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RESEARCH ARTICLE

The response of the scleractinian coral *Turbinaria reniformis* to thermal stress depends on the nitrogen status of the coral holobiont

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SUMMARY

The physiological response of the scleractinian coral *Turbinaria reniformis* to ammonium enrichment (3 μmol l^{-1}) was examined at 26°C as well as during a 7 day increase in temperature to 31°C (thermal stress). At 26°C, ammonium supplementation had little effect on the coral physiology. It induced a decrease in symbiont density, compensated by an increase in chlorophyll content per symbiont cell. Organic carbon release was reduced, likely because of a better utilization of the photosynthesized carbon (i.e. incorporation into proteins, kept in the coral tissue). The δ^{15}N signatures of the ammonium-enriched symbionts and host tissue were also significantly decreased, by 4 and 2‰, respectively, compared with the non-enriched conditions, suggesting a significant uptake of inorganic nitrogen by the holobiont. Under thermal stress, coral colonies that were not nitrogen enriched experienced a drastic decrease in photosynthetic and photoprotective pigments (chlorophyll *a*, β-carotene, diadinoxanthin, diatoxanthin and peridinin), followed by a decrease in the rates of photosynthesis and calcification. Organic carbon release was not affected by this thermal stress. Conversely, nitrogen-enriched corals showed an increase in their pigment concentrations, and maintained rates of photosynthesis and calcification at *ca.* 60% and 100% of those measured under control conditions, respectively. However, these corals lost more organic carbon into the environment. Overall, these results indicate that inorganic nitrogen availability can be important to determining the resilience of some scleractinian coral species to thermal stress, and can have a function equivalent to that of heterotrophic feeding concerning the maintenance of coral metabolism under stress conditions.

Key words: coral, nitrogen, symbiosis, temperature.

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INTRODUCTION

Scleractinian corals owe their success in nutrient-poor tropical waters to their association with photosynthetic dinoflagellate symbionts of the genus *Symbiodinium*. These symbionts, located in the coral host tissue, can take up and retain dissolved inorganic nutrients, such as phosphorus and nitrogen, from the surrounding seawater (Muscatine, 1980; Falkowski et al., 1993; Grover et al., 2002; Godinot et al., 2009). They also recycle host metabolic wastes into new molecules (Falkowski et al., 1984; Rahav et al., 1989). Nutrients, coupled to the products of photosynthesis (photosynthates), support both the symbionts and host metabolism because most of the photosynthates are translocated to the host for its own nutrition (Muscatine et al., 1981). In this way, corals are considered to be autotrophic organisms. However, predation on zooplankton by the animal host (heterotrophy) allows the symbionts to acquire the rest of their nutrient needs (Houlbrèque and Ferrier-Pagès, 2009). Together with essential nutrients, nitrogen and phosphorus, acquired both through autotrophy and heterotrophy, enter into the composition of many biological molecules and are important for biochemical mechanisms, growth and reproduction (Godinot et al., 2009).

Under optimal growth conditions, there is a close coupling between inorganic nutrient acquisition and the equilibrium of the symbiotic association. Excessive amounts of inorganic nutrients in seawater, especially nitrogen, can disrupt the equilibrium by inducing an overgrowth of the symbionts, at the detriment of the coral host metabolism and calcification in particular (Marubini and Davies, 1996; Ferrier-Pagès et al., 2000; Renegar and Riegl, 2005). Eutrophication is also usually associated with a significant loss of coral cover and diversity (Renegar and Riegl, 2005). However, inorganic nutrient shortage can also be detrimental to the symbiotic association, which in this case will not have the building blocks necessary to increase its biomass or achieve maximal rates of photosynthesis and calcification (Wiedenmann et al., 2013). In this last study, a lack of phosphate in nitrogen-rich seawater induced bleaching. Therefore, it was shown that moderate seawater enrichment in nitrogen (or phosphorus) can enhance coral photosynthesis and calcification (Tanaka et al., 2007; Godinot et al., 2009). Corals also have thicker tissue in nutrient-rich waters (Sawall et al., 2011).

