

# The role of host-based color and fluorescent pigments in photoprotection and in reducing bleaching stress in corals.

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**Abstract** Coral tissue colors result from both the intracellular symbiotic dinoflagellates and the host's own cellular pigments. The brownish colors are due to the symbionts' photosynthetic pigments; the bright purple-blue and fluorescent colors are produced by the coral host and are proteins closely related to Green Fluorescent Protein (GFP). One of the documented biological functions of GFP-like pigments in corals is light optimization. When light is excessive, GFPs reduce symbionts' photoinhibition and photo-damage to coral's tissues. Here we explored further the ecological roles of coral coloration. Spectral properties of GFP-like pigments of many common, shallow reef corals were found to match the absorption of dinoflagellate pigments, indicating suncreening by photon removal/deflection. The survey of depth-related distribution of fluorescent and purple-blue color-morphs further confirmed the role of GFPs in photoprotection. These color-morphs were most abundant at high-light shallow depths and their numbers dropped with increasing depths, however, at deeper sites, numbers of fluorescent color-morphs increased, suggesting a reversal of GFP function from suncreening to that of light amplification in light-limited habitats. We also examined the degree of bleaching damage in different color-morphs during and following the 2002 mass bleaching event. We found that low-GFP-pigmented morphs had significantly higher degrees of damage to their symbionts, especially to photosynthesis and a higher degree of partial colony mortality than in high-GFP-pigmented morphs. These results further substantiate earlier findings that GFP-like pigments reduce photoinhibition and the severity of bleaching-related physiological damage of corals.

## Introduction

Tropical coral reefs are some of the most colorful assemblages of organisms. Reef building corals, the organisms largely responsible for reef formation, have an abundance of color pigments and exhibit diverse intra-specific color variations (i.e., polymorphism), ranging from bright pink or purple-blue colors to intensely fluorescent greens, yellows and reds (e.g., Kawaguti, 1944; Mazel, 1995; Salih *et al.* 1998a, 2000; Dove *et al.* 2001; Veron 2000). Research of color pigmentation in reef anthozoans recently came into prominence with a breakthrough discovery that each pigment color is determined by a sequence of a single family of proteins, closely related to the green fluorescent protein (GFP) (Matz *et al.* 1999). GFP, first isolated from the jelly-fish *Aequorea victoria* (Shiomomura *et al.*, 1962), is one of the most biotechnologically famous proteins and, together with a range of its color variants, GFPs are used extensively in fluorescence-based microscopy and other technologies as fluorescent markers for medical and biological research (Tsien, 1998). The GFP-type host-based colors of corals, anemones, zoanths and relatives are mainly of two types – the fluorescent pigments (**FPs**) that may or may not be visible in daylight (Matz *et al.* 1999; Weidenmann *et al.* 2000, 2004; Labas *et al.* 2002; Mazel *et al.* 2003); and the non-fluorescent, chromophoric (**CPs**), the pink, purple or blue colors, prominently visible in daylight (Weidenmann *et al.* 2000; Lukyanov *et al.* 2000; Dove *et al.* 2001; Gurskaya *et al.* 2001), sometimes referred to as “pocilloporins” (Takabayashi & Hoegh-Guldberg, 1995; Dove *et al.* 1995). These GFP-based colors are highly abundant on reefs; for example, a study of fluorescent corals found that in the shallowest reef zones of the Great Barrier Reef most colonies are pigmented at varying degrees, with up to 97% of corals containing fluorescent pigmentation (Salih, 2000; Salih *et al.* 2000).

The biological function of GFP in the jellyfish is believed to be as an acceptor of blue bioluminescence emissions from the  $\text{Ca}^{2+}$ -triggered photoprotein aequorin (e.g., Ward 2002). There is still, however, no consensus on the functional role of the astonishing color diversity in non-bioluminescent marine organisms such as reef corals. Early suggestions of a suncreening function of coral coloration (Kawaguti, 1944) were substantiated by later research that provided the first direct evidence that FPs function in light-optimisation of coral tissue and in shallow corals are photoprotective (Salih *et al.* 1998a, 2000, 2001; Dove *et al.* 2001, 2004; Gilmore *et al.* 2003). The suggested mechanism of photoprotection is via absorption of high energy solar radiation and fluorescence dissipation by FPs and by transfer of light energy among spectral variants of GFP via radiative energy transfer and dissipation (Salih *et al.* 1998a, 2000; Dove *et al.* 2001; Salih, 2004) and via the more efficient, the non-radiative FRET (Förster resonance energy transfer) (Förster, 1948) among intracellular

assemblies of GFP-like proteins (Gilmore *et al.* 2003; Salih *et al.* 2003, 2004; Cox & Salih, 2005). In many corals dense pigment layers can even function in light screening by photon scattering and broadband reflection (Salih *et al.* 1998a, 2000; Gilmore *et al.* 2003), accounting for the frequently observed white or silvery coloration of coral tissues of some intertidal genera e.g., *Favia*, *Platygyra*, *Goniastrea* (e.g., *Faviidae*, Veron, 2000). The photoprotective function of coral FPs may be reversed – FPs enhance light in low-light conditions (Schlichter *et al.* 1990; Salih *et al.* 1998a, 2000).

