. Nevertheless, it highlights some interesting aspects of GFP-like protein and anthozoan biology. Firstly, this study provides the first identification of a domain on a GFP-like protein and highlights the need to investigate aspects of the protein, other than the chromophore, when exploring GFP-like protein functionality.

Secondly, the quenching of fluorescence has implications for the use of fluorescence proteins as biomarkers. The use of these proteins as biomarkers for reef health assumes that the pigmentation is related to the protein concentration, however, if other factors such as metals can change this behaviour, the results may be unreliable without understanding the full suite of potential quenchers. Lastly, although it does not appear to have a biological function in the scleractinians, the presence of the domain in the actinarians warrants further study. Although closely related, the presence of the domain only in certain actinarian proteins could represent different strategies between the two organisms, potentially in their mechanisms for detoxification of heavy metals. In the face of increasing pollution on reefs worldwide (Hughes et al. 2003; Fabricius 2005), understanding the physiological responses used by different organisms is important for predictions of future reef health and metal accumulation by actinarian RFPs may represent one possible detoxification strategy.

3.5.5.2.Applications of GFP-like proteins

This study provides the first identification and characterisation of a metal binding domain in a GFP-like protein. The knowledge of this domain will enable the development of new markers that can potentially be used to study diseases related to metal homeostasis such as Alzheimer's disease. In addition, the mutagenesis strategy employed in this study generated a RFP mutant with reduced aggregation tendencies. As aggregation is a common problem with RFPs *in vivo*, a similar mutagenesis strategy could vastly improve RFPs enabling improved localisation of marker molecules.

3.6. Supplementary figures

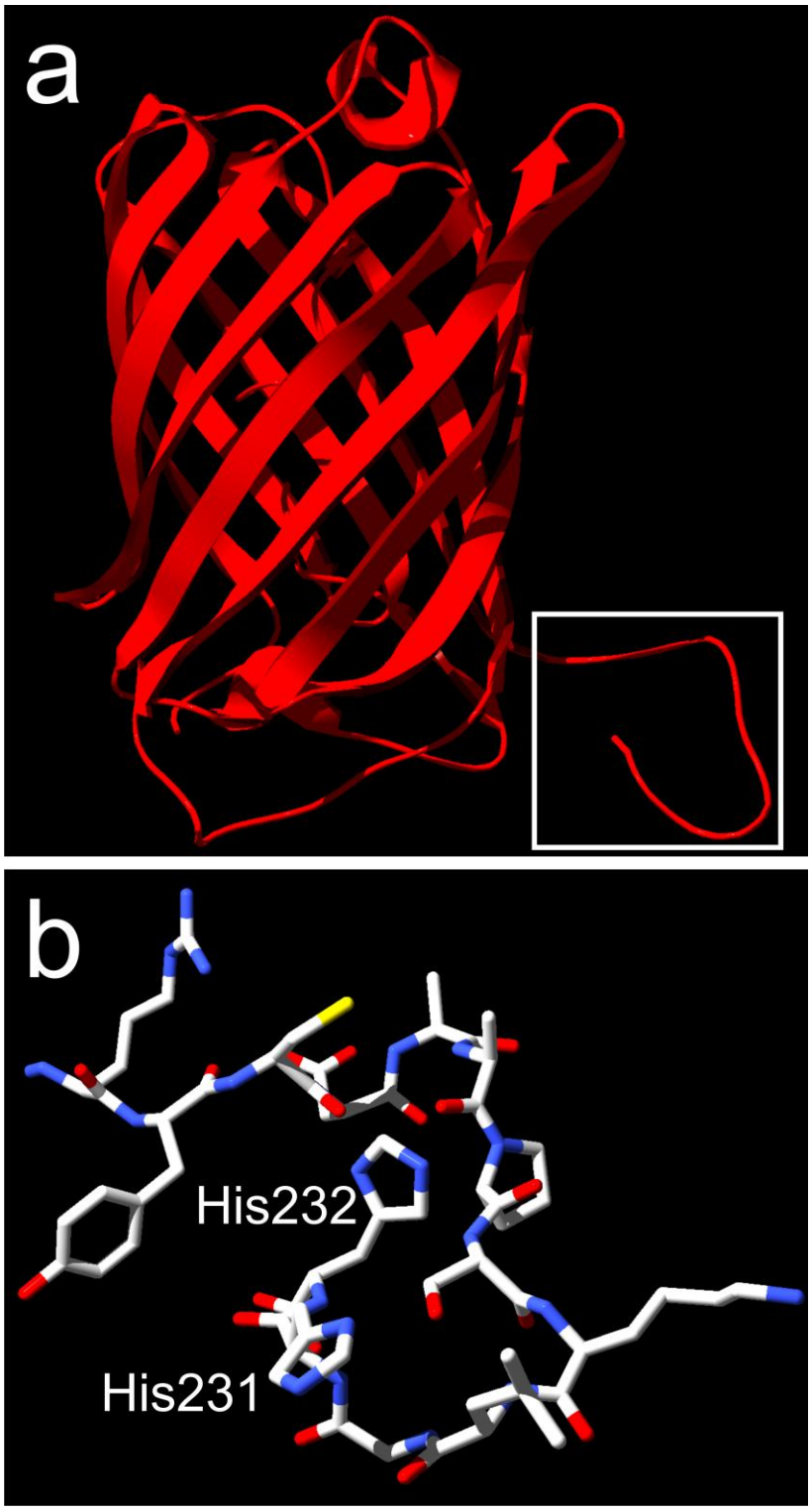

Figure S3.1 – Three dimensional structure of asFP595 (PDB: 2A50; Andresen et al., 2005), a protein homologous to asFP595/1. (a) Ribbon diagram showing an asFP595 monomer. The white box highlights the C terminus. (b) Ball and stick model of the C terminus. The C terminus of asFP595 was mutated *in silico* (N232R) to demonstrate the C terminus and binding motif found in asFP595/1.

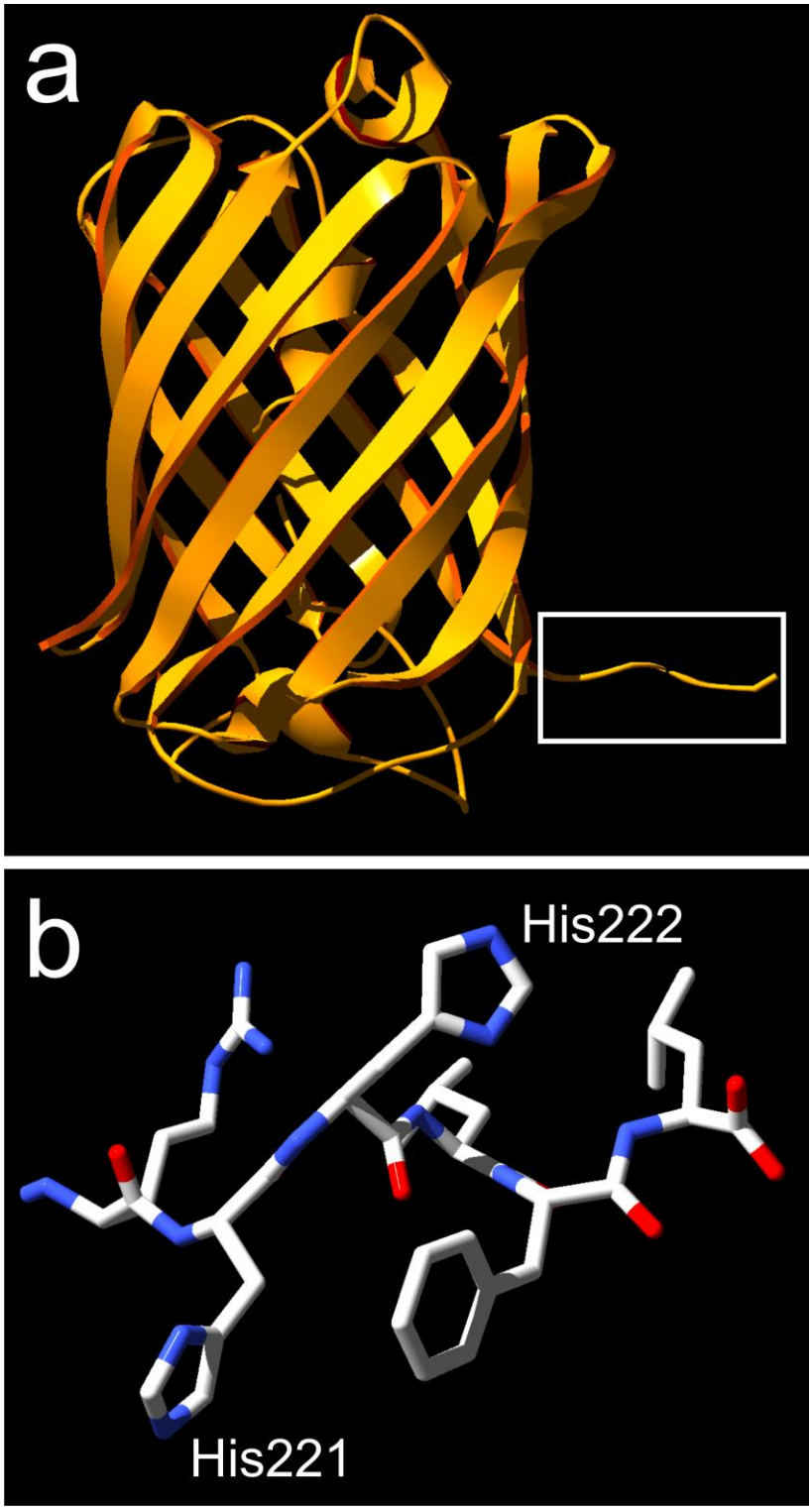

Figure S3.2 – 3D structure of DsRed (PDB: 1GGX; Wall *et al.*, 2000). (a) Ribbon diagram of a DsRed monomer with the C terminus highlighted by the white box. (b) Ball and stick model showing the His-His motif in the C terminus of DsRed.

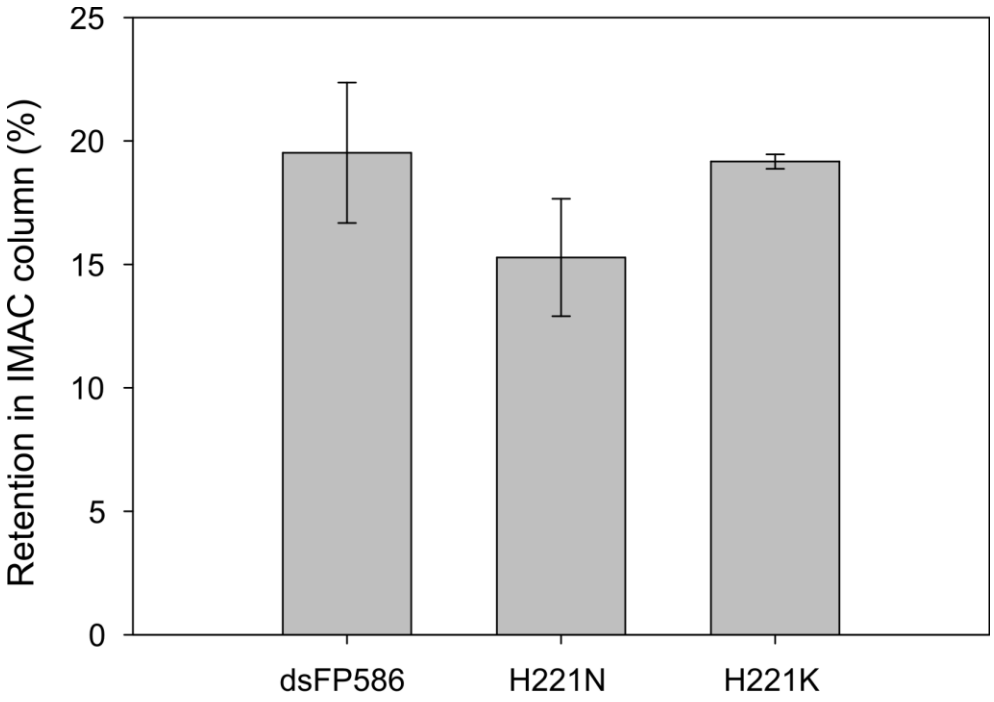

Figure S3.3 – Retention of dsFP586 and its C terminus mutants in the IMAC binding assay. Error bars indicate the standard deviation of triplicate measurements.

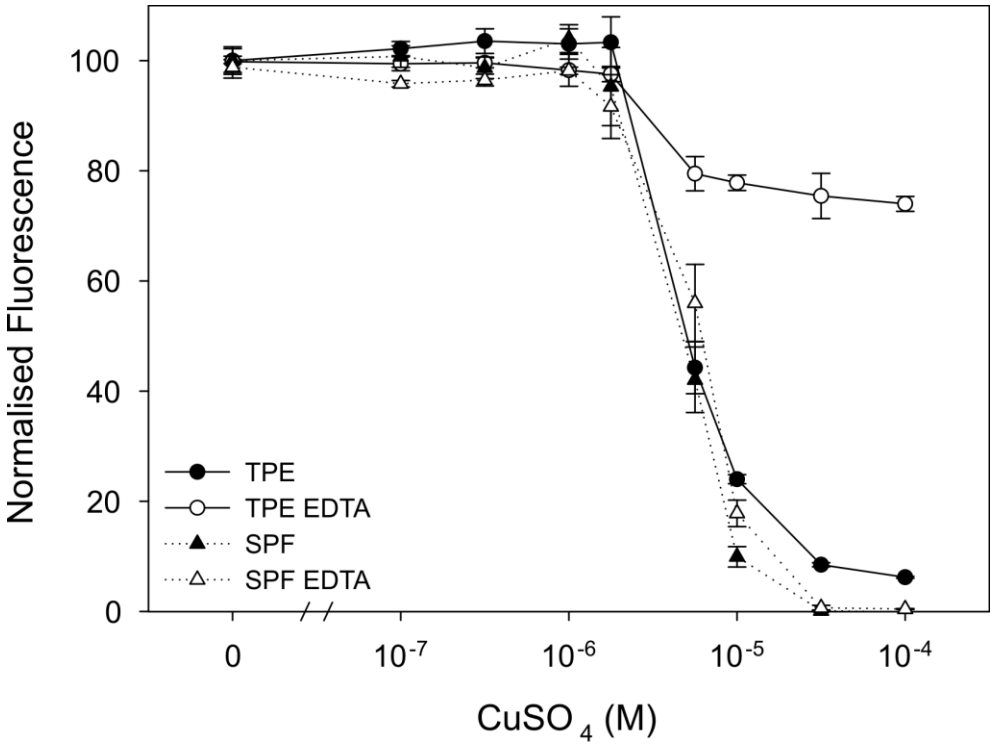

Figure S3.4 – Fluorescence intensity of asFP595/1 in the presence of copper sulphate. The untreated and EDTA treated TPE (circles) and SPF (triangles) are show by filled and empty shapes respectively. Error bars show the standard deviation of triplicate measurements.

CHAPTER 4. Screening by coral GFP-like chromoproteins supports a role in photoprotection of the zooxanthellae.

4.1. Abstract

Green fluorescent protein (GFP)-like pigments are responsible for the vivid colouration of many reef building corals and have been proposed to act as photoprotectants. Their role remains controversial, however, as the functional mechanism has not been experimentally elucidated. Here, this study provides direct evidence to support a photoprotective role of the non-fluorescent chromoproteins (CPs) that form a biochemically and photophysically distinct group of GFP-like proteins. Motivated by observations of *Acropora nobilis* from the Great Barrier Reef, the photoprotective role of CPs was explored by analysing five species of corals under controlled laboratory conditions. *In vitro* and *in hospite* analyses of chlorophyll excitation demonstrate that screening by CPs leads to a reduction in the excitation of chlorophyll corresponding to the spectral properties of the specific CPs present in the coral tissues. In the range from 562nm to 586nm, at which the CPs show their maximal absorption, the analyses revealed a reduction of chlorophyll excitation by up to 50%. The observed screening behaviour was consistent for established and regenerating tissue and amongst symbiont clades A, C and D. Moreover, it was found that in two differently pigmented morphs of *Acropora valida* grown under identical light conditions and hosting subclade type C3 symbionts, high CP expression correlated with reduced photodamage under acute light stress. It is proposed that CPs represent a host-based strategy to help minimise light stress in conditions where existing photoprotective mechanisms may be overwhelmed.

4.2. Introduction

Corals inhabit a range of light environments spanning two orders of magnitude of irradiance from the shallow waters to the deeper mesophotic reefs (Lesser et al. 2010). Consequently, corals and their symbionts need mechanisms for acclimation and adaption to different irradiances, chromaticity and temporal variability in the light field. Even at the scale of a single shallow water colony, there are large variations in light exposure of the coral's tissues controlled by factors such as seasonal and diel changes in surface irradiance, changes in the absorbing and scattering materials in the

water column, orientation of the tissue surface relative to the incident irradiance and wave focusing effects (Falkowski et al. 1990; Veal et al. 2010; Kaniewska et al. 2011). The shallow water environments where reef building corals thrive are typified by irradiances often exceeding photon fluxes of ~300-750µmol m^{-2} s^{-1} that have been shown to saturate photosynthesis of shallow water corals' algal symbionts *in hospite* (Gorbunov et al. 2001; Levy et al. 2004; Hennige et al. 2008). As such, some shallow water corals can be considered to live in an environment where light is in excess. In addition, other factors, such as temperature stress, may act to reduce the photosynthetic capacity of the zooxanthellae thus increasing the excess of excitation energy (Iglesias-Prieto et al. 1992; Warner et al. 1996; Smith et al. 2005). Exceeding the capacity of the photosynthetic machinery of the algal symbionts can result in photoinhibition and the production of reactive oxygen species (ROS) (Smith et al. 2005) that can cause damage to a variety of cellular components including lipids, proteins and DNA (Lesser 2006). Consequently, both the coral and the symbionts possess mechanisms to reduce light stress. In fact, light stress in combination with elevated water temperatures is considered a fundamental cause of coral bleaching due to the increased production of ROS and/or inhibition of PSII repair mechanisms (Takahashi et al. 2004; Tchernov et al. 2004; Smith et al. 2005; Lesser 2006). Furthermore, light is a key driver of nutrient-stress mediated coral bleaching (Wiedenmann et al. 2012b).

Corals' endosymbiotic algae optimise their light absorption and usage in response to the incident irradiance. The mechanisms employed include long term acclimation strategies such as changes to the size and/or number of photosynthetic units (PSUs) (Falkowski and Dubinsky 1981; Hennige et al. 2009) as well as acclimatory mechanisms designed to address the supraoptimal irradiances brought about by short term changes such as diurnal variability. The short term responses employed include down regulation of the PSII reaction centres (Gorbunov et al. 2001), xanthophyll conversion (Brown et al. 1999), cyclic electron transport (McCabe Reynolds et al. 2008) and dissociation of antenna complexes from PSII (McCabe Reynolds et al. 2008). The degree with which different acclimatory mechanisms are employed, and their relative contribution to photoprotection, appears to be, at least in part, associated with the genetic identity of the symbiont clade and hence, there is evidence that some symbiont clades/subclades are better suited to certain environments than others (Iglesias-Prieto et al. 2004; McCabe Reynolds et al. 2008). Nevertheless, in spite of extensive protection afforded by non-photochemical quenching, photodamage to PSII has been shown to occur in shallow water corals on cloud-free days (Gorbunov et al. 2001) and under thermal stress (Warner et al. 1999).

While some host-based responses to light stress are the same as strategies employed by the symbiont, such as the production of MAAs and antioxidant enzymes, others remain unique to the host. A photoprotective function has been proposed for the host-based family of green fluorescent protein (GFP)-like pigments that are frequently expressed by anthozoans (Salih et al. 1998; Wiedenmann et al. 1999; Salih et al. 2000; Dove et al. 2001). The host pigments of corals and other anthozoans can be divided into two major groups: the fluorescent proteins (FPs), responsible for cyan to red hues and the non-fluorescent chromoproteins (CPs; synonym: pocilloporins), that produce the intense pink, purple and blue pigmentation in corals (Dove et al. 2001; Salih et al. 2006; Alieva et al. 2008; D'Angelo et al. 2008). The CPs and FPs are thought to protect the symbionts from high irradiances by absorbing photons or distributing them away from the algal pigments by re-emission or reflectance (Salih et al. 1998; Wiedenmann et al. 1999; Salih et al. 2000; Dove et al. 2001; Salih et al. 2006). It is proposed that these pigments act through screening (Salih et al. 2000), a process whereby the light received by the photosynthetic pigments has previously passed through a layer or compartment containing the photoprotective pigments (Merzlyak et al. 2008). As such, screening is distinct from other photoprotective systems where the photoprotectants and the photosynthetic pigments directly compete for light absorption (Merzlyak et al. 2008).

The high abundance of fluorescent colour morphs in shallow water, the resistance of their zooxanthellae against photoinhibition, the stability and slow turnover of the GFP-like pigments and their light-regulated expression support a photoprotective function (Wiedenmann et al. 1999; Salih et al. 2000; Leutenegger et al. 2007b; D'Angelo et al. 2008). Nevertheless, the exact mechanism through which GFP-like proteins function is controversially discussed as no reduction due to GFPs absorption was evident in the chlorophyll excitation spectrum of zooxanthellae in Caribbean corals (Mazel et al. 2003b). Also, the high-level expression of GFP-like proteins in growth zones of corals that are free from zooxanthellae and in several azooxanthellate anthozoans from habitats with low light stress, including the deep sea, sheds doubts whether all of these pigments fulfil a photoprotective function (Wiedenmann et al. 2004a; D'Angelo et al. 2008; Vogt et al. 2008; D'Angelo et al. 2012). In addition to the questions over the photoprotective role of the FPs, the direct effects of CPs on symbiont photosynthesis remain unclear as the peaks of CP absorption are outside the major absorption bands of the photosynthetic pigments (D'Angelo et al. 2008; Dove et al. 2008). The aforementioned observations together with the results of molecular phylogenetic analyses of the protein sequences suggest that distinct groups of GFP-like proteins could fulfil various functions among different taxonomic groups (Alieva et al. 2008; D'Angelo et al. 2008).

An improved knowledge of the function of this important family of host pigments is required to determine their potential in protecting reef corals from the increased environmental perturbations predicted for the next centuries (Hughes et al. 2003). GFP-like proteins possess features that make them promising biomarkers of coral health (D'Angelo et al. 2008; D'Angelo et al. 2012). Yet, their value as intrinsic indicators of environmental stress could be further increased by an improved understanding of their potential functions.

Blue colour morphs of *Acropora nobilis* with notably higher zooxanthellae and photosynthetic pigment concentrations than their brown counterparts growing adjacently were observed in a shallow water reef setting at Heron Island (Electronic Supplemental Material, ESM Fig. S4.1). The increased density and pigment content of zooxanthellae could be interpreted as a shade acclimation due to screening effects by the blue chromoprotein which represents the dominant host pigment in these individuals. This observation was the motivation for the present in-depth study into the role of CPs in the photobiology of corals. This study set out to test the hypothesis that corals express CPs to reduce light stress on their algal symbionts. In testing this hypothesis, the following questions are addressed Firstly, do CPs screen certain wavelengths of visible light that would otherwise be absorbed by the photosynthetic pigments of the zooxanthellae? Secondly, are the symbionts screened by CPs less susceptible to high irradiances in the relevant spectral range? Lastly, is screening relevant to growth zones where symbiont densities are low?

