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Light-dependent fluorescence in the coral *Galaxea fascicularis*

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Abstract Light in the sea is one of the major factors influencing corals, with changes in light being rapid along the depth gradient. Those changes can be a potential stress factor for coral-reef organisms and affect different aspects of the coral's physiology, including its fluorescence. Fluorescence is a physical phenomenon, comprising the emission of light by a substance that has absorbed light with a different wavelength. Major hypotheses concerning the role of coral fluorescence include that of photoprotection and the facilitation of photosynthesis. We sought to further investigate some ecophysiological aspects of coral fluorescence. We focused on the effect of different light conditions on fluorescence of the coral *Galaxea fascicularis* and used photography, confocal microscopy, and spectral measurements to assess changes in its fluorescence. We show that fluorescence is significantly influenced by light and, therefore, by depth. Coral fluorescence increased with the increase in light intensity and when the spectrum of light was broader. Hence, we support the "sunscreen" hypothesis and conclude that fluorescence plays a role in the coral's defense mechanism against harmful radiation. However, multiple fluorescent proteins, as found in different locations of the coral tissue, might suggest more than one functional role of fluorescence in the coral's physiology.

Keywords Fluorescence · Mesophotic coral ecosystems (MCEs) · *Galaxea fascicularis* · GFP · Coral · Red Sea

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Introduction

Coral reefs are among the most diverse and important marine ecosystems in the world, providing a source of income (Salvat, 1992), protective structures near shorelines (Sheppard et al., 2005), and a habitat for many marine organisms (Nagelkerken et al., 2000). The last few decades have seen a dramatic decline in coral-reef health due to disease (Aronson & Pratchtt, 2001; Rosenberg & Loya, 2004; Willis et al., 2004; Bruno et al., 2007), climate change (Hoegh-Guldberg, 1999; Hoegh-Guldberg et al., 2007), and human-related activities such as sedimentation, pollution, and diving pressure on the reef (Fishelson, 1973; Edinger et al., 1998; Loya et al., 2004; Wiedenmann et al., 2013; D'Angelo & Wiedenmann, 2014). Corals, belonging to the phylum Cnidaria, can establish an

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endosymbiotic relationship with dinoflagellates from the genus *Symbiodinium*, also known as zooxanthellae. These provide the corals with photosynthesis products (Muscatine & Cernichiari, 1969) and give them their brown color (Kawaguti, 1944; Jeffrey & Haxo, 1968; Trench, 1993). This relationship has been intensively studied in many aspects, following the impact of the interruption of this symbiosis, known as coral bleaching (Loya et al., 2001; Douglas, 2003; Pratchett et al., 2008). Light in the sea is one of the major factors determining coral distribution and affecting coral physiology (Kawaguti, 1944; Jeffrey & Haxo, 1968; Trench, 1993), growth (Highsmith et al., 1983; Huston, 1985; Marubini et al., 2001), and morphology (Einbinder et al., 2009). The spectrum and intensity of light available to corals and their symbionts change rapidly along the depth gradient. Light intensity decreases and the spectrum becomes narrower as we move down the slope (Jerlov, 1968; Lesser et al., 2009), constituting a potential stress factor in both shallow water (very intense light) and deep water (absence of light). As noted, this change in light affects different aspects of the coral physiology, including its fluorescence (Vermeij et al., 2002b). Fluorescence is a physical and optical phenomenon that has been reported in many marine organisms, including invertebrates and fishes (Mazel et al., 2004; Haddock et al., 2005; Michiels et al., 2008; Haddock et al., 2009; Sparks et al., 2014). Basically, fluorescence is the emission of light by a substance that has absorbed light or other form of electromagnetic radiation. Emitted light has a longer wavelength, and therefore lower energy, than the absorbed radiation (Stokes, 1852). These properties, and the fact that corals are among the most fluorescent organisms in the sea, have led to different hypotheses regarding the role of fluorescence in corals, such as its function as antioxidant, facilitator of photosynthesis and photoprotectant (Salih et al., 2000; Bou-Abdallah et al., 2006; Dove et al., 2008; Roth & Deheyn, 2013). Although there are several contributors to coral fluorescence, such as the red chlorophyll fluorescence from the symbiotic zooxanthellae (Warner et al., 2010) and the bioluminescence of other symbionts (Lesser et al., 2004), the main contribution to coral coloration and fluorescence can be attributed to the fluorescent and non-fluorescent proteins of the coral itself (Dove et al., 2001; Oswald et al., 2007; Smith et al., 2013). The best known fluorescent protein is the green fluorescent protein (GFP) isolated from the jellyfish *Aequorea victoria* (Shimomura et al., 1962); and the GFP-like proteins, among several other fluorescent proteins isolated from different marine species are already being used in molecular biology as visual markers (Wenck et al., 2003; Nishizawa et al., 2006; Kumagai et al., 2013). Due to the non-invasive properties of fluorescence, it is a rising star in the field of coral-health monitoring (D'Angelo et al., 2012; Hume et al., 2013; Roth & Deheyn, 2013; Hume et al., 2014). However, despite the intensive research and interest in those proteins, their biological role is still unclear and under debate. Due to the fluorescent proteins optical characteristics, their biological role may be related to the zooxanthellae in more than one way. Two major hypotheses regarding the coral–zooxanthellae symbiosis have been posited: one is that the fluorescent proteins might constitute a protective layer, preventing the zooxanthellae from being exposed to excessive light by means of physically preventing light from penetrating the tissue (Salih et al., 2000) or by converting short wavelengths to longer, less harmful wavelengths (Schlichter et al., 1994); while the second hypothesis suggests that the fluorescent proteins might facilitate photosynthesis by broadening the light spectrum available for photosynthesis at depths that receive only a narrow light spectrum (Schlichter et al., 1994). In this study, we focused on the coral *G. fascicularis*. *Galaxea fascicularis* is an hermatypic reef-building coral found along a depth gradient in the Gulf of Aqaba. This coral possesses both zooxanthellae and green fluorescence, which make it ideal for examining a possible connection between depth, zooxanthellae, and fluorescence.

