Green fluorescent protein-like pigments optimize the internal light environment in symbiotic reef building corals

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Abstract

Pigments homologous to the Green Fluorescent Protein (GFP) have been proposed to fine-tune the internal light microclimate of corals, facilitating photoacclimation of photosynthetic coral symbionts (Symbiodiniaceae) to life in different reef habitats and environmental conditions. However, direct measurements of the *in vivo* light conditions inside the coral tissue supporting this conclusion are lacking. Here, we quantified the intra-tissue spectral light environment of corals expressing GFP-like proteins from widely different light regimes. We focus on (1) photoconvertible red fluorescent proteins (pcRFPs), thought to enhance photosynthesis in mesophotic habitats via wavelength conversion, and (2) chromoproteins (CPs), which provide photoprotection to the symbionts in shallow water via light absorption. Optical microsensor measurements indicated that both pigment groups strongly alter the coral tissue light environment. Estimates derived from light spectra measured in pcRFP-containing corals showed that fluorescence emission can contribute to >50% of orange-red light available to the photosynthetic symbionts at mesophotic depths. We further show that upregulation of pink CPs in shallow-water corals during bleaching leads to a reduction of orange light by 10-20% compared to low-CP tissue. Thus, screening by CPs has an important role in mitigating the light-enhancing effect of coral tissue scattering during bleaching. Our results provide the first experimental quantification of the importance of GFP-like proteins in fine-tuning the light microclimate of corals during photoacclimation.

Introduction

Tropical coral reefs are solar-powered oases of marine biodiversity, where scleractinian corals are responsible for building the reef structures that host over 30% of all named marine multicellular species (Fisher et al. 2015). Corals rely heavily on photosynthesis by their dinoflagellate endosymbionts (Symbiodiniaceae) to meet their metabolic requirements (Muscatine 1990). The distribution of reef-building corals is therefore constrained by the availability of photosynthetically active radiation (PAR, 400-700 nm), which is in turn determined by physical factors such as latitude, seasonality, water depth and the optical properties of the water body (Kirk 1994; Kleypas et al. 1999; López-Londoño et al. 2021). Variability in these parameters affects both the quantity and spectral quality of irradiance, exposing corals to a large range of light environments even across small spatial scales (Brakel 1979; Anthony and Hoegh-Guldberg 2003; Eyal et al. 2015). On shallow reef flats in clear water regions, corals are routinely exposed to excess irradiance that supersaturates symbiont photosynthesis (Jimenez et al. 2012; Wangpraseurt et al. 2014b), with short light flashes reaching over 3900 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ due to the lensing effect of waves (Veal et al. 2010). Additionally, shallow water corals are exposed to a broad irradiance spectrum covering ultraviolet (UV) to near infrared (NIR) radiation (Wangpraseurt et al. 2014b). At the other

extreme, photosynthetic corals on mesophotic reefs at water depths between 50-150 m can receive as little as 1% of surface PAR, largely concentrated in the blue-green region of the spectrum and greatly reduced for UV and red wavebands (Lesser et al. 2009; Eyal et al. 2015; Tamir et al. 2019). Light conditions can also vary for individual colonies from the same water depth depending on colony morphology (Kaniewska et al. 2011; Wangpraseurt et al. 2014b) and the reef structure that surrounds them (Falkowski and Dubinsky 1981; Wangpraseurt et al. 2014b).

Corals have evolved a number of mechanisms for optimising symbiont photosynthesis in the presence of spatial and temporal heterogeneity in light availability. Some of these mechanisms occur at the symbiont cell level, while others involve changes in coral host morphology both at the colony and polyp-scale (Kramer et al.; Hoogenboom et al. 2008; Todd 2008). Coral symbionts can modify their light harvesting and energy quenching over timescales ranging from seconds to months. For example, non-photochemical quenching allows rapid dissipation of excess irradiance in the photosynthetic apparatus of symbionts, preventing photodamage in high light environments (Hoegh-Guldberg and Jones 1999; Gorbunov et al. 2001). Changes in photosynthetic pigment concentrations and symbiont cell numbers enable corals to acclimate to changes in irradiance over longer time periods (Falkowski and Dubinsky 1981; Titlyanov et al. 2001); specifically, corals can increase their symbiont numbers as well as the per cell photosynthetic pigment content in response to a reduction in light levels, and vice versa (Falkowski and Dubinsky 1981; Titlyanov et al. 2001).

Coral host morphology is an important factor controlling the irradiance available for symbiont photosynthesis (Helmuth et al. 1997; Ralph et al. 2002; Einbinder et al. 2009; Kaniewska et al. 2011; Nir et al. 2011; Wangpraseurt et al. 2014b; Kahng et al. 2019). Coral colonies from low light environments maximize their interception with the downwelling irradiance and assume plate-like morphologies that are more conducive to light gathering, while shallow water colonies assume more self-shading morphologies, such as vertical plates or branches (Anthony et al. 2005; Kaniewska et al. 2011). Morphological adaptations make some species depth-specialists, such as the mesophotic coral *Leptoseris* which can host photosynthetic symbionts down to depths of well over 100 m (Rouzé et al. 2021). Growth form can also exhibit a great degree of phenotypic plasticity in many coral species, as evident from changes in morphology when colonies are transplanted across depth gradients (Willis 1985; Tamir et al. 2020). Such plasticity is one of the factors that enables depth generalist corals, such as *Montastraea cavernosa* in the Caribbean, to thrive across light gradients of several orders of magnitude (Lesser et al. 2010).