4.3. Methods

4.3.1. Coral samples

Experimental corals were cultured and propagated by fragmentation in the experimental mesocosm of the Coral Reef Laboratory at the National Oceanography Centre Southampton. Briefly, corals were grown under 12hrs light:dark cycle provided by 250W metal halide lamps with burners producing a high proportion of blue light (ESM Fig. S4.2; Aqualine 10000 (13000K), Aqua Medic, Germany), with the temperature kept at 24˚C (+/-0.5ºC) during the course of the experiments. The full details of the system are described elsewhere (D'Angelo and Wiedenmann 2011). Five corals were selected based on their CP expression: *Acropora valida*, *Acropora polystoma*, *Acropora millepora*, *Seriatopora hystrix* and *Montipora foliosa*. *S. hystrix* (D'Angelo et al. 2008) and *A. polystoma* (D'Angelo et al. 2012; Wiedenmann et al. 2012b) of unconfirmed geographical origin were purchased from the ornamental trade. The other species were

collected from Fijian reefs and were acquired through the Tropical Marine Centre (London, UK) (D'Angelo et al. 2012). Corals were acclimated to tank conditions for at least 18 months prior to the experiments. CP pigmented morphs of *A. valida*, *A. millepora* and *S. hystrix* were analysed for comparison of the optical properties of light exposed and shaded tissue. *A. valida* colonies were also used to compare photophysiological responses to light stress between two colour morphs (brown and purple). *M. foliosa* and *A. polystoma* were incorporated into the study to explore the effects of localised CP expression in growth zones.

Fragments of *A. valida*, *A. millepora* and *S. hystrix* were grown perpendicular to the incident irradiance for >1 month to enable acclimation to the light field. As CPs are upregulated in response to light, they are essentially absent in corals exposed to low light, but their tissue content increases in proportion to photon flux densities beyond 100µmol m^{-2} s^{-1} (D'Angelo et al. 2008). As a consequence, coral branches grown at an orthogonal angle to the incident light, show high expression of CPs in their upper side, whereas the underside is essentially free from these pigments enabling comparison of CP pigmented and non-CP pigmented tissue. Measurements with a Li-Cor LI-250A indicated that the upper sides of the coral branches were exposed to photon fluxes of ~200µmol m^{-2} s^{-1}. The reflected light, from the bottom of the tank, reaching the underside of the branches was ~10µmol m^{-2} s^{-1}.

4.3.2. Determination of symbiont density and phylotype

Coral tissue was removed from the skeleton using a scalpel. The branch was subsequently photographed (Canon G9) and the area sampled was determined by image analysis (ImageJ). Coral tissue was homogenised by osmotic disruption in MilliQ water and vortexing. Zooxanthellae were pelleted by centrifugation (500 x g for 5 min). After separation from zooxanthellae, a clarified protein extract was obtained from the supernatant by centrifugation at 20,000 x g for 1 h.

The zooxanthellae pellet was washed in filtered seawater by three repeated centrifugation-resuspension cycles at 500 x g for 5min. Zooxanthellae were quantified in a haemocytometer using a fluorescence microscope. Restriction fragment length polymorphism (RFLP) analyses of the amplified small subunit ribosomal DNA (SSU rDNA) revealed clade C as the dominant zooxanthellae clade in the purple and brown colour morphs of *A. valida* used for the light stress experiment. Analyses of the PCR amplified internal transcribed spacer region 2 (ITS2) (LaJeunesse 2002) identified the dominant zooxanthellae population in both morphs to subclade C3. The amplified fragments analysed in this study were cloned using the Strataclone PCR Cloning Kit

(Agilent Technologies) and three clones from each colour morph were sequenced (Macrogen) (Genbank Accession: KC405646-KC405651).

4.3.3. Characterization of CPs

In order to characterise the spectral properties of the CPs encountered in this study, independent tissue samples were taken from the respective corals and homogenised as outlined above. The clarified tissue extracts were subjected to isopropanol precipitation as previously described (Wiedenmann et al. 2000) and further purified by size exclusion chromatography, where required (Wiedenmann et al. 2002). The CP spectra were measured on a Cary absorption spectrophotometer (Varian, USA). Three CPs from *A. millepora* (Genbank Accession: KC349891, KC411499, KC411500; D'Angelo et al. 2008) and a CP from *Anemonia sulcata* (Genbank Accession: AF322222) were cloned as described (Wiedenmann et al. 2000; D'Angelo et al. 2008), expressed and purified according to published protocols (Wiedenmann et al. 2002).

4.3.4. Protein quantification

Tissue concentrations of avalCP580 and apolCP582 were required to guide the *in vitro* experiments and to compare between high light and low light growth zones, respectively. In order to enable quantification of avalCP580 and apolCP582, the molar extinction coefficients of these CPs were determined using the alkaline denaturation method (Kredel et al. 2008). As the amino acid sequence for these CPs were unknown, the molecular weight of CPs was estimated by averaging the molecular weights of five acroporiid CPs [GenBank: AAU06853.1, AAU06856.1, AAU06855.1, AAU06854.1, ACH089425.1 (Alieva et al. 2008; D'Angelo et al. 2008)] calculated with Lasergene's Protean software (DNASTAR, USA). Considering the low standard deviation (±0.23%) of the individual molecular weights from the mean (25 kDa), the estimates can be expected to be representative.

The CP concentration in clarified protein extracts from the purple morph of *A. valida,* and from the growth zones of *A. polystoma* under two light conditions was quantified by spectral unmixing using the method of least squares (Settle and Drake 1993; Hedley et al. 2004). Although spectral unmixing is usually performed on reflectance, this study uses absorption as according to Beer's Law, the absorption of a mixture can be considered as the additive sum of the weighted absorption coefficients of the components within the mixture (Kirk 1994). The endmember spectra used in the two-endmember unmixings were the purified CP spectrum and the spectrum of a clarified protein extract taken from an independent tissue sample from the respective species

(*A. valida* – brown morph; *A. polystoma* – low light). All spectral unmixings were performed on clarified tissue extracts with absorption readings less than 1.0 to ensure linearity. CP concentrations were calculated using the extinction coefficients and were normalised to the total soluble protein concentrations (BCA assay, Pierce).

4.3.5. Reflectance measurements

Reflectance spectra were recorded according to Mazel and co-workers (2003b) using a fibre optic reflectance probe connected to an Ocean Optics USB4000 spectrometer and a tungsten halogen HL-2000-HP light source (Ocean Optics, USA) using Spectralon 99% diffuse reflectance standard as a reference (Labsphere, USA). These measurements were performed on *S. hystrix*, *M. foliosa* and *A. polystoma*. For all reflectance measurements, five independent measurements were taken from different locations on the colony. Reflectance measurements (R) were used to estimate absorbance (D_e) using the formula $D_e = \log(1/R)$ (Enriquez et al. 2005; Rodríguez-Román et al. 2006). This method has been shown to give comparable results to absorbance measurements performed on coral laminae using the opal glass technique (Enriquez et al. 2005).

4.3.6. Fluorescence spectroscopy

Chlorophyll excitation spectra were measured as the fluorescence excitation of PSII (680nm) emission. The fluorescence spectra were performed on all species using a Varian Cary Eclipse fluorescence spectrophotometer with a fibre optic attachment as described previously (Leutenegger et al. 2007b; D'Angelo et al. 2008). The excitation spectrum of PSII emission at 680nm was measured from 400-620nm with excitation and emission slits widths of 10nm and 5nm, respectively. Initial fluorescence excitation and emission measurements were conducted on all studied species to ensure that emission at 680nm was solely due to chlorophyll emission (ESM Fig. S4.3) with no interference from other fluorophores such as red fluorescent proteins. For comparison of coral tissues with different pigmentation, the chlorophyll excitation spectra were normalised to the 460nm peak under the assumption that excitation at 460nm is unaffected by host pigments (Mazel et al. 2003b).

Excitation difference spectra were calculated to explore the spectral nature of the pigments driving the observed changes in chlorophyll excitation spectra. Here, the chlorophyll excitation of CP pigmented tissue was subtracted from the non-CP pigmented tissue and difference was normalised to the excitation at that wavelength of the brown morph. As an example, an excitation difference value of 0.5 would represent 50% lower excitation of chlorophyll in the CP pigmented tissue compared to

the non-CP pigmented tissue at that specific wavelength. The excitation difference spectra peaks were consistent across the chlorophyll emission spectrum (ESM Fig. S4.3c).

4.3.7. *In vitro* CP screening experiment

An *in vitro* experiment was performed to assess the potential for CPs to provide screening for zooxanthellae in the absence of other potentially confounding variables. Symbionts were extracted from the tissues of *Anemonia sulcata* (clade A) (Visram et al. 2006) and two different colonies of *A. valida* (clade C and D) according to the aforementioned protocol and were resuspended in sterile filtered seawater. Samples of each freshly isolated symbiont clade were loaded into different wells in an 8-well culture chamber and allowed to settle for 15 minutes. Two millimetre-deep chambers containing different CPs, and a PBS control, were placed between the settled symbionts and the fibre optic probe of the fluorescence spectrometer. The chlorophyll excitation spectra were subsequently recorded. The measurement for each CP/PBS filter was measured on the same population of the respective clade to ensure that measurements enabled comparisons of identical populations with the same physiology and photobiology. The zooxanthellae concentrations (1.0×10^6 cells per cm^{-2} of the bottom area of the wells) used in this experiment are comparable to the symbiont density of the blue morphs of *Acropora nobilis* at ~1.5m at Heron Island (ESM Fig. S4.1). The CPs selected for analysis were asulCP562, amilCP580 and amilCP601 to enable demonstration of the impact of CPs over their range of absorption peaks. The concentrations of each CP filter were calculated to provide the same peak absorbance as the areal tissue concentrations of CP found in a purple morph of *A. valida* (0.09mg cm^{-2})

4.3.8. Light stress experiment

Brown and purple colour morphs of *A. valida*, both hosting subclade C3 symbionts and grown under 200µmol m^{-2} s^{-1} on the light exposed sides, were exposed to an additional 1000 µmol m^{-2} s^{-1} of orange light (peak spectral irradiance = 595nm; ESM Fig. S4.2) provided by an array of LEDs (P4 Amber Star, Seoul Semiconductor, Korea) for 2 days with a 12:12hrs light:dark cycle. Chlorophyll fluorescence parameters were recorded using a Diving PAM according to previous recommendations (Warner et al. 2010) including exposure to dim light (<5µmol m^{-2} s^{-1}) prior to and during measurements taken after 12 hour dark acclimation to minimise the impacts of chlororespiration. The maximum quantum efficiency of photosystem II (Fv/Fm) was recorded prior to the experiment and at the end of second dark cycle. The effective quantum yield (ΔF'/Fm')

was measured at the end of the second light cycle. Due to the slow relaxation time of chronic photoinhibition (Gorbunov et al. 2001) and the short duration of the experiment, any reduction in Fv/Fm from the start of the experiment to the end of each dark cycle should indicate damage to PSII.

4.3.9. Assessment of CP expression in growth zones

A. polystoma and *M. foliosa* were used to assess the effects of CPs in growth zones as CP expression is localised to these regions in these species. Approximately 1 cm^2 of tissue was removed from replicate colonies of *A. polystoma* to enable assessment of CP expression in regenerating tissue. Half of the colonies were moved to low light conditions (~60µmol m^{-2} s^{-1}) whereas others were left under higher light intensities (~200µmol m^{-2} s^{-1}). After 10 days, spectroscopic characterisations of the regenerating areas were made and tissue samples were taken for analysis of proteins and zooxanthellae content.

Replicate colonies of two colour morphs of *M. foliosa* were grown under ~150µmol m^{-2} s^{-1}. The purple morph expresses the CP, mfolCP577, exclusively in the growth margins of the colony whereas the CP expression is essentially absent from the brown morph. Spectroscopic measurements were taken on both the grown margins and central regions of the colonies.

4.4. Results

4.4.1. Spectral properties of the CPs

The CPs isolated from the studied coral species had absorption peaks ranging from 562-586nm (ESM Fig. S4.4). The pink appearance of *S. hystrix* is due to the accumulation of shysCP562 with an absorption maximum at the 562nm (D'Angelo et al. 2008), the shortest wavelength of the studied set of CP pigments. The *M. foliosa* and *A. valida* colonies contain CPs with peak absorptions at 577nm and 580nm, respectively, (herein referred to as mfolCP577 and avalCP580). Consequently, the pigmented regions of the corals appear purple-violet. The three CPs cloned from *A. millepora* (amilCP580, amilCP586 and amilCP601) had absorption maxima at 580nm, 586nm and 601nm. The absorption spectrum of partially purified tissue extracts peaked at 586nm suggesting that the bluish colour of the morph used in this study results from a dominant expression of amilCP586. To enable an estimation of tissue CP content of the tissue, molar extinction coefficients were approximated for two proteins, avalCP580 (183,000 M^{-1} cm^{-1}) and apolCP584 (103,000 M^{-1} cm^{-1}). Absorption

spectra and the molar extinction coefficients of the studied pigments are typical for the range of commonly found anthozoan CPs (Alieva et al. 2008).

4.4.2. *In vitro* CP screening experiment

The three *Symbiodinium* clades showed similar chlorophyll excitation spectra with major peaks at around 460nm and 525nm (Fig. 4.1). While the excitation spectra for clades C and D were almost identical, the contribution of the 525nm peak in the clade A spectra was reduced, with a maximum difference between the spectra at around 520nm. The latter was likely due to differences in the zooxanthellae pigment composition as this peak had been previously attributed to peridinin (McCabe Reynolds et al. 2008). When a CP solution was placed between the zooxanthellae and the fibre optic probe, mimicking the localisation of CPs in pigmented coral's tissue, there was a clear decrease in the excitation of chlorophyll. Chlorophyll excitation was reduced by up to 18% over the 400-620nm spectrum measured and the wavelength specific reduction was up to 55% at 580nm for the amilCP580. This decrease in the excitation of chlorophyll was consistent with the absorption characteristics of the CP, with excellent agreement between the difference spectra and the absorption spectra of the CP. The agreement was consistent for all three clades studied.

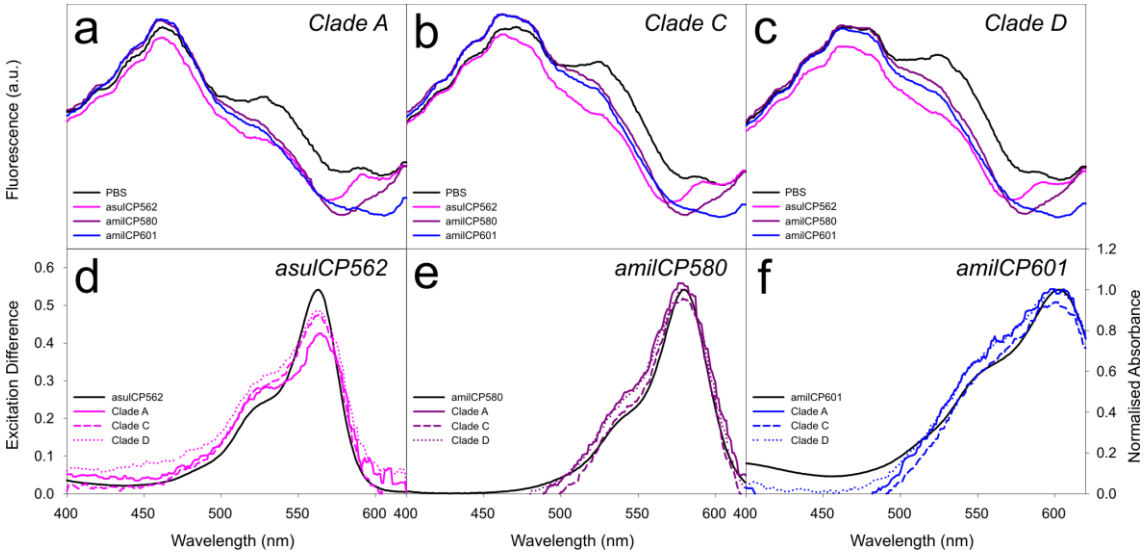

Figure 4.1. *In vitro* assessment of screening by chromoproteins. (a-c) Chlorophyll excitation spectra of freshly isolated zooxanthellae of clades A (a), C (b) and D (c) through solutions of PBS, asulCP562, amilFP580 and amilCP601. (d-f) Excitation difference spectra through the asulCP562 (d), amilCP580 (e) and amilCP601 (f) solutions from populations of zooxanthellae of clades A , C and D. The absorption spectra of the respective CPs are shown for reference and are normalised to a peak value of 1.0. All spectra are the means of five independent measurements.

4.4.3. Impact of CPs on the optical properties of *S. hystrix*

To confirm the applicability of the *in vitro* experiment, spectroscopic measurements were performed on a pink colony of *S. hystrix* that expressed a CP absorbing at 562nm (Figure 4.2). In agreement with the *in vitro* study, the presence of a CP in the light exposed tissue resulted in a measured decrease in the excitation of chlorophyll that corresponded to the absorption properties of the CP.

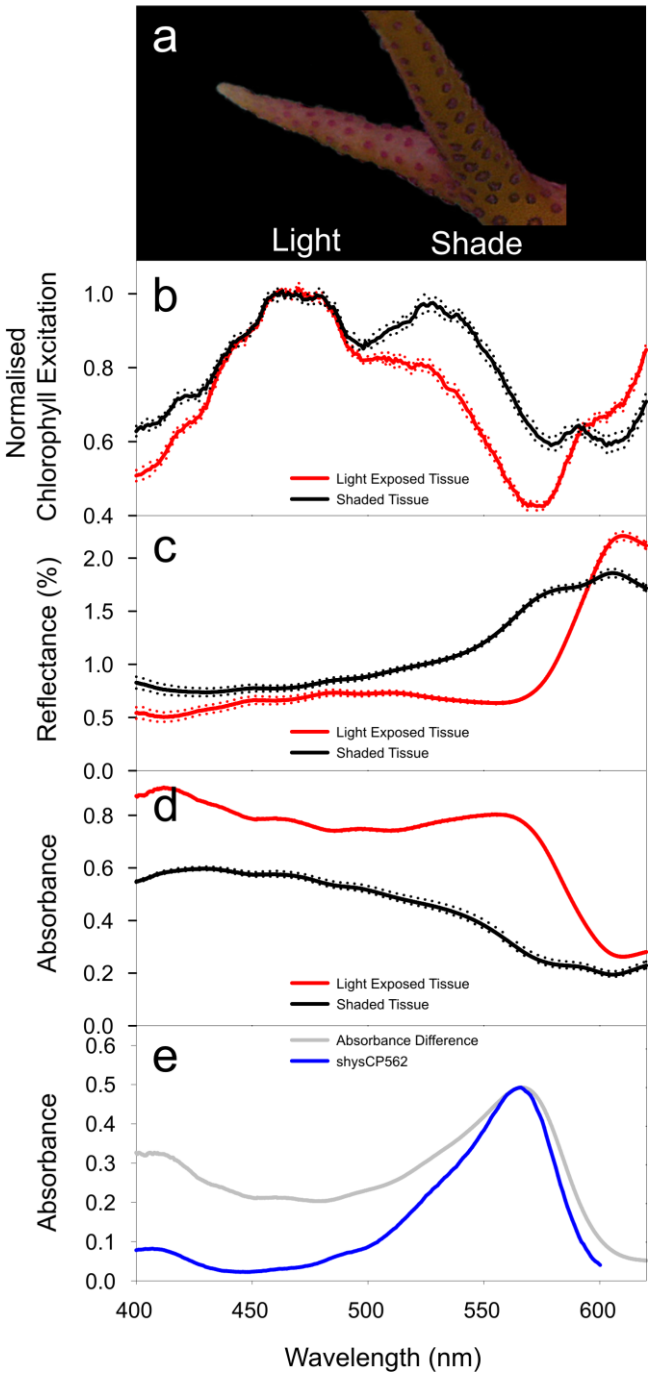

Figure 4.2. Impact of shysCP562 on the optical properties of *Seriatopora hystrix*. (a) Photograph showing the accumulation of shysCP562 in the light exposed tissues of *S. hystrix*. (b-d) Normalised chlorophlyll excitation (b), reflectance (c) and estimated absorbance (d) spectra of the light exposed and shaded surfaces. Solid lines are the mean of 5 measurements, with the ±SD shown by the dotted lines. (e) The difference between absorbance of light exposed and shaded tissues. The absorption spectrum of shysCP562 is shown for reference.

In terms of the optical properties of the corals, the reflectance spectra of the upper and lower sides of the *S. hystrix* colony fall into the two different reflectance categories described by Hochberg and co-workers (2004) in a study of corals in the field. The shaded undersides of the branches yield reflectance spectra showing maxima at around 575, 600, and 650nm typical of "brown" corals (Hochberg et al. 2004). In contrast, the reflectance spectra of the light exposed, pigmented tissue shows features characteristic of "blue" corals: the 575nm maximum is absent, resulting in a plateau-like shape between 600 and 650nm (Hochberg et al. 2004). Converting these data into estimated absorbance highlights an absorption peak in the spectrum at approximately 555nm. Comparisons of the difference between the absorbance of the upper and lower tissues with the absorption spectrum of shysCP562 reveals a strong similarity between the absorption feature at 566nm in the difference spectrum with the CP absorbing at 562nm.

4.4.4. Relationship between excitation difference and CP absorption

To further investigate screening by CPs of symbionts *in hospite*, the excitation difference spectra of light exposed vs shaded sides was compared against the absorption properties of the respective CP in *S. hystrix*, *A. valida* and *A. millepora* (Figure 4.3). Not only did the shapes of the difference spectra match the CP absorption spectra but the peak locations showed good agreement across the 24nm range of the CPs' absorption maxima (i.e. the peaks ranging from 562-586nm). By compiling the data taken from five different species and different types of comparison (light vs shaded tissue, growth zones vs non-growth zones and CP-pigmented morph vs non-CP pigmented morph) a strong linear relationship (r^2 = 0.96) between the peak of excitation difference spectra and the CP absorption maxima was identified.