Materials and methods

Coral collection and maintenance

Galaxea fascicularis was chosen in order to carry out the different light condition experiments since it is abundant in the Gulf of Aqaba, found along a broad depth gradient, is easy to fragment into individual polyps and presents a strong fluorescent pattern that has been previously studied (Karasawa et al., 2003). For the light treatment experiments, 5 cm (ca. 10 polyps) samples from *G. fascicularis* were collected from the mesophotic coral ecosystems (MCEs)

offshore from the Interuniversity Institute (IUI) and Dekel beach, Eilat, Israel at 50 m depth. Fragments were collected during technical diving, and samples from each colony were put into Ziploc bags (SC Johnson, USA) filled with seawater and transferred immediately to running open-circuit seawater aquaria at the IUI in Eilat. Corals were fragmented into individual polyps and were given 2 weeks to acclimate under a blue light filter; "Deep blue" (Lee filters, UK) that mimics light conditions at 40 m depth, before being transferred to the experiment conditions.

Controlled light experiments

Corals were exposed to four different light conditions, simulating different depths in the sea, for 120 days. The first group was the ambient light group, which was exposed to full natural sunlight and represented 3 m depth. Two lighting filters, "Zenith blue" and "Deep blue" (Lee Filters, UK), were used to mimic 20 and 40 m depths, respectively (D'Angelo et al., 2008), and a dark room was used to deprive corals of light. Each fragment was placed inside a 2-l glass container with running seawater supply.

Photography

For each coral sample, two images were taken at each sampling time: one with full light spectrum flash and no filters (i.e., non-fluorescent image); and the other with an underwater fluorescence excitation filter (NightSea, USA) on the flash and Y12 LP filter (Hama, UK) on the camera lens to capture a fluorescent image. Corals were photographed inside the glass container, through water, next to a fluorescent caliber (NightSea, USA). The green channel of each image was separated using Photoshop CS6 software (Adobe, USA), and pixel intensity of the polyp area from the green channel of each image was analyzed using ImageJ software (Abràmoff et al., 2004; Roth & Deheyn, 2013).

Spectral analysis

The spectrum of *G. fascicularis* fluorescence was recorded using an USB2000 portable spectrometer (Ocean Optics, USA). The optical sieve was equipped with a Yellow LP barrier filter (NightSea, USA) to block the excitation light. Excitation was established using an external light source equipped with a blue excitation filter (NightSea, USA), which produced a blue light peaking at 450 nm. Spectra were recorded and analyzed using the SpectraSuit software (Ocean Optics, USA).