Understanding the coral–nutrient coupling is even more important today, as coral reefs are declining globally as a result of a combination of direct and indirect human impacts (Hughes et al., 2003). In particular, the rise in ocean temperature and increased frequency of strong temperature anomalies will have several detrimental effects on coral health. Indeed, climate models predict that increasing sea surface temperatures will decrease the availability of inorganic nutrients (Hutchins et al., 2007; Beman et al., 2011). It also already induces mass coral bleaching events, a phenomenon where corals lose a significant amount of their symbionts and/or their algal photosynthetic pigments (Jokiel and Coles, 1990; Hoegh-Guldberg, 1999; D'Croz et al., 2001). As a consequence, unless they are able to increase their predation on zooplankton (Grottoli et al., 2006; Ferrier-Pages et al., 2010), corals also lose, *via* the loss

of their symbionts, their ability to acquire essential inorganic nutrients (Godinot et al., 2011). Long-term bleaching therefore leads to a decrease in many coral physiological processes, such as calcification, gametogenesis and fertilization (Szmant et al., 1990; Rodrigues and Grottoli, 2006; Moya et al., 2008).

Despite the importance of inorganic nutrients in the maintenance of the symbiosis, the effect of nutrient addition on the response of thermally stressed corals has never been studied. However, this effect has been investigated in experiments manipulating seawater levels of the partial pressure of CO_2 (P_{CO_2}) levels. They showed a positive effect of a moderate nitrogen addition on the growth of corals maintained under high P_{CO_2} (Langdon and Atkinson, 2005; Holcomb et al., 2010; Chauvin et al., 2011), or at least no detrimental effect (Marubini and Atkinson, 1999; Renegar and Riegl, 2005). It was indeed suggested that boosting the energetic status of corals by adding organic (zooplankton feeding) or inorganic nitrogen (nitrate or ammonium) helped to offset the negative impact of ocean acidification (Cohen and Holcomb, 2009).

In this study, we therefore tested the effect of moderate ammonium enrichment in presence of normal phosphate concentrations ($0.07\,\mu mol\,l^{-1}$) (Wiedenmann et al., 2013) on the physiological response of the scleractinian coral *Turbinaria reniformis* Bernard 1896 to a thermal stress inducing bleaching. Two previous experiments performed on this species (Ferrier-Pagès et al., 2010; Hoogenboom et al., 2012) showed that both the light reactions of photosynthesis and the carbon fixation (i.e. the dark reactions of photosynthesis) were impaired by thermal stress. However, colonies supplied for several weeks with zooplankton (organic food) during thermal stress maintained higher rates of photosynthesis compared with unfed colonies, probably as a result of a positive effect of increased nutrient availability, a thicker tissue or a higher supply of energy. This study was therefore performed to test the first hypothesis, i.e. that a supply of inorganic nitrogen would reduce the effect of bleaching in colonies of *T. reniformis* exposed to thermal stress. We thus hypothesized that the response of *T. reniformis* to a thermal stress will depend on the nutritional status of the coral holobiont. Heat-stressed colonies maintained in nitrogen-poor waters will decrease their symbiont concentration, chlorophyll levels and rates of photosynthesis and calcification, as observed previously (Hoegh-Guldberg, 1999; Baker, 2001; Tchernov et al., 2004). Conversely, nitrogen-enriched colonies may be able to keep their symbiont population and their rates of photosynthesis at an optimal or at least suitable level to ensure their resilience to thermal stress. In addition, we also investigated the xanthophyll de-epoxidation process in nitrogen-enriched and non-enriched corals submitted to a thermal stress. In many algae, this process quenches energy from excess light and is therefore activated during exposure to high irradiance levels or in the presence of some photoinhibitors (Ebbert et al., 2001; Brown et al., 2002; Matsubara and Chow, 2004; Dove et al., 2006; Warner and Berry-Lowe, 2006).

MATERIALS AND METHODS

Experimental setting

Five colonies of the scleractinian coral *T. reniformis* (identified with electronic chips), originally sourced from the Red Sea, were cut into 120 nubbins of similar size (24 per parent colony). Nubbins were maintained for 3 weeks of recovery, under the same culture conditions as the parent colonies: temperature of 26±0.2°C, irradiance of $200\,\mu mol\,photons\,m^{-2}\,s^{-1}$ (on a 12 h:12 h light:dark cycle), continuous seawater renewal (eight times per day), and fed once a week with *Artemia salina* nauplii at repletion.