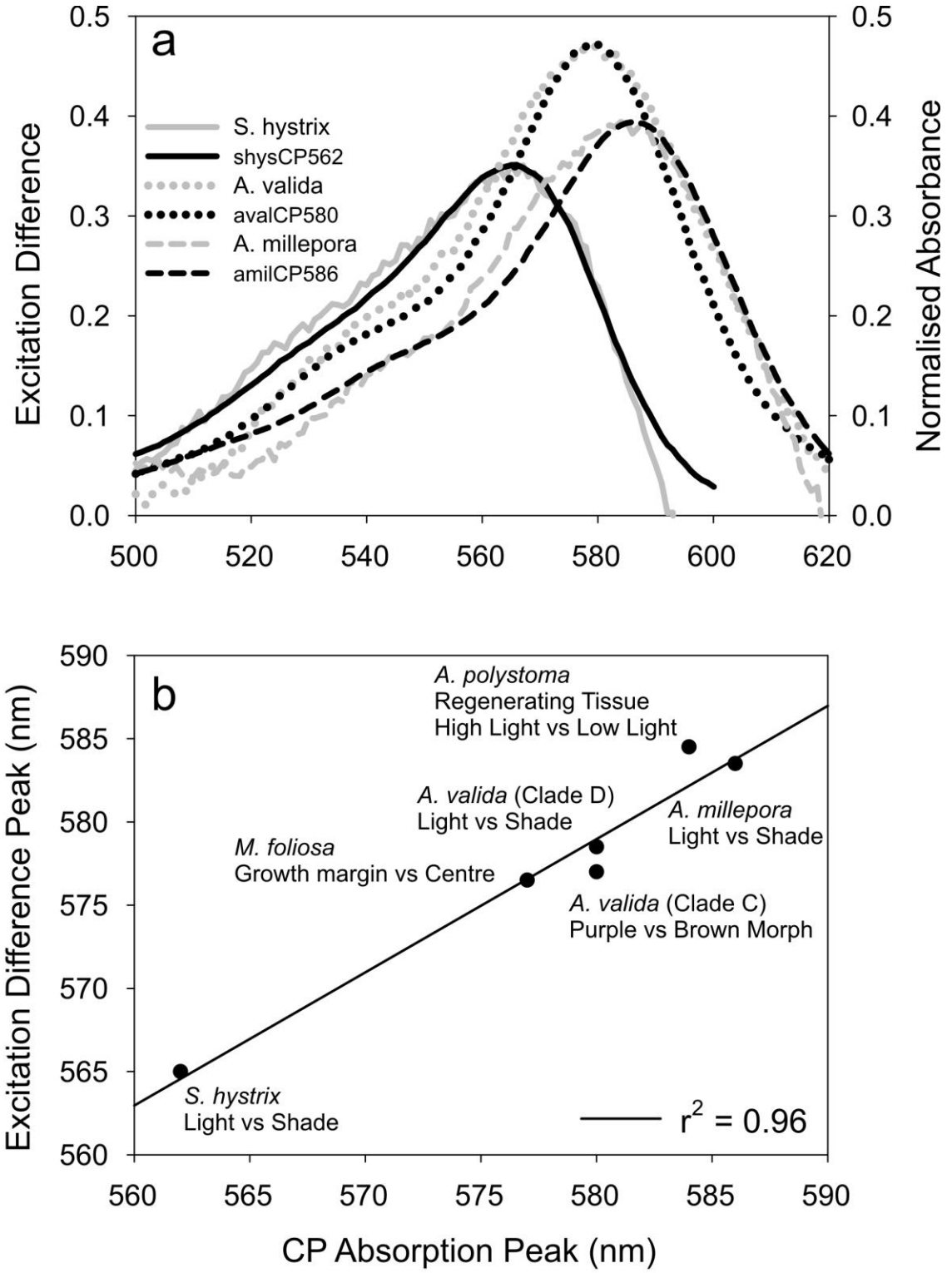

Figure 4.3. The relationship between the excitation difference spectra and the respective CP absorption. (a) Comparison between the excitation difference spectrum of *Seriatopora hystrix*, *Acropora valida* and *Acropora millepora*, and the respective CPs (shysCP562, avalCP580, amilCP586) absorption spectrum. (b) Linear relationship between the CP absorption peak and the peak of the excitation difference spectrum. Each point represents the comparison between pigmented and non-pigmented tissue from five difference coral species (*S. hystrix*, *A. valida*, *A. millepora*, *Acropora*

polystoma and *Montipora foliosa*). *A. valida* is represented twice from comparisons between light and shaded tissue of a purple morph hosting clade D symbionts and between two colour morphs (brown vs purple) hosting clade C symbionts.

4.4.5. Response of different colour morphs to high irradiances

Using the purple and brown morph of *A. valida*, the response of CP pigmented and non-CP pigmented tissues to high irradiances was compared to explore the impacts of CP screening on the light stress response of zooxanthellae (Fig. 4.4). Both colour morphs host subclade C3 zoxanthellae as the dominant symbiont strain. Prior to experimentation, the corals were grown side-by-side under the same irradiance (200µmol m^{-2} s^{-1}) to ensure that they experienced comparable light histories. The morphs differed in appearance due to the light-driven expression of avalCP580 in the ectodermal tissue of the branch uppersides of the purple morph (Figure 4.4; ESM Figure S4.5). Differences in the response to high irradiance were observed when the uppersides of the branches of the brown and purple colour morphs were exposed to intense orange light (1000µmol m^{-2} s^{-1}). Both colour morphs showed a clear reduction in the maximum quantum yield of the upper branch sides over the course of the light stress experiment although the decline was significantly greater in the brown morph (Mann-Whitney U test, $U = 0.000$, $n = 5$, $p < 0.05$). While in the purple morph the Fv/Fm was reduced by 21%, the brown morph showed an almost 50% reduction. Similarly, the purple morph also showed a higher effective quantum yield in the light than the brown morph. When the shaded sides of the colonies, both without CP pigmentation, were exposed to the same conditions, the decline in the maximum quantum yield was considerably larger than for the light acclimated upper sides of the branches with the reduction in Fv/Fm exceeding 75% for both morphs. Importantly, the decrease was comparable between both two morphs (Mann-Whitney U test, $U = 0.850$, $n = 5$, $p = 0.421$) and this trend was also consistent for the effective quantum yield.

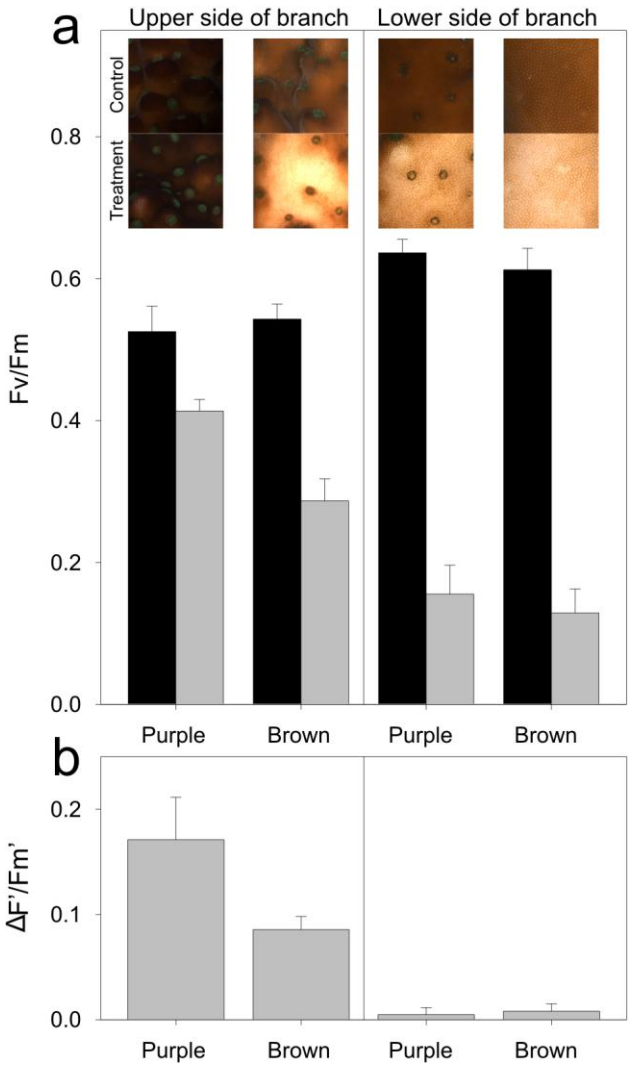

Figure 4.4. The impacts of light stress on the photobiology of brown and purple *Acropora valida* colour morphs hosting clade C symbionts. (a) Comparison of maximum quantum yield before (black bars) and after (grey bars) the light stress treatment for light acclimated upper (left panel) and shade acclimated lower (right panel) tissues. (b) The effective quantum yields are shown by the light grey bars and were performed prior to the end of the light stress treatment. The error bars show the ±SD of 5 measurements. Inset: Photographs showing the visual appearance of control and treatment corals.

4.4.6. CP expression in growth zones

Regenerating tissue of *A. polystoma* grown under higher light contained very low symbiont densities, whereas the new tissue of the low light sample was fully colonized by zooxanthellae (Figure 4.5). Under the higher light conditions, the tissue content of apolCP584 contributed ~6% to the total soluble protein of the high light regenerating

tissue, representing a ~30-fold greater CP content than the new tissue grown under low light. The difference in CP concentration is reflected in the chlorophyll excitation spectra, with the higher CP content in the high light tissue resulting in a relative reduction in the excitation of chlorophyll peaking at 582nm. The absorption by the CP was again evident in the absorption spectrum in the high light regenerating tissue. In agreement with the findings for *M. foliosa* (Figure S4.6), the lack of absorption by algal pigments in regenerating tissue under high light results in a high reflectance in the blue-green spectral range (Figure S4.7).

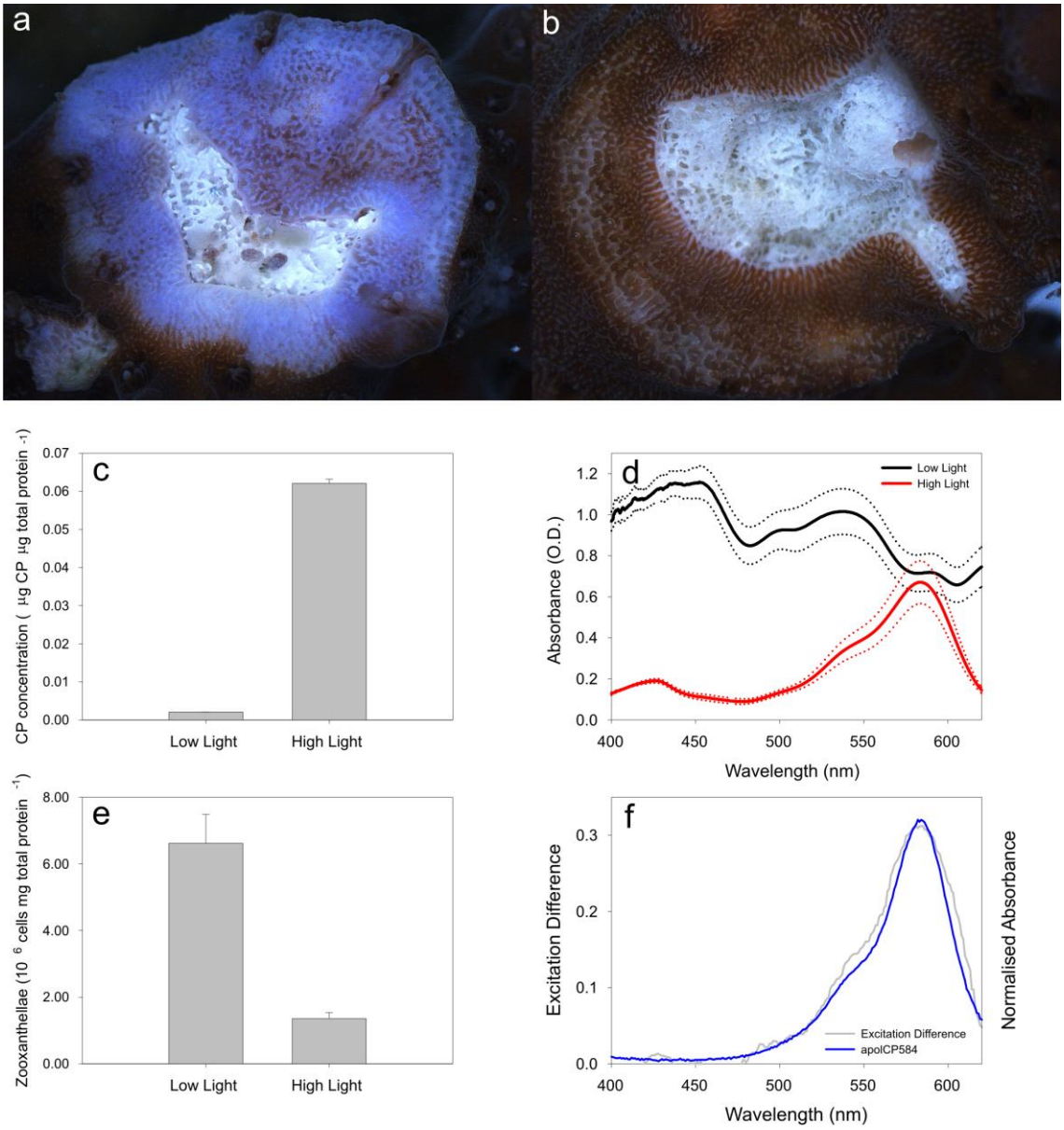

Figure 4.5. Regulation of CP expression in regenerating tissue of *Acropora polystoma* under different light conditions. (a,b) Photographs of *A. polystoma* regeneration zones grown under high (a; ~200 µmol m^{-2} s^{-1}) and low (b; ~60µmol m^{-2} s^{-1}) light. (c,e) CP (c) and zooxanthellae (e) content of regenerating tissue under the two light conditions. Error bars indicate the ±SD of triplicate measurements. (d) Mean estimated absorbance

for the low and high light tissue. Dotted lines show the ±SD of 5 independent measurements. (f) The excitation difference spectrum of high vs low light regenerating tissue. The absorption spectrum of apolCP584 is shown for reference and is normalised to the height of the difference spectrum.

4.5. Discussion

The family of GFP-like proteins represents an important group of host pigments in reef building corals and other anthozoans (Matz et al. 1999; Wiedenmann et al. 1999; Salih et al. 2000; Wiedenmann et al. 2000; Dove et al. 2001; Wiedenmann et al. 2002; Wiedenmann et al. 2004b; Oswald et al. 2007; Alieva et al. 2008). It was proposed that these pigments exert a photoprotective function in corals and sea anemones by absorbing photons or redistributing them away from the major absorption bands of the photosynthetic pigments of the algal symbionts (Wiedenmann et al. 1999; Salih et al. 2000; Dove et al. 2001). However, a general function of GFP-like proteins as photoprotectants has been controversially discussed (Mazel et al. 2003b; D'Angelo et al. 2008) and observations suggest functional heterogeneity among members of this large protein family (Alieva et al. 2008; D'Angelo et al. 2008; Vogt et al. 2008). In the present study, the non-fluorescent CPs were tested for their potential to screen light that would be destined for absorption by the zooxanthellae, whether this screening provides relief from light stress and if screening is applicable to regions of low symbiont density.

4.5.1. Screening by CPs

A previous study of 19 Caribbean coral species concluded that coral GFPs were not serving a photoprotective function as the FPs did not significantly alter the chlorophyll excitation spectrum nor did they increase the coral reflectance (Mazel et al. 2003b). In contrast, this study has found that CPs have a clear effect on the chlorophyll excitation spectrum, with a clear decrease in excitation around the absorption maxima of the CPs. This was evident in the analyses of both the zooxanthellae *in vitro* and *in hospite*.

The *in vitro* analyses provide a unique standpoint from which to monitor the effects of CPs on the chlorophyll excitation spectrum. Here, measurements were taken on identical populations of each clade of isolated zooxanthellae with different CP filters and therefore, differences between the excitation spectra could be attributed solely to the CPs rather than differences in zooxanthella photobiology. While an *in vitro* experimental setup may not accurately represent the complex light environment of a

coral's tissues (Kuhl et al. 1995; Enriquez et al. 2005), the close agreement in the degree of chlorophyll excitation reduction (~ 50% at 580 nm) between the *in vitro* experiments (clade D, amilCP580) and the *A. valida* (clade D, avalCP580) colony from which the CP concentrations were calculated demonstrates that the results are indeed relevant to the *in hospite* functioning of the CPs. Furthermore, the results show the screening occurs across the maxima range of CPs from 562nm to 601nm. The measurable impact of CPs can be attributed to their high molar extinction coefficients which tend to be significantly greater than those of their fluorescent counterparts, especially of GFPs (Salih et al. 2006; Alieva et al. 2008; Nienhaus and Wiedenmann, 2009).

The spectral analyses of the zooxanthellae *in hospite* support the findings of the *in vitro* experiment, whilst furthering our knowledge of how CPs alter the optical properties of the coral. The excellent agreement between the excitation difference and CP absorption spectra clearly demonstrates the screening by CPs. It is important to stress that the screening occurring in coral tissue is due to the physical separation of the two absorbing species, the zooxanthellae and the CPs. The light received by the zooxanthellae, located in the endoderm, is directly dependent on the absorption and scattering processes occurring in the ectoderm that separates them from the external light field. The CPs, located in the ectoderm, absorb certain wavelengths of light and therefore modify the light reaching the endoderm and consequently, the algal symbionts. The screening behaviour is evident from the optical measurements recorded in this study as CPs increase the absorption by the coral holobiont but not only is this absorbed light energy not passed on to chlorophyll or the accessory pigments and therefore not contribute to the chlorophyll excitation, but it also reduces the availability of those wavelengths for the symbionts below, which is manifested by a reduction in the chlorophyll excitation. The screening by CPs acts presumably in a similar manner to the epidermal anthocyanins in higher plants (Liakopoulos et al. 2006; Merzlyak et al. 2008).

CPs absorb photons in a spectral range that lies mostly outside the major absorption bands of photosynthetic pigments (D'Angelo et al. 2008; Dove et al. 2008). Therefore, it has been argued that any photoprotection afforded by CPs is likely to be indirect, for instance, by a blue light enrichment of the internal light field that promotes chlorophyll retention, increased antioxidant pools and promoting the expression of photoprotective Early Light-inducible Proteins (ELiPs) production (Dove et al. 2008). While these assertions are likely to be entirely valid, the results presented here demonstrate that the role of CP absorption in photoprotection could also be direct, particularly as the *in vitro* experiment demonstrated that total chlorophyll excitation

between 400-620nm could be reduced by up to 18%. The chlorophyll excitation spectra determined for zooxanthellae of five coral species show clearly that light use efficiency is less spectrally discriminant than absorption spectra of the pigments in dilute solutions would suggest. These results are consistent with photosynthetic action spectra from other studies measured by fluorescence (Lesser et al. 2000; Mazel et al. 2003b) and oxygen sensors (Kuhl et al. 1995) where the intensity parameter is incident irradiance. This effect results from strong scattering and pigment packaging occurring at the level of the symbiont cells and inside the coral tissue (Kirk 1994; Stambler and Dubinsky 2005). As a result, the contribution of wavelengths outside the major pigment absorption bands to the excitation of the symbiont population's chlorophyll fluorescence is substantial.

The photosynthetic antennae may not be the only site of photoinhibition as the manganese complex of the oxygen evolving complex (OEC) has also be implicated in photoinhibition (Hakala et al. 2005; Baird et al. 2009; Takahashi et al. 2010). Hakala et al. (2005) report that the direct excitation of an OEC Mn ion results in its release into the thylakoid lumen. The OEC is subsequently unable to donate an electron to P680+ resulting in damage to the PSII reaction centre (Hakala et al. 2005). Although predominantly absorbing in the UV region of the spectrum, the high photon fluxes throughout the visible spectrum are sufficient to cause damage to the OEC (Hakala et al. 2005). Indeed, Takahashi and co-workers (2010) found that light in the yellow-orange region of the visible solar spectrum to be the most damaging for higher plants despite low absorption in this spectral range by the photosynthetic pigments. Despite the stronger attenuation of light at longer wavelengths by seawater (Smith and Baker 1981; Kirk 1994), yellow-orange light remains an important region of underwater light field. For example, Dustan (1982) found that even at 3m, the spectral irradiance on a Caribbean reef contained more light at 567nm than at blue wavelengths (<500nm). As other existing holobiont photoprotective mechanisms, such as MAAs, act outside the yellow orange region of the spectrum, the CPs in shallow water corals might provide protection in a vulnerable spectral window, functioning in a similar manner as anthocyanins in higher plants (Merzlyak et al. 2008). Finally, important physiological processes in corals, including regulation of genes encoding GFP-like proteins, are controlled by blue light (Levy 2003; Levy et al. 2006; D'Angelo et al. 2008; Kaniewska et al. 2009). A photoprotective mechanism that removes longer wavelengths appears to be a sensible strategy to avoid interference with the blue light regulated mechanisms.

The species selected for the CP analyses in this study are fast growing branching and plating corals that are relatively short lived in comparison to genera such as *Porites*.

Nevertheless, CPs are also present in these slow growing species (e.g. Kawaguti, 1944; Salih et al. 2006). The screening behaviour demonstrated in this work should be consistent for corals of different species and morphologies to those described here as the organisation of CPs within the tissues is consistent amongst these different corals (Kawaguti, 1944). In a similar manner, the results should be consistent for corals hosting different zooxanthellae clades and this is supported by observations on *A. valida* (different colonies hosting clades C and D) and the *in vitro* experiment.

4.5.2. CP screening correlates with reduced photodamage

The potential for CP screening to afford relief from light stress was assessed by subjecting two colour morphs of *A. valida* to high photon fluxes of yellow-orange light (1000µmol m^{-2} s^{-1}). It was found that yellow-orange light, in excess, is indeed damaging. The measure of photodamage used in this study was the reduction in the maximum quantum yield after light stress, in spite of 12 hours recovery in the dark. This is indicative of chronic photoinhibition rather than dynamic photoinhibtion as the dynamic processes should relax within around 3 hours whereas photodamage can take between 30-50 hours (Gorbunov et al. 2001). This measure of photodamage has been used in previous studies and correlates with other biomarkers of photodamage such as D1 concentration and symbiont cell viability (Warner et al. 1999; Gorbunov et al. 2001; Franklin et al. 2004). The CP-containing tissues of the branch uppersides of the purple morph of *A. valida* suffered less photodamage under high light stress compared to the brown morph. Contrastingly, comparable damage was observed when the CP-free tissue of the branch undersides of both morphs was subjected to the light stress treatment. Hence, the finding that zooxanthellae in tissue containing high concentrations of CPs show reduced damage under the impact of light stress strongly supports an ecologically relevant photoprotective function of CPs and enables us to reject the null hypothesis that CPs do not reduce light stress on the zooxanthellae. It is important to highlight, however, that the expression of CPs is certainly not vital for shallow water corals, otherwise they would be ubiquitous. Instead, they are likely to represent a strategy for extending the ecological niche of a species. It is well established that zooxanthellae have mechanisms that are very effective in protecting the photosynthetic apparatus under supraoptimal irradiances (Gorbunov et al. 2001) and, therefore, it has been questioned whether there is a need for additional host-based photoprotectants (Mazel et al. 2003b). However, there is evidence that under shallow water light conditions on a cloud free day and under thermal stress, the symbionts' capacity for photoprotection can be exceeded and photodamage can occur (Iglesias-Prieto et al. 1992; Warner et al. 1999; Gorbunov et al. 2001). It is proposed that under these conditions, where the symbionts protective strategies are

overwhelmed, the expression of CPs may help the coral to minimise the light stress in addition to other strategies such as the expression of antioxidants or the association with light tolerant *Symbiodinium* clades. Moreover, the CPs could additionally act to promote survival or during occasional periods of extraordinary stress.

A photoprotective role for GFPs has been questioned as bathymetric studies of the GFP content of *Montastrea faveolata* and *Montastrea cavernosa* showed no significant correlation between depth and also other species lacked the light-dependent regulation of GFP-like proteins that would be expected for a typical photoprotectant (Mazel et al. 2003b; Wiedenmann et al. 2004a; Leutenegger et al. 2007a; Vogt et al. 2008). However, D'Angelo and co-workers (2008) clearly demonstrated a blue light controlled regulation of the tissue content of FPs and CPs in shallow water corals. In agreement with the findings of D'Angelo et al. (2008), a pronounced accumulation of CPs in the upper side of branches both in field samples and in experimental corals grown under controlled light conditions was observed in this study. Apparently, not all corals demonstrate a light regulated expression of GFP-like proteins (Leutenegger et al. 2007b). However, the high light induced production of CPs supports a photoprotective function and helps to explain the depth dependent decrease in morphs with pronounced CP colouration observed in a previous study (Salih et al. 2006). The co-existence of CP-containing and CP-free colour morphs in shallow water supports the assertion that protection provided by the pigments is not vital but potentially offers more subtle benefits enabling corals to acclimate to a broader range of conditions in habitats characterised by a highly dynamic light field.