Light availability to the coral symbionts depends on the optical properties of the surrounding host tissue and the underlying skeleton (Wangpraseurt et al. 2012, 2019; Marcelino et al. 2013). Coral tissue and skeleton scatter incident light with a strength and directionality that

are highly variable across species and even between different structures within a single colony (Marcelino et al. 2013; Wangpraseurt et al. 2016a, 2019; Enríquez et al. 2017). The interplay between tissue and skeleton scattering and reflection and algal absorption modulate coral photosynthesis (Marcelino et al. 2013; Brodersen et al. 2014; Wangpraseurt et al. 2016a, 2019; Scheufen et al. 2017). In some corals, this can lead to outstanding photosynthetic quantum efficiencies that are close to the theoretical maximum (Brodersen et al. 2014). Optical properties of coral colonies exhibit ontogenetic plasticity, which enables some coral species to occupy a wider niche (Anthony et al. 2005). For example, on mesophotic reefs the depth-generalist corals *Paramontastrea peresi* and *Porites lobata* tend to assume growth forms with enhanced light exposure and skeletons with higher reflectivity compared to their shallow water conspecifics, resulting in higher absorption by the symbionts (Kramer et al. 2020).

The coral host can also modulate incident light by synthesizing host pigments. The most widespread coral host pigments belong to the Green Fluorescent Protein (GFP)-like superfamily, a group of homologous pigments which are ubiquitous among the Cnidaria (Shimomura et al. 1962; Matz et al. 1999; Alieva et al. 2008). Pigments in this family share a similar structure based on a tripeptide chromophore capable of absorbing visible or UV radiation (Nienhaus and Wiedenmann 2009). However, despite having similar structure, cnidarian GFP-like proteins exhibit great variability in their optical properties and regulation mechanisms, indicating that they are functionally diverse (Salih et al. 2000; Leutenegger et al. 2007; Alieva et al. 2008; D'Angelo et al. 2008; Nienhaus and Wiedenmann 2009; Smith et al. 2013; Aihara et al. 2019).

GFP-like proteins with inducible expression are found predominantly in corals from high light environments, and they are transcriptionally regulated by blue light intensity (Salih et al. 2000; D'Angelo et al. 2008). GFP-like proteins with light-induced expression include fluorescent proteins (FPs) with emission in the cyan, green and red spectral regions (CFPs, GFPs and RFPs), which absorb light and re-emit it as red-shifted radiation, and non-fluorescent chromoproteins (CPs), which absorb light but have no or little fluorescence emission (Alieva et al. 2008; D'Angelo et al. 2008). These pigments accumulate in high concentration in colony parts with low symbiont densities, often areas of active growth, such as skeletal ridges, the tips of branching corals, the margins of plating corals, and areas affected by epibionts and mechanical damage (Salih et al. 2000; D'Angelo et al. 2012). The increased photon fluxes inside the host tissue caused by the absence of light absorption by the symbionts and light reflection and scattering by skeleton and host tissue components (Enríquez et al. 2005; Wangpraseurt et al. 2017) have been suggested to cause the high-level pigment expression in these colony parts (Smith et al. 2013; Bollati et al. 2020). Additionally, blue light-induced upregulation as part of an optical feedback loop has been shown to drive strong accumulation

of these pigments in bleached corals during episodes of mild heat stress or in heat-free bleaching, caused, for instance, by nutrient stress (Bollati et al. 2020).

FPs and CPs with light-induced expression are usually localised ectodermally, and have been shown to exert a photoprotective function on the underlying symbiont layer (Salih et al. 2000; Smith et al. 2013; Gittins et al. 2015; Quick et al. 2018). Upregulation of these pigments in growing parts of colonies and in bleached tissue has been proposed to promote (re)colonisation of host tissue by symbiont cells via the reduction of internal light stress in areas with low symbiont densities (D'Angelo et al. 2012; Smith et al. 2013; Bollati et al. 2020). GFP-like proteins are also widely distributed in corals that are depth generalists or even mesophotic specialists (Oswald et al. 2007; Eyal et al. 2015), where they are constitutively expressed, often at high levels, across a wide range of irradiance regimes (Leutenegger et al. 2007). It has long been proposed that fluorescent pigments in low light habitats could enhance coral photosynthesis by modulating the spectrum and intensity of light that reaches the symbiont cells (Schlichter et al. 1994; Salih et al. 2000). A light-enhancement mechanism has been suggested for a particular group of constitutive GFP-like proteins known as photoconvertible RFPs (pcRFPs), which are common in thick-tissue depth generalist and mesophotic coral species (Ando et al. 2002; Wiedenmann et al. 2004; Oswald et al. 2007; Eyal et al. 2015). pcRFPs emit green fluorescence when first synthesised, but their chromophores are irreversibly converted to a red-emitting species upon absorption of a UV photon ~390 nm (Ando et al. 2002; Wiedenmann et al. 2004). As coral GFP-like proteins are typically arranged in tetramers, UV-induced post-translational modification leads to the formation of tetramers that contain both green and red subunits; they can therefore absorb light in the blue-green range and re-emit it in the orange-red range via highly efficient Förster Resonance Energy Transfer (FRET) between adjacent subunits (Wiedenmann et al. 2004; Bollati et al. 2017). The orange-red waveband is scarce on mesophotic reefs, yet it can stimulate chlorophyll fluorescence deeper in the tissue compared to blue-green wavelengths because it is poorly absorbed by photosynthetic pigments, an effect analogous to green light driven photosynthesis in plant leaves (Vogelmann and Evans 2002; de Mooij et al. 2016; Smith et al. 2017). Indeed, pcRFP-containing corals have been shown to survive better under a simulated mesophotic light spectrum as compared to non-pigmented conspecifics (Smith et al. 2017).