Nubbins were then equally divided into eight 20 l tanks (three nubbins per colony and per tank), with a renewal rate of eight times per day. Feeding was stopped to avoid any interaction with the addition of inorganic nitrogen (Grover et al., 2002), and light was maintained at $200\,\mu mol\,photons\,m^{-2}\,s^{-1}$. Temperature was kept at 26±0.2°C. Nubbins were maintained for 4 weeks under these new conditions. Half of the tanks (hereafter called N-26) were then enriched, for four additional weeks, with $3\,\mu mol\,l^{-1}$ ammonium (NH_4), while the other half (hereafter called C-26) remained un-enriched. For ammonium enrichment, a batch tank of 100 l seawater was prepared every day with an NH_4 concentration of $200\,\mu mol\,l^{-1}$. A peristaltic pump continuously delivered ammonium-enriched seawater to the experimental tanks in order to reach, after mixing with normal seawater, a final concentration of $3\,\mu mol\,l^{-1}$. Dissolved inorganic nutrients were monitored twice a week in each tank using an autoanalyser (Proxima, Alliance Instruments) and according to Treguer and Le Corre (Treguer and Le Corre, 1975): in the non-enriched tanks, total nitrogen concentration (ammonium, nitrate and nitrite) remained below $0.7\,\mu mol\,l^{-1}$ and phosphorus concentration remained below $0.5\,\mu mol\,l^{-1}$. In the enriched tanks, total nitrogen concentration varied between 3 and $3.8\,\mu mol\,l^{-1}$ whereas phosphorus concentration remained below $0.5\,\mu mol\,l^{-1}$.

At the end of the 4 weeks of nitrogen enrichment, temperature was gradually (over 3 days) increased to 31±0.2°C in half of the enriched (hereafter called N-31) and non-enriched tanks (hereafter called C-31) while the others remained at 26°C. After 7 days under this thermal stress, five nubbins (from the five different colonies) were sampled for each set of measurements described below. Four sets of samples were therefore produced, corresponding to the following four conditions: N-26 and N-31 for nitrogen-enriched samples maintained at 26 and 31°C, and C-26 and C-31 for non-enriched or control samples maintained at 26 and 31°C. The use of replicate genotypes across treatments removed the genotypic variation between treatments.

Protein and lipid quantification

Coral tissue was removed from the skeleton in 20 ml of 0.45-μm-filtered seawater using a Water Pik (Water Pik International, Castle Court, Surrey, UK) at 4°C. The tissue slurry was homogenized using a Potter tissue grinder (Wheaton Industries, Millville, NJ, USA). For protein determination, a 0.5 ml sub-sample was treated with 0.5 ml NaOH ($0.5\,mol\,l^{-1}$) for 5 h at 60°C. Total protein content was measured on the supernatant using the BCA assay kit (Interchim, Montluçon, France) (Smith et al., 1985) and a Xenius spectrofluorometer (Safas, Monaco). Lipid measurement was performed using the method of Bligh and Dyer (Bligh and Dyer, 1959), adapted by Hoogenboom et al. (Hoogenboom et al., 2010). Samples were normalized to the skeletal surface area.

PSII fluorescence and P700 absorbance

The photosynthetic efficiency of photosystems I (PSI) and II (PSII) was assessed using a Dual PAM (Walz, Effeltrich, Germany) and according to Hoogenboom et al. (Hoogenboom et al., 2012) (see this paper for detailed description of the PSI and PSII parameters). Colonies were thus placed in a small glass container inside a dark box to acclimate for 10 min. Dark-adapted PSII rapid-light curves (RLC) and recovery were measured for each colony according to Ralph and Gademann (Ralph and Gademann, 2005) at irradiances of 0, 11, 18, 27, 42, 75, 131, 221, 665, 1033 and $1957\,\mu mol\,photons\,m^{-2}\,s^{-1}$, and then in the dark for 10 min for the recovery period. Colonies were then allowed to ‘rest’ for 10 min before PSI RLC were measured at the same light intensities, on a different region of the colony than PSII measurements to avoid potential effects of prior exposure to saturating light pulses on PSI photochemistry. PSI and PSII parameters obtained during the RLC,

such as the maximal electron transport rate (ETR_{max}) or the maximal and minimal non-photochemical quenching (NPQ), were used in a principal component analysis (PCA) described below.