4.5.3. Screening by CPs in regions of low symbiont density

In cases of constrained distribution of FPs to structural features such as skeletal ridges, the pigments were typically found where the concentration of zooxanthellae is lowest (Mazel et al. 2003b). It was concluded that the physical segregation from the algae would preclude the FPs from having a role in symbiont photobiology (Mazel et al. 2003b). Moreover, the high-level expression of CPs in growth zones of corals that are essentially free from zooxanthellae (D'Angelo et al. 2012) called for further analyses of the function of GFP-like proteins in these regions. Here, it was investigated whether the presence of CPs could afford screening to growth regions such as coral tips or margins, regenerating colony parts or areas in which an infestation with epibionts has stimulated a growth-mediated innate immune response (D'Angelo et al. 2012). In the regenerating tissue of *A. polystoma* grown under low light, the expression of CPs was largely absent whereas high light colonies amassed a ~30-fold increase in CP concentration, confirming a light-driven upregulation of CP expression (D'Angelo et al.

2008). The constrained localisation of the CPs in the growing areas devoid of zooxanthellae can be explained by the high internal light fluxes in the blue-green spectral range that result from the lack of absorption of the incident light by the photosynthetic pigments of the symbiont and its reflection by the coral skeleton, potentially amplified by multiple scattering (Enriquez et al. 2005). The comparison of reflectance spectra of tissue areas with and without zooxanthellae suggests a 5-7-fold increase in internal fluxes of blue-green light in growing regions of the colony. An increase in light intensity in this order was previously proven to stimulate an enhanced production of GFP-like proteins (D'Angelo et al. 2008).

Once the symbiont population becomes established, the CP expression decreases due to the reduction of the internal blue light fluxes. Consequently, the light driven up-regulation and the transient appearance of CPs in the growth zones could enable certain corals to provide screening to colony parts with greatest need, without compromising the overall productivity. Furthermore, considering that axial polyp development is blue light dependent (Kaniewska et al. 2009), the absorption characteristics of the CPs in growth zones are ideally suited to provide screening without interfering with blue light regulated growth processes.

In agreement with the spectral analyses of established tissues, CP expression in regenerating tissues is clearly capable of screening the symbionts. It is postulated that the presence of CPs in growth zones assists in the colonisation of new coral tissue of pigmented morphs by providing photoprotection from the high internal light fluxes associated with multiple scattering in the coral skeleton (Enriquez et al. 2005; Terán et al. 2010). Considering that a photoprotective role of GFP-like proteins has been proposed for azooxanthellate octocorals (Kahng and Salih 2005), this photoprotection may not only be beneficial for establishing a symbiont population but also protect the developing host tissue. While this study has demonstrated a correlation between CP screening and photoprotection of zooxanthellae in established coral tissue, the impact of screening in growth zones and on host tissue needs further investigation.

4.5.4. Conclusions

Coral reefs are under threat and it is forecast that winners and losers will emerge as the conditions on reefs change in the future (Hughes et al. 2012). The key for reef managers is to identify the winners, the species most likely to survive future conditions, enabling the implementation of management strategies that afford greater protection to these more tolerant corals. Here, it is demonstrated that CP expressing morphs have a greater tolerance to light stress than their brown counterparts and

therefore, these colour morphs should be targeted for greater protection. Additionally, reef rehabilitation efforts could benefit from the selection of CP colour morphs as they are well suited to shallow water nurseries and the light regulation of the CPs should enable replantation in different light environments.

4.6. Supplementary Methods

4.6.1. Field Samples

Field coral samples (branch fragments ~10 cm length) of brown and blue colour morphs of *Acropora nobilis*, growing adjacent to each other, were collected at ~1.5 m depth at low tide on the outer reef slope of the Heron Island, Great Barrier Reef under a Marine Parks Permit (Nr. G26894.1). Care was taken to collect samples of similar light histories, selecting the most light-exposed branches for further analysis. Zooxanthellae density was calculated as described in the methods section and extraction of zooxanthellae pigments was performed according to a previous protocol (Leutenegger et al. 2007a). After extraction, the absorption of the clarified extracts was determined in a Cary spectrophotometer (Varian, USA) and concentrations of chlorophyll a, chlorophyll c2 and peridinin were calculated using the formulas provided by Jeffrey and Humphrey (1975) and Parsons and Strickland (1963).

4.7. Supplementary Figures

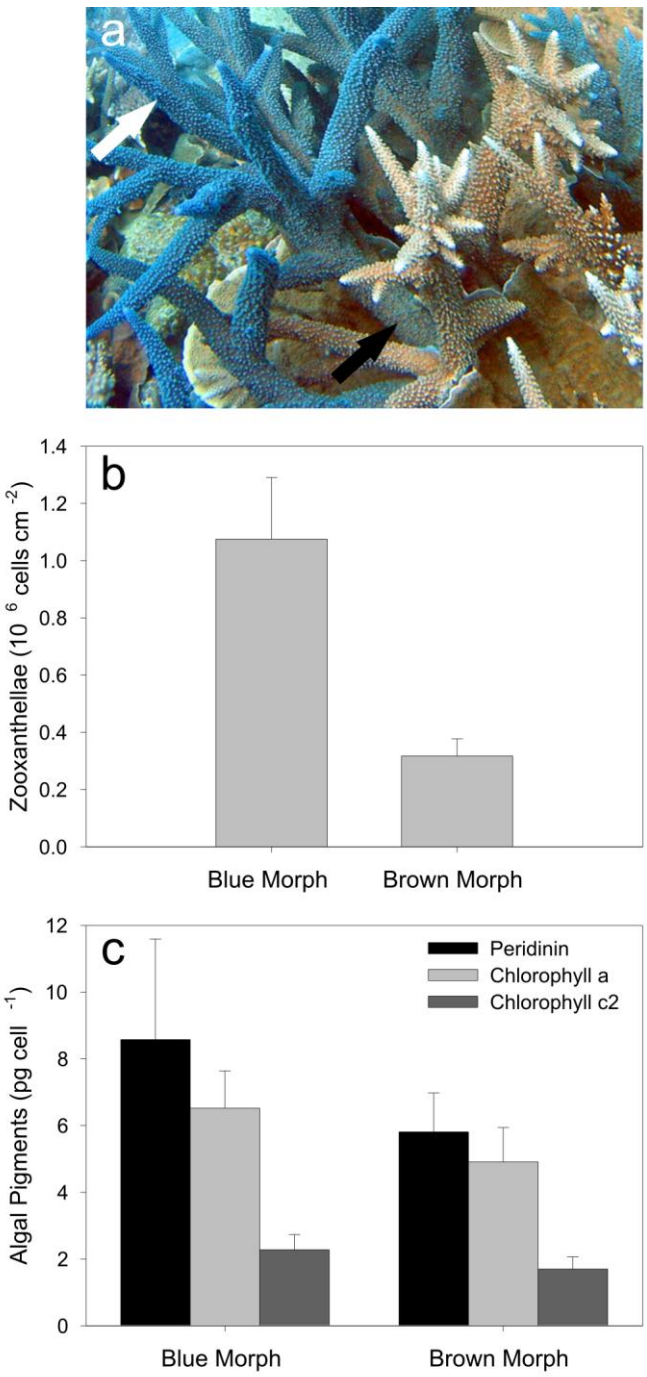

Figure S4.1. Analysis of blue and brown morphs of *Acropora nobilis* from the Great Barrier Reef. (a) Photograph of the two morphs side-by-side on the reef. The white arrow indicates a light exposed branch whereas the dark arrow shows a branch that is subject to self shading within the blue morph colony. (b,c) Mean zooxanthellae densities (b) and pigment concentrations (c) from five replicate colonies collected at 1.5m. Error bars indicated the ±SD of the mean.

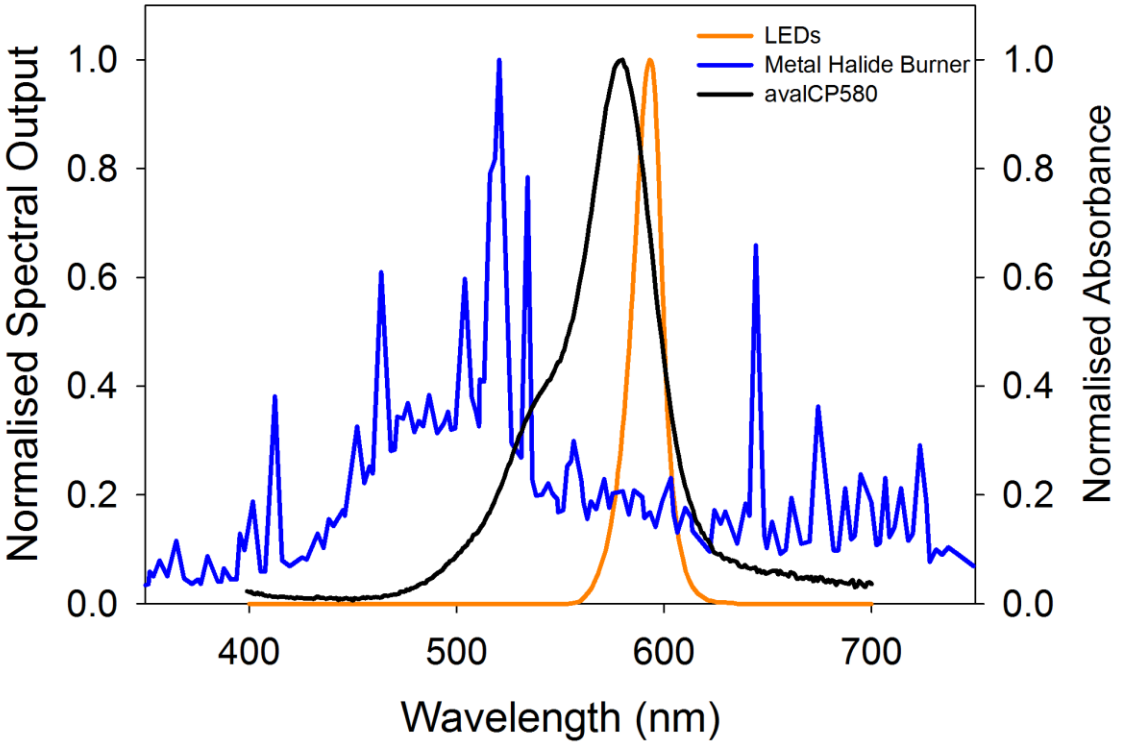

Figure S4.2. Optical properties of the light sources used in this study. The normalised spectral output of the metal halide burners used for growing the aquarium corals and the amber LEDs used for the high irradiance experiments. The absorbance spectra for avalCP580 is shown to demonstrate the overlap between the LED output and the absorption of the CP.

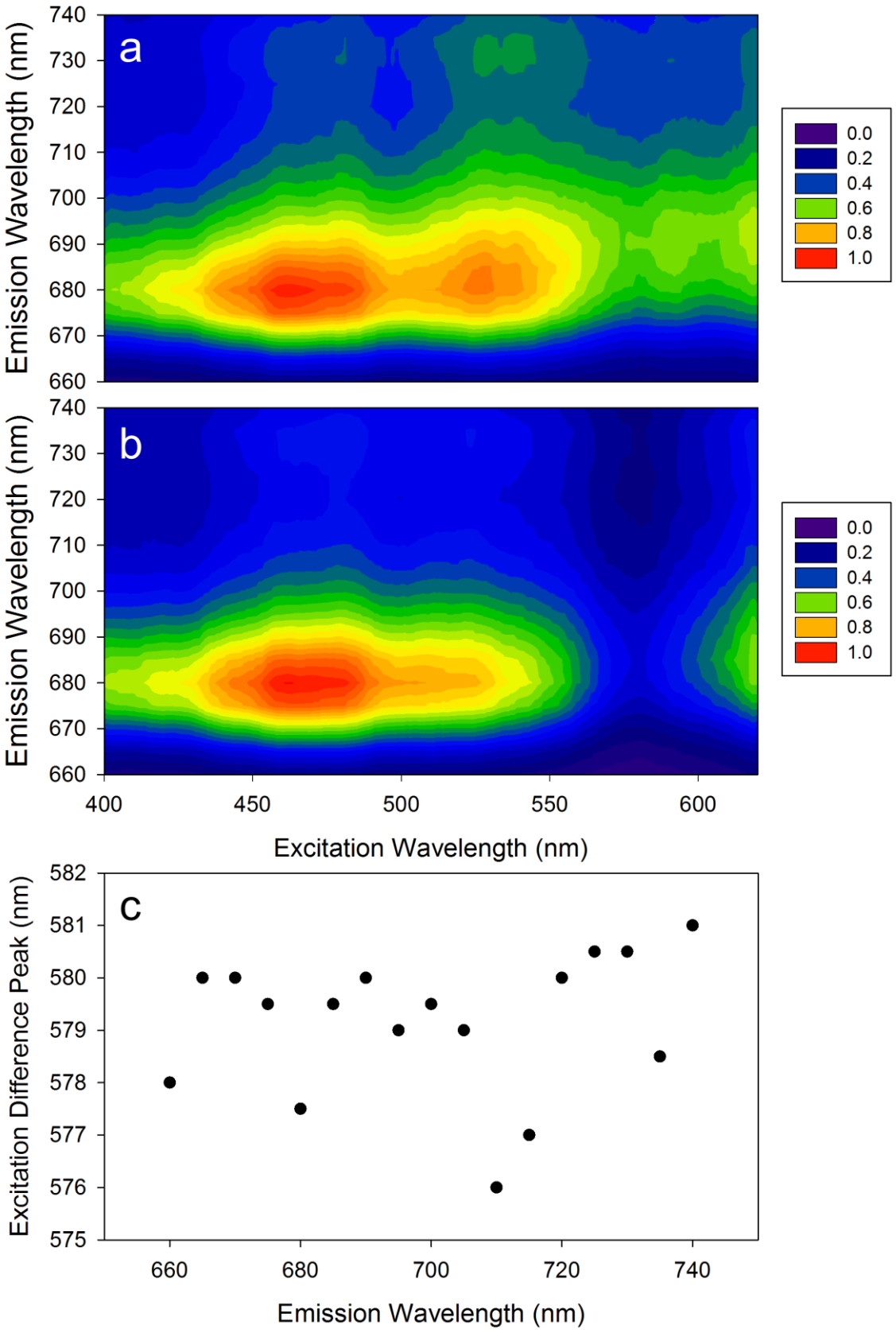

Figure S4.3. Excitation and emission properties of the brown and purple colour morphs of *Acropora valida*. (a,b) Excitation-emission plot for the brown (a) and purple (b)

morphs. The excitation spectra are sampled at 1nm intervals and the emission spectra are sampled every 5nm. (c) Difference spectrum peak values across the chlorophyll emission range. The mean peak wavelength of the difference spectrum is 579nm with a standard deviation of 1nm and the respective CP absorption peak is at 580nm.

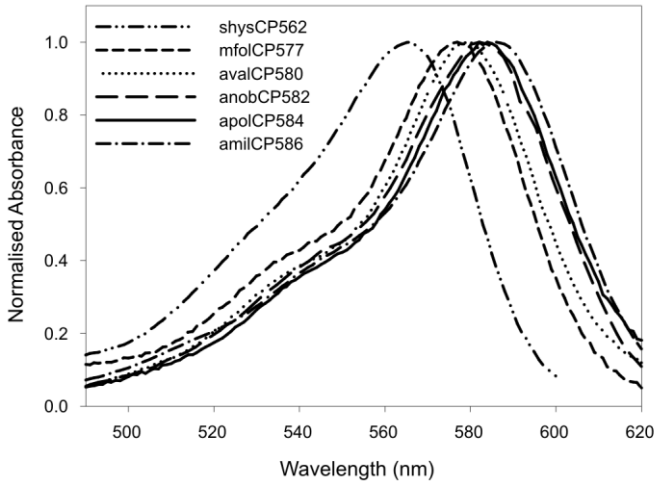

Figure S4.4. Absorption spectra of CPs isolated and purified from the corals *Seriatopora hystrix* (shysCP562), *Montipora foliosa* (mfolCP577), *Acropora valida* (avalCP580), *Acropora nobilis* (anobCP582), *Acropora polystoma* (apolCP584) and *Acropora millepora* (amilCP586). Spectra are normalised to a peak height of 1.0.

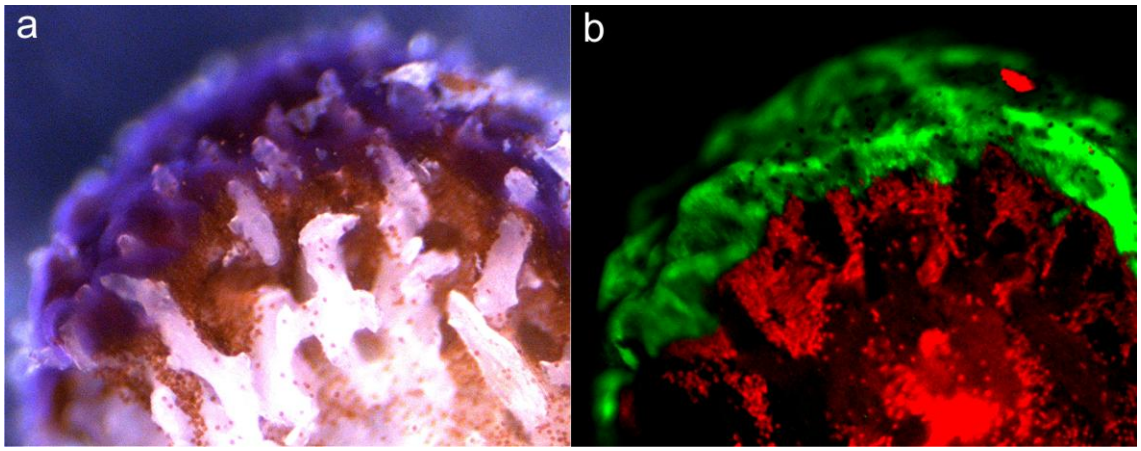

Figure S4.5. Localisation of avalCP580 in the purple morph of *Acropora valida*. (a) Micrograph of a cross section of an *A. valida* tip showing the localisation of the purple chromoprotein. (b) Fluorescent image of the corresponding cross section showing the presence of ectodermal green fluorescent proteins (green) and chlorophyll fluorescence from the zooxanthellae (red).

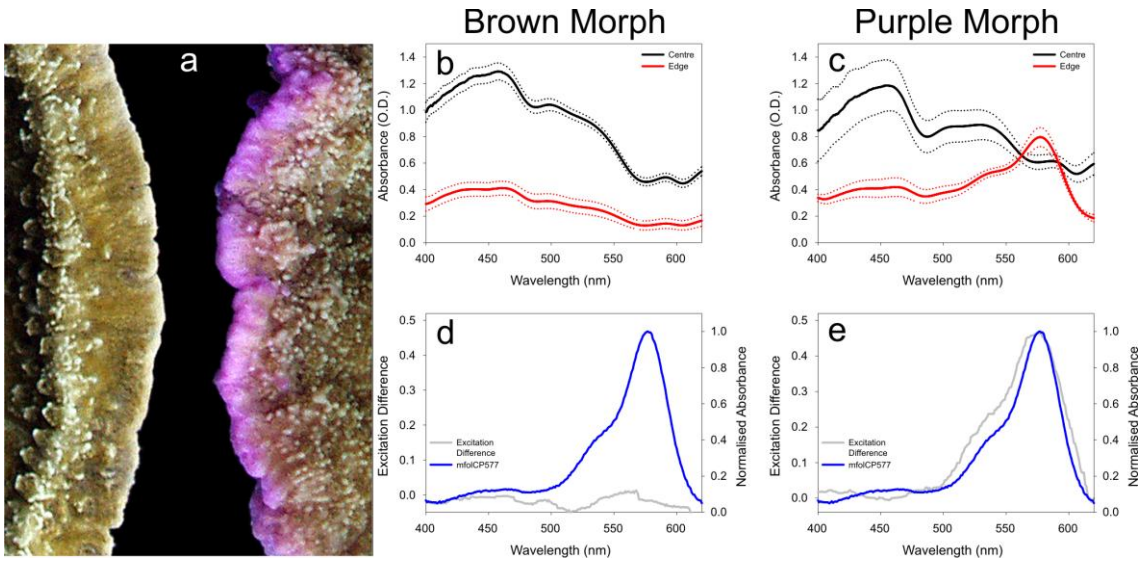

Figure S4.6. Characterisation of chromoproteins in growth regions. (a) Photograph of two *Montipora foliosa* morphs expressing (right) and not expressing (left) chromoproteins in the colony margins. (b,c) Mean estimated absorbance spectra of the brown (b) and purple morphs (c) at the centre and growth margins of the colonies. Dotted lines indicate the ±SD of 5 independent measurements. (d,e) Excitation difference spectra for the brown (d) and purple (e) morphs. The absorption spectra of mfolCP577 are shown for reference.

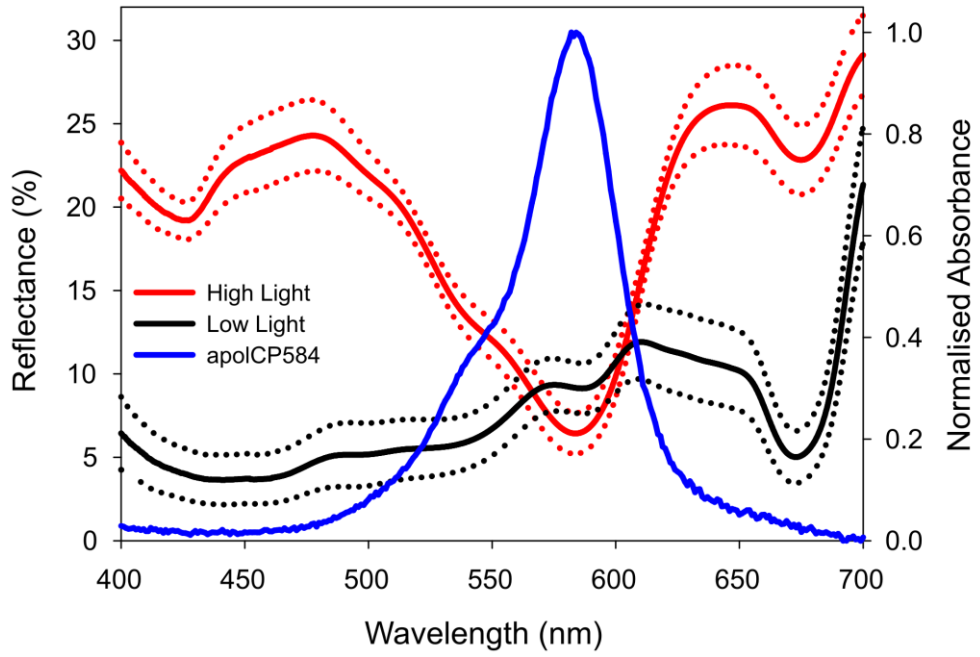

Figure S4.7. Reflectance spectra of regenerating zones in *Acropora polystoma*. The solid lines show the mean reflectance of the high and low light regeneration zones with the standard deviations of 5 measurements is shown by the dotted lines. The absorption spectrum of apolCP584 is shown for comparison.

CHAPTER 5. Internal light modification by photoconvertible red fluorescent proteins as a potential mechanism for depth acclimation in corals.