Confocal microscopy

Live corals were imaged using META LSM 510 (Carl Zeiss, Germany) to determine internal location and spectral properties of the fluorescent proteins and zooxanthellae. Excitation was achieved with a Diode lamp 405 nm and Argon lamp 488 nm (for excitation in the UV and blue spectrum). Images were acquired using 420–480 nm BP, 505–550 nm BP, or 583–754 nm BP filters to obtain cyan, green, and red fluorescence at 512 × 512 pixel resolution and 10× magnification. Corals were placed in a glass-bottom dish (MatTek Corporation, USA), immersed in 0.22 µ filtered seawater to reduce background noise from the seawater. Images were analyzed and color channels were separated using Photoshop CS6 software (Adobe, USA).

Coral physiology tests

Fragments of *G. fascicularis* were tested immediately after collection ($n = 11$) and at the end of the light experiment ($n = 6$ in each light treatment) for protein concentration, zooxanthellae density, and chlorophyll concentration to assess the health of the corals. Tissue was removed with an airbrush, and proteins and zooxanthellae were separated by centrifuging and homogenizing the tissue. Coral surface area was determined using the paraffin wax method (Stimson & Kinzie III, 1991). Protein concentrations were determined using Micro BCA protein assay kit (Pierce, Thermo Fisher Scientific, USA) according to the manufacturer's protocol (D'Angelo et al., 2008, 2012) and were normalized to coral surface area. Chlorophyll was extracted with acetone 90% and measured spectrometrically at 630, 664, and 750 nm with a Multiskan spectrum microplate spectrophotometer (Thermo Fisher Scientific, USA). Chlorophyll concentration was determined as previously described (Jeffrey, 1975) and normalized to zooxanthellae cells. Zooxanthellae were counted with a hemocytometer under a microscope and normalized to coral surface area.

Statistical analysis

Statistical analysis was performed using R software (RDCT, 2008). Data were checked for normality (Shapiro–Wilk normality test) and homogenity of variance (F test) and tested accordingly with parametric one-way ANOVA with permutation or a repeated measure ANOVA. Post hoc tests were done by Tukey test. P values < 0.05 were considered statistically significant.

Results

Coral fluorescence

Results from measurements of the green channel of coral images (Fig. 1) show a significant effect of light on coral fluorescence over time. The corals show a dramatic decrease in fluorescence during the first 20 days of the experiment followed by an increase up to the end of the experiment in all treatments except the 40 m treatment. The most fluorescent corals were those under the "shallow" or 3 m light conditions, followed by the 20 m corals; while the least fluorescent were those under the 40 m conditions and dark. At 3 m there was a 4.3 ± 0.4% increase in fluorescence relative to the beginning of the experiment or a 30.9 ± 6% increase from the lowest fluorescence (on day 20). Under the 40 m treatment, there was a 40 ± 0.2% decrease in fluorescence from the beginning of the experiment followed by a steady fluorescence from day 20. Fluorescence in the 3 and 20 m treatments was elevated after day 20 but remained constant in the 40 m and dark treatments. Results are represented as averages ± SDs. Repeated-measure ANOVA, $df = 12$, $P < 0.001$, $F = 9.467$, $n = 9, 9, 12, 12$ polyps for each treatment (3, 20, 40 m, dark), respectively. Differences in the level of fluorescence in beginning of the experiment (day 0) and the end of it (day 119) were statistically significant in all treatments, except the 3 m treatment (Tukey Test, $P < 0.001$).

Fluorescence spectra

Figure 2 presents the excitation spectrum and emission spectrum of *G. fascicularis*. Measurements were taken with a blue excitation light source that peaks at 450 nm (black line). The general fluorescence spectrum of *G. fascicularis* (gray line) shows several peaks: the first