Photosynthesis, respiration and symbiont density

Measurements were performed with a set of temperature-controlled respirometry chambers (50 ml volume), filled with 0.45-μm-filtered seawater, coupled with a Strathkelvin oxygen electrode system (Strathkelvin 928 oxygen meter with computer interface; Strathkelvin Instruments, North Lanarkshire, UK). Electrodes were calibrated at the incubation temperature using N_2 and air-bubbled seawater as 0 and 100% oxygen saturation values, respectively. Temperature was maintained at 26 or 31°C using a water bath and the chambers were stirred using magnetic stirrers. A metal halide lamp was used as a light source. For each sample, photosynthetic rates were assessed at 200 μmol photons $m^{-2} s^{-1}$ for 15 min. Respiration (oxygen consumption) was obtained after 25 min incubation in darkness. Rates were normalized either per skeletal surface area or per chlorophyll content as described below. Surface area was estimated using the wax technique according to Stimson and Kinzie (Stimson and Kinzie, 1991).

To estimate symbiont density and chlorophyll concentration, tissue was removed from the skeleton using an Air Pick (compressed air ABAC, LP30, ABAC, Valence, France). Samples were collected in a beaker with 10 ml of 0.45-μm-filtered seawater. The tissue slurry was homogenized using a Potter tissue grinder and an aliquot (1 ml) was taken for symbiont counts using an inverted microscope (Leica, Wetzlar, Germany) and Histolab 5.2.3 image analysis software (Microvision, Every, France). The rest of the slurry was centrifuged at 8000 ***g*** for 10 min at 4°C. Subsequently, the supernatant was removed and the symbiont pellet was re-suspended in 5 ml acetone for extraction of chlorophylls *a* and c_2. Samples were extracted in darkness at 4°C for 24 h. Chlorophyll content was determined according to Jeffrey and Humphrey (Jeffrey and Humphrey, 1975).

Pigments analysis

All steps for pigment extraction (β-carotene, diadinoxanthin, diatoxanthin and peridinin) were performed at 4°C in the dark. Tissue was removed from the skeleton in 5 ml of 0.45-μm-filtered seawater using an Air Pick. The tissue slurry was homogenized using a Potter tissue grinder and centrifuged at 8000 ***g*** for 10 min at 4°C. Subsequently, the supernatant was removed, the symbiont pellet was rinsed three times in filtered seawater, and was then frozen in liquid nitrogen before freeze-drying. For extraction of pigments, pellets were then grounded in a mortar with methanol/methylene chloride (20/1, v/v) and centrifuged at 12,500 ***g*** for 5 min. The supernatant was filtered on PTFE membrane (0.2 μm) (Acrodisc, Pall, Saint-Germain-en-Laye, France) and dried under a stream of nitrogen. Pigments were dissolved in a mixture of methylene chloride/distilled water (50/50, v/v) and after decantation the non-pigmented aqueous phase was discarded to remove the salt. The pigment phase was then evaporated under a stream of nitrogen and re-dissolved with methanol just before injection. Reversed-phase HPLC was performed according to Arsalane et al. (Arsalane et al., 1994) using a 32 Karat Gold system equipped with a diode array detector (Beckman Coulter, Villepinte Roissy CDG, France) on a C18 Allure column (Restek, Lisses, France). Extinction coefficients used for external calibration of the system were as in Berkaloff et al. (Berkaloff et al., 1990). Samples were expressed as μg mg^{-1} dry mass. The de-epoxidation ratio was calculated as:

$$DR = \frac{Dt}{Dd + Cis\text{-}Dd + Dt}, \quad (1)$$

where Dd, Cis-Dd and Dt are diadinoxanthin, cis-diadinoxanthin and diatoxanthin concentrations, respectively.