While GFP-like proteins are likely to have additional functions for the coral holobiont (Aihara et al. 2019; Id et al. 2021), optimisation of the symbiont light environment is arguably one of their primary roles. The effects of GFP-like proteins on the light environment experienced by coral symbionts are not simply dependent on the spectral properties of the isolated protein; rather, they are a product of the environmental and physiological regulation of the protein within its three-dimensional optical context. For example, microsensor measurements and

optical modelling have shown that scattering by GFP-like proteins can enhance light penetration at low GFP concentrations, while high concentrations of GFP increase backscattering and reduce light penetration through the coral tissue (Lyndby et al. 2016; Parkins et al. 2021). Variability in the distribution and arrangement of these proteins – diffuse or granular, ectodermal or endodermal – also affects how they interact with light (Salih et al. 2000; Wangpraseurt et al. 2019), further indicating that spectrally similar GFP-like proteins can be involved in different light modulation mechanisms.

Studies examining the *in vivo* internal light environment of coral colonies at the microscale, particularly during environmentally driven changes in their GFP-like protein complement, are therefore an essential step towards understanding the role these proteins play in photoacclimation of symbiotic corals. Here, we investigated how environmental regulation of GFP-like proteins affects the intra-tissue light microenvironment of depth-generalist and shallow water corals. We focused on the effects of i) UV-driven photoconversion in corals containing pcRFPs, and ii) blue light-driven upregulation of inducible CPs during bleaching and in active growth colony margins. Our study provides direct evidence for the importance of GFP in optimizing the coral-algal symbiosis under extreme light regimes.

Results and Discussion

Photoconversion and pcRFPs in depth generalist corals

Measurements of scalar irradiance taken at the tissue surface of *M. cavernosa* under white illumination showed an enhancement of 120-180% of the incident irradiance across the visible spectrum. This enhancement was detected for all specimens regardless of the degree of photoconversion (Fig. 1a). Vertical light attenuation was strongest between 410-470 nm (covering chlorophyll *a* absorption) and weakest in orange-red wavebands (Fig. 1b).

Figure 1. Spectral scalar irradiance in *M. cavernosa* under broad spectrum light. a) $E_0(\lambda)$ at coenosarc surface, with excitation and emission peaks of unconverted and converted McavRFP highlighted (colorful arrows), as well as absorption peaks of Symbiodiniaceae photosynthetic pigments (black arrows). Mean (solid lines) + s.d. (dashed lines); n=3 measurements on 2 fragments. b) Spectral attenuation coefficient of $E_0(\lambda)$ from coenosarc surface to skeleton (average thickness = 560 μ m). Mean (solid lines) $\pm$ s.d. (dashed line), n=3.

The enhancement of scalar irradiance commonly observed at the surface of coral colonies is a result of light scattering by the underlying tissue and skeleton (Kühl et al. 1995; Wangpraseurt et al. 2014a), which displays great interspecific variability (Marcelino et al. 2013; Wangpraseurt et al. 2019). Depending on the scattering strength of the two compartments, light in deeper aboral tissue layers can be strongly reduced (Wangpraseurt et al. 2012) or enhanced (Szabó et al. 2014; Wangpraseurt et al. 2020), relative to the top (oral) tissue layers. Our measurements of the tissue attenuation coefficient for *M. cavernosa* indicate shading of the deeper tissue areas, particularly in the violet-blue range (Fig. 1b). Additionally, previous measurements of optical scattering suggest that light enhancement due to skeleton backscatter is limited for this species compared to other coral taxa (Marcelino et al. 2013). While these properties may confer bleaching resistance and provide photoprotection for the symbiont population in shallow water (Wangpraseurt et al. 2012; Marcelino et al. 2013), tissue

shading greatly reduces carbon fixation rates in symbiont cells found in deeper tissue layers (Wangpraseurt et al. 2016b), which might negatively affect colonies in light-limited environments; yet, *M. cavernosa* is able to thrive at mesophotic depths (Lesser et al. 2010). At the coral surface, spectral changes due to conversion of the pcRFP pool were observed at the absorption and emission peaks of the green (507 and 514 nm) and red chromophore (571 and 582 nm) (Fig. 1a). Surface light enhancement by GFP-like proteins in corals can be attributed to a combination of elastic scattering (i.e. scattering without modifications of photon energy) and inelastic scattering (i.e. scattering with modification of photon energy, such as the case of absorption followed by fluorescence emission) (Lyndby et al. 2016). Under white light, the degree of photoconversion of the pcRFP pool did not appear to significantly affect the spectral attenuation coefficient (Fig. 1b), indicating that the effects of pcRFP scattering on attenuation of the incident are relatively small compared to the effects of tissue scattering and photosynthetic pigment absorption. Host pigment-derived enhancement of light in pcRFP-containing corals in shallow water environments therefore appears a minor component for the overall tissue light microclimate, albeit with some variability due to water depth and the optical properties of the water column, as well as spectral differences between our white measuring light and sunlight. In contrast, corals on mesophotic reefs experience a much more narrow incident spectrum, where blue-green wavelengths that efficiently excite pcRFPs are abundant, while light in the orange-red spectral range is greatly reduced (Lesser et al. 2009; Eyal et al. 2015; Tamir et al. 2019). Under these conditions, the relative contributions of light scattering and fluorescence emission by pcRFPs are therefore likely very different from those measured under white light.