5.1. Abstract

Changes in the chromaticity and intensity of light with depth are the primary controls on the vertical extent of symbiotic corals. Corals living in deeper reefs (>20m) experience a light environment that is distinctly different from their shallow water counterparts. Therefore, characterising the mechanisms used by the holobiont to the changing intensity and spectral composition is fundamental to our understanding of the ecology of deeper reefs that may serve as refugia from disturbance for corals and the corals that are mostly to benefit from these refugia. Host pigments, namely GFP-like proteins, have been proposed to perform functions related to the light adaptation and acclimation in corals although the biological function of red fluorescent proteins has received little attention. Considering that a distinct group of red fluorescent proteins, the photoconvertible red fluorescent proteins (PCRFPs), are typically associated with depth generalist corals and species typical found in deeper reefs, this study set out to explore the vertical distribution of red fluorescent corals and the potential role of PCRFPs in the photobiology of corals. Belt transects from 6 different depths, at two sites in the Gulf of Eilat, show the proportion of red fluorescence increases with depth, down to the maximum depth studied (45m). Furthermore, spectroscopic analyses and chlorophyll fluorescence imaging reveal that the spectral properties of PCRFPs are dependent on the light regime and would shift abundant wavelengths under deep water conditions, to wavelengths that provide greater penetration through symbiont populations. It is proposed that the PCRFPs in deeper water corals may act to redistribute the light within the coral tissue to enable a more even distribution of light across their symbiont population and that the photoconversion of these proteins enables regulation by the prevailing light conditions. The results are discussed in the context of the challenges faced in evaluating the proposed hypothesis and recommendations for future work are made. This study provides the first insight into the ecology of red fluorescent morphs and highlights the importance of the spectral quality of light on the internal light field of corals.

5.2. Introduction

The success of reef building corals has been attributed to the symbiosis with dinoflagellates of the genus *Symbiodinium*, enabling high productivity in tropical low nutrient waters. As photosynthesising organisms, the symbionts are dependent on light and therefore zooxanthellate corals are largely restricted to the euphotic zone (Falkowski et al. 1990). The penetration of light in clear tropical waters, in which corals reside, is mainly controlled by the optical properties of the water molecules as concentrations of chlorophyll and dissolved organic matter are typically, however not always, low (Kirk 1994; Lesser et al. 2009). Zooxanthellate corals are found in habitats spanning over two orders of magnitude in irradiance (Falkowski and Dubinsky 1981), from exposed reefs in shallow waters to shaded habitats and mesophotic (>30m) reefs. The spectral quality of visible light is also variable, particularly with depth, where attenuation by the water column is strongest in the red region of the spectrum (Smith and Baker 1981) resulting in an underwater light field that is increasingly dominated by blue-green light with depth (Mass et al. 2010). Consequently, corals living below depths of 15-20m are mostly deprived of red light.

The responses of the coral holobiont to changes in the light field due to attenuation of light with depth can be separated into the responses of the symbiont and those of the host. There are symbiont types that appear to be adapted to certain light regimes and consequently play an important role in the vertical distribution of some corals (Iglesias-Prieto et al. 2004; Frade et al. 2008), although the identification of shallow water generalist symbionts in corals from 67-100m suggests that the symbiont community may not be the only factor involved (Chan et al. 2009). Acclimation to low light conditions generally involves an increase in pigmentation either through increasing the number/size of the photosynthetic units(Falkowski and Dubinsky 1981; Hennige et al. 2009) and/or the density of the symbionts (Titlyanov et al. 2001). Increasing pigment densities is an effective strategy at increasing light collection although not efficient due to the packaging effect as the efficiency of light collection per unit pigment decreases (Duysens 1956; Stambler and Dubinsky 2005). Nevertheless, algae residing in corals growing in the extremes of their habitat range do not necessarily always show the expected photoacclimatory response to low light. In the Red Sea, the coral *Leptoseries fragilis* shows a reduction in symbiont densities and pigment per unit surface area with increasing depth from 100-135m (Fricke et al. 1987). Similarly, *Leptoseris* spp. colonies at 68-113m in Au'au Channel, Hawaii, possessed lower areal pigment concentrations than shallow water (2-15m) *Porites* spp. (Kahng et al. 2012) and in both cases, host based mechanisms have been proposed as enabling these

corals to inhabit these depths (Schlichter et al. 1986; Fricke et al. 1987; Kahng et al. 2012).

Host based mechanisms are an important component in facilitating zooxanthellate corals existence in deeper waters. Both the macro- and micro-scale morphology of the colony are key to light collection. A dense branching or plating colony is ideal for high light environments, providing efficient shading from the high irradiances but it is too inefficient for low light environments (Anthony et al. 2005; Stambler and Dubinsky 2005). In the deeper/light limited environments, there is a preference for more planar and encrusting growth forms maximising the surface area intercepting the incident irradiance (Fricke et al. 1987; Anthony et al. 2005; Stambler and Dubinsky 2005). It is not only the macroscale morphology of the colony that is important in light collection, the presence and microscale morphology of the coral skeleton is important. It has been shown that the skeleton can enhance light collection by multiple scattering and consequently, corals can absorb equivalent number of photons to higher plants but with a much lower concentration of chlorophyll (Enriquez et al. 2005). Indeed, Kahng and co-workers (2012) found that the microscale architecture of *Leptoseris* spp. skeletons enabled these mesophotic corals to capture more light than the shallow water *Porites* spp. colonies but with less pigment per unit area.

Host pigments have been proposed to play a role in the acclimation/adaptation to light. Fluorescent proteins (FPs) homologous to the GFP have been proposed to perform a photoprotective function in shallow waters (Kawaguti 1944; Wiedenmann et al. 1999; Salih et al. 2000). Salih and colleagues (2000) found the highest concentrations of fluorescent morphs (97% of corals) in the shallow reef flats and that colour morphs experienced less photoinhibition than their non fluorescent counterparts. Certain members of the GFP-like protein family are upregulated in response to increasing light intensities (D'Angelo et al. 2008) and demonstration of screening by CPs also supports a photoprotective function (Smith et al. 2013 – Chapter 4). The photoprotective hypothesis for the FPs remains controversial, however, as other authors have not found a trend with FP content and depth (Mazel et al. 2003b) and neither significant removal of photons by the FPs from the chlorophyll excitation spectrum or reflectance spectrum is observed (Mazel et al. 2003b). A photoenhancing role has also been proposed. Schlichter and colleagues (1986) postulated that gastrodermal cyan fluorescent pigments of host origin may enhance photosynthesis in deeper water species, *L. fragilis*, by converting UV light into wavelengths more suitable for photosynthesis and increasing scattering within the tissue. Salih and co-workers (2000) noted the presence of green FPs associated with the zooxanthellae in the endoderm and also suggested that they could be involved in enhancing

photosynthesis. A non-radiative transfer of energy between FPs and zooxanthellae has been discounted due to the distance dependency of fluorescence resonance energy transfer (FRET) and the absence of evidence for FRET in fluorescence lifetime studies (Gilmore et al. 2003a). In addition to the absence of FRET between FPs and PSII, Gilmore and co-workers (2003a) could not find evidence for radiative energy transfer either in blue and green shallow water corals.

Much of the attention on coral fluorescence has focused on the cyan and green fluorescence rather than the red fluorescence. Corals possess two different types of red fluorescent proteins, delineated by their chromophore types, the DsRed-type and the photoconvertible Kaede-EosFP-type chromophores. The red FPs with a DsRed chromophore are characterised by a single excitation peak and a broad emission peak (Alieva et al. 2008) and are upregulated in response to increasing light intensities (D'Angelo et al. 2008). In contrast, the Kaede-type red FPs are constitutively expressed (Leutenegger et al. 2007b). These photoconvertible red fluorescent proteins (PCRFPs) irreversibly switch from green to red fluorescence under irradiation by UV/violet light due to a break in the peptide backbone (Nienhaus et al. 2005). PCRFPs are responsible for pigmentation in a range of species, such as *Montastrea cavernosa*, *Catalaphyllia jardinei*, *Lobophyllia hemprichii* and *Trachyphyllia geoffroyi* (Ando et al. 2002; Wiedenmann et al. 2004b; Leutenegger et al. 2007b; Oswald et al. 2007), and can contribute up to 7% of the total soluble protein content (Oswald et al. 2007). Furthermore, their unique spectral properties have enabled insights into the energetic costs associated with host GFP-like protein pigmentation (Leutenegger et al. 2007b).

Corals residing in deeper waters tend to be sheltered from the impacts of shallow water disturbances and consequently it has been hypothesised that mesophotic reefs (>30m) may act as refugia for corals (Glynn 1996; Bongaerts et al. 2010). The benefits of the refugia may be limited to 'depth generalist' species (Bongaerts et al. 2010) and therefore it is important to understand the mechanisms employed by corals to acclimate and adapt to deeper waters. Previous studies have shown that PCRFPs are found in *M. cavernosa*, an extreme depth generalist (shallow water to lower mesophotic reefs) (Bongaerts et al. 2010), and other species typically found in low light and outside the shallowest parts of the reef. These observations suggest that the ecology of the PCRFPs is distinctly different to the CPs (Chapter 4) despite absorbing in a similar region of the spectrum. Therefore, functional heterogeneity between the proteins may relate to the differences in other spectral properties of these proteins. The PCRFPs emit light (~580nm) rather than thermally dissipate excitation energy and they undergo photoconversion in contrast to the CPs. As such, these properties are the focus of this study. Considering that spectral irradiances outside of the absorption

peaks of the photosynthetic pigments is important for driving photosynthesis at the deeper depths within leaves of higher plants (Nishio 2000; Vogelmann and Evans 2002), it is postulated that the emission of PCRFPs (~580nm) will enable greater penetration of light within the tissues of corals, particularly in deep water conditions where these wavelengths are largely absent. In order to advance this theory, the following hypotheses were tested. Firstly, the proportion of red fluorescent morphs increases with respect to decreases in red light irradiances. Secondly, in the absence of transcription regulation (Leutenegger et al. 2007b), the photoconversion of these proteins enables tuning of the red fluorescence excitation to the coral's prevailing light environment. Finally, that the wavelengths of PCRFP emission correspond to the wavelengths of greatest tissue penetration.

5.3. Methods

5.3.1. Red colour morph distribution

The depth distribution of red fluorescent morphs was assessed using a total of 40 belt transects performed across six different depths and two reef locations in the Gulf of Eilat (Inter University Institute and Mirador). The optical properties of these waters with depth are well characterised (Schlichter et al. 1986; Einbinder et al. 2009; Mass et al. 2010) and closely approximate oligotrophic waters for the majority of the year (Mass et al. 2010). The reefs are typified by heterogenous cover in the shallow water compared to continuous reef at 45m. While the shallow waters have higher species richness, the deeper reef areas have larger colonies comprised of predominantly encrusting and massive forms.

The belt transects were 25m in length and 10cm in width due to bottom time limitations at the deeper sites and to enhance the possibility of recording rare specimens, most notably, red fluorescent colonies at the shallow sites. For each transect, the proportion of red fluorescent colonies was recorded and calculated relative to the total number of fluorescent coral colonies. Only hermatypic corals were surveyed. The transects in the shallow sites (1m, 5m, 12m and 20m) were performed at night using a blue torch (NightSea) for excitation and yellow filters to mask the excitation light. Due to the predominance of blue light in the underwater light field at depths of 30-45m, the contrast of the red fluorescence was sufficient for transects to be performed during the day without the need for excitation using the blue torches.

5.3.2. Mesocosm Experiments

The corals used for this study were maintained in the experimental mesocosm system at the National Oceanography Centre (D'Angelo and Wiedenmann 2011). Colonies of *M. cavernosa*, *Oxypora sp.* and *Echinophyllia sp.* were fragmented and grown under two different light conditions. Half of the colonies were subject to 200µmol m^{-2} s^{-1} white light whereas the other half were grown under ~5µmol m^{-2} s^{-1} of blue light (Figure S5.1) and the two conditions are herein referred to as the shallow water and deep water conditions respectively.

5.3.3. Spectroscopy

In vivo fluorescence spectroscopy was performed on the live colonies using the fibre optic attachment of a fluorescence spectrophotometer (Varian Cary Eclipse) (D'Angelo et al. 2012). The fibre optic probe was placed 0.5cm from the coenosarc tissue and the excitation and emission properties of the corals were recorded. The excitation and emission slit widths were 10nm and 5nm, respectively.

To obtain the zooxanthellae pigment absorption spectrum, zooxanthellae were isolated from the red morph of *Oxypora sp.* growing in the shallow water conditions. Zooxanthellae were extracted as described previously (Hartle-Mougiou et al. 2012) and resuspended in sterile filtered seawater. In order to obtain a pigment spectrum representative of the *in vivo* pigments, the extraction was performed in sterile filtered seawater as opposed to solvent extractions which involve shifts in the spectra. The zooxanthellae were sonicated on ice at 30 second intervals for 5 minutes. The solution was subsequently centrifuged for 40 minutes at 20,000xg. Absorption spectra were recorded using a Cary Absorption spectrophotometer (Varian, USA).

5.3.4. Tissue modelling

A one-dimension model of a coral tissue was developed as a series of plane-parallel pigmented solutions. In a similar manner to Teran and coworkers' (2010) "flat layer model", if it is assumed that the zooxanthellae are arranged in a layer that is index matched and free of scattering, the transmittance (T) through a tissue layer of depth z, when illuminated perpendicular to the surface, is given by:

$$T(\lambda) = 1 - e^{a(\lambda)z}$$

Where $a_{(\lambda)}$ is the absorption coefficient of the medium. $a_{(\lambda)}$ was calculated using the measured packaged chlorophyll specific absorption spectrum for *Symbiodinium* clade A2 grown under low light (Hennige et al. 2009). Clade A2 was selected as it exhibited the greatest degree of packaging amongst 7 subclades grown under low light (Hennige et al. 2009). It should be noted that subclade A2 is not typically associated with low light environments and is much larger than other clade A symbionts when grown in culture (Brading et al. 2011), however, the extent of its packaging is the most extreme available and therefore represents the most conservative spectrum available for the model. The chlorophyll concentrations of zooxanthellae used to build the model were taken from values obtained for shaded *Stylophora pistilata* in the Red Sea (8.3pg chl $cell^{-1}$) (Falkowski and Dubinsky 1981). The volumetric chlorophyll concentration of the model tissue was determined using the volumetric chlorophyll concentration of a symbiont cell (diameter = 10µm; Figure S5.2) and therefore a tissue depth of 10µm would be the equivalent of one homogenous layer of low light symbiont cell pigmentation. Nevertheless, it should be stressed that the depth of the tissue reported here should be treated as arbitrary, akin to optical depth, as changes in coral pigmentation typical of different species (compared to the *S. pistilata* data used in this study) and depth acclimation, either zooxanthellae numbers or pigment concentration, would alter the physical depth reported here.

The modelled ectoderm layer uses the extinction coefficients and quantum yields previously reported for the *M. cavernosa* PCRFP (Oswald et al. 2007) and ectoderm concentrations and depth calculated in this study for *Oxypora* sp.. The model assumes isotropic emission of fluorescence and thus 50% of the fluorescence is considered to be emitted in the upper hemisphere and therefore away from the zooxanthellae.

The model was run with measured irradiances from different depths on a Caribbean reef (interpolated from Dustan 1982). The intensity of the irradiance within the tissue, relative to the incident irradiance was recorded at 0.5µm intervals.

5.3.5. Chlorophyll fluorescence distribution

Chlorophyll fluorescence distribution with different excitation wavelengths was measured in a suspension of zooxanthellae (see Supplementary Methods) and in a brown colour morph of *Discosoma* sp. with weak cyan fluorescence. A corallimorpharian was selected due to the ease of dissection, in the absence of a skeleton, and because it possesses a tissue thickness similar to coral species studied.

The corallimorpharian was anesthetised by the gradual addition of $MgCl_2$ to seawater over 4 hours to a final concentration of 3.75% to avoid contraction and then fixed in 1% paraformaldehyde overnight. The paraformaldehyde solution was prepared with sterile filtered seawater rather than MilliQ water to maintain the pH and osmolarity.

Fixed tissue cross sections were cut with dissecting scissors and submerged in a petri dish of sterile seawater. The fibre optic probe from a fluorescence spectrophotometer was aligned to illuminate the surface of the tissue and provided 20nm wide spectral bands, centered at 400 to 660nm. The chlorophyll fluorescence distribution was recorded using a Canon EOS600D camera equipped with a 710nm (±25nm) filter. The integration times for the images were adjusted according to the fluorescence intensity at the sample wavelength to ensure sufficient dynamic range of the red channel while avoiding saturation. Integration times for the images ranged from 4 to 12 minutes.

The red channel from each image was post-processed using a background blank image to remove systematic noise and a median filter to remove random noise using Matlab.

5.4. Results and discussion

5.4.1. Depth distribution

A survey of coral colonies in the Gulf of Eilat revealed that the proportion of red fluorescent colonies within the coral assemblage increases with depth. While completely absent in the shallowest surveyed depth of 1m, the proportion of red fluorescent colonies increases almost linearly with depth below 10m, to 30% at 45m (Figure 5.1). There is a significant negative correlation (Spearman's Rank Correlation, $r = 0.92$, $p<0.05$) between the log transformed red irradiance and the proportion of red fluorescent morphs, supporting the rejection of the null hypothesis that the two variables are unrelated.

The red fluorescent colonies encountered at the deeper depths (30m and 45m) belonged to colonies of genera such *Favia*, *Fungia* and *Mycedium*, a genus where a PCRFP has previously been isolated (Alieva et al. 2008). Previous work on corals expressing DsRed-type RFPs have shown that these FPs are upregulated in response to light and are virtually absent in low light (D'Angelo et al. 2008) and therefore it is highly probable that the red fluorescence found in deeper waters is due to PCRFPs as they are constitutively expressed (Leutenegger et al. 2007b). Indeed, it has previously been shown that the relative abundance of the red colour morph of *M. cavernosa*, shown to host a red PCRFP (Oswald et al. 2007), increases with depth in a Caribbean

reef (Lesser et al. 2007). The distribution of this red fluorescence is interesting in light of the proposed photoprotective function of fluorescent proteins (Kawaguti 1944; Wiedenmann et al. 1999; Salih et al. 2000) as the distribution is opposite to what would be expected for a photoprotectant. In line with the findings of other studies, these results support the notion that functional heterogeneity exists amongst the difference groups of GFP-like proteins (Alieva et al. 2008; D'Angelo et al. 2008; Vogt et al. 2008).

Unlike the expression of other GFP-like proteins, such as the GFPs and the CPs, the expression of PCRFPs appears to be restricted to certain coral taxa. The PCRFPs previously identified and reported in this study are typically expressed by species located in deeper reefs (>20m) or depth generalist species (sensu. Bongaerts et al. 2010). The red fluorescent colonies encountered at the deeper depths (30m and 45m) in this survey belonged to colonies of genera such *Favia*, *Fungia* and *Mycedium*, a genus where a PCRFP has previously been isolated (Alieva et al. 2008). All corals currently identified as expressing PCRFPs belong to the families Favidae, Mussidae, Pectiniidae, Trachyphylliidae, Caryophyllidae and Fungidae (This study; Ando et al. 2002; Wiedenmann et al. 2004; Oswald et al., 2007; Alieva et al. 2008). These corals are typically fleshy corals that have massive or encrusting growth forms and, to date, PCRFPs have not been reported in fast growing, shallow water corals such as acroporids. It is proposed that the typical external light environment inhabited by these deeper water corals and the internal light environment inside their voluminous tissues results in limited light penetration (see sections 5.4.2 and 5.4.4) and therefore the need for PCRFPs.

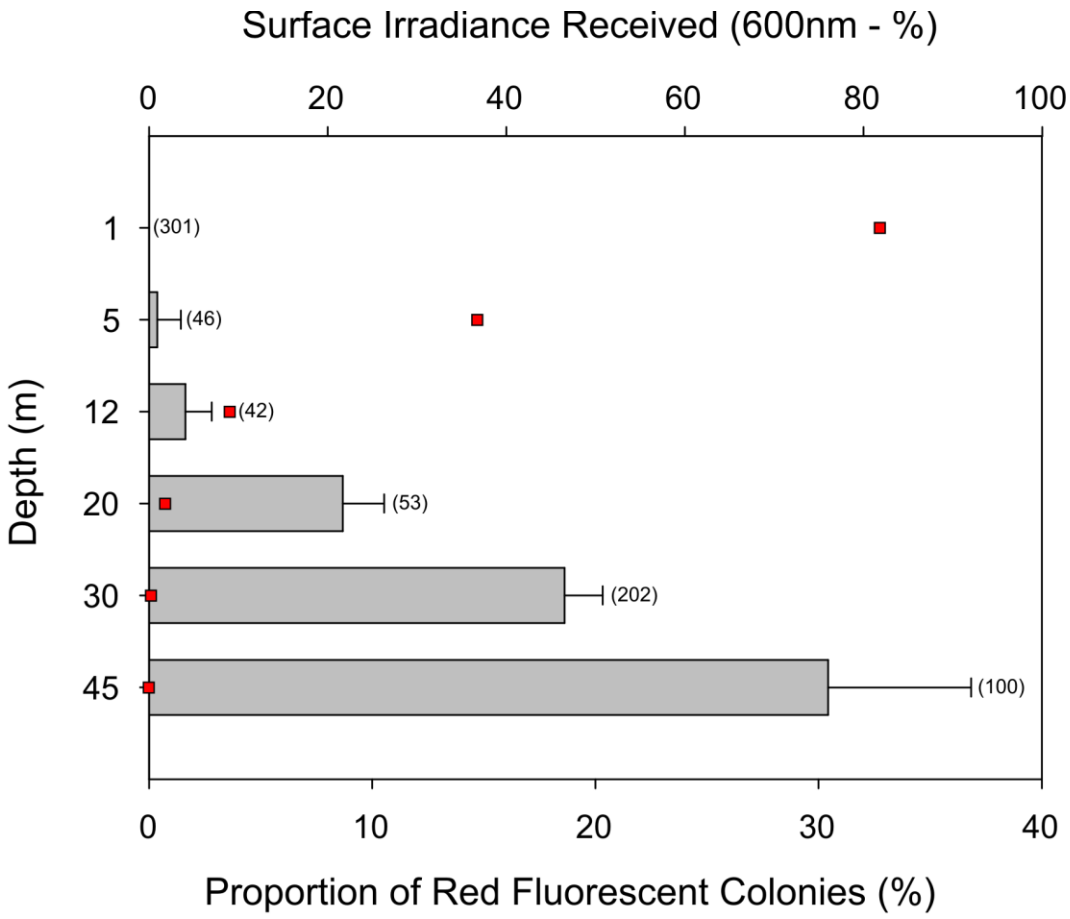

Figure 5.1: Distribution of red fluorescent coral colonies with depth. Bars indicate the mean proportion of red fluorescent colonies to the total number of fluorescent colonies at six different depths in the Gulf of Eilat. Error bars show the standard deviation of the means. The mean number of colonies sampled per transect is given in brackets. The approximate proportion of red light (600nm) received at that depth is shown by the red squares. The data was interpolated from averaged Kd values for the Gulf of Eilat in Stambler (2006).

5.4.2. Impact of light regime on PCRFPs

To explore the factors that could be driving the depth distribution of red fluorescence in the Red Sea corals and evaluate whether photoconversion acts to 'tune' the excitation properties to the prevailing light conditions, this study evaluated the impact of light regime on the spectral properties of the red fluorescent colour morph of *M. cavernosa*. The reflectance spectra under the two simulated light conditions (shallow and deep) both show the contribution of the red fluorescent protein peaking at ~580nm (Figure S5.3). The contribution of red fluorescence to the *M. cavernosa* reflectance spectra has previously been observed in the field by Mazel (2006). The excitation and emission properties of the *M. cavernosa* PCRFP (Figure 5.2a) are

relatively consistent with PCRFPs from other species (Table 5.1 and Oswald et al. 2007). In all three species analysed in this study (*Echinophyllia* sp., *Oxypora* sp. and *M. cavernosa*) the emission is characterised by a peak at 581nm with a vibronic sideband at around ~630nm. Due to intratetrameric FRET, the excitation properties of the FPs are comprised of two components, the unconverted green form (excitation peak at 507nm) and the photoconverted red form (excitation peak at 571nm). The relative contribution of the two forms depends on the degree of photoconversion and thus the exposure to light of wavelengths ranging from ~360-430nm (Wiedenmann et al. 2004b). The corals grown under the deep water conditions have a greater contribution of the unconverted green form to their excitation spectrum due to the low photon fluxes and reduced availability of UV-violet light required for photoconversion. Nevertheless, there are differences between different species grown under the same conditions and therefore, it appears to be a combination of the prevailing and the conversion efficiency that influences the excitation properties of these proteins. It is postulated that the propensity of the PCRFP to photoconvert serves as an additional control on the proportion of the two forms (Table 5.1). This is most likely due to structural differences in the proteins as retracing the evolution of the ancestral forms of a PCRFP found differences in the photoconversion rate between different mutants (Field and Matz 2010). In addition, observations of *M. cavernosa* in the Caribbean show that the contribution of the green form to the excitation spectrum in the field can exceed the values observed under the deep water conditions used in this study (Lesser et al. 2004).