It is widely known that high irradiances exacerbate thermal bleaching (Brown 1997; Jones *et al.* 1998; Salih *et al.* 1998b; Brown *et al.* 2000). Consequently, good photoprotective strategies also provide a significant defense against thermal stress and mass coral bleaching (reviewed, Coles & Brown, 2003). A significant correlation between corals' FP concentration and the degree of the resistance to bleaching was recorded following the 1998 mass bleaching event on the Great Barrier Reef (GBR), accounting for some of the observed inter- and intra-specific differences in bleaching (Salih, 2000; Salih *et al.* 2000). In general, studies of the hierarchy in species susceptibility to bleaching find that pocilloporids and acroporids are highly susceptible (Marshall & Baird 2000; McClanahan *et al.* 2004) and these often have relatively low densities of GFPs (Salih *et al.* 1998a, 2000), while faviids and other slower growing, massive corals which are often highly resilient to bleaching (Baird & Marshall 2002), have relatively high GFP concentrations (Salih *et al.* 1998a, 2000). By modulating the light that reaches the symbiotic algae, GFP-like pigments confer a better resistance to temperature-induced bleaching in comparison to non-fluorescent morphs (Salih *et al.* 2000).

The present study investigated further the photoprotective function of coral colors and their role in enhancing bleaching resistance. This was done by evaluating the cellular localization of FPs and dinoflagellates; characterizing the spectral properties of GFP-like pigments; surveying the depth distribution of coral color-morphs; and by comparing the effects of bleaching on dinoflagellate photoinhibition, dinoflagellate degradation and colony mortality of GFP-pigmented and non-pigmented morphs.

## Materials and Methods

**Sample collection** - Scleractinian coral samples were collected at the inter-tidal lagoon, reef flat and inner and outer slope of Heron Island (23°26'S, 151°55'E), One Tree Island (OTI) (23°30'S, 152°06'E), Ribbon Reefs (No.14.139 14°39'378"; 145°38'515"; No.14.140 14°39'831", 145°39'807"; No.14.041 15°23'791", 145°45'852", No.15.050 15°30'101", 145°47'243"; No.14.151 14°54'932", 145°41'468"; No.14.152 - 14°55'60", 145°40'80"; No.15-072 15°30'766", 145°46'643" & Tracy Wonderland, 15°30'46.1"S, 145°46'37.5"E) of the Great Barrier Reef and Osprey reef (13°53.244, 146°33'435"; 13°48'086", 146°32'729"; 13°54'143", 146°33'714") in the Coral Sea, Australia, at sites 0.2-40 m depths. Samples were brought back in seawater and maintained in aquaria at One Tree or Heron Island research stations, or on-board the Undersea Explorer vessel or at the marine aquaria facility of the Electron Microscope Unit, University of Sydney. They were also frozen or fixed in glutaraldehyde, using a fixation protocol (2.5% glutaraldehyde, 0.1M phosphate buffer and stored at 4°C degrees Celsius until analysis) that was found to preserve cell structural integrity, as well as the fluorescence characteristics of GFP-like proteins (Salih *et al.* 1998a).

**Host-based GFP pigment spectral characterization** - The excitation and emission spectra of FPs were determined using small samples taken from live, frozen or glutaraldehyde-fixed corals. *In vivo* excitation and emission spectra of the major FPs were obtained using fluorescence spectrophotometer (Varian Cary Eclipse) with a fiber-optic probe attachment, which was immersed in seawater and placed immediately above coral surface. The fiber-optic coupler, in combination with the remote read probe, enabled spectral characterization of live corals without the need for protein extraction. Spectra were

collected at 5 nm slit width resolution. For each coral, a full complement of excitation/emission spectra was determined for as many FPs as was possible to resolve by this method. Non-fluorescent purple-blue pigments were characterized by the absorption spectra either in 0.1 phosphate buffer using UV-Vis absorption spectrophotometer.

**Fluorescence, confocal microscopy & micro-spectrophotometry** - Tissues dissected from live or glutaraldehyde-fixed coral polyps were imaged using a Nikon Eclipse E800 microscope and standard epifluorescence system with a 100W mercury lamp, using filters to excite blue FP (Ex 330-380nm; DM400nm; BA420nm), green FPs (Ex 450-490nm; DM505 nm; BA520nm) and red FPs (Ex510-540nm) and fitted with a cooled CCD camera (Sensicam). FPs in excised live tissues were also imaged by Leica TCS spectrophotometer confocal inverted epifluorescence microscope (Leica, Heidelberg, Germany) at excitation with Ar (458, 488, 514 nm lines) and green HeNe lasers (543 nm line). All fluorescence emission spectra from cells were obtained by directing the emissions into a PMT1 at 8 bit resolution (256 greyscale levels) and measuring the entire spectral data (400-700nm). Spectral characteristics of cells were determined by selecting 6-12 areas of interest.

Bleaching-related degradation of dinoflagellates in color-morphs was investigated in *Acropora intermedia* (n = 15) sampled during the 2002 mass bleaching at Chinaman Reef, GBR. Samples were visually assessed for bleaching and for the purple-blue pigmentation. Sub-samples were fixed in glutaraldehyde (Salih *et al.* 1998a) and the degree of their host-based fluorescence subsequently categorized as low, medium or high using epi-fluorescence microscopy at blue (450-490nm) excitation on a Nikon Fluorescence microscope. Algae isolated from sun-exposed sample surfaces were imaged by Leica confocal microscope at 488 nm excitation. Bleaching related degradation of algal symbionts were identified as in Salih *et al.* (1998b).

**Transmission Electron Microscopy (TEM)** – TEM of dinoflagellates from the above-mentioned samples was used to evaluate the degradation processes at a higher resolution. Glutaraldehyde-fixed tissue from each *A. intermedia* sample were fixed in 1% Osmium tetra oxide, dehydrated in ethanol series and embedded in Spurr's resin. Thin (90 nm) sections were cut by Leica Ultracut UCT Microtome (Germany), mounted on un-coated 200 mesh grids, contrasted with Uranyl Acetate (14 minutes) and lead citrate (12 minutes) and examined with a Biofilter CM120 TEM and a Zeiss 902 Transmission Electron Microscope.