Calcification rates

Calcification rates were measured using the total alkalinity (TA) technique, assuming a consumption of 2 moles alkalinity for every mole of calcium carbonate produced (Smith and Kinsey, 1978). Five colonies per treatment were incubated during 4 h in glass beakers containing 100 ml of 0.45-μm-filtered seawater. Three additional beakers, containing only seawater, were run in parallel for blank measurements. Beakers were sealed, mixed using a magnetic stirring bar and 3 h incubations were performed in a temperature-regulated water bath (26 or 31°C) under the same irradiance at which colonies were grown (200 μmol photons $m^{-2} s^{-1}$). At the beginning and end of incubations, 50 ml seawater was sampled in each beaker and refrigerated in darkness prior to analysis of TA, determined in three to five replicate Gran titrations with 0.1 mol l^{-1} HCl using a Metrohm Titrando 888 titrator (Metrohm USA, Riverview, FL, USA). The difference in TA between the beginning and end of incubation was used to estimate calcification rates (Tentori and Allemand, 2006). Data were normalized to the skeletal surface area.

Total organic carbon

The total organic carbon (TOC) flux between seawater and corals was measured using the beaker incubation method according to Wild et al. (Wild et al., 2005) and Naumann et al. (Naumann et al., 2010). Prior to utilization, all beakers were washed with filtered (0.45 μm) normal or nitrogen-enriched seawater depending on the treatment. Nubbins were incubated for 6 h under a constant temperature (either 26.0±0.2 or 31.0±0.2°C) in a water bath, and under the same irradiance as in the culture conditions. Three additional beakers, containing only seawater, were run in parallel as controls. Stirring was carried out using Teflon-coated magnetic stir bars. After 6 h, corals were removed from the incubation beakers and transferred back to the maintenance tanks prior to sampling of the incubation media. At the beginning and end of the incubations, three seawater subsamples per beaker (17 ml) were drawn by sterile syringe from the thoroughly homogenized incubation media of coral and control beakers to quantify the TOC concentration. They were then transferred into pre-combusted (450°C, 5 h) glass vials, acidified with phosphoric acid (20%, 250 μl) to pH <2 and kept frozen (−20°C) until analysis by high-temperature catalytic oxidation using a TOC analyser (Shimadzu TOC-VCPH; CV maximum ≤1.5%, i.e. ±1 μmol C l^{-1}, certified reference materials, Hansell Laboratory, University of Miami, Miami, FL, USA). TOC fluxes were estimated from the difference in TOC concentrations at the beginning and end of the incubation in the beakers containing corals. These fluxes were corrected by the fluxes obtained in control beakers. Results were normalized to the skeletal surface area and incubation time.

$\delta^{15}N$ and $\delta^{13}C$ determination

Tissue was removed from the skeleton in 10 ml of 0.45-μm-filtered seawater using an Air Pick. The tissue slurry was homogenized using a Potter tissue grinder and symbiont cells were separated from the animal tissue by three centrifugations at 8000 ***g*** for 10 min at 4°C. Both fractions (supernatant containing the animal tissue and pellet containing the symbionts) were individually freeze-dried until subsequent analysis. Samples were then analyzed for $\delta^{15}N$ using a Geo-20:20 isotope ratio mass spectrometer (Sercon, Crewe, UK). International reference materials (IAEA-600 and IAEA-CH6, International Atomic Energy Agency, Vienna, Austria) were used for scale calibration of results to air, and two different analytical

quality control samples were also analysed with each batch for quality control purposes. Precision, as determined by repeat analysis of the reference materials and quality controls, was better than ±0.20‰ for measured $\delta^{15}N$ and $\delta^{13}C$ values. Data are expressed in the standard δ unit notation where:

$$\delta X = [(R_{sample}:R_{reference})-1\ ]\times 10^3\,, \qquad (2)$$

where R=^{15}N:^{14}N for nitrogen, reported relative to air N_2, or R=^{13}C:^{12}C for carbon, reported relative to Vienna Pee Dee Belemnite.