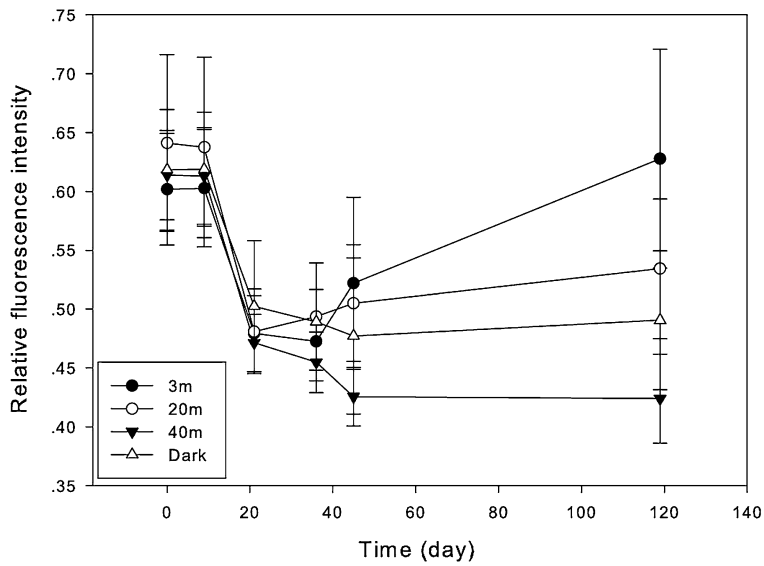

Fig. 1 Image analysis of change in fluorescence in the coral *Galaxea fascicularis* under four different light treatments (i.e., depths): 3 m depth (*black circles*), 20 m depth (*white circles*), 40 m depth (*black triangle*), dark (*white triangle*), after 9, 21, 36, 45, and 119 days. Fluorescence is influenced by light and an overall decrease in fluorescence can be seen in the 40 m and dark treatments, while an increase is shown in the other light treatments (3 and 20 m). Results are represented as averages ± SD. Repeated-measure ANOVA, $df = 12$, $P < 0.001$, $F = 9.467$, $n = 9, 9, 12, 12$ polyps for each treatment (3, 20, 40 m, *dark*), respectively.

was at ~515 nm in the green spectrum, probably as a result of the host GFP-like protein; the second peak was at 680 nm; and the third (shoulder) was at 720 nm, represent the symbiont's chlorophyll fluorescence (Krause & Weis, 1991).

Coral physiology

Several indexes of the coral physiology were examined at the beginning and end of the experiment in order to evaluate the effect of the different light treatments on coral health. The host (*G. fascicularis*) protein concentration was normalized to the coral surface area and is shown in Fig. 3. A significant difference (one-way ANOVA with permutation, $df = 4$, $P < 0.001$, $F = 18.85$, n = 11,6,6,6,6 at each treatment) in host protein concentration was found between the control group and the different light treatments (3, 20, 40 m, and dark), but no significant difference was found between the different light treatments (Tukey test). Protein concentrations (μg cm^{-2}) were 381.3 ± 63.33 261.75 ± 32.46, 238.95 ± 43.47, 225.66 ± 43.2 and 205.96 ± 13.57 in the control group, 3, 20, 40 m, and dark treatments, respectively.

Figure 4 presents the results of the zooxanthellae density counts and chlorophyll concentration measurements. A statistically significant difference in zooxanthellae density (one-way ANOVA with permutation, $df = 4$, $P < 0.001$, $F = 6.425$, n = 11,6,6,6,6 for each treatment,) and chlorophyll concentration (one-way ANOVA with permutation, $df = 4$, $P < 0.001$, $F = 12.45$, n = 11,6,6,6,6 at each treatment) was observed between the treatments. Although values of zooxanthellae density and chlorophyll concentration under the different light treatments were lower than those of the control group, typical reactions of corals to changes in light conditions or depth were observed. While there were fewer cells (zooxanthellae) per cm^2 of coral surface area when exposed to less intense light or to a narrower light spectrum, there was an increase in the content of chlorophyll of each individual cell, and vice versa.

Fluorescent protein location and distribution

Using confocal microscopy we assessed the location and distribution of fluorescent proteins in live polyps of *G. fascicularis* from 50 m depth. We found two distinct fluorescent proteins, presented in Fig. 5. Both proteins (P1 and P2) emit in the green light spectrum (GFP-like proteins) but are represented in blue (P1) in Fig. 5a and green (P2) in Fig. 5b for easy discrimination. Zooxanthellae are represented in red in Fig. 5c. The proteins are located in the endodermal tissue of the corals (P2) and above the zooxanthellae (P1) as shown in Fig. 5d.