**Bathymetric survey of color-morphs** – Depth-related distribution of color morphs was determined at 3 sites at Osprey Reef in the Coral Sea. This oceanic atoll has very clear waters and steep slopes, presenting a good opportunity to assess the effect of depth and, consequently light, on the distribution of CPs and FPs. Coral samples were randomly collected from corals at 1, 5, 10, 20, 30 and 40 m depths. Purple-blue coloration of each sampled coral was recorded based on visual observation. The degree of fluorescent pigmentation in samples was established using epifluorescence microscopy at UVA, blue and green light excitation. From combined results of visual and microscopic analyses, each coral was categorized as purple-blue, fluorescent or non-fluorescent morph, based on the degree of its dominant pigmentation.

**Bleaching & post-bleaching visual surveys** – A mass bleaching event occurred on most of the GBR between January and March 2002. At Heron Island corals experienced temperatures at  $<3.2^{\circ}\text{C}$  and irradiance of  $<2.0 \times 10^4 \text{ m m o l q u a n t a m}^{-2} \text{ s}^{-1}$  (Berkelmans *et al.* 2003; Dove, 2004). Reefs of the Coral Sea also bleached. At each site, a qualitative visual assessment of the degree of bleaching and the susceptibility of different color-morphs was made in January and February 2002 at Heron Island reefs, in March 2002 at Ribbon GBR and Osprey Coral Sea reefs. Most sites were surveyed by day-time, as well as at night-time, using blue-light torches (NightSea, US) to excite FPs, photographed and filmed using an underwater video camera with water-proof casing.

**Bleaching-related photoinhibition of color-morphs** – During the 2002 mass bleaching event at Osprey Reef, we investigated whether host-based pigments influenced the degree of bleaching-related damage of dinoflagellate photosynthesis in different color-morphs. The reduction of photosynthetic efficiency of symbionts was analyzed by dark-adapted quantum yield of chlorophyll fluorescence ( $F_v/F_m$ ) measurements using Diving-PAM (pulse amplitude modulation) fluorometer. The analysis was made at night-time using randomly selected corals ( $n = 42$ ). Following the  $F_v/F_m$  analysis, corals were illuminated by blue-light torches (NightSea, US) and their host-based fluorescence determined. Non-fluorescent, purple-blue coloration of each colony was visually determined at white light illumination. Based on this visual examination, the degree of host-based pigmentation, including both the purple-blue and the fluorescent pigmentation, was categorized as highly pigmented (HP), medium pigmented (MP) or non/low pigmented (LP). In cases where corals were low-fluorescent or medium-fluorescent but strongly CP-colored, they were categorised as HP. The degree of bleaching was visually assessed and all colonies categories as: 0 – unbleached; 1-very mildly bleached; 2–medium bleached; 3–almost completely bleached, except for shaded areas; 4-completely bleached. Significant differences between the degree of bleaching and  $F_v/F_m$  of color-morphs were tested by one-way ANOVA and Tukey test ( $P < 0.05$ ).

**Bleaching-related mortality of color-morphs** – During the severe mass bleaching at Chinaman Reef, GBR, a quantitative survey was conducted to investigate whether purple-blue pigmentation affected the degree of bleaching and the resultant tissue mortality of branching species. This site was selected because bleaching was sufficiently severe to cause rapid partial colony mortalities. Randomly selected colonies were photographed and their color-morph status defined as purple-blue pigmented if their tissues were visibly purple-blue, or non-pigmented, if tissues were cream, beige or brownish. Samples from each were taken for epifluorescent determination of tissue fluorescence. Samples from some cream-beige colonies were found to be cyan fluorescently pigmented and the latter were excluded from the subsequent analysis so that only purple-blue (+/- fluorescent) versus non-pigmented morphs were compared. The degree of bleaching was visually divided into 5 categories as described above. The amount of partial colony tissue mortality was visually estimated *in vivo* as percentage of dead colony area. Bleaching-related tissue mortalities were classed as all those in which dead tissues were surrounded by visibly bleached tissues and the dead parts were “freshly dead” and not thickly overgrown with endolithic algae so as to differentiate bleaching-related mortalities from mortalities prior to bleaching. One-way ANOVA was used to compare the degree of bleaching and mortality in the two color-morph groups (JMP).

Another survey was conducted to determine the post-2002 bleaching partial colony mortality of massive corals at Osprey Reef. The survey was carried out 18 months after the bleaching event (as part of CoReLL2003 workshop, Aug-Sept 2003) at a depth of 5 m at 2 sites - inside the lagoon and at outer reef slope. For analysis, data from 2 sites was pooled. The survey was at night-time to enable the fluorescence characteristics of surveyed colonies to be established by blue-light illumination. Randomly selected colonies were first illuminated by blue-light NightSea torch and were categorized as either highly fluorescent, medium fluorescent or non-fluorescent. Colonies were next illuminated with white light, photographed, taxonomically identified and the percentage of dead tissue area of each colony estimated. Differences between the 3 color-morph groups were determined by one-way ANOVA and Student's t-test (JMP).