Coral	Green Excitation Peak	Red Excitation Peak	Emission peak	Shallow Conditions Green:Red Excitation Ratio	Deep Water Conditions Green:Red Excitation Ratio
Montastrea cavernosa	507nm	571nm	581nm	0.7	1.3
Oxypora sp.	508nm	570nm	581nm	0.5	0.9
Echinophyllia sp.	509nm	570nm	581nm	0.5	0.5

Table 5.1 Spectroscopic properties of photoconvertible red fluorescent proteins from three different coral species. Excitation ratios are the mean of five independent measurements taken from different areas on single colonies (~25cm^2 – 80cm^2) grown under the each light condition.

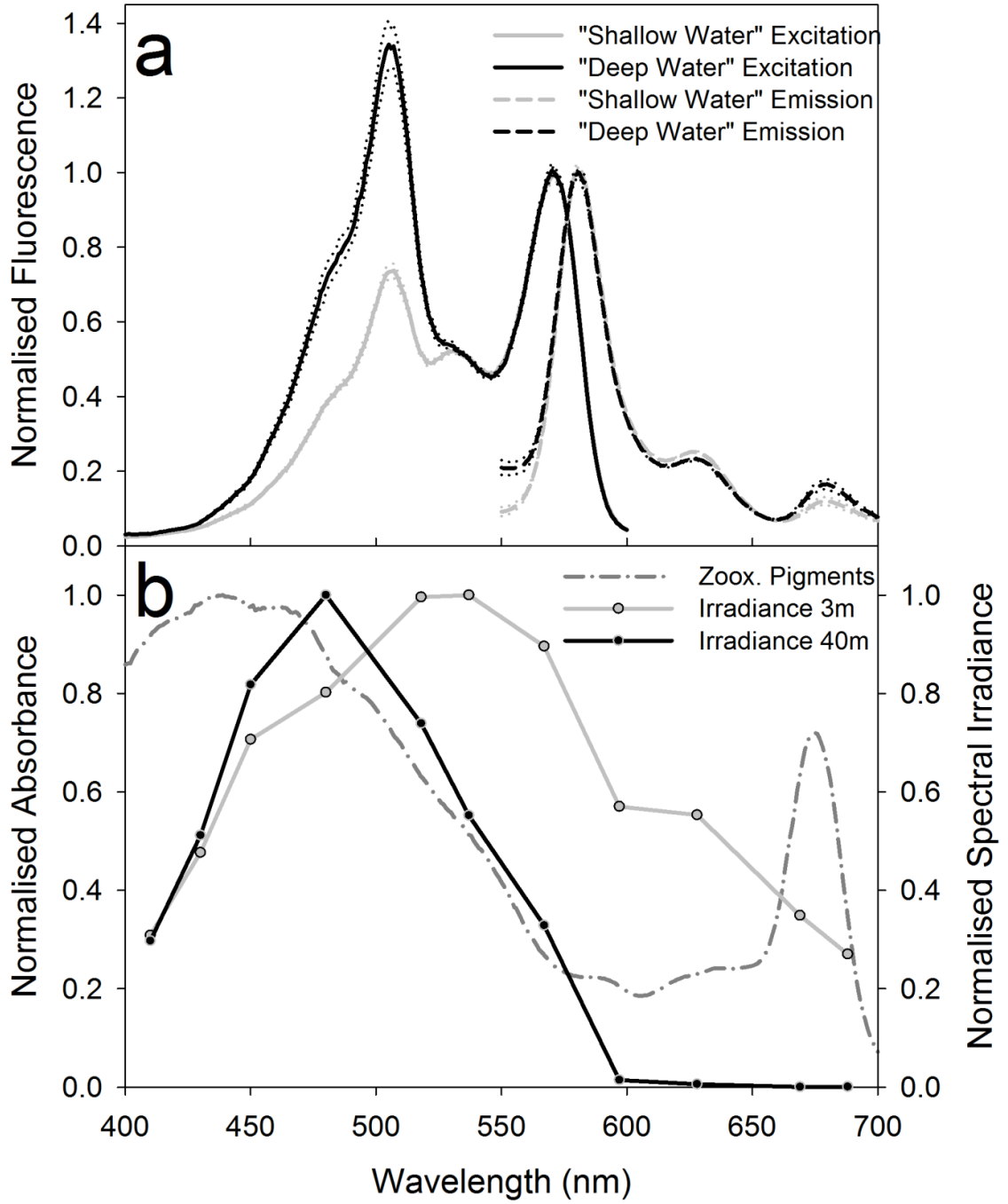

Figure 5.2. Optical properties of *Montastrea cavernosa* red fluorescence in relation to the underwater light field and absorption by the algal symbionts' pigments. (a) The *in vivo* excitation (emission at 630nm) and emission (excitation at 485nm) spectra of *M. cavernosa* red fluorescence under shallow and deep water conditions. The spectra are the mean of 5 independent measurements taken from different areas on single colonies (~25cm^2 – 80cm^2) grown under the each light condition and the ±SD are shown by the dotted lines. The excitation spectra are normalised to the red excitation peak. The emission spectra are normalised to the peak FP emission at 581nm. (b) Absorption of a symbiont pigment extract from *Oxypora* sp., a coral expressing a

PCRFP with similar properties to *M. cavernosa* (see Table 5.1) and representative irradiance at 3m and 40m depth (Dustan, 1982). The spectra are normalised to a maximum value of 1.0.

5.4.3. Spectral suitability of PCRFPs

The PCRFPs represent a unique group of coral FPs with distinct spectral and regulatory patterns. In order to understand the potential function, it is necessary to consider the optical properties of the FP in the context of the underwater light environment and the corals' internal light environment.

The underwater light field changes dramatically with depth due to the selective attenuation of different wavelengths of light (Kirk 1994). Corals in deeper waters are subject to light predominantly in the blue-green region of the spectrum due to the strong attenuation of red light by seawater (Figure 5.2b) (Smith and Baker 1981). PCRFPs emit in wavelengths that are largely absent in deeper reef waters and it is interesting to note that the excitation spectrum is shifted towards green dominated excitation in the deep water conditions. This shift represents a shift towards wavelengths that are more abundant at depth compared to the wavelengths required to excite the red excitation band. In essence, the weaker photoconversion in deeper waters results in the transformation of light that is abundant at depth into wavelengths that are less abundant/absent.

The chromaticity of the internal light field is largely controlled by the absorption of the algae's photosynthetic pigments (Kuhl et al. 1995). The shape of the absorption spectrum of the algal pigments isolated from *Oxypora* sp. is similar to that of the underwater light field at depth (Figure 5.2b), with strong absorption by the pigments at the most abundant wavelengths and weaker absorption between 550nm and 650nm. The red chlorophyll absorption band is a region of the electromagnetic spectrum that is strongly absorbed by seawater and therefore is unlikely to be directly excited except in the shallowest waters. The emission of the FP is located between the major absorption peaks of the algal pigments and therefore raises questions as to the purpose of diverting photons away from the major absorption bands to a region that is weakly absorbed. It has been observed in higher plants that the green 'window' in the absorption spectrum enables increased penetration of light into the deeper tissue layers (Nishio 2000; Vogelmann and Evans 2002). As a typical response of the coral holobiont to decreasing light intensities is to increase the concentration of algal cells and/or their pigments (Falkowski and Dubinsky 1981; Titlyanov et al. 2001), then the

reduction in light penetrating these optically dense tissues could become a limiting factor. The poor penetration of light in these tissues could be enhanced as deeper water corals (>20m) live in a light environment that is dominated by wavelengths that correspond to the peak wavelengths of light absorption by the algal pigments and therefore penetrate poorly in the tissue. This may limit the extent with which a coral can pack its tissues with algal symbionts/their pigments as there needs to be sufficient light penetration to maintain the symbiont population. Poor penetration of light may help to explain the observed decreases in symbiont pigment concentrations at more extreme depths (Fricke et al. 1987; Kahng et al. 2012) and the requirement for efficient multiple scattering in these individuals (Kahng et al. 2012).

It is postulated here that the PCRFPs modify the internal light field to improve light penetration in the coral tissue by shifting poorly penetrating wavelengths abundant at depth to those that will penetrate deeper in the host tissues. The post-translation modification of the chromophore would enable acclimation to the different spectral light regimes found at depth and could potentially enable depth generalist species such as *M. cavernosa* (Bongaerts et al. 2010; Lesser et al. 2010) to inhabit a wide range of light environments.

5.4.4. Light penetration in anthozoan tissue

5.4.4.1.Tissue Model

To theoretically evaluate whether PCRFPs emit at wavelengths of greater tissue penetration and if PCRFPs were capable of altering the penetration of light in coral tissues, a 1D tissue model was developed using parameters measured in this and other studies (see Materials and Methods). Firstly, different wavelengths of light were passed through the model tissue without a PCRFP and intensity of light recorded at different tissue depths (Figure 5.3). It can be seen from the distribution of light within the tissue that light which is weakly absorbed by the photosynthetic pigments penetrates deeper, as observed in higher plants (Vogelmann and Evans 2002). Notably, the penetration of light used to predominantly excite the PCRFP at depth (~500nm) penetrates much shallower compared to the emission wavelength of the PCRFP (~580nm) with 12% and 45% of light transmitted at 10µm (the equivalent of one “shaded”-zooxanthellae layer), respectively.

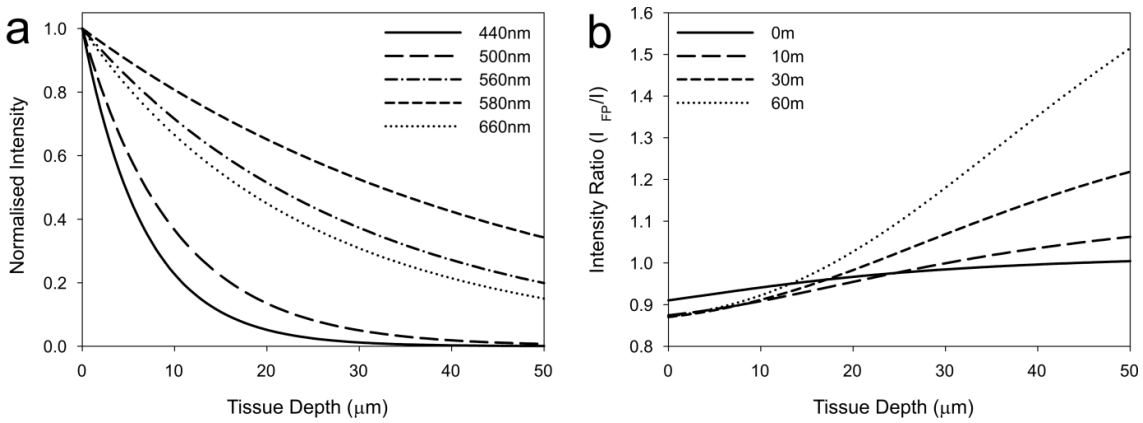

Figure 5.3. Modelled light penetration through coral endodermal tissue. (a) Penetration of different wavelengths through the modelled coral tissue without FP in the ectoderm. (b) Ratio of intensity at different tissue depths between coral tissue expressing a PCRFP and control tissues without the PCRFP. The ratios are shown under 4 different irradiances associated with different depths.

The model was subsequently used to compare the effect of PCRFPs on the penetration of light in tissues exposed to irradiances from different depths (Figure 5.3b). At shallow tissue depths, the intensity of light within the tissue is less for the PCRFP expressing tissues compared to the tissue where the PCRFP is absent i.e. less than 1.0. This is due to the absorption of light by the PCRFP in the ectoderm and despite reemission, it is lower than the PCRFP free tissue due to the following three factors. Firstly, light that would otherwise be strongly absorbed in the upper tissue layers is re-emitted at wavelengths that will penetrate deeper lowering the initial absorption. Secondly, fluorescence is an inefficient process and photons are lost as heat (i.e quantum yield<1). Lastly, the model assumes isotropic emission of fluorescence and consequently 50% of photons are emitted in the upper hemisphere away from the symbionts. Nevertheless, anisotropic emission has been observed in GFP-like proteins (Knight et al. 2002) and ectoderm structure may alter the proportion of fluoresced photons emitted into the external environment.

At the deeper tissue depths, the response of the model tissue differs between water depths. At the shallow water irradiances, the intensity of light penetrating deeper in the FP expressing tissue is similar to the tissue without the PCRFP. However, as the incident irradiance becomes spectrally narrower with depth, the presence of the PCRFP enables greater penetration of light with the greatest penetration relative to the control tissue under the 60m irradiance. It is important to consider that the model does not incorporate scattering, however, scattering would act to increase the pathlength and as

it optimally amplifies wavelengths of weak absorption by the zooxanthellae pigments (Kuhl et al. 1995), the effects of the PCRFP may be underestimated.

5.4.4.2.Experimental evaluation

The outcomes of the model were tested experimentally on cross sections of the corallimorpharian *Discosoma* sp. (Figure 5.4) and zooxanthellae in suspension (Figure S5.4) using chlorophyll fluorescence as an indicator of the distribution of absorption by the zooxanthellae. *Discosoma* was selected for analyses as it had a tissue thickness similar to the studied species and the absence of a skeleton enabled easy dissection and sample preparation. The absence of a skeleton will remove the effects of skeletal amplification of the internal light field by multiple scattering (Enriquez et al. 2005) but under these experimental conditions, the chlorophyll fluorescence was very low/absent in the lower tissue zooxanthellae layer. Nevertheless, as previously discussed, amplification by multiple scattering would actually serve to increase the effect of FP emission on the redistribution of light as amplification is greatest for wavelengths that are most weakly absorbed (Kuhl et al. 1995).

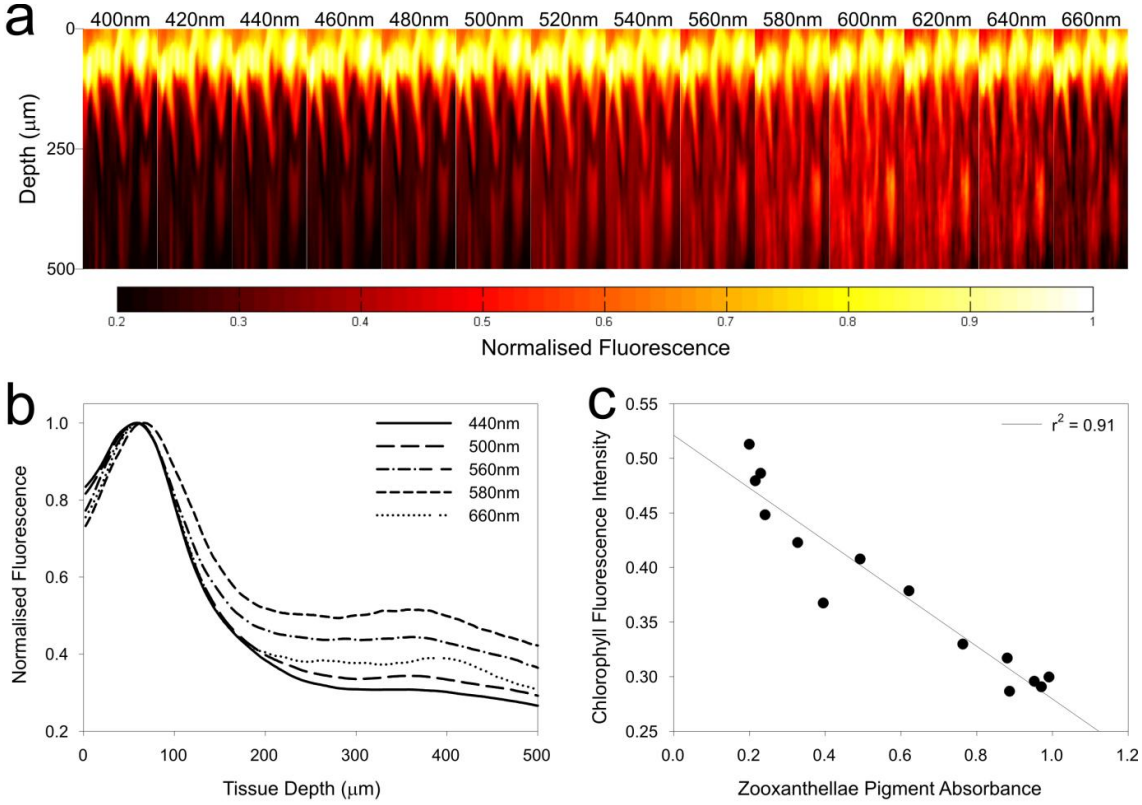

Figure 5.4. Distribution of tissue chlorophyll fluorescence in response to different excitation wavelengths. (a) Images of chlorophyll fluorescence distributions in the brown *Discosoma* sp. morph under different wavelengths. Intensities are normalised to a maximum value of 1.0. (b) Mean chlorophyll distribution at different depths

throughout the tissue. (c) Relationship between mean chlorophyll fluorescence intensity between 0.25-0.5mm depth in the tissue and the absorption of the symbionts' pigments. The absorption of the zooxanthellae pigments is normalised to a maximum value of 1.0. The original maximum absorbance was less than 1.0 and therefore within the linear range of the Beer-Lambert law.

Under shorter wavelengths of light (400-500nm) the chlorophyll fluorescence distribution in the *Discosoma* tissue is largely concentrated in dense aggregations of zooxanthellae at the oral ectoderm/endoderm boundary with relatively little chlorophyll emission deeper in the tissue cross section (Figure 5.4a). Under longer excitation wavelengths around 580-620nm, there is a greater proportion of fluorescence deeper in the tissue which decreases as the illumination source approaches 660nm. The variation in chlorophyll absorption with tissue depth, indicated by fluorescence, correlates with the absorption spectrum of the photosynthetic pigments of zooxanthellae (Figures 5.4c) and shows a similar pattern of the wavelength distribution with depth as seen in zooxanthellae in suspension (Figure 4) and the modelled tissue (Figure S5.4). As the zooxanthellae are largely responsible for the optical properties of corals (Hochberg et al. 2004), this is to be expected and agrees with the findings of Kuhl and co-workers (1995) who found that the internal light field of a *Favia* sp. colony at 0.1-0.2mm is dominated by orange-red wavelengths. Nevertheless, the impact of changing spectral quality of incident irradiance with increasing depth on the corals' internal light environment has previously received little attention. The data presented here, particularly in the context of the underwater light field, suggest that the light penetration and the impact of water depth will place restrictions on a coral's symbiont populations and their associated pigmentation.

The findings of the *in silico* analyses and the experimental analyses on *Discosoma* sp. tissue and zooxanthellae suspensions support the hypothesis that the wavelengths of PCRFP emission penetrate deeper in the coral's tissue and it is evident that this is impacted by the prevailing light conditions on deeper reefs. The next challenge is to overcome the technical difficulties associated with directly assessing the impact of PCRFP on the internal light field.

5.4.5. Challenges in experimentally testing the impact of PCRFPs

Analyses of differences in light penetration in higher plants have typically used similar methodologies employed in this study (Vogelmann 1993; Vogelmann and Evans 2002). These methodologies are limited, however, in their suitability for assessing the effects

of PCRFPs on the penetration of light due to cross-talk between the two fluorophores. Spectral decomposition of the two fluorophores is not possible due to the absorption of FP fluorescence by the photosynthetic pigments and the reabsorption of chlorophyll fluorescence, while chlorophyll fluorescence imaging also detects a dominant FP emission signature (See Appendix section "Overlap of red FP and chlorophyll emission"). Future work should focus on measurement of carbon fixation tissue profiles with radiocarbon (Evans and Vogelmann 2003) as it should enable experimental assessment of the proposed PCRFP light modification without the challenges posed by the spectral overlap of the two fluorophores.

5.4.6. Biological significance

There are two important questions that need to be answered regarding the proposed function; is the redistribution of light occurring and does it have any biological significance. As PCRFPs appear to be solely expressed by certain coral families, it is postulated that the typical external light environment inhabited by these deeper water corals and the internal light environment inside their tissues necessitates the need for a mechanism to improve penetration of light through the symbiont population. Based on the observations made here regarding the penetration of different wavelengths through symbiont pigmentation and the ability of other GFP-like proteins to alter the light field experienced by the symbionts (Smith et al., 2013 – Chapter 4), it certainly seems possible that PCRFPs could modify the internal light field, although this requires further testing once the experimental challenges associated with these measurements can be overcome. The second question, is it biologically significant, is arguably more important, however, much harder to experimentally test. It is evident from the modelling exercise that the proposed redistribution of light by PCRFPs could enhance the availability of light deeper in the coral tissue under deep water conditions but it is unclear whether the amount of light redistributed is sufficient to enhance deeper-tissue photosynthesis and if it is worth the associated costs. It is important to consider that for a given number of photons absorbed by the PCRFP, a fraction in the order of 30-50% will be lost due to the quantum efficiency of the fluorescence and a further 50% will be lost if it is assumed that emission is isotropic as half of the emission will be directed to the upper hemisphere, i.e. away from the zooxanthellae. Consequently, between ~25-35% of absorbed photons will be converted into red fluorescence in the direction of the zooxanthellae and depending on tissue concentration, could be considered a considerable sink for light energy. In contrast to previous assertions that pigmentation in corals is cheap (Leutenegger et al. 2007b), the potential losses of light energy by PCRFPs suggest that expression of PCRFPs could be expensive for low light corals. Considering the possible expense of these proteins, their adaptive evolution

(Field and Matz 2010) and the observed ecological distribution in the Red Sea, it is conceivable that these proteins perform an important function in low light and mesophotic corals, highlighting the need for further research into the proposed function and other potential functions of these proteins.

5.4.7. Wider Implications

Mesophotic refugia have been proposed as a safe haven for corals in the face of future climate change and could act as potential sources of larvae for future shallow water reefs under stress (Glynn 1996; Bongaerts et al. 2010). This hypothesis has recently been examined and it was concluded that only certain species are capable of inhabiting reefs ranging from the shallow waters to the deeper mesophotic reefs (Bongaerts et al. 2010) and this is likely to result from the challenges of acclimating to the different light environments. This study has identified that it is not only the change in total irradiance that is important but also the spectral composition of the irradiance at deeper depths and that the change in chromaticity impacts light penetration through the coral's tissues. It is proposed that the expression of the PCRFPs and their photoconversion extend the ecological niche of these species enabling them to acclimate to a range of light environments. The species expressing PCRFPs could potentially be the corals most likely to benefit from mesophotic refugia in the future. In addition, under the backdrop of the changing water quality on reefs (Fabricius 2005; Hennige et al. 2010), the ability to acclimate to a range of light environments will become increasingly important for the survival of corals and could further benefit species expressing PCRFPs.

5.5. Supplementary methods

5.5.1. Reflectance spectroscopy

Reflectance spectra were recorded using a reflectance fibre optic probe connected to an Ocean Optics USB4000 and a tungsten halogen light source (HL-2000-HP). The reflectance measurements were recorded according to a published protocol (Mazel et al. 2003b) and using a Spectralon 99% diffuse reflectance standard as a reference. Replicate measurements were taken from different regions of the colony.