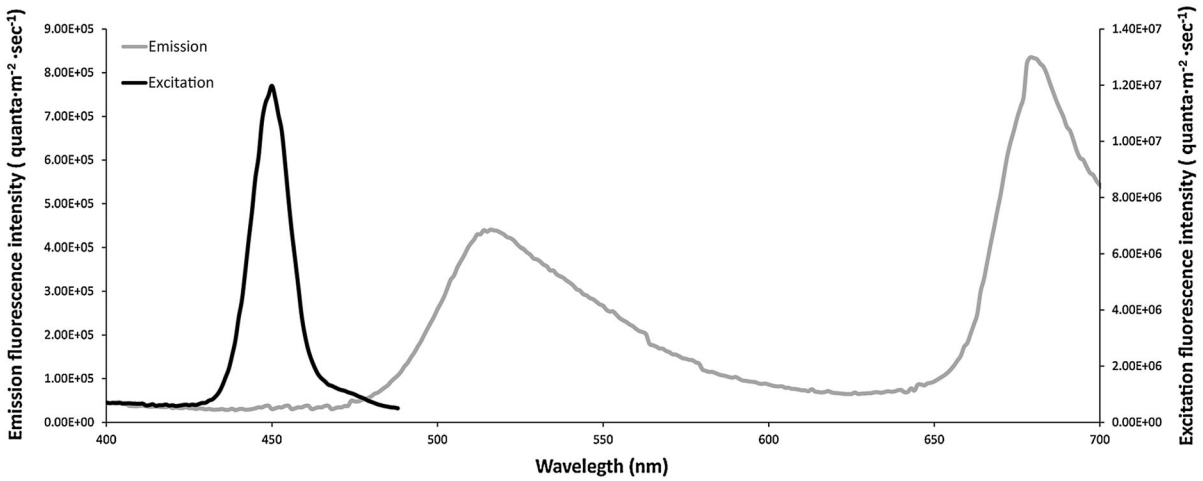

Fig. 2 General *G. fascicularis* excitation (*black line*) and emission (*gray line*) fluorescence spectrum: Coral fluorescence was excited with a 450 nm light source and fluorescence spectra were recorded using a spectrometer. Two clear emission peaks are shown at 515 and 680 nm. The peak at 515 nm is a green fluorescence probably a result of a GFP-like protein, and the peak at 680 is a *red* chlorophyll fluorescence associated with the symbiotic algae

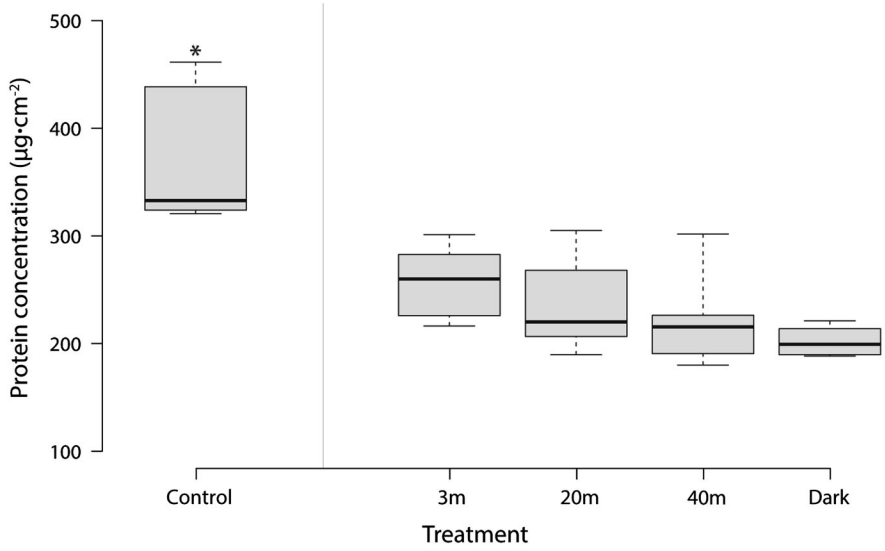

Fig. 3 Host (*G. fascicularis*) protein concentrations. Center lines show the medians; *box limits* indicate the 25th and 75th percentiles; whiskers extend to minimum and maximum values. A significant difference between the control group and the light treatments groups was found (one-way ANOVA with permutation, $df = 4$, $P < 0.001$, $F = 18.85$, $n = 11, 6, 6, 6, 6$ polyps for the control group and each treatment, respectively) but no effect was found in the different light treatments on protein concentration (Tukey test) and therefore no effect of host protein concentration on the fluorescence levels of the corals