## Results

### Spectral properties of GFP-type pigments.

Excitation and emission spectra collected from live corals immersed in seawater showed that almost all sampled colonies ( $n = 61$ ) were fluorescently pigmented: the majority contained medium to high FP concentrations and only relatively few corals contained low FP concentrations. Most corals had more than one spectral FP type and usually had one major and 1 to 7 minor FPs. In total, there were over

40 FP spectral variants, with emissions in blue, green and red, from 470 to 640 nm, although these may not necessarily represent distinct GFP-like proteins (**Fig. 1**, showing representative spectra). The most abundant FPs were blue and cyan ( $\lambda_{ex}=380-446$ ;  $\lambda_{em}=470-500$ nm) and greens ( $\lambda_{ex}=446-515$ nm/ $\lambda_{em}=502-535$ nm). Yellow ( $\lambda_{ex}=480-540$ ;  $\lambda_{em}=540-568$ nm) but red FPs ( $\lambda_{ex}=550-578$ ;  $\lambda_{em}=570-640$  nm) were commonly present in low concentrations in many corals. In shallow water, many corals, such as *Acropora intermedia*, *A. cytheria*, *A. pulchra* that form large monospecific stands, or *Styloporora pistillata*, *Favia* spp and other genera, contained blue and green FPs with excitation peaking in the UVA-blue (380-446nm) or blue-green (460-515 nm) range (**Fig. 1 a-e**). The excitation of these FPs was extremely well matched to the excitation peaks for dinoflagellate pigments (**Fig. 1f**), spectrally characterized using live corals that did not possess FPs or using freshly extracted algae from coral tissues. Absorption spectra of buffer extracts of CPs from purple-blue pigmented tissues of *Acropora*, *Styloporora*, *Montipora*, *Porites* spp showed maximal absorption in the green-orange region at 530-590 nm. In many cases, however, these buffer extracts also contained fluorescent pigments when analysed fluoro-spectrophotometrically.

### **In vivo imaging and microspectral characterization of GFP-like pigments.**

Imaged by epifluorescence microscopy FPs had variable inter- and intra-specific distribution patterns and had different in localization of dominant FPs within and between colonies. For example, in *Faviidae*, FPs were uniformly distributed in the tissues, or localized between polyps or, most frequently, concentrated in the oral discs of polyps (**Fig. 2 b-c**) but were reduced in tentacles. Polyps of fleshy corals, such as *Goniopora* spp, were often strongly pigmented, with both fluorescent (**Fig. 2e-f**) and purple-blue pigments concentrated in the oral disc and tentacle tips. In faviids, FPs were usually localized within pigment granules in specialized pigment cells (**Fig. 2h**). Other genera with granular FPs include *Poritidae*, especially *Goniopora* spp, several *Agariciidae* spp, etc. FPs in many other common families, such as *Acroporidae*, *Pocilloporidae*, *Pectiniidae*, *Fungiidae*, *Mussidae*, etc., were concentrated either in or between polyps (**Fig. 2d**), in tentacles, and light-facing parts of corals, such as calyces, branch tips, etc.; were not granular or contained within specialized cells.

Confocal optical sectioning of pigmented tissues showed that FPs occurred both in cells of the ectoderm and the endoderm (**Fig. 2g-h**), but not the mesogloea. There was usually a strong correlation between the most fluorescently pigmented tissues and the dinoflagellate concentrations – algal densities were frequently highest underneath pigmented oral discs, tentacles and tentacle tips. The exception to this was the presence of low algal numbers in *Acropora* colony tips which contained highly pigmented (either purple-blue CPs or FPs or mixtures) tissues but few algae.

We also performed microspectrometric analysis of FP-containing cells dissected from coral tissues in several common species in order to characterize the full complement of FPs at cellular level. This technique resolved an even higher spectral diversity than previous macro-spectrophotometric FP analyses. In many species, up to 12 spectrally distinct FPs were recorded not only in different color-morphs of that species but also at the level of a single colony and in many cases, within a cell. Notably, different emitters in cells occurred in progressively red-shifted light emission spectral series. Our analyses suggested that blue, green and red FPs within cells had donor/acceptor fluorophore spectral properties, so that emissions of one FP overlapped the excitation spectrum of the neighbouring FP forming pigment assemblies that transferred energy from one to another.

### **Depth distribution of color-morphs.**

At 0.5-1.2 m the abundance of non-pigmented morphs was lower than at other depths, while the abundance of fluorescent and color-morphs was highest (**Fig. 3**). When the bathymetric distribution was examined for all color-morphs, i.e., pooled data for purple-blue and fluorescent versus non-pigmented morphs, there was a significant trend of decreasing abundance of pigmented morphs with depth to 30 m. Thus, an inverse relationship of the abundance to depth was recorded for purple-blue morphs (log. regression  $R=0.833$ ) and fluorescent morphs (log. regression  $R^2=0.747$ ), but increasing numbers of non-pigmented cream or beige color-morphs (log. regression  $R^2=0.945$ ). Below 30 m, however, fluorescent morph numbers increased significantly while the non-pigmented morphs

decreased, when compared to shallower depths. The difference between the abundance of fluorescent and non-pigmented morphs was only significant between the shallow depth 0.5-1.2m and 30m, but was not significant between 5, 10 and 20m. Purple-blue color-morphs were also most abundant at shallow 1 and 5 m depths, with no significant differences between the two, and their abundance decreased with depth to a minimum at 40m, with significant differences when shallow depths were compared to 20m and 30m depth; 10m being intermediate between them. At 40 m, the deepest sites surveyed, there were very few purple-pink-blue color-morphs although very light blue or blue-tipped morphs were present.

#### **Visual bleaching surveys.**

The most severe bleaching at Heron Island reef occurred at the upper reef slope, at 1.5 to 10m, where branching *Acropora* spp form large mixed-species stands. Despite the very bleached appearance of these stands, many acroporids were only partially bleached. Bleaching in the inter-tidal lagoon was widespread and affected most genera, was less severe than at the upper slope. Highly fluorescent colonies, many visibly green-colored, were visually less bleached than their non-green conspecifics. Bleaching of purple-blue color-morphs was very variable, was species and color-pigment dependent. Cream or blue-tipped *Acropora* (*A. hyacinthus*, *A. tenuis*, *A. humulis*, *A. aspera*, *A. pulchra*, *A. millepora*, *A. intermedia*, *A. muricata*) were severely bleached, while neighboring colonies with overall deep purple-blue pigmentation were partially bleached. Stands of the purple-blue *A. intermedia* and *A. muricata* at 1-3 m depth, monitored using permanent transects (data not presented) since the 1998 mass bleaching, were less bleached than the non-pigmented neighboring conspecifics and in 2003 suffered almost no post-bleaching mortalities. The widespread occurrence of the White Syndrome disease, discovered to affect the acroporids following the 1998 bleaching was found more prominent in patches that had bleached severely in 2002, but the purple-blue stands were least affected.