Data analysis

Statistics were performed using Systat 13 (Systat Software, Chicago, IL, USA). All parameters are expressed as means ± s.e.m. Data were checked for normality using a Kolmogorov–Smirnov test with the Lilliefors correction, and for variance homoscedasticity using a Levene's test. Preliminary partly nested ANOVAs were used to test the tank effect, with three factors (ammonium addition, temperature and tank), according to the procedure described in Underwood (Underwood, 1997). Because the tank effect nested within ammonium addition and temperature was not significant, it was not considered in further analyses. The two tanks per condition were therefore pooled. An ANOVA was used to assess the effect of ammonium enrichment and temperature on the physiological parameters tested (ammonium and temperature as fixed effects, and genotype as random effect). When significant differences were found, the analyses were followed by *a posteriori* tests (Tukey's test). There was, however, no genotype effect in this study.

Fluorometry measurements generated large data sets with many correlated variables that explain different, yet related, components of the photophysiology. For this parameter, we therefore used PCA to detect which variables contributed most strongly to the observed variance in photochemistry, and with which factors these variables were associated. The PCA included 11 variables, as follows (Hoogenboom et al., 2012; Klughammer and Schreiber, 2008): for PSII: fluorescence-based maximum quantum yield (Φ_f or Y_{maxII}), relative maximum electron transport rate ($rETR_{maxII}$), maximum unregulated non-photochemical quenching (Φ_{NO} or Y_{NO}), maximum quantum yield of regulated heat dissipation (Y_{NPQ}; a loss process serving for protection), maximum non-photochemical quenching (NPQ), maximum coefficient of non-photochemical quenching (qN), minimum photochemical quenching (qP; defined as the fraction of fluorescence that is quenched by oxidized quinone, Q_A), and minimum fraction of overall PSII reaction centers open (qL); and for PSI: maximum quantum yield (Y_{maxI}), relative maximum electron transport rate ($rETR_{maxI}$), and maximum donor-limited quenching (Y_{ND}).

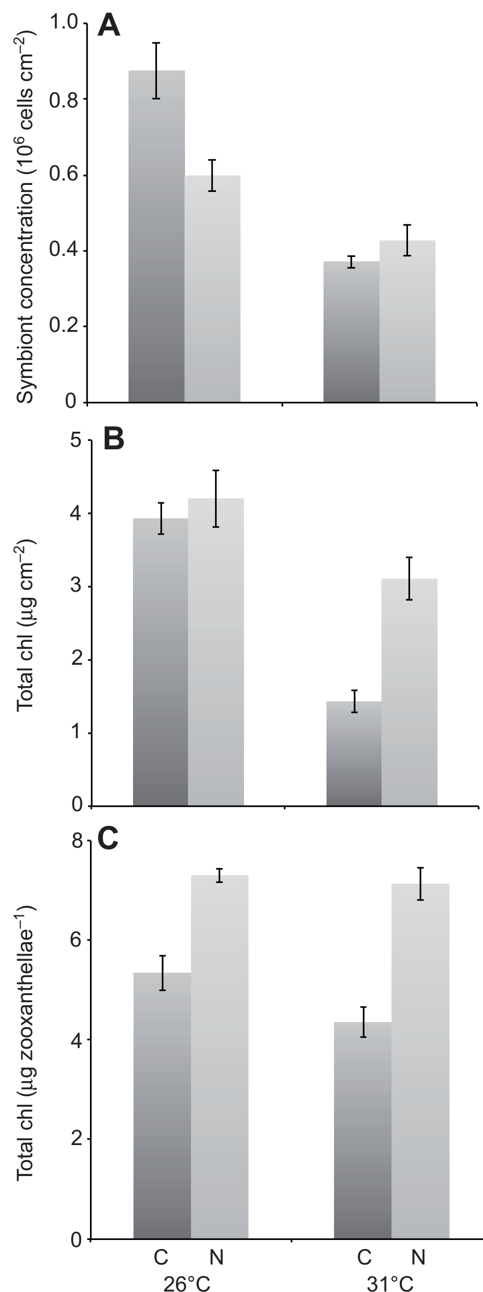

Fig. 1. (A) Symbiont concentration, (B) total chlorophyll concentration and (C) chlorophyll content per symbiont in *Turbinaria reniformis* colonies incubated at 26 and 31°C in the presence (N) or absence (C) of nitrogen. Data are means ± s.e.m. of five samples.

RESULTS

Protein, lipid, symbiont and pigment concentrations

There was no significant effect of nitrogen enrichment or temperature on the areal protein and lipid contents (ANOVA, P>0.05). Mean protein concentration was equal to 2.50±0.14 µg cm^{-2}, while mean lipid concentration reached 400±100 µg cm^{-2}.