To isolate the contribution of fluorescence emission by the pcRFP pool to the tissue light microenvironment of *M. cavernosa*, we collected fluorescence emission profiles in the 505-700 nm range using a blue (455-505 nm) excitation light source (Fig. 2, Fig. S1). At all stages of photoconversion (signified by the decreasing 514:582 nm emission ratio and defined as *unconverted*, *partially converted* and *converted*, Fig. 2a,b), fluorescence emission from both the unconverted and converted chromophore was highest at the surface and decreased towards the skeleton. However, attenuation of light emitted by the unconverted chromophore was more pronounced relative to attenuation of the converted chromophore emission (Fig. 2a,c). This is in agreement with the white light attenuation coefficient (Fig. 1b), which was weaker in the orange-red range. The peridinin-chlorophyll *a*-protein (PCP) light harvesting complex efficiently absorbs blue, green and red wavelengths (Jiang et al. 2012), resulting in strong attenuation by symbionts in the oral gastrodermis (Fig. 1); conversely, poor absorption of orange light by symbiont pigments allows this waveband to penetrate deeper into the tissue (Fig. 1).

Figure 2. Host pigment fluorescence distribution in the tissue of *M. cavernosa* undergoing photoconversion. Scalar irradiance measurements collected under blue excitation 455-505 nm (Fig. S1c). **a)** 514:582 nm emission ratio, indicating the degree of photoconversion for each coral (*unconverted* >1, *partially converted* ~1, *converted* <1). **b)** Fluorescence micrographs of representative photoconversion stages. **c)** Normalised fluorescence emission profiles (arbitrary units, a.u.).

Smith et al. (2017) showed that orange light has a higher potential to stimulate photosynthesis in symbiont cells found deeper in the coral tissue as compared to blue-green light. This effect is analogous to what is observed in plant leaves, where enhanced illumination with green light increases photosynthesis in deeper cell layers (Vogelmann and Evans 2002; de Mooij et al. 2016). Therefore, it was proposed that orange fluorescence emission by pcRFPs could provide an energetic advantage for symbiotic corals in greater water depths dominated by blue light (Smith et al. 2017). Indeed, colour morphs with pcRFPs survived longer than non-pigmented conspecifics in simulated deep water light environments (Smith et al. 2017). Here, we directly quantified the impact of pcRFPs on the light environment experienced by deep symbionts *in hospite* in corals. We used the blue light excited fluorescence emission profiles from *M. cavernosa* (Fig. 2c) at different stages of photoconversion (Fig. 3a-c) to calculate the number of photons generated by fluorescence emission integrated over the green (Fig. 3d) and red (Fig. 3e) spectral range. Fluorescence emission by a converted pcRFP pool provides an amount of orange-red photons that ranges between 1% (close to the skeleton) and 5% (at the coral surface) of the blue incident intensity, with intermediate values of around 3% at a tissue depth of 400 μ m (Fig. 3e). This is a more than three times higher contribution by the converted pcRFP, as compared to measurements in the same waveband on *unconverted* coral. Our direct measurements of the coral tissue light field thus provide experimental proof that orange-red fluorescence from host pigments penetrates coral tissues better than green fluorescence, and that ectodermal pcRFPs can improve the illumination of otherwise shaded tissue areas despite the narrow mesophotic light spectrum. Thus, under a mesophotic light spectrum, the symbionts harboured by conspecific fluorescent morphs experience a spectrally different light environment depending on the pigment complement expressed by their host (Ben-Zvi et al. 2021). We obtained a similar result for a second species, *Echinophyllia* sp., by comparing specimens that contain a highly converted pcRFP to conspecific morphs that lack pcRFPs entirely (Fig. S3). This confirms the relevance of our findings to other pcRFP-containing corals from mesophotic environments (Eyal et al. 2015).

Figure 3. Photon scalar irradiance of host pigment fluorescence in the tissue of *M. cavernosa* at representative photoconversion stages. a) Tissue surface emission spectra of *unconverted*, *partially converted* and *converted* specimens (ex = 455-505 nm). b) Integrated green (505-560 nm) and red (560-650 nm) fluorescence emission along a tissue depth profile, measured under blue excitation (455-505 nm, Fig. S1c).

To predict how spectral modifications by pcRFPs affect *in situ* light harvesting on mesophotic reefs, we used a published dataset of downwelling spectral irradiance from a coral reef in Eilat (Eyal et al. 2015) (Fig. 4a) and simulated the *in vivo* emission of orange-red light along a depth gradient from 10 to 130 m, using the *converted M. cavernosa* as a model (Fig. 4b). The incident 455-505 nm light required to excite the pcRFP pool is relatively abundant down to 100 m water depth ($1.5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), however the amount of 560-650 nm light is only in the range of $1 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 50 m and attenuates rapidly to about $0.1 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 80 m (Fig. 4a). As a consequence, the pcRFP pool between 50 and 80 m depth can provide at least as many 560-650 nm photons as incident sunlight, effectively doubling the scalar irradiance in this waveband compared to corals without pcRFPs (Fig. 4b). On mesophotic reefs beyond 80 m water depth, fluorescence emission by the pcRFP becomes the dominant source of 560-650 nm light for *M. cavernosa*. This calculation yielded similar results when applied to a dataset of modelled irradiance in the Bahamas (Lesser et al. 2009) (Fig. S4), a natural habitat of this species, suggesting that our findings are relevant to mesophotic reef systems independent from potential differences in the underwater light environment.