Bleaching at Ribbon reefs (GBR) was very variable, with some reefs showing little bleaching, other experiencing severe bleaching. Many sites in close vicinity to reef channels and oceanic currents had low bleaching at 5-20%, restricted mainly to the vulnerable species such as *Seriatopora hystrix*, *P. damicornis*, *Montipora* spp, and finely branched *Acropora* spp. Other reefs were severely bleached at 50% to ~85%. GBR bleaching was more widespread in shallower than deeper reef zones. Post-bleaching (2003) surveys found good recovery of corals at most sites, except where bleaching severity was <80%, e.g., Chinaman Reef, where many of corals died. Corals at sites with moderate or severe degree of bleaching were found increasingly susceptible to diseases. Visual surveys of bleaching in color and non-pigmented morphs did not identify clear differences but post-bleaching surveys found large numbers of apparently fully recovered purple-blue and fluorescent acroporids at sites that bleached strongly.

The extend of bleaching at Osprey Reef sites (Coral Sea) was also site dependent, with some sites exhibiting over 65% bleaching, others only mildly bleached. The bathymetric distribution was different from GBR reefs, with corals at deeper sites more bleached than at shallower depths. Many massive corals, usually considered to be more resistant to bleaching than branching corals, were severely or partially bleached and in all cases, bleaching was most severe on the uppermost, light-exposed surfaces. Many acroporids had dark colored undersides indicating that dinoflagellates were sheltered from bleaching in shaded colony parts. Most branching corals were affected by bleaching with the exception of *Porites nigrescence* and *P. rus* which did not show signs of bleaching, regardless of coloration. These, apparently very resilient species, were excluded from the analysis of photoinhibition differences in branching color-morphs (below). Surveys performed 12 and 18 months following bleaching showed good coral cover and high abundance of bleaching-susceptible branching species of all color-morphs, although many dead colonies were present at sites that bleached heavily. Many massive corals had large lesions of dead tissue at the most sun-exposed surfaces (**Fig. 4**).

**Bleaching-related photoinhibition of color-morphs** – There was some correlation between the visual assessment of the degree of bleaching and  $F_v/F_m$ , but no significant correlation between the

former and color i.e. between the degree of visually assessed bleaching in the low, medium and highly pigmented color-morphs. Some colonies appeared strongly fluorescent under blue-light but appeared bleached at white light illumination and *vice versa*. The *in situ* comparison of the photosynthetic efficiency ( $F_v/F_m$ ) of dinoflagellates of different color-morphs, on the other hand, revealed highly significant differences between low-pigmented ( $0.346 \pm 0.315$ ) and high-pigmented corals ( $0.522 \pm 0.0219$ ) morphs, with the former at 34% lower than the latter.  $F_v/F_m$  values of medium-pigmented corals were intermediate ( $0.475 \pm 0.036$ ) and were not significantly different from the other 2 color-morph groups.

**Bleaching-related partial mortality of color-morphs** – When the bleaching survey was restricted to only branching corals (*Acropora*, *Montipora*, *Stylophora*, *Pocillopora* spp), almost all showed varying degrees of visible bleaching, however, color-morphs were significantly less bleached ( $p = 0.0078$ ) than the non-pigmented morphs. Visual estimates of the partial colony mortalities revealed that the surface area of dead tissues in the purple-blue morphs was 77.6% less than in non-pigmented morphs (**Fig. 2a, 4d**).

In another survey conducted at Osprey reef 18 months after bleaching, 92 massive colonies were compared for the degree of partial colony mortality and fluorescent pigmentation (**Fig. 4 a-b**). 52% were categorized as being highly fluorescent and of the rest, one half were medium and another half were low or none fluorescent. Most of the lesions were localized at the most sun facing colony parts (**Fig. 4 a-b**). The size of dead lesions was significantly different among corals of each group and especially prominent between high and low fluorescent corals, with 28.4 % compared to 65% of surface area dead, respectively (**Fig. 4c**). Medium fluorescent corals had intermediate degree of damage at 45.5 %. In each color-morph category, however, there were cases (23% of corals surveyed) that did not conform to the trend, with low/medium fluorescent morphs exhibiting low tissue mortalities (>30% dead area) or highly fluorescent morphs exhibiting high tissue mortalities (<50% dead area).

**Microscopic imaging of dinoflagellates in bleached corals** - Confocal imaging of dinoflagellates from highly fluorescent corals found typical 'healthy-looking' cells, with compact and thick chloroplast lobes and no sign of chloroplast degradation (**Fig. 4a,b**). The majority of dinoflagellates in low fluorescent corals appeared degraded. Features of degradations included those previously defined (Salih *et al.* 1995, 1998b) - the disruption of cell components, breakdown of chloroplast lobes, separation of thylakoid membranes, increased cell vacuolation, appearance of plastoglobuli (**Fig. 4c**). TEM analysis of dinoflagellates from highly fluorescent corals showed the presence of multi-lobed chloroplasts, with closely packed thylakoid membranes, indicative of 'healthy' cells (**Fig. 4d**). Dinoflagellates from low fluorescent corals had markedly reduced chloroplast lobes, dissociated thylakoid lamellae and severe cell vacuolation (**Fig. 4f**). Dinoflagellates from medium-fluorescent corals appeared to have intermediate degree of degradation (**Fig. 4e**).