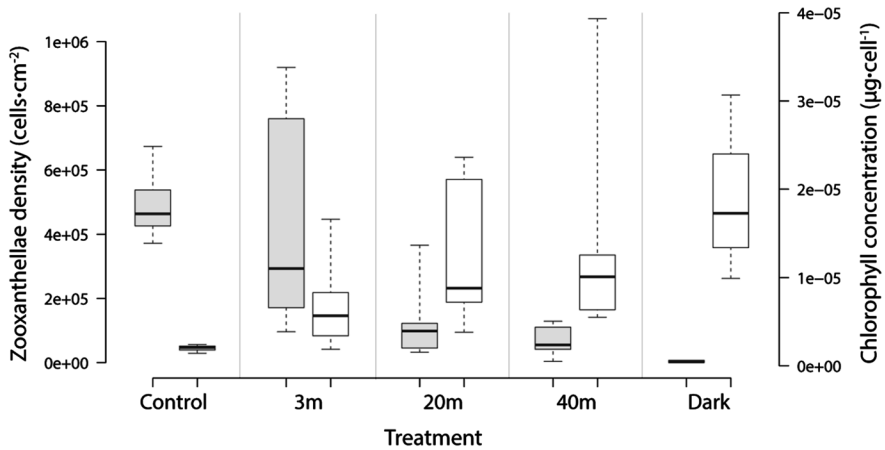

Fig. 4 Zooxanthellae density and chlorophyll concentration: An acclimation pattern of zooxanthellae (*gray bars*) and chlorophyll (*white bars*) to changes in light, as zooxanthellae density decreases with depth and chlorophyll concentration increases with depth. *Center lines* show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to minimum and maximum values; width of the boxes is proportional to the square root of the sample size (one-way ANOVA with permutation, $df = 4$, $P < 0.001$, $F = 12.45$, for the zooxanthellae density and $df = 4$, $P < 0.001$, $F = 6.425$ for chlorophyll concentration, $n = 11, 6, 6, 6, 6$ polyps for the control group and each treatment, respectively)

Discussion

Coral fluorescence along a depth gradient

This study began from the personal visual impression that deep-water corals exhibit higher fluorescence than that of shallow-water corals, and from reports showing that in deep water where there are very low light conditions and almost complete darkness, corals and other marine organisms present fluorescent colors (Schlichter et al., 1985; Haddock et al., 2005). From the results shown in our work (Fig. 1) and in previous