We note that our analysis could lead to overestimation of predicted red fluorescence at shallow depths, where high light levels may saturate the chromophore population thus resulting in a

non-linear relationship between incident light and fluorescence emission. However, the relationship between fluorescence emission and excitation light was linear up to at least 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (of incident 455-505 nm light) (Fig. S5, Table S1), corresponding to the incident irradiance (455-505 nm) measured at 48 m depth in the Red Sea (Fig. 4a). Our calculations of fluorescence emission were thus considered accurate below 48 m water depth.

Figure 4. Spectral photon irradiance and wavelength conversion in corals with photo-convertible host pigments along a depth gradient. a) Integrated photon irradiance in the 450-505 nm and 560-650 nm range measured on a mesophotic reef in Eilat, Red Sea (Eyal et al. 2015). **b)** Measured 560-650 nm light generated by pcRFP emission and estimated relative contribution to the total amount of 560-650 nm light available to the coral holobiont. Error bars represent range between measurement at the coral skeleton (1%) and measurement at the coral surface (5%).

Even though this constitutes a dramatic spectral alteration in relative terms, it is important to note that the absolute number of photons generated by pcRFP fluorescence is small compared to what is provided by ambient light in shallow waters. The benefit for coral photosynthesis may thus be negligible under higher light conditions in comparison to the metabolic trade-off of having to maintain a highly concentrated pcRFP pool (Leutenegger et al. 2007; Oswald et al. 2007; Quick et al. 2018). Nonetheless, a small advantage can become significant under strong light-limitation, as shown by the increased long term survival of pcRFP-containing coral morphs compared to their brown conspecific kept side-by-side under

a simulated mesophotic spectrum (Smith et al. 2017). Such small fitness advantage could have contributed as a driving factor in some of the multiple instances of convergent evolution of red emitting homologues from green ancestor proteins (Shagin et al. 2004; Ugalde et al. 2004; Field et al. 2006; Gittins et al. 2015). Furthermore, the benefits and trade-offs associated with high level expression of pcRFPs may have contributed to the rise of colour morphs among individuals from genera such as *Trachyphyllia*, *Lobophyllia*, *Echinophyllia* and *Montastraea* (Field et al. 2006; Oswald et al. 2007; Gittins et al. 2015) as a result of balancing selection as previously suggested for GFP-like proteins in both shallow and deep water corals (Quick et al. 2018).

Photoprotective chromoproteins and bleaching in shallow water corals

To evaluate the effect of high ectodermal concentrations of CPs on the light environment of coral symbionts *in hospite*, we performed spectral scalar irradiance measurements on colonies of *Pocillopora damicornis* and *Montipora foliosa*. Pink colour morphs of *P. damicornis* express a CP with absorption maximum at 565 nm (Dove et al. 1995), which can accumulate in high concentrations following bleaching (Bollati et al. 2020) (Fig. 5a). Brown morphs of this species do not produce detectable amounts of CP (Fig. 5a). The bare white skeleton of *P. damicornis* enhanced spectral scalar irradiance by 140-150% across the entire visible spectrum (Fig. 5a). In brown morphs, the enhancement was reduced to <120% in the wavebands corresponding to the absorption peaks of symbiont photosynthetic pigments, while the enhancement remained close to 130% in the orange region of the spectrum (Fig. 5a). Bleached corals expressing high pink CP concentrations experienced a surface light enhancement in the blue spectral region comparable to what was measured over bare skeleton; however, this was reduced to ~123% in the orange waveband corresponding to the absorption peak of the CP (Fig. 5b). Measurements of the spectral attenuation coefficient from colony surface to skeleton showed that brown unbleached tissue is light-attenuating in the blue and red regions of photosynthetic pigment absorption, but light-enhancing in the orange-red region; conversely, blue light is enhanced in bleached pink tissue, while orange light is attenuated (Fig. 5c).

M. foliosa has several colour morphs, one of which produces high concentrations of a purple CP localised in areas of low symbiont density at the colony margins, while the inner area has low CP content and appears brown (D'Angelo et al. 2012) (Fig. 5d). Measurements of surface spectral scalar irradiance on tissue found in the inner region of the colony showed similar spectral properties to those of brown *P. damicornis* and exhibited 140% light enhancement, which was reduced to 110% in the symbiont photosynthetic pigments absorption wavebands (Fig. 5e). In the high CP area at the colony margin, scalar irradiance showed a strong enhancement (>150%) comparable to what measured for bleached tissue in a CP-free morph

(Fig. 5e); however, this was heavily spectrally modified by the presence of the purple CP, which reduced light enhancement by up to 25% within its absorption spectrum compared to other spectral bands (Fig. 5e).

The *P. damicornis* skeleton exhibits an intermediate level of microscale fractality and an intermediate scattering coefficient (mass-fractal dimension ~ 1.75 and reduced scattering coefficient of $\sim 7.5 \text{ mm}^{-1}$ (Marcelino et al. 2013)), which implies a relatively efficient redistribution of light over the tissue (Marcelino et al. 2013). While this type of skeletal structure can help healthy corals optimise photosynthesis, during bleaching this can amplify the irradiance increase derived from the loss of photosynthetic pigment absorption (Enríquez et al. 2005; Marcelino et al. 2013; Wangpraseurt et al. 2017). Our data confirms that bleached *P. damicornis* tissue indeed experiences a strongly light enhancing microenvironment, resulting from enhanced skeletal scattering due to reduced tissue absorption (Wangpraseurt et al. 2017). Such strongly enhanced light microhabitat can also be found in healthy corals such as in the growing plate margins of *M. foliosa*, where the symbiont population is less abundant compared to the inner areas (Fig. 5d). While blue light is attenuated in unbleached corals, the absence of symbionts in bleached corals or tissue areas with low symbiont densities (e.g. in areas of active tissue growth), results in the enhancement of blue light possibly leading to transcriptional upregulation of CPs (D'Angelo et al. 2008). This supports the notion that the optical feedback loop is a key mechanism underlying the colourful bleaching phenomenon (Bollati et al. 2020). Our data also shows that high CP concentrations counteract the light-enhancing effects of bleaching within the CP absorption spectrum, as signified by the relative light enhancement measured in bleached corals in the orange region, a waveband known to result in light stress and bleaching for various coral species when provided at high irradiance (Smith et al. 2013; Wijgerde et al. 2014; Quick et al. 2018). Thus, optimisation of the light microenvironment by CPs in symbiont-depleted tissue could provide the necessary conditions for (re) colonisation by symbiont cells (D'Angelo et al. 2012; Smith et al. 2013; Bollati et al. 2020).