## Discussion

This study provides further evidence that coral host-based pigments perform an important biological function and we again demonstrate that a major part of this function is as sun-screens in reducing the photophysiological effects of light stress and also in reducing the impacts of thermal stress and the associated post-bleaching mortalities. This light/thermal stress protective mechanism is additional to the UV-B protective mechanism provided by an active mycosporin-like amino acid (MAA) system (Dunlop & Shick, 1998) and an active xanthophyll cycle that can dissipate up to 80% of excess radiation (Gorbunov *et al.*, 2001). However, clearly these and other photoprotective mechanisms can be overwhelmed as happens during instances of solar bleaching (Brown *et al.* 1999) or when thermal and light stress interact to cause mass coral bleaching (review, Coles & Brown, 2003). Nevertheless, under such conditions, GFP-like pigments appear to play a key role.

Despite the increasing evidence of the photoprotective function of GFP-like pigments, several

studies claim to have found little evidence for such processes. In some cases, spectral studies of CPs and FPs appeared to show that these compounds lack peak absorption in the UV range or at peak excitation wavelengths of dinoflagellate photosynthetic pigments, concluding that they were neither UV-sunscreens nor photoprotectants in dinoflagellates (Dove *et al.* 1995; Takabayashi & Hoegh-Guldberg, 1995; Mazel *et al.* 2003). Examination of the spectral properties of coral host-based pigments in our study, however, demonstrates strong evidence for the photoprotective hypothesis, viz. that GFPs function in photoprotection by absorbing high energy, damaging solar radiation and many FPs spectrally fall into the range of UVA absorbers (**Fig. 1**), having peak excitation at 380 nm. These FPs are present in many shallow acroporids, pocilloporids, poritiids and faviids and consequently may act to complement sunscreening by MAAs of dinoflagellates and of coral's own tissues. This fact may explain their presence in coral tissues lacking the dinoflagellates, such as oral discs overlying the gonads, or in growing edges of colonies, that are known to have high cell division rates (Salih *et al.* 1998a). Cyan, green and yellow FPs with peak excitation by blue and green wavelengths at 440-530 nm are also commonly present in corals and directly overlap the excitation spectra of dinoflagellate chlorophylls and peridinin (Larkum, 1996). By absorbing the high energy blue light, known to cause bleaching (Fitt & Warner, 1995), they may reduce bleaching susceptibility. Maximal absorption peaks of non-fluorescent purple-blue CPs fall into the region of 530-590 nm, thereby increasing light absorption at wavelengths not absorbed by FPs and reducing the photo-stress by removing excess light. Thus, the green-yellow light absorbing FPs and CPs may reduce the risk of damage due to excessive absorption of green wavelengths, just as anthocyanins do in plants (Barnes *et al.* 2002).

The sun-screening properties of coral host pigments are increased by energy dissipation between pairs of spectrally overlapping pigments, blue-green, green-yellow, yellow-red (Salih *et al.* 2000, 2003; Gilmore *et al.* 2003). In the present study we confirmed the presence of such spectral pairs in many corals by micro-imaging and spectroscopic detection. One of the explanations for the presence of such pigment diversity in corals must be related to their energy channeling and dissipative properties, operating both via the radiative and non-radiative FRET-type mechanisms (Salih, 2000, Salih *et al.* 2000-2004; Gilmore *et al.* 2003; Cox & Salih, 2005). It is now evident that even single color corals possess multiple color pigments and emissions of the shorter-wavelength FPs may couple efficiently to longer-wavelength ones via FRET – for example, emissions from ‘donor’ blue FPs directly coupling to ‘acceptor’ greens, with no visible blue emissions. The presence of multi-color spectral variants in tissues of even single color corals has been confirmed by the molecular analysis - in *Montastrea cavernosa* genes coding for cyan, two greens and red emission colors were found (Kelmanson & Matz, 2003).

Dove *et al.* (2004) argued that the principal property of a photoprotectant is the ability to absorb harmful energy, whilst the mechanisms of the disposal of absorbed energy, i.e., fluorescence in the case of corals, are of secondary importance (Dove *et al.* 2004). FPs were considered to be less efficient in photoprotection than CPs because the former absorb little light in comparison to CPs (i.e., have low extinction coefficients). The available data of extinction coefficients for various cloned anthozoan proteins, whilst highly variable, show relatively high values of up to 72,500-120,000 cm<sup>-1</sup> M<sup>-1</sup> (e.g., Matz *et al.* 1999; Miywaki, 2002; Shagin *et al.* 2004) compared to those recorded for coral CP (pocilloporin) of 31,950 cm<sup>-1</sup> M<sup>-1</sup> (Dove *et al.* 1995). It is also important that light transmitting properties of pigments are determined *in vivo* because their properties in solution are altered. For example, the reflectance spectra measured on live coral tissues with granular FPs had high blue light absorption and broad-band reflectance properties, which at the same time, were highly dynamic: polyp expansion and contraction in response to light either reduced or increased the light-blocking properties, respectively (Salih *et al.* 1995, 1998, 2000). Thus, when light is excessive, coral tentacles or polyps contract (**Fig. 2d,e**), thereby concentrating FPs into smaller volume and increasing light absorption and scattering. Polyp expansion leads decreased concentrations of FPs and deeper light penetration into tissues. In deep water, where light intensity is low and reduced in spectral diversity, FPs may well increase the available light by fluorescence and scattering as originally proposed by Schlichter *et al.* (1990) and possibly extend the light wavelength via fluorescence coupling and re-emission for the absorption of the dinoflagellate peridinin-chlorophyll-protein complex (at 440-520 nm). In summary, FPs and CPs dynamically modify the intensity and the quality of light available for dinoflagellates and

**Comment:** Don't buy into the resonance transfer issue. It is largely irrelevant here. The main point is the sunscreening properties due to backscattering.