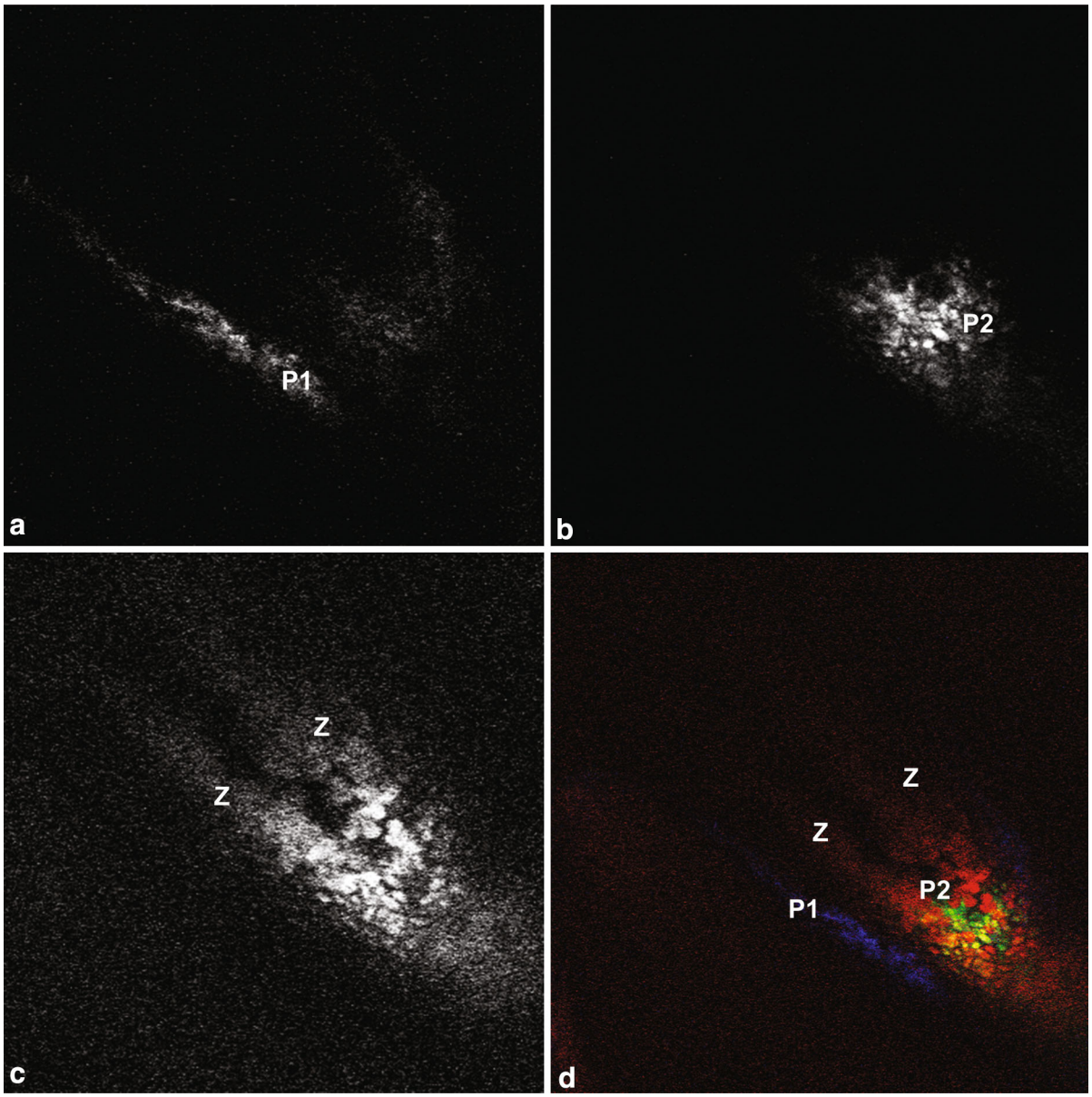

Fig. 5 2D confocal images of a *G. fascicularis* tentacle: Location of the fluorescent proteins (P1 and P2) and zooxanthellae (Z) appears to differ for each protein. **a** P1 is located in the ectodermal tissue of the coral, while **c** P2 is located in the coral's endoderm tissue. Excitation was achieved with a Diode lamp 405 nm and Argon lamp 488 nm. Images were captured with 420–480 nm BP, 505–550 nm BP, or 583–754 nm BP filters at 512 × 512 pixel resolutions and ×10 magnification. The different locations might suggest a different role for each protein

work (Gleason, 1993; Salih et al., 2000; Vermeij et al., 2002a; D'Angelo et al., 2008; Smith et al., 2013), we conclude that fluorescence is significantly influenced by changing light conditions, and hence by depth. There was a decrease in fluorescence along the simulated depth gradient that we created and, therefore, the suggested role of coral fluorescence as a photoprotectant can be considered as its major role, or at least the major role of the green fluorescence in *G. fascicularis*, since this was the main fluorescence observed in this work. The fact that (a) the most fluorescent corals (under the 3 m light condition) were those under the

most intense light conditions, and that (b) fluorescence was reduced under the 40 m and dark treatments, in which the corals received very low light or no light at all, offers additional support for this hypothesis. It should be recalled that although past works have revealed no correlation between depth and fluorescence (either positive or negative), those studies were restricted to certain geographical areas (such as the Caribbean) or to certain species (Mazel et al., 2003) and therefore, we assume it is species specific.

Although corals were given 2 weeks to acclimate under the 40 m light filter before placed under the experimental light conditions (i.e., the different lighting filters), we found that the corals probably suffered from a stress response, as indicated by the steep decrease in fluorescence in all treatments between days 0 and 20 and the difference in coral physiology between the control group and the other groups. Hence, this period of stress response (days 0–20) should be taken into account when interpreting the results of such an experiment. Changes in coral fluorescence can occur after only a short period under temperature stress experiments (Smith-Keune & Dove, 2008; Roth & Deheyn, 2013), but when changing experimentally the light conditions, as performed in our experiment, a short period of acclimation time might be insufficient to represent the real changes in coral fluorescence. It has been shown that although transcript levels can be elevated after only 8 h of light treatment, the maximal levels of fluorescence are reached only after 4 weeks (D'Angelo et al., 2008).