Figure 5. Effects of photoprotective chromoproteins on coral light environment. a) Representative photographs of unbleached brown and bleached pink *P. damicornis*. **b)** Spectral scalar irradiance ($E_0(\lambda)$) measurements at the coenosarc surface of unbleached brown *P. damicornis*, bleached *P. damicornis* with high CP content, and *P. damicornis* skeleton. **c)** Attenuation coefficient of $E_0(\lambda)$ for unbleached brown and bleached pink *P. damicornis*, calculated from surface to skeleton (tissue thickness = $\sim 300 \mu\text{m}$). **d)** Compilation of representative photographs of the unbleached high CP morph, unbleached no CP morph and bleached no CP morph of *M. foliosa*. **e)** Surface $E_0(\lambda)$ measurements of unbleached inner region (low CP), unbleached margin (high CP) and bleached no CP morph of *M. foliosa*. In **a** and **d**, scale bar = 5 mm. In **b** and **e**, mean (solid lines) $\pm$ s.d. (dashed lines), $n=3$. Arrows indicate peak absorption wavelength in nm for symbiont photosynthetic pigments (black) and CPs (pink).

While the pcRFPs and CPs examined in this study are characterised by very different optical properties, environmental regulation and ecological distribution, both coral host pigments can regulate internal light fluxes in the orange-red spectral range. Enhancement of this waveband can be a double-edged sword for corals; on one hand, poor absorption by photosynthetic pigments allows better penetration (Smith et al. 2017), which can be exploited by pcRFP-containing corals to illuminate deeper tissue areas under light limitation. On the other hand, light amplification in symbiont-free tissue produces excess irradiance in this waveband, which can cause photodamage unless counteracted by CP screening or other photoprotective mechanisms (Smith et al. 2013; Wijgerde et al. 2014; Wangpraseurt et al. 2017; Quick et al. 2018). Spectral regulation by GFP-like host pigments is thus a flexible

mechanism to achieve fine spectral regulation of the intra-tissue light field of corals, enabling them to optimise light capture for their algal symbionts in response to environmental light gradients. Future investigations into this diverse and ubiquitous protein family will likely reveal further mechanisms through which the coral host supports light capture and facilitates the photosymbiosis along the steep light gradients that characterise coral reef habitats.

Materials and Methods

Coral culture and aquarium set up

Coral colonies of *M. cavernosa*, *Echinophyllia* sp., *Pocillopora damicornis* and *Montipora foliosa* were asexually propagated for >10 years in the experimental coral aquarium facility of the Coral Reef Laboratory, National Oceanography Centre, Southampton, UK (D'Angelo and Wiedenmann 2012).

Spectral scalar irradiance measurements

Microscale spectral light measurements were conducted as described in Wangpraseurt et al. (2017) using scalar irradiance microsensors with a spherical tip diameter of 60 μm (Rickelt et al. 2016) and connected to a fiber-optic spectrometer (QE65000, Ocean Optics, USA). Microsensor profiles were performed from the coral skeleton upwards towards the tissue surface in vertical increments of 20-100 μm using a motorized micromanipulator (Pyroscience GmbH, Germany) controlled by dedicated software (Profix, Pyroscience GmbH, Germany). The step size was adjusted according to the total coral tissue thickness with step sizes of 20 μm for *P. damicornis*, 70 μm for *M. cavernosa* and *Echinophyllia*. For *M. foliosa*, only surface spectra were collected as the tissue was very thin (< 60 μm). Scalar irradiance measurements were performed using vertical illumination from above, while the scalar irradiance microsensor was inserted into coral tissue at an angle of 45° relative to the incident light (Fig. S1a). White LED illumination was provided by a fiber-optic lamp (KL-1600 LED, Schott GmbH, Germany) (Fig. S1a,b), producing 360-900 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the measurement distance.

For *M. cavernosa* and *Echinophyllia*, we also measured the spatial distribution of emitted fluorescence. For this, narrow-waveband excitation in the blue spectral range was provided vertically incident by a xenon arc lamp equipped with a band-pass filter (Leica GFPplus filter, $\lambda=455\text{-}505\text{nm}$, Fig. S1c) and scalar irradiance measurements in the 505-650 nm range were used to estimate fluorescence emission. The light source output (455-505 nm) at the measurement distance was 12-46 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. No emission filter was used on the detector side as bleed-through of excitation light in this spectral range was minimal (<0.5%). Scalar irradiance microsensor measurements were calibrated against a spectroradiometer (MSC15, Gigahertz-Optik, Germany) by measuring the incident downwelling irradiance from

the white LED light source over a black light absorbing surface first with the microsensor in water, then with the spectroradiometer at the same distance from the light source in air (Wangpraseurt et al. 2012; Brodersen et al. 2014).