**Comment:** Once again avoid the notion that energy is transferred in the green to photosynthetically active pigments. If other people want to push this let them do th work. For us it is better not to have to argue this one.

reduce photodamage of coral tissues.

Another argument presented against the photoprotective function of coral pigments was based on the apparent lack of light-related, depth distribution of pigmented morphs (Takabayashi & Hoegh-Guldberg, 1995; Gleason, 1998; Mazel *et al.* 2003). Color expression in aquaria-grown corals in response to bright light is, however, a property of corals well known to aquarists. In the field, visible light was shown to influence color expression of purple-blue colors (Takabayashi & Hoegh-Guldberg, 1995; Salih, 2000), while FP concentrations were greater in high-light than in self-shaded parts of corals (Salih, 2000). The present study identified a clear correlation of the abundance of GFP-pigmented morphs and depth. The trend of diminishing distribution of GFPs with depth, however, was only significant between ~1m and 30 m depth and there were no significant differences at intermediate depths of 5, 10 and 20 m. These results are in agreement with the bathymetric survey of GFP protein concentration in the Caribbean corals, in which no significant differences were found for the effect of depth in either *Montastrea faveolata* or *M. cavernosa* over the range of 3 to 30 m (Mazel *et al.* 2003). Since UV and blue light penetrate to considerable depth, especially in clear reef waters (Jerlov, 1968), corals even down to a depth of 20 m may be exposed to photo-stress during peak irradiances. This may account for the lack of significant variations of fluorescent color-morph abundance between 3-5 to 20 m depths.

This study showed an important role of colors in reducing coral mortalities as a result of coral bleaching. A survey of massive corals identified significant differences between tissue mortality in low compared to high fluorescent corals (65% vs 28.4%). Our analysis of  $F_v/F_m$  of different color-morphs during bleaching found the low-pigmented morphs (with CPs and/or FPs) to be 34% less photosynthetically competent than the high-pigmented morphs. This analysis did not address the question of whether purple-blue CPs alone reduce photo-damage because of the difficulty of finding purple-blue colonies without FPs. Therefore, the role of CPs in bleaching is not completely clear, although our Heron Island, Chinaman reef and Osprey reef surveys found non-pigmented and light purple-blue *Acropora spp* to be more bleached than the dark purple-blue morphs. An experiment to test the influence of thermal stress on bleaching of different purple-blue morphs (i.e., CPs or pocilloporins), compared the sensitivity to thermal stress of dark blue, light blue and cream colour morphs of *Acropora aspera* at Heron Island, GBR (Dove, 2004). The study did not examine bleaching of fluorescent (green) color-morphs, finding them too rare at Heron Island sites. Whilst we also found green *A. aspera* to be rare, our surveys of Heron Island color-morphs found many cream colored *Acropora spp*, including *A. aspera*, to be highly cyan-fluorescent, even though in daylight their tissues had no obvious coloration (Salih, 2000; Salih *et al.* 2000; present study). The presence of cyan-fluorescent morphs in the Dove's (2004) experiments and surveys is the likely reason for their finding that the photosynthetic capacity of the cream colored morphs and the symbiont densities in field-bleached and experimentally bleached cream-color morphs were higher than those of the other color morphs. Consequently, the Dove (2004) results show no conflicting evidence to our results.

Inter-colony variability in the level of photoinhibition and bleaching can clearly be a result of not only the host-based GFP-like pigments but relate to other photoprotective strategies. This would explain our observations that a proportion of surveyed corals (23%) lacked a correlation between colony color and bleaching tolerance – some corals lacking GFPs, remained unbleached. Such differences in bleaching may reflect the defences associated with the presence of light or thermally resistant dinoflagellate clades (e.g., Buddemeier & Fautin, 1993; Rowan *et al.* 1997; Ulstrup & Van Oppen, 2003). During high light and thermal stress, thylakoid membranes in the chloroplasts of dinoflagellates become sites for the formation of reactive oxygen species, causing membrane lipid peroxidation and leakage of toxic products into host cells (Salih *et al.* 1998b; Salih, 2000; Tchernov *et al.* 2004). Confocal imaging and staining for lipid-peroxidation of dinoflagellates from bleached fluorescent versus non-fluorescent corals revealed found the former with lower levels of chloroplast damage and lipid peroxidation (Salih *et al.* 1998b; Salih, 2000). In the present study, confocal imaging and TEM studies of dinoflagellates from bleached GFP-pigmented and non-pigmented corals also found reduced photodamage of symbionts' thylakoid membranes by GFPs. The alternative hypothesis, that dinoflagellate clades with thermal stress-resistant thylakoid membranes are present in color-morphs is

also possible, although studies of dinoflagellate diversity in at least one polymorphic species *Acropora aspera*, found only a single clade in different color morphs (van Oppen *et al.* 2001; Dove *et al.* 2004).

Other host-mediated physiological defences, such as MAAs, antioxidants, heat shock proteins, etc., may also account for the interspecific differences in bleaching (reviewed Coles & Brown, 2003). We also do not rule out that some GFP-type pigments have functions other than light regulation and that many may be multi-functional, such as in photoreceptor signalling (Salih *et al.* 2004) and as antioxidants (Salih, 2000; Mazel *et al.* 2003; Salih *et al.* 2004).