5.5.2. Chlorophyll fluorescence distribution in a zooxanthellae suspension

Clade C zooxanthellae were isolated from *Euphyllia paradivisia* grown under the deep water conditions. Tentacles were cut from two polyps and the tissue was disrupted using a potter homogeniser. The tissue slurry was filtered using a 0.1mm mesh. The homogenate was then centrifuged at 700xg for 5 minutes to pellet the symbionts. The symbiont was pellet was resuspended in 50ml of sterile filtered seawater and centrifuged again. This wash step was repeated twice. After the wash steps, the zooxanthellae pellet was resuspended in 1ml and the concentration of zooxanthellae was determined using a haemocytometer and epifluorescent microscope. The concentration of the zooxanthellae suspension was then adjusted to $5x10^6$ cells cm^{-3}.

The zooxanthellae suspension was loaded into a cuvette and the fibre optic probe of a fluorescence spectrophotometer was inserted into the suspension to provide 20nm wide wavebands of excitation with central wavelengths ranging from 400 to 660nm. The zooxanthellae were kept in suspension by gentle mixing with a magnetic bead. Images of chlorophyll fluorescence were taken with a Canon EOS600D through a 710nm (±25nm) filter.

5.5.3. Confocal microscopy

A coenosarc tissue sample of *Oxypora* sp. was fixed in 2% paraformaldehyde in sterile filtered seawater for 24 hours at 4˚C. After fixation, the tissue sample was washed three times in sterile filtered seawater for 15 minutes. The sample was cut using a scalpel and imaged on a Leica SP5 confocal microscope using a x20 objective. The fluorescence was imaged using two laser lines, 561nm for the red fluorescent protein

(Em: 570-630nm) excitation and 633nm for the chlorophyll excitation (Em: 670-740nm).

5.6. Supplementary Figures

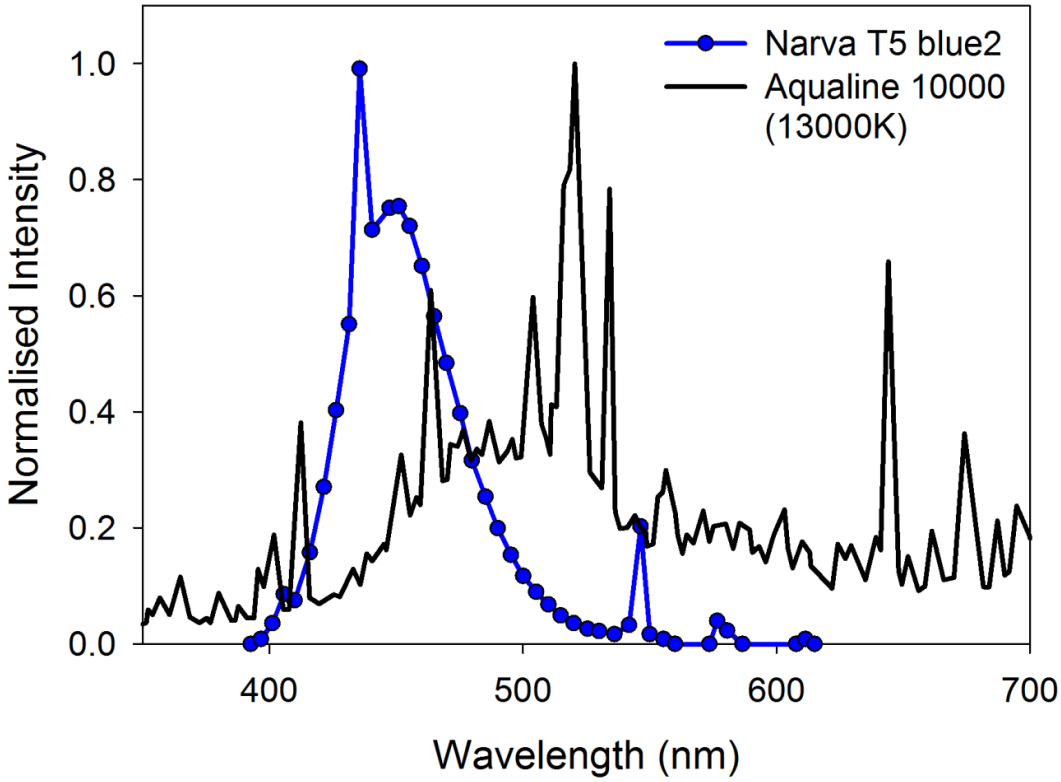

Figure S5.1. Normalised spectral output of the light sources used in this study. The Aqualine 10000 (13000K) was used for the shallow water light conditions whereas the Narva T5 blue2 was used for the deep water conditions.

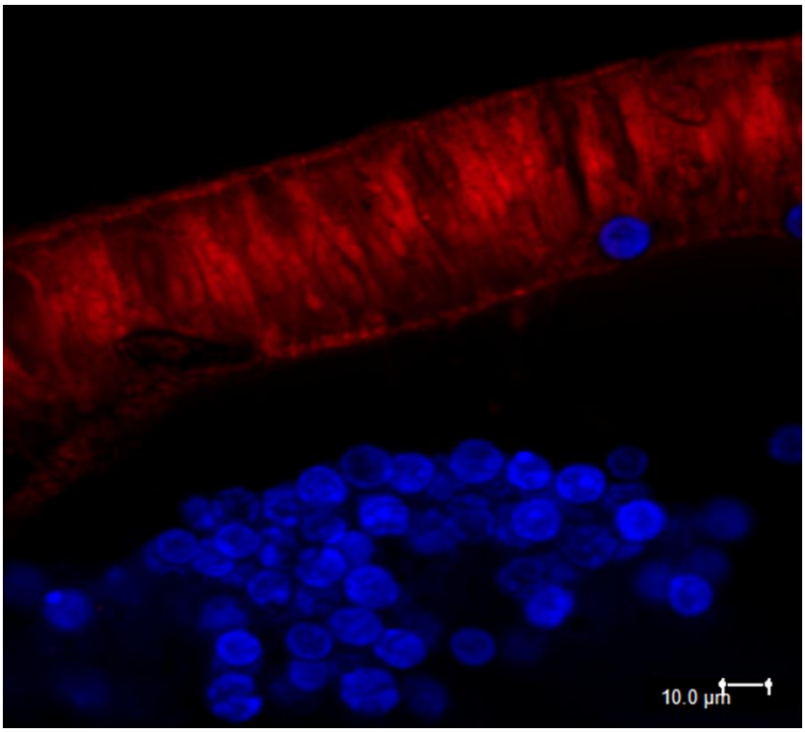

Figure S5.2. Confocal imaging of *Oxypora* sp. ectoderm and upper endoderm. The red channel shows the red fluorescent protein emission (570-630nm) and chlorophyll emission (670-740nm) is shown by the blue channel.

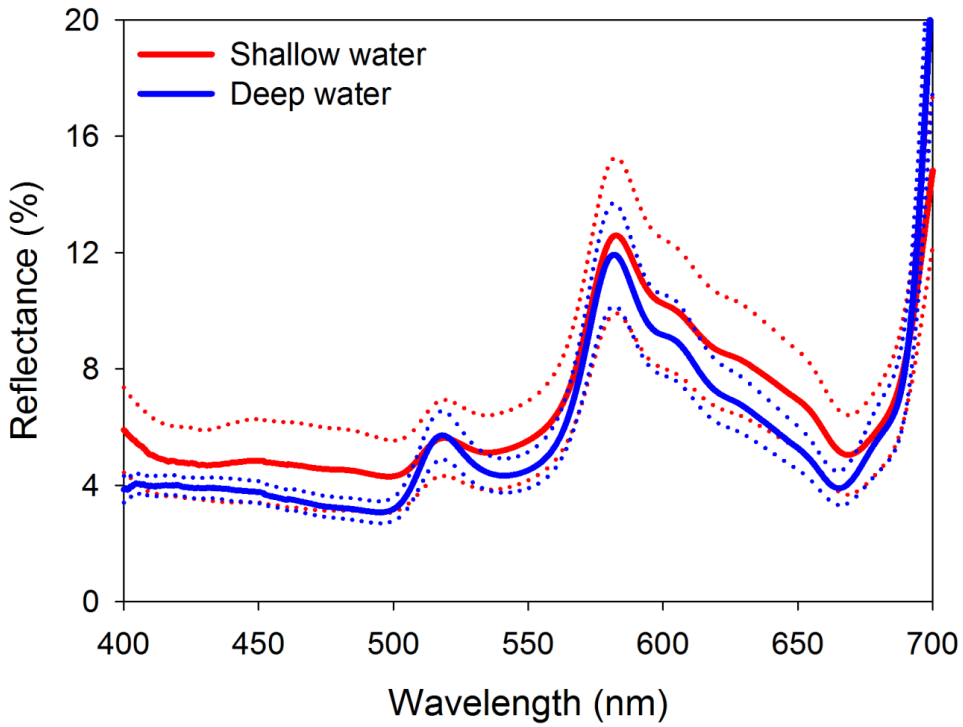

Figure S5.3. Reflectance spectra of two red *Montastrea cavernosa* colonies grown under simulated shallow and deep water light regimes. Spectra are the mean of 5 independent measurements and the ±SD are shown by the dotted lines.

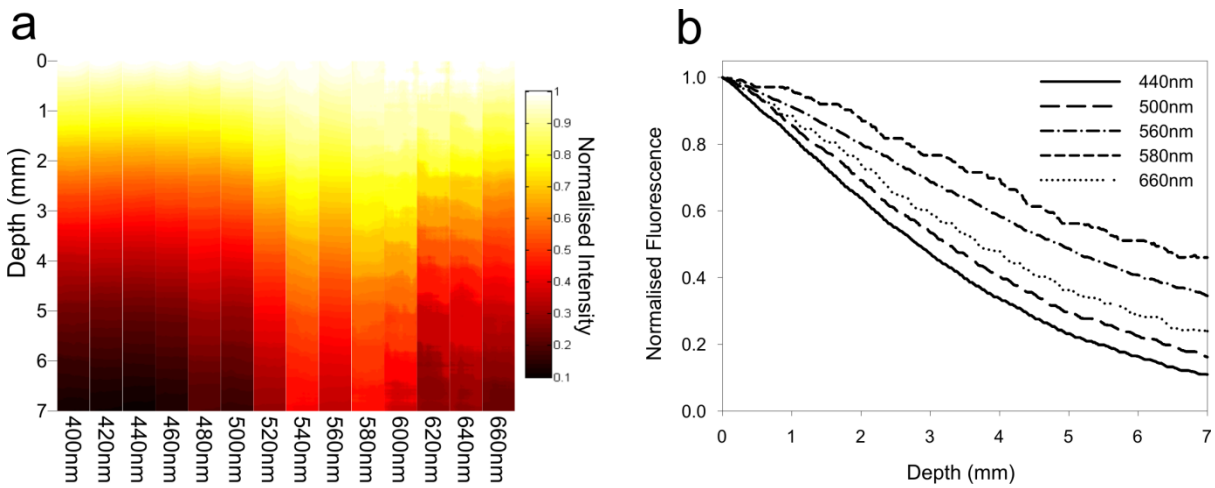

Figure S5.4. Chlorophyll fluorescence through a zooxanthellae suspension. (a) Images of chlorophyll fluorescence distribution under different excitation wavelengths. Intensities are normalised to a maximum value of 1.0. (b) The corresponding mean chlorophyll fluorescence at different depths within the suspension under different excitation wavelengths.

CHAPTER 6. Conclusions

6.1. Identification and analysis of a metal binding site in the red fluorescent protein asFP595/1

6.1.1. How prevalent is metal binding amongst GFP-like proteins?

This study identifies, for the first time, the specific amino acids responsible for cobalt chelation in a naturally occurring protein from the GFP family. A combination of *in silico* analysis and site directed mutagenesis enabled identification and experimental testing of the two histidine amino acids (His230 and H231) in the C terminus of the FP, asFP595/1, from *A. sulcata*. The binding site was confirmed by both forward and reverse mutations on asFP595/1 and asulCP, respectively. It should be noted, however, that there may be other amino acids involved although motifs comprising of two histidines have been identified in other cobalt binding proteins (Feese et al. 2001; Hsin et al. 2008).

The C terminus motif was present in three GFP-like proteins from *A. sulcata* and alignments with other proteins from this family show that it is also present in two FPs from *Discosoma* spp. (DsRed and dsFP483) and a CP from *Actinia equina* (aeCP597). Overall, the low prevalence of the binding site indicates that metal binding may not be widespread amongst the naturally occurring GFP-like proteins and therefore not represent a widespread biological function. Furthermore, the weak binding observed in the DsRed homolog, dsFP586, in this study indicates that the exposure of the motif is an important factor in determining chelation to immobilised metal ions and is in agreement with a previous study attempting to engineer histidine based metal affinity sites to a FP (Tansila et al. 2008). As the extended C terminus is not a feature of coral and corallimorpharian GFP-like proteins, it appears that metal binding in these proteins may be confined to certain taxonomic groups.

6.1.2. How do interactions with transition metals impact on the proteins properties?

Previous studies on DsRed have shown that the presence of transition metals, namely copper, can influence the intensity of the protein's fluorescent emission (Eli and Chakrabartty 2006; Sumner et al. 2006). Here, it is demonstrated that cobalt, in addition to copper, can influence of the emission of asFP595/1. One of the main

findings of Chapter 3 was that the binding site associated with affinity to the immobilised metal ions appears to be different from the site responsible for quenching. This is unsurprising considering the distance of the binding site from the chromophore, however, affinity and copper IMAC retention in DsRed were assumed to originate from the same, yet unknown, site (Rahimi et al. 2007a). Consequently, it is possible that other GFP-like proteins may possess the unexposed metal affinity sites that whilst not binding to immobilised ions, may still interact and quench in the presence of ions such as copper.

Another important finding was that the proteins aggregate in the presence of metal ions and that the aggregation tendencies are different amongst the three mutants generated in this study. The exact mechanism for the binding is unclear considering that the two non-binding mutant proteins (according to the IMAC assay) show very different aggregation tendencies and requires further exploration. While previous work to reduce aggregation in GFP-like proteins has focused on the N terminus of the protein (Yanushevich et al. 2002), the results presented here demonstrate that mutations in the C terminus can reduce aggregation.

6.1.3. Limitations

The main limitation of the metal binding in GFP-like proteins study is that the motif identified appears not to be the only site involved in metal interactions. The motif was identified using immobilised metal affinity as a screening method for metal binding amongst 11 GFP-like proteins. The IMAC assay is a novel application of a standard protein purification technique; however, it is limited to screening for exposed motifs. Despite mutants with reduced metal affinity, metal-induced quenching was present in the two mutant proteins, highlighting that metal interactions were occurring elsewhere in the protein. Further work is required to assess whether the quenching assay could serve as a screening method for the quenching motif.

The biological interpretation of the metal binding could be limited by the choice of cobalt as the immobilised metal ion. Cobalt was selected due to the higher specificity of the matrix compared to copper as copper resins can bind proteins with a single exposed histidine on the protein's surface (Gaberc-Porekar and Menart 2001) and therefore are unlikely to select for a more specific binding site. As such, the cobalt resin may underestimate the strength and prevalence of binding, however, it is considered acceptable as it reduces the risk of false positives. Nevertheless, while cobalt is found in association with proteins, there is a higher prevalence of

metalloproteins binding to copper (Hsin et al. 2008) and therefore could be considered as a more biologically relevant metal to study.

6.2. Screening by coral GFP-like chromoproteins supports a role in photoprotection of the zooxanthellae

6.2.1. Do CPs provide screening for corals' algal symbionts?

With the same experimental approach used to challenge the photoprotective function in GFPs, this study shows a clear and wavelength specific reduction in chlorophyll excitation associated with their non-fluorescent homologs, the chromoproteins. This is the first time that a direct reduction in chlorophyll excitation has been demonstrated for a group of GFP-like proteins.

CPs were selected as a target for this study due to their typically larger extinction coefficients compared to the GFPs (Alieva et al. 2008), reports of enhanced fitness of CP morphs under high light (Dove 2004) and that all absorbed photons are dissipated as heat rather than re-emitted. In this study, the screening by CPs is shown for CPs spanning the range of their peak absorption (562-601nm) and for symbiont clades A, C and D. The reduction in the chlorophyll excitation shows remarkable agreement with the absorption properties of the respective CP and can reduce chlorophyll excitation by up to 50% at the wavelength of peak CP absorption.

6.2.2. Are the wavelengths absorbed by the CPs damaging?

The proposed photoprotective function for the CPs has been questioned largely due to the absorption of the CPs occurring in a region of the spectrum where the absorption by the zooxanthellae's photosynthetic pigments is weak. The results of this study demonstrate that the excitation of chlorophyll by yellow-orange (560-610nm) light is much greater than would be anticipated by pigment data alone, consistent with expectations based on pigment packaging (Duysens 1956; Stambler and Dubinsky 2005) and multiple scattering within the skeleton (Enriquez et al. 2005). This is also supported by measurements of photosynthetic action spectra (Halldal 1968; Kuhl et al. 1995), indicating that these wavelengths are important in the photobiology of corals. Furthermore, the light stress experiment demonstrates that supraoptimal fluxes of orange light were sufficient to induce photodamage and bleaching.

6.2.3. Limitations

Studies into the effects of any GFP-like protein or other host based mechanisms on the photobiology of corals will generally be limited by the availability of adequate controls due to the numerous confounding variables such as symbiont and host genotypes, symbiont pigmentation and antioxidant defences. In this study, it was necessary to evaluate the impact of the expression of CPs on the chlorophyll excitation spectrum against suitable control tissues. After extensive consideration, it was felt that no single control was 'ideal' due to potential differences in the aforementioned variables and consequently a combination of different control tissues was used for the assessment of screening by CPs. The demonstration of screening was consistent across comparisons with various controls (light vs shaded tissue, CP pigmented morph vs brown morph, growth margin vs central tissue etc), supporting the assertion that the changes observed resulted from CP expression rather than other variables.

While the response of the different controls was remarkably consistent in terms of the shape of the chlorophyll excitation spectrum, the majority of these controls were unsuitable for the light stress assay. Corals grown under different light regimes or hosting different symbiont clades will behave differently under high light intensities and therefore, the only suitable control available was to use two colour morphs of the same species (purple and brown) that hosted the same symbiont clade. Although comparisons of physiological fitness amongst different colour morphs of the same species have been used before (Kawaguti 1944; Takabayashi and Hoegh-Guldberg 1995; Salih et al. 2000; Dove 2004; Salih et al. 2006; Leutenegger et al. 2007a; Dove et al. 2008), it should be noted that due to the potential for other confounding variables, more extensive comparisons (i.e. using colour morphs of multiple species) are required before a photoprotective function can be assigned.

Lastly, the light stress experiment used in this study is rather extreme in comparison to the conditions normally experienced by shallow water corals. The intensity of 1000µmol m^{-2} s^{-1} is certainly less than the midday irradiances in shallow waters but as it was constant over the 12 hour light cycle, it represents a light regime different to field conditions (e.g. Gorbunov et al. 2001). Furthermore, corals in shallow water would not experience a light field comprised solely of the narrow waveband provided by the LEDs. The narrow spectral range was selected to enable experimentation of the specific effects of the CP (which absorbs in this region of the spectrum) and therefore, while providing a means of testing the direct effects of the CP expression, it does not relate to a 'real world' light climate. Other wavelengths of light outside of the LED emission spectrum, most notably blue light, could have influenced the corals' response

to the light stress as certain physiological processes are regulated by blue light (Levy 2003; Levy et al. 2006b; D'Angelo et al. 2008; Kaniewska et al. 2009).

6.3. Internal light modification by photoconvertible red fluorescent proteins as a potential mechanism for depth acclimation in corals.

6.3.1. What is the ecological distribution of red fluorescence morphs?

The depth distribution of red fluorescent morphs has received little attention in the literature and this is likely to result from their low abundance in shallow waters. After noting that the photoconvertible RFPs are generally associated with species whose range typically extends into low light and mesophotic habitats, this study set out to assess the depth distribution of these colour morphs. The results presented in Chapter 5 demonstrate that red fluorescent morphs are more abundant at deeper depths, with a linear increase in the proportion of red fluorescent morphs below 10m, down to the maximum depths surveyed. The results are currently limited to two sites from the Gulf of Eilat and therefore require further surveys to explore the depth distributions in less oligotropic waters where the spectral light distribution is likely to differ from the current sites. This is particularly important considering the forecasted decline in water quality on reefs associated with expansion of coastal populations (Fabricius 2005). The dramatic effects of coastal populations on the water quality of reefs is already evident (Hennige et al. 2010) and this may act to alter the depth distribution of the red fluorescent morphs seen here. Nonetheless, considering the rate of increase is constant below 10m up to the maximum depth studied, it would be interesting to explore deeper depths although this is largely limited by the technicalities of diving at these depths.

6.3.2. How do the optical properties of the photoconvertible red fluorescent proteins relate to their environment? Do these provide clues to their biological function?

The requirement of UV-violet light for post-translational modification of PCRFPs results in a strong contribution of the green form of the protein to the excitation of red fluorescence in deeper waters. For corals in deeper (>20m) water environments, this causes the absorption of wavelengths of light that are dominant at depth and transfers

them to wavelengths which are virtually absent. Analysis of light penetration through symbiont populations *in vitro* and *in vivo* demonstrates that the wavelengths of light abundant in deeper waters could limit penetration of light in coral tissues. This thesis proposes a novel mechanism by which corals in deeper environments use their PCRFPs to modify the internal light field to enable greater penetration of light, creating a more even distribution of the light resource across their symbiont population.

6.3.3. Limitations

The main limitation of the study on light modification by PCRFPs is that whilst a new function is proposed for these proteins, it is not experimentally demonstrated. The proposition of the function is based on theoretical evaluation of the spectral properties of the proteins and experimental observations of light penetration in anthozoan tissues, however, it was not experimentally tested due to the limitations outlined below.

The methods used to assess fluorescence distribution in suspension and in *Discosoma* tissues relied on the assumption that the only source of fluorescence emission in the imaged spectral window (710nm ±25nm) was due to chlorophyll fluorescence and this was checked prior to each experiment with a fluorometer. However, when assessing a red fluorescent morph expressing a PCRFP, there is substantial overlap between the emission of the PCRFP and chlorophyll emission and it is not possible to separate the two signals with sufficient confidence. The inability to separate the two signals also prevents measurements using fibre optic probes to deduce differences in the penetration of light using chlorophyll fluorescence.

An alternative approach to the problem was tested using measurement of oxygen evolution. As the proposed function relates to the penetration of light, it was necessary to use fibre optic optodes to record changes in oxygen evolution in the deeper regions of the tissue under different wavelengths of light. This method, however, was unsuccessful due to the inability to maintain a constant environment due to contraction and expansion of the tissue. Considering the long experimental durations required, anesthetising the coral was not a viable option. A possible alternative for future investigation will be discussed in the further work section.

Other limitations to consider in this work include the wider applicability of the underwater survey and the simplicity of the tissue model. The underwater survey of red fluorescent distribution is limited to two sites in the Gulf of Eilat and the restrictions in survey time for dives down to depths of 45m. Consequently, further work is required

to explore whether the observed trends are evident for other regions. The tissue model is simple in that it is one dimensional and assumes no scattering within the model; it can be considered similar to the "flat tissue model" used by Teran and co-workers (2010), except with the inclusion of the FP containing ectoderm. However, Teran and co-workers (2010) demonstrate that including scattering will alter the absorption of light within the tissue. Incorporation of multiple scattering by the skeleton is unlikely to alter the interpretation of the results of the simple model used in Chapter 5 as multiple scattering will act preferentially on the light that penetrates deep enough to be scattered (i.e. the wavelengths corresponding to the fluorescent emission). In addition, the skeleton absorbs most strongly in the blue region of the spectrum and therefore will further reduce the penetration of the wavelengths that already penetrated poorly.