Induction of photoconversion and measurements of pcRFP-containing corals

We performed scalar irradiance measurements in *M. cavernosa* at different stages of photoconversion to assess how near-UV driven photoconversion of the pcRFP pool affects the symbiont light environment. We defined the stages of photoconversion by the relative proportions of green (514 nm) and red (582 nm) fluorescence emission, measured with 455-505 nm excitation at the colony surface. A 514:582 nm ratio >1 indicates a higher contribution of the green chromophore (defined as *unconverted* coral), a ratio ~1 indicates substantial contributions from both the green and the red chromophore (defined as *partially converted* coral), and a ratio <1 indicates a higher contribution of the red chromophore (defined as *converted* coral).

To obtain *unconverted* corals, we placed two colonies derived from fragmentation of the same mother colony under green incident irradiance provided by LED strips (Lumiled Luxeon Rebel Green, λ =530 nm, FWHM=60 nm; Fig. S2a) for 40 days. This treatment resulted in the accumulation of the unconverted pcRFP (Leutenegger et al. 2007), evidenced by a 514:582 nm emission ratio >1. We then used one of these colonies to induce localised photoconversion by illuminating the coral tissue surface (spot diameter 3 mm) with a xenon arc lamp of a fluorescence microscope (Leica MZ10 equipped with a UV bandpass filter (λ =355 nm, FWHM=55 nm; Fig. S2b) for 6 hours (Eyal et al. 2015); this waveband only partially overlaps with the action spectrum of pcRFP photoconversion (Wiedenmann et al. 2004) and is therefore less effective in inducing the photochemical reaction. In combination with the limited duration of the exposure, the UV treatment resulted in a mix of unconverted and converted pcRFP, as evidenced from the 514:582 nm ratio ~1 (*partially converted* coral). The second *M. cavernosa* colony was exposed to near-UV irradiance provided by LED strips (Aquaray NUV, λ =412, FWHM=18.5 nm; Fig. S2c) for 10 hours; this treatment induces rapid photoconversion of the pcRFP pool (Bollati et al. 2017), resulting in a 514:582 nm emission ratio <1 (*converted* coral). All photoconversion experiments were performed in a flow-through system at a water temperature of 26°C regulated by a 25 W aquarium heater (Tunze).

We also assessed the contribution of fluorescence emission by pcRFPs to spectral scalar irradiance against other GFP-like proteins by performing measurements on two morphs of *Echinophyllia* sp.. Both morphs had been kept under broad spectrum illumination long term, side by side in our mesocosm. The green morph contained a non-inducible GFP, while the

red morph contained a CFP and a highly converted pcRFP (green:red emission ratio <<1) (Alieva et al. 2008; Bollati et al. 2017; Smith et al. 2017).

Experimental bleaching and measurements of corals containing inducible CPs

To test how bleaching affects irradiance in corals containing inducible CPs, we performed measurements on healthy and bleached *P. damicornis* and *M. foliosa*. A *P. damicornis* colour morph capable of expressing a pink CP with absorption maximum at 565 nm (Dove et al. 1995; D'Angelo et al. 2008; Bollati et al. 2020) was bleached by exposure to 400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of red LED light (Lumiled Luxeon Rebel Deep Red, $\lambda=660 \text{ nm}$, FWHM=40 nm; Fig. S2d) for 7 days. This treatment induces bleaching without inducing the expression of the light-sensitive genes responsible for CP production (D'Angelo et al. 2008; Smith et al. 2013; Wijgerde et al. 2014; Bollati et al. 2020). Bleaching was confirmed by visual comparison with untreated specimens. Bleached fragments were then exposed to blue light (100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; Lumiled Luxeon Rebel Royal Blue, $\lambda=450 \text{ nm}$, FWHM=40 nm; Fig. S2e) for 20 days to stimulate upregulation of the pink CP, resulting in a “colourful bleaching” response (D'Angelo et al. 2008; Bollati et al. 2020) (Fig. S2). Alongside the bleached specimen, we performed spectral scalar irradiance measurements on i) a *P. damicornis* skeleton and ii) an unbleached brown morph of the same species, which expresses an inducible CFP but no CP.

For *M. foliosa*, we measured a morph capable of producing a CFP and a pink CP, with the latter accumulating in high concentrations at the colony margins (D'Angelo et al. 2012; Bollati et al. 2020). We compared this healthy, unbleached coral with a *M. foliosa* morph that does not produce CPs, bleached by phosphate starvation (Wiedenmann et al. 2013; Rosset et al. 2017; Bollati et al. 2020) after 65 days in our high nitrate / low phosphate mesocosm (D'Angelo and Wiedenmann 2012). Both colonies were kept under broad spectrum illumination (200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; Aquamedic metal halide lamp).

Data processing

All spectral data were corrected for spectrometer dark noise readings obtained under dark conditions and calibrated against a spectroradiometer as described above. For spectral measurements performed under incident white light, the spectra were normalised to the incident irradiance as described previously (Wangpraseurt et al. 2012).