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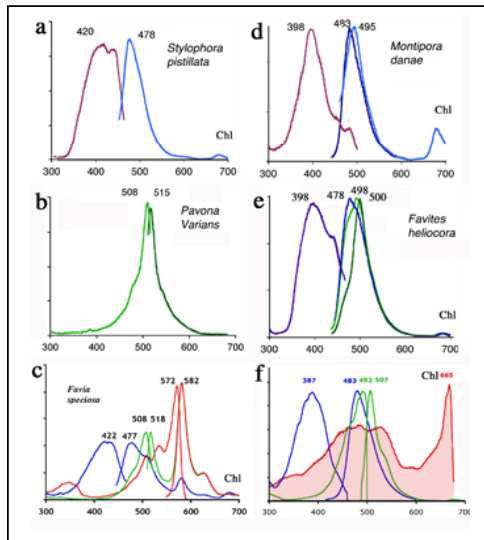
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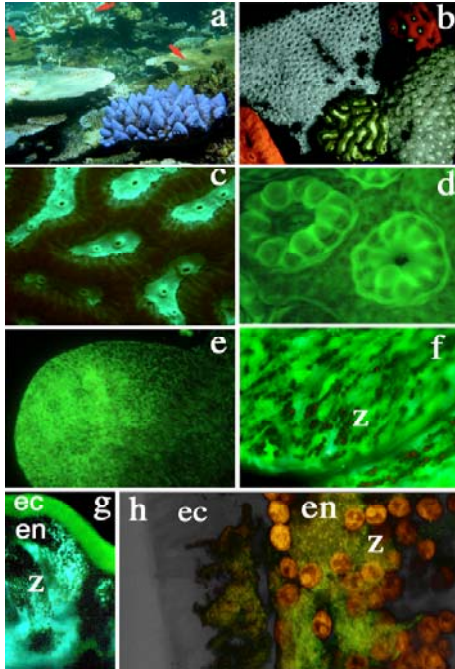
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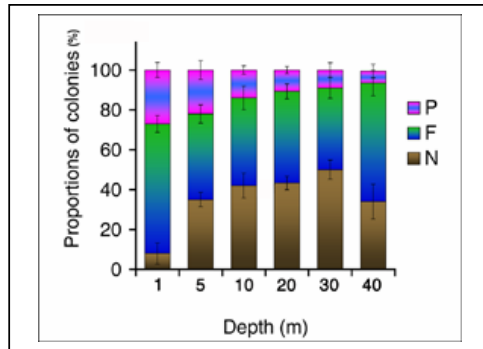
**Figure 1.** FP spectra in coral tissues. (a, b, d, e) - 1st curve in each panel shows the major excitation spectrum; the other 1-3 curves are blue, green FP emission spectra. (c) Excitation & emission spectra for minor blue & green & major red FPs in a red morph. (f) Excitation spectrum of live symbionts (red shading) due to chlorophyll & peridinin; & excitation/emission spectra of common blue & green FPs. Numbers - spectral peaks. Chl – emission peak due to chlorophyll. X-axis – wavelengths (nm); y-axis – normalized fluorescence intensity.



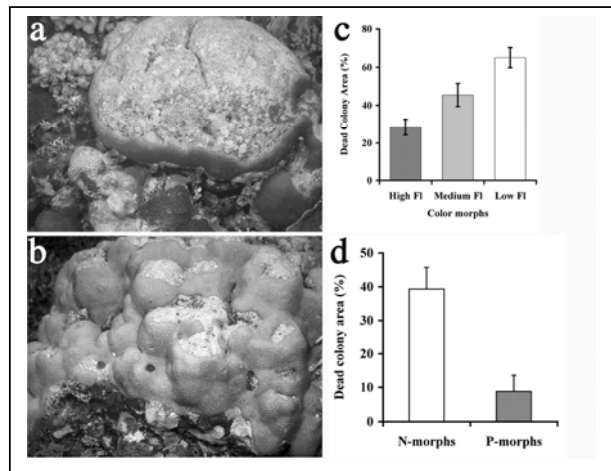


**Figure 2.** Coral pigments. (a) Mildly bleached purple-blue *Acropora* next to strongly bleached & partially dead (arrows) non-GFP pigmented acroporids. (b) Fluorescent Faviidae seen in blue light; (c) Dense FPs in oral discs of faviid; (d) FPs in & between polyps of *P. damicornis* are denser in contracted tentacles; (e) Tentacle tip of *Goniopora* with dense FPs; (f) Close up of tip with FP-containing cells & symbionts; (g) Green ectodermal & blue endodermal FPs in *Lobophyllia*; (h) Confocal 3D imaging of live *Goniopora* tissue – ectodermal & endodermal FPs. Arrows – dead tissues; z – zooxanthellae; ec – ectoderm; en - endoderm

**Figure 3.** Bathymetric distribution of purple-blue (P), fluorescent (F) and non-pigmented (N) color-morphs at different depths at Osprey



**Figure 4.** Post-bleaching partial colony mortality of corals. Colonies with massive morphologies showed bleaching of the most light-facing parts of colonies and subsequent tissue death (**a**) non-fluorescent *Goniastrea edwardsi* and (**b**) fluorescent *Leptoria phrygia*. (**c**) Comparison of the degree of post-bleaching damage in highly fluorescent, medium fluorescent and low-fluorescent massive colonies estimated as dead area of colony surface of each coral. (**d**) Comparison of the dead area of colony surface of purple-blue versus non-pigmented branching corals. Error bars are  $\pm$  standard error of the mean.



**Figure 5.** Symbiotic dinoflagellates in bleached *Acropora* colonies: (a-c) Confocal and (d-f) TEM imaging. (a) Cells showing no sign of degradation in tissues of highly fluorescent color-morph; (b, d) cells from fluorescent color-morph with compact chloroplast lobes and no sign of cell degradation; (e) cell from medium fluorescent color-morph with mild signs of chloroplast degradation - slight dissociation of thylakoid lamellae and vacuolation; (c, f) severely degraded cells from non-fluorescent color-morph – reduced, vacuolated chloroplast lobes, dissociated thylakoid lamellae, abundant plastoglobuli (marked by arrows). Note globular vesicle within chloroplast in (c), typically indicative of cell break down (Salih, 2000).