Location and distribution of fluorescent proteins in *G. fascicularis*

The confocal images (Fig. 5) reveal more than one fluorescent protein in *G. fascicularis*. Similar observations have been made in various coral species (Dove et al., 2001; Mazel et al., 2003; D'Angelo et al., 2008). The presence of multiple proteins may suggest that fluorescence has more than one functional role in the coral, and that each protein might play a different role in the coral's physiology (Alieva et al., 2008; D'Angelo et al., 2008; Vogt et al., 2008). Although the fluorescent proteins are usually reported as located in the coral epidermis (Kawaguti, 1944; Mazel, 1995; Salih et al., 2000; Oswald et al., 2007), there have been some reports of endodermal location (Schlichter et al., 1985, 1994; Salih et al., 1997). Here we found three major locations in the tissue: internal and external (relative to the zooxanthellae), and at the tips of the coral tentacles. The different locations of each protein also support the hypothesis that the proteins might have different roles. For example, P2 was found in the endodermal tissue of the coral and is located beneath the zooxanthellae, and might expand the narrow blue spectrum found in 50 m depth (400–500 nm) (Jerlov, 1968) into a broader spectrum by converting blue light into green light, and emit the resulting light to the chlorophyll and additional pigments (e.g., carotenoids) for photosynthesis (Beer et al., 2014). This hypothesis has been previously raised by others (Schlichter & Fricke, 1991; Schlichter et al., 1994), and here we provide indirect evidence to support it as well as the "sunscreen" hypothesis. On the other hand, because P1 is located in the ectoderm tissue of the coral, it might protect both the coral and the zooxanthellae from strong and harmful radiation and serve as a natural sunscreen by being the first layer to absorb this radiation and release it in a less energetic form, possibly even by simply reflecting it away from the delicate coral tissue (Salih et al., 1997, 2000). Judging from the confocal microscopy results (Fig. 5) and the fluorescent images, it appears that the proteins are concentrated where the zooxanthellae are present in low densities, or in individuals that are more bleached than others (i.e., corals from the dark treatment that became bleached). This conclusion should be approached with caution, however, since this may be simply a side effect of the reduced shade spread by the zooxanthellae over the fluorescent proteins, and no correlation has previously been found between the zooxanthellae and FP's distribution (Nir et al., 2011). If the zooxanthellae do shade the fluorescent proteins, this might be another aspect of the coral–symbiont relationship. The shading may protect the fluorescent protein from undergoing photobleaching, since fluorescent proteins are known to degrade or change their emission spectra when exposed to intense or prolonged light (Salih et al., 2006; Leutenegger et al., 2007).

Galaxea fascicularis physiology under different light conditions

The findings from the coral physiology tests at the end of the experiment indicate several points. First, even under extreme light conditions the corals displayed a

typical light acclimation response, as previously described (Anthony & Hoegh Guldberg, 2003; Mass et al., 2007). The fact that photoacclimation did occur provides evidence that the filter system effectively mimics a gradient of depths and can be used as a controlled environment for depth gradient experiments. Unsurprisingly, the values of zooxanthellae density and chlorophyll concentration (Fig. 4) in the control group were higher than those of the light treatments groups, since the corals were transferred to new light conditions and might have suffered from stress, as can also be noticed in the dip in fluorescence that was recorded at the beginning of the light experiment (Fig. 1). These reactions could be connected to the fluorescent proteins by means of enhanced photosynthesis; or not connected to the fluorescent proteins at all. If there is a connection between the algae and fluorescent proteins it is probably through the broadening of the available light spectrum by the fluorescent proteins that, as noted before, can convert the short wavelengths found in deep water into longer wavelengths. The host protein concentrations (shown in Fig. 3) in the light treatments groups were lower than those in the control group (again, due to the stress response), but we did not find a significant effect between the different light treatments on the coral's protein concentrations when examining its physiology. This suggests that the changes in fluorescence recorded in these light experiments were due to a specific reaction of the fluorescent proteins of either degradation or production, and not due to a total change in the host protein concentration resulting from stress.

Conclusions

The extant color diversity of coral fluorescent proteins evolved under positive natural selection, indicating a functional importance that is still not fully understood (Field et al., 2006). We sought to investigate the influence of light on coral fluorescence and examine other potential aspects of this phenomenon. While we could not reach a definite conclusion regarding the biological role of fluorescence, we have demonstrated that fluorescence might be affected by light and, therefore, by depth: When the corals are exposed to intense light, their fluorescence is elevated; whereas when deprived of light, their fluorescence was decreased—hence supporting the "sunscreen" hypothesis. Although the latter might be the major role of green fluorescence in *G. fascicularis*, the results also revealed the presence of more than one fluorescent protein, and different locations were observed for each protein, hence suggesting more than one role for fluorescence in this coral.

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