The spectral attenuation coefficient of scalar irradiance ($K_0(\lambda)$) was calculated from measurements obtained under white light following Kühl (2005):

$$K_0(\lambda) = \frac{\ln \left(\frac{E_0(\lambda)_1}{E_0(\lambda)_2} \right)}{z_2 - z_1}$$

where $E_0(\lambda)_1$ and $E_0(\lambda)_2$ are the spectral scalar irradiance measured at tissue depths z_1 and z_2 . $K_0(\lambda)$ was calculated from surface to skeleton using the average tissue thickness (deduced from the mean number of equal steps performed by the micromanipulator from the skeleton to the upper tissue surface: 560 μm for *M. cavernosa*, 300 μm for *P. damicornis*). For fluorescence emission measurements under blue excitation, integrated photon irradiance was calculated for different portions of the visible spectrum (incident: 455-505 nm; green emission: 505-560 nm; red emission: 560-650 nm) after converting spectral scalar irradiance to spectral photon scalar irradiance using Planck's equation (see (Lichtenberg et al. 2017)). Integrated photon irradiance over the same incident (455-505 nm) and red (560-650 nm) spectral ranges was also calculated for a dataset of field spectral scalar irradiance collected from Eilat, Israel (Eyal et al. 2015) and for a dataset of spectral scalar irradiance simulated from surface measurements collected in the Bahamas (Lesser et al. 2009).

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Data availability

Data used in this study is available from <https://doi.org/10.5061/dryad.0gb5mkm1z>

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Supplementary Information file

Green fluorescent protein-like pigments optimize the internal light environment in symbiotic reef building corals

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Figure S1. The scalar irradiance measurement set up (**a**) and spectra of the two light sources used for measurement (KL-1600 LED, **b**; xenon arc lamp + GFPplus filter, **c**)

Figure S2. Spectra of light sources used for photoconversion (a-c) and bleaching (d,e) experiments (a, Lumiled Luxeon Rebel Green; b, xenon arc lamp + UV bandpass; c, Aquaray NUV; d, Lumiled Luxeon Rebel Deep Red; e, Lumiled Luxeon Rebel Royal Blue)

Figure S3. Photon scalar irradiance in the tissue of green and red morphs of *Echinophyllia*. **a)** Fluorescence micrographs (**a**) and surface fluorescence emission (**b**) of green and red morphs. **c)** Integrated green (505-560 nm) and red (560-650 nm) fluorescence emission along a tissue depth profile, measured under blue excitation (455-505 nm, Fig. S1). Spheres show means $\pm$ s.d., $n=3$.

Figure S4. Relative contribution of fluorescence emission by pcRFPs and downwelling irradiance to the total 560-650 nm light available to pcRFP-containing corals along a depth gradient in the Bahamas (modelled irradiance data from ref [13]).

Figure S5. Relationship between blue (455-505 nm) excitation light photon irradiance and emitted green (505-560 nm, **a**) or red (560-650 nm, **b**) fluorescence at 10 tissue depths, obtained with a set of neutral density filters. Trendlines represent fitted linear regression (adjusted $p < 0.05$, table S1).

Incident intensity calibration (Fig. S5 Supplementary Methods)

To determine whether the relationship between excitation light intensity and emitted fluorescence was linear within our measurement range, we performed a calibration profile on a *converted M. cavernosa* sample using a set of neutral density filters. At each depth, we collected fluorescence emission measurements under 455-505 nm excitation with five combinations of neutral density filters placed between the excitation light and the specimen. For each measurement, we then calculated the integrated photon scalar irradiance in the incident range (455-505 nm), the integrated green fluorescence emission (505-560 nm, fig. S5a) and the integrated red fluorescence emission (560-610 nm, fig. S5b). A linear regression was fitted for each tissue depth and each chromophore, with intercept set to zero (Fig. S5); p-values were corrected for multiple testing using the Bonferroni adjustment.

Table S1. Linear regression results for Fig. S5.

Green (505-560 nm) fluorescence emission						
Depth	Slope	Std Error	t value	R² adjusted	P	Adjusted P
0	0.00902626	9.03E-05	99.9875929	0.99950008	6.00E-08	6.00E-07
70	0.0087601	7.62E-05	114.967832	0.99962183	3.43E-08	3.43E-07
140	0.01156811	0.00021343	54.2017461	0.99830038	6.94E-07	6.94E-06
210	0.01619148	0.00017715	91.4024378	0.9994018	8.59E-08	8.59E-07
280	0.02494033	0.00019703	126.581943	0.99968803	2.34E-08	2.34E-07
350	0.03021069	0.00018083	167.069327	0.99982089	7.70E-09	7.70E-08
420	0.03351092	0.0003004	111.554686	0.99959834	3.87E-08	3.87E-07
490	0.033718	0.00022538	149.603347	0.99977664	1.20E-08	1.20E-07
560	0.02842214	0.00042339	67.1300277	0.99889146	2.95E-07	2.95E-06
630	0.01999956	0.00028305	70.6577889	0.9989993	2.40E-07	2.40E-06
Red (560-650 nm) fluorescence emission						
Depth	Slope	Std Error	t value	R² adjusted	P	Adjusted P
0	0.01719483	8.20E-05	209.593244	0.99988619	3.11E-09	3.11E-08
70	0.01510699	6.92E-05	218.286085	0.99989507	2.64E-09	2.64E-08
140	0.01881416	0.00019939	94.3604722	0.9994387	7.56E-08	7.56E-07
210	0.0266481	8.29E-05	321.256566	0.99995155	5.63E-10	5.63E-09
280	0.04096949	0.00025024	163.718467	0.99981349	8.35E-09	8.35E-08
350	0.05367283	0.00046253	116.040816	0.99962879	3.31E-08	3.31E-07
420	0.07300642	0.00047553	153.525421	0.9997879	1.08E-08	1.08E-07
490	0.09251997	0.00093074	99.4042684	0.99949419	6.14E-08	6.14E-07
560	0.08291338	0.00118633	69.8904363	0.99897723	2.51E-07	2.51E-06
630	0.06235948	0.00053652	116.22921	0.99962999	3.29E-08	3.29E-07