6.4. Wider Implications

The findings of this study support functional diversity within the GFP-like proteins. By treating photophysically and biochemically distinct GFP-like proteins separately in analysis of the biological function, different ecological patterns emerged and helps explain why attempts to assign a universal function to GFP homologs in corals have provided conflicting results. The studies presented in Chapters 4 and 5 clearly demonstrate substantial differences between distinct types of proteins from the GFP – like protein family. The CPs provide screening of the symbionts (Smith et al. 2013 – Chapter 4), are upregulated at the transcript level in response to light (D'Angelo et al. 2008) and are predominantly found in shallow waters (Salih et al. 2006). In contrast, the PCRFPs are constitutively expressed at the transcript level (Leutenegger et al. 2007b), however, show light regulation at the post-translational level (Smith et al. *In prep* – Chapter 5) and increase in relative abundance with depth up to 45m (Smith et al. *In prep* – Chapter 5). Considering that both of these proteins have emerged multiple times in the evolutionary tree of the GFP-like proteins (Alieva et al. 2008) and that GFP-like proteins are under adaptive evolution (Field et al. 2006), it supports functionary heterogeneity between these proteins.

While the effects of the spectral composition of the underwater light field on coral photobiology have been dealt with before, the impacts it might have on light penetration within the tissue have not been discussed. The imaging of chlorophyll fluorescence distribution in anthozoan tissues and suspensions of zooxanthellae show the poorer penetration of blue and green wavelengths suggesting that the chromaticity of light in deeper waters could serve to limit the size of the symbiont population (Smith et al., *In prep* – Chapter 5). This may help explain why symbiont densities

decrease at more extreme depths and why alternative mechanisms may be required (Schlichter et al. 1986; Kahng et al. 2012).

Light in the yellow-orange (560-610nm) region of the visible spectrum receives relatively little attention in the coral literature largely due to its weaker absorption by the photosynthetic pigments and poor penetration in the water column. Nevertheless, the results presented here show two examples where these wavelengths are important in coral photobiology. Firstly, these wavelengths are absorbed by the zooxanthellae to a greater degree than would be expected by looking at the pigment spectra. This results from pigment packaging (Duysens 1956; Stambler and Dubinsky 2005) and multiple scattering within the skeleton (Enriquez et al., 2005) which both act to increase the contribution of these wavelengths. It is this improved penetration and amplification that could be damaging in high light environments in the shallow waters but also beneficial to deeper water corals with dense pigmentation. Secondly, yellow-orange light has been shown to be the most photoinhibiting component of the solar spectrum in higher plants, despite its low absorption by the photosynthetic pigments (Takahashi et al. 2010). It is proposed that this photoinhibition is caused by light induced damage to the manganese cluster of the oxygen evolving complex. The role of GFP-like proteins in protecting from damage to the Mn cluster has not been studied although the spectral suitability of the CPs should be noted and could be particularly useful in shallow water environments where these wavelengths are abundant. The reduction in chlorophyll excitation observed in response to CP expression demonstrates an alteration to the internal light field that would also impact light absorption by other components within the algal cells, such as the Mn cluster.

Previous analyses into the evolution of GFP-like proteins have noticed the presence of certain sites within the protein structure that could be involved in interacting with a binding partner (Field et al. 2006). The analyses presented here of metal binding amongst GFP-like proteins suggest that metal binding may not be a universal function, particularly in coral FPs. It should be noted, however, that this study only assessed binding to immobilised ions which will be less accessible than metal ions in solution and could potentially require less amino acids for coordination. Accordingly, it is difficult to attribute a physiological function to the metal binding observed here and further investigations would be required to explore a potential function. The observation of metal binding in GFP-like proteins does highlight the need for further analyses into the potential for GFP-like proteins to be interacting with other molecules and the benefit of using our considerable knowledge about their biochemistry to learn more about their biological function.

The focus of these studies has been on the impacts of the GFP-like protein light modification on the photobiology of the symbionts, however, they may serve a function directly relating to the host. The potential for light, outside of the UV, to damage host tissues has not been experimentally examined although a role for GFP-like proteins to provide host protection has been proposed (Kahng and Salih 2005). As such, it is difficult to assess the impact of GFP-like proteins on the host without using measures that could also be altered by the symbionts' light response, such as the parameters relating to reactive oxygen species (ROS concentration, antioxidant activity, extent of lipid peroxidation). This remains a challenge for the field but demonstration of a screening effect of CPs sufficiently strong enough to impact on chlorophyll excitation provides evidence that protection of host chromophores should be possible. Likewise, for the spectral redistribution by PCRFPs, it may be possible that they serve a host function and a potential candidate for exploration would be a function related to larval settlement as red light may act as a cue (Mason and Cohen 2012).

A final but fundamental point to be addressed concerning the expression of GFP-like proteins is why, if they serve important functions, are coral GFP-like proteins not expressed by all corals? The answer is likely to be that they represent one of many strategies that could be employed to address the same problem and therefore are not *essential*. If the proposed photoprotective function is taken as an example, it can be seen that the strategy of the coral is to reduce light stress. The coral holobiont possesses many different mechanisms to directly reduce light stress or its consequences of which the CPs may represent one option. The conditions where one mechanism (or combination of mechanisms) provides enhanced fitness over others will depend on a multitude of environmental factors (e.g. light intensity and composition, temperature, flow etc) that may be highly variable both spatially and temporally and hence results in the heterogeneity amongst strategies employed by different species and individuals within a species. This can be seen for other photoprotective mechanisms such as xanthophylls cycling, with different degrees of cycling between species attributed to different photoprotective strategies (Warner and Berry-Lowe 2006).

6.5. Summary

In summary, this thesis supports the hypothesis that different groups of GFP-like proteins have distinct functions. The results presented here indicate that metal binding is not a function of scleractinian GFP-like proteins. Nevertheless, the identification of the metal binding domain in some actinarian RFPs is an important discovery as it emphasises the need to consider the whole protein molecule when assessing the

biological function of these proteins, rather than only the chromophore, and supports the classification of the actinarian FPs/CPs as a distinct group of GFP-like proteins, with potentially distinct biological functions.

It is demonstrated that coral chromoproteins reduce light stress on their symbionts, increasing their tolerance of excessive irradiances. In contrast, the first study into the biology of PCRFPs shows that they are associated with low light environments where light penetration in coral tissues is limited and it is proposed that they alter the internal light field to enable maintenance of coral population in deeper waters. In combination, these studies highlight how the coral host can influence the light environment experienced by its symbiont and helps explain why symbiont photoacclimation cannot fully explain the ecological distribution of corals in relation to their light environments. The results presented here suggest that the role of the host in the photobiology of the symbiosis may have been underestimated and that the host plays an important role in the bleaching tolerance of corals by extending their ability to deal with sub- and supraoptimal irradiances.

The work presented here extends beyond scientific interest as it has practical implications for reef managers and policy makers. The advancement in our knowledge of GFP-like protein expression and functionality can be used to guide management efforts to protect reefs. Considering that future reefs are likely to suffer more frequent bleaching events (Hoegh-Guldberg 1999) and further degradation of water quality (Hughes et al. 2003; Fabricius 2005), the demonstration that corals expressing CPs and PCRFPs are able to extend their light niches suggests that they should be targeted for greater protection and used in restoration schemes.

6.6. Future Work

The research presented in this thesis will stimulate new research into the role of GFP-like proteins but also in the wider field of coral photobiology. Three potential studies in particular, are outlined below.

6.6.1. Assessment of spectral redistribution by PCRFPs

As previously discussed, experimentally testing to investigate if PCRFPs enable greater chlorophyll excitation at deeper depths has proved troublesome. However, it may be possible to experimentally test the hypothesis by looking at the distribution of carbon fixation within the tissue using radiocarbon labelling. This approach has been used to measure light distribution in spinach leaves (Evans and Vogelmann 2003) although

applying the methodology to explore the effect of PCRFPs will require identification of an appropriate control in order to assess changes in penetration.

6.6.2. Experimental assessment of different protein groups

This study set out to look at different GFP-like protein groups separately rather than attempt to assign a function to the whole coral GFP-family (Salih et al. 2000; Palmer et al. 2009). The experimental approach used here is supported by evolutionary evaluations of the protein family (Field et al. 2006; Alieva et al. 2008), the different regulation patterns (D'Angelo et al. 2008; Leutenegger et al. 2007; Smith et al. *In prep* – Chapter 5), the distinct spectral properties (Alieva et al. 2008; Smith et al. 2013 – Chapter 4; Smith et al. *In prep* – chapter 5) and ecological distributions (Salih et al. 2006; Smith et al. 2013 – chapter 4). Further studies using a similar approach, identifying and dealing with distinct groups of GFP-like proteins separately will enable a greater understanding of these proteins and provide a basis from which to evaluate the wider hypothesis that different GFP-like protein groups have different biological functions in corals.

6.6.3. Using coral photobiology to improve estimates of productivity

The ultimate goal of evaluating GFP-like proteins and their role in the photobiology of corals should be to use the knowledge to improve assessments of the health of coral reefs and the change in reef health in response to environmental degradation. The use of coral fluorescence as a potential tool for monitoring of reefs is slowly being realised (Mazel et al. 2003a; D'Angelo et al. 2012), however, our knowledge of these proteins could also be incorporated into a wider project aiming to improve estimates of coral productivity. There have been attempts to model coral productivity from remote sensing data (Hochberg and Atkinson 2008) although the approach is limited by lack of incorporation of photobiological processes occurring at a sub-community scale. Other fields, such as terrestrial ecosystems studies, use models that take account of the processes occurring at different scales (within leaf to canopy) to evaluate radiative transfer (Vogelmann 1993; Lewis and Disney 2007) and this would greatly benefit coral reef science. Recently, the field of coral photobiology has greatly benefitted from the development of models to test hypotheses and explain experimental results (Enriquez et al. 2005; Terán et al. 2010). The simple model presented in Chapter 5, while limited in scope, provides theoretical results that were consistent with the general trends of light penetration observed in the experimental analyses. This highlights the power of such models and the development of more advanced tissue models, incorporating other factors including the skeletal and tissue structure will allow for more robust

assessments of coral photobiology and productivity. Obviously understanding how GFP-like proteins alter the internal light field will be an important component in such assessments and the results of this study provide the basis from which to work, particularly the demonstration that chlorophyll excitation reduced by CPs. Further spectroscopic and physiological measurements could be used to build and validate a tissue model. The tissue level data could then be combined with existing three dimensional underwater radiative transfer models (Hedley 2008) to form a unique model that would enable large scale assessments of light use and productivity by corals as well as enabling *in silico* investigations into the role of individual parameters, such as FPs, on the photobiology in different environments.

CHAPTER 7. Appendix

7.1. Tables

Function	Description	Examples	Metal Ion
Structural	Configuration of protein tertiary and/or quaternary structure	Zinc finger domain	Zinc
Electron Transfer	Uptake, release, and storage of electrons	Iron-Sulphur Proteins Blue Copper Proteins	Iron Copper
Dioxygen Binding	Metal-O_2 coordination and decoordination	Haemoglobin Myoglobin Haemerythrins Haemocyanin	Iron Iron Iron Copper
Transport and Storage	Uptake, binding, and release of metals in soluble form	Transferrins Ferritins Metallothioneins	Iron Iron Zinc, Cadmium, Copper, Gold, Silver, Mercury, Platinum
Catalytic	Substrate binding, activation and turnover	Superoxide Dismutases (SODs)	Copper, Zinc, Manganese, Iron
		Oxidases	Iron, Copper
		Oxygenases	Iron, Copper
		Oxotransferases	Iron, Molybdenum
		Nitrogenases	Iron, Molybdenum
		Hydrogenases	Iron, Nickel

Table 1. Roles of metal binding in biological systems. The table summarises the classification and descriptions of the structural and functional aspects of metal binding provided by Holm and co-workers (Holm *et al.*, 1996)

7.2. Overlap of red FP and chlorophyll emission

This section will briefly discuss the challenges posed by the overlap between the red FP and chlorophyll emission using an example of fluorescent emission from *Oxypora* sp. The fluorescent emission spectrum was recorded by illuminating the dorsal surface of an *Oxypora* individual growing on a microscope slide, enabling the transmitted fluorescence emission to be recorded from below. The measurement was performed using a 500nm (±10nm) excitation light and the emission spectrum was recorded using a collimating lens attached to an Ocean Optics USB2000 spectrometer.

The transmitted emission spectrum of the *Oxypora* individual appears to show little overlap between the two fluorophores (Figure A1a). However, when compared with the emission of a homologous protein, EosFP (Wiedenmann et al. 2004b), it is clear that the spectrum is modified (Figure A1b). A typical solution to a mixed signal in fluorescence and reflectance spectroscopy is to perform a linear unmixing (Dickinson et al. 2001; Hedley et al. 2004). This assumes that the mixture is the sum of the spectra of each component (endmember) within the mixture weighted by its concentration (see Chapter 3 methods). However, in the case coral tissue, the fluorescent emission of the FP is absorbed, to some degree, by the algal pigments of the zooxanthellae and therefore the endmember spectrum for the FP is modified. This is demonstrable by modelling the transmission of EosFP through pigmented layers of algal pigments using the equation:

$$T(\lambda) = 1 - e^{a(\lambda)z}$$

Where T is the transmitted fluorescence, a is the absorption coefficient of the algal pigments and z is the depth of the layer. It can be seen that as the concentration is increased (i.e. from concentrations 1 to 4), the shape of the emission spectrum is modified (Figure A1c). By modelling the effects of the absorption of the PCRFP, the PCRFP endmember can be estimated (Figure A1d).

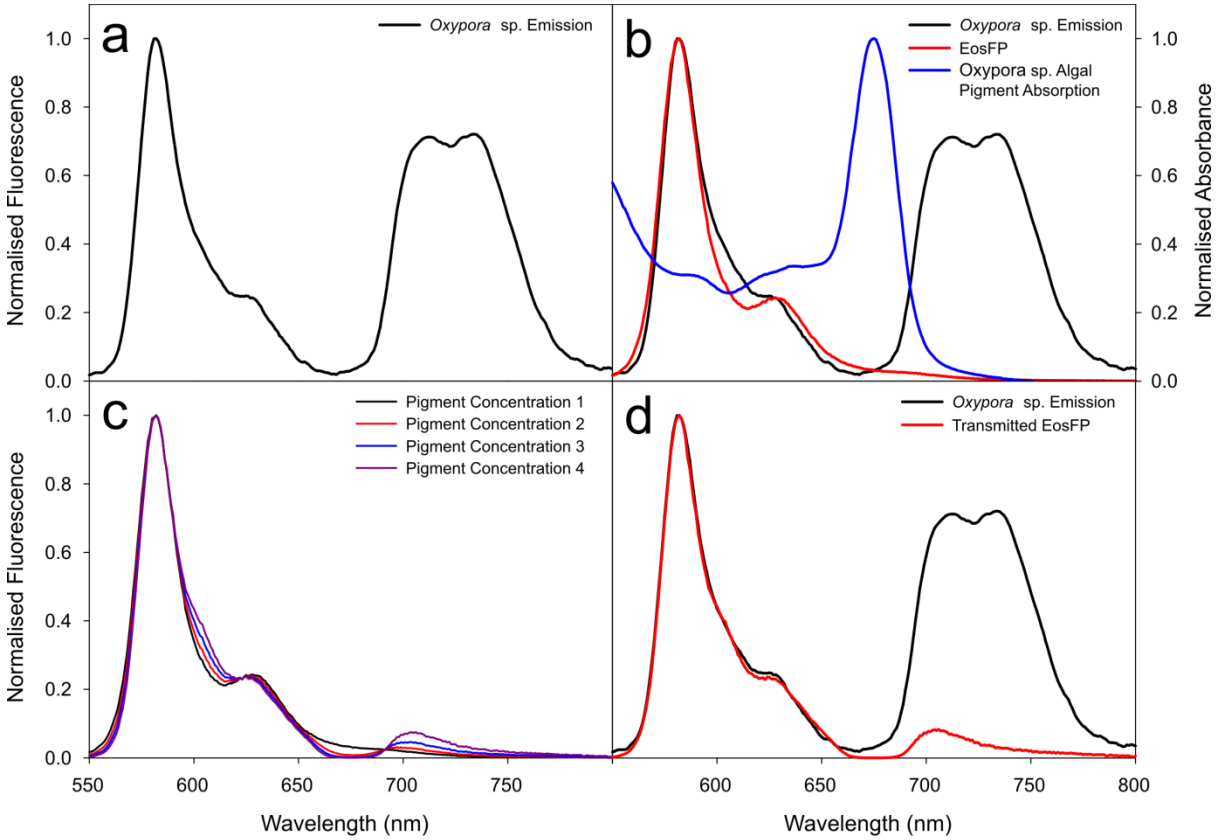

Figure A1. The impact of spectral overlap between photoconvertible red fluorescent protein and chlorophyll emission. (a) Measured transmitted *Oxypora* sp. emission. (b) Comparison of *Oxypora* sp. transmitted fluorescence with the emission of purified EosFP and the absorption spectrum of pigments extracted from *Oxypora*'s zooxanthellae. (c) The impact of absorption by the algal pigments on the shape of EosFP emission. The concentrations are arbitrary and increase linearly from 1 to 4. (d) Comparison of *Oxypora* sp. transmitted fluorescence and the modelled EosFP endmember. All spectra are normalised to a maximum value of 1.0.

The emission of chlorophyll fluorescence is also reabsorbed and modifies its emission spectrum. This has been observed in other studies and has potential to provide information regarding the penetration of different wavelengths of light (see review by Buschmann, 2007). Nevertheless, the modified emission of the PCRFP will alter the shape of the chlorophyll emission due to the minor peak at ~705nm and therefore limits the potential for using the modified chlorophyll emission to provide information about how the PCRFPs alter the fluorescence distribution in the tissues.

The spectral overlap between the red FP and chlorophyll emission also prevented the application of the tissue chlorophyll fluorescence imaging to red fluorescence morphs. This was tested with a red fluorescent morph of *Discosoma* sp. (see Chapter 4 for methods), however, the high tissue concentrations and the overlap resulted in a signal

that was dominated by the red FP fluorescence from the ectoderm rather than the chlorophyll fluorescence in the endoderm (Figure A2). It should be noted that the red FP in *Discosoma* is not a PCRFP but the spectral overlap would affect assessment of PCRFPs in the same manner.

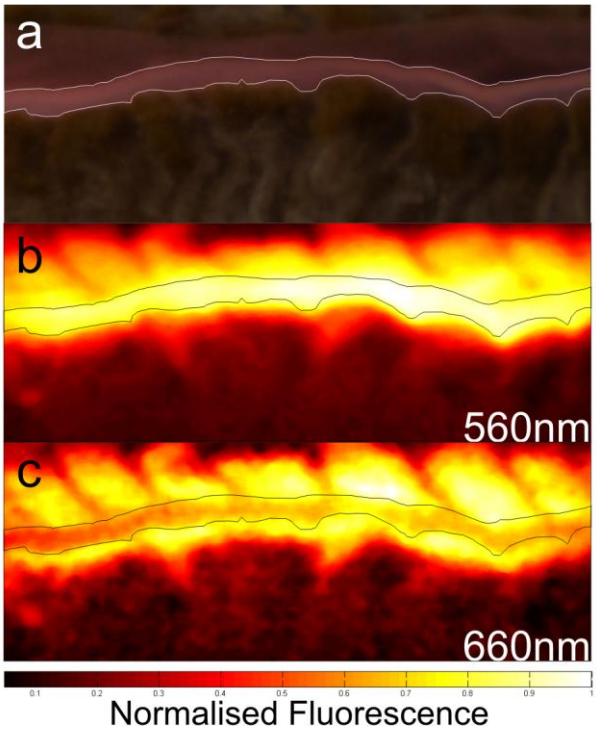

Figure A2. Fluorescence distribution in the tissue of a red *Discosoma* sp. morph. (a) White light image of the *Discosoma* cross section. The ectoderm is outlined in white. (b) Fluorescence image under 560nm excitation. The fluorescence is located primarily in the ectoderm, outlined in black. (c) Fluorescence distribution under 660nm excitation. The fluorescence in this image is located under the ectoderm, corresponding to the region of high zooxanthellae concentrations.

7.3. Protocols

7.3.1. IMAC assay protocol

1. Load 500µl of Talon Metal Affinity Resin (Clonetech) solution into a capped spin column.
2. Centrifuge the column at 4000 x g for 1 minute to remove storage buffer (final resin volume = 250µl).
3. Add 300µl of extraction buffer to the resin.
4. Mix by inversion and centrifuge at 4000 x g for 1 minute.
5. Repeat steps 4 and 5 twice.
6. Seal spin column outlet with Parafilm.
7. Load 300µl of the protein extract to the column.
8. Close cap and mix by inversion.
9. Mix by inversion every 10 minutes for a total of 40 minutes.
10. Centrifuge at 4000 x g for 1 minute to remove unbound protein. Keep the flow through.
11. Add 300µl of extraction buffer (50mM sodium phosphate buffer, 300mM NaCl; pH 7.2) to the matrix. Mix by inversion and leave for 5 minutes.
12. Centrifuge at 4000 x g for 1 minute and keep the flow through.
13. Repeat steps 11 and 12 five times.
14. Repeat steps 11 and 12 three further times using elution buffer (50mM sodium phosphate buffer, 300mM NaCl, 300mM Imidazole; pH7.2) rather than extraction buffer.
15. Load 100µl of the flow through from each stage to individual wells in a 96 well plate. Add 100µl of extraction and elution buffers to individual wells to serve as blanks.
16. Place the 96 well plate into a fluorescence spectrophotometer and record the fluorescence emission spectrum for each well (Selection of the appropriate excitation and emission wavelengths will be specific to the individual protein assayed. Nevertheless, it is important to ensure that the excitation and emission wavelength and slit widths are the same for each well).
17. The fluorescence intensity of the respective blank should be subtracted from the intensity of each well. The protein concentration in each well can subsequently be calculated as the proportion of the well fluorescence to the sum of fluorescence across all wells.

7.3.2. Fluorescence reduction analysis protocol

The following protocol is for the assessment of a single concentration of the target metal (in this case CoCl2). Multiple reactions can be performed in parallel to assess a range of concentrations.

1. Add 100µl of the protein solution (2µM) to a microcentrifuge tube.
2. Add 100µl of the CoCl2 solution of known concentration (stock solution must be twice target concentration due to the dilution factor). Mix using the pipette and label the tube as the total protein extract (TPE).
3. Remove 100µl of the mixture and add to a new microcentrifuge tube. Label the tube as the soluble protein fraction (SPF).
4. Leave the solutions at room temperature of seven hours.
5. Centrifuge the SPF sample at 12000 x g for 10 minutes.
6. In the meantime, load the TPE sample into a 96 well plate and read the fluorescence emission spectrum. The selection of excitation and emission parameters will be protein dependent but must be consistent for both the TPE and SPF fractions and for all concentrations (if multiple concentrations are being assayed).
7. After centrifugation of the SPF, carefully remove the supernatant, avoiding any pellet that may have formed on the microcentrifuge tube wall. Add the supernatant into a 96 well plate well and read the emission spectrum using the same parameters as the TPE. Keep the microcentrifuge tubes for analysis of precipitates.
8. Add 200µl of EDTA (0.5M) to each well. Incubate at 4˚C overnight.
9. Repeat readings on the fluorescence spectrophotometer with the EDTA treated samples.

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