Symbiont and total chlorophyll concentrations (per skeletal surface area) presented significant differences among treatments (ANOVA, P<0.05). At 26°C, symbiont concentration was significantly higher in C-26 than in N-26 corals (Tukey test, P<0.01; Fig. 1A). However, there was no difference in total chlorophyll content between the two treatments (Tukey test, P>0.05; Fig. 1B) because N-26 symbionts had significantly higher chlorophyll content per cell than C-26 symbionts (Tukey test, P<0.001; Fig. 1C). After 7 days, thermal stress significantly decreased symbiont and chlorophyll concentrations in both C-31 and N-31 corals (Tukey test, P<0.007; Fig. 1A). However, while chlorophyll concentration decreased by more than 60% in C-31 samples, it only decreased by 25% in N-31 samples, leading to a significantly higher concentration in these latter corals (Tukey test, P<0.001; Fig. 1B,C). Nitrogen enrichment thus reduced the total loss of chlorophyll.

Concerning the other pigments monitored, there was a significant effect of temperature, ammonium enrichment and their interaction on pigment composition (Tukey test, P<0.001). The maximal amount for all pigments (β-carotene, diadinoxanthin, diatoxanthin

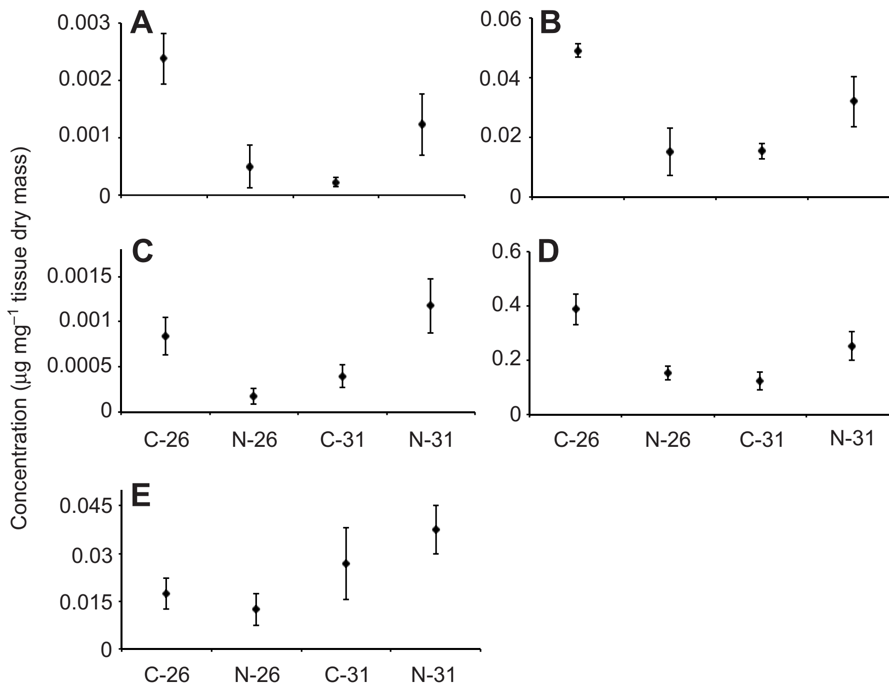

Fig. 2. (A) β-carotene content, (B) diadinoxanthin content, (C) diatoxanthin content, (D) peridin content and (E) de-epoxidation ratio in corals maintained at 26 and 31°C in the presence (N) or absence (C) of nitrogen. Data are means ± s.e.m. of five samples.

and peridinin; Fig. 2) was obtained in C-26 nubbins. These nubbins significantly decreased their pigment concentrations at 31°C, by a factor of 2 to 10 (Tukey test, $P<0.005$). Ammonium addition induced a significant decrease in all pigments at 26°C compared with C-26 corals (Tukey test, $P<0.001$), and a significant increase at 31°C (Tukey test, $P<0.001$). The de-epoxidation ratio presented a tendency to increase from 26 to 31°C in both enriched and un-enriched nubbins; however, the increase was significant only in enriched nubbins (Tukey test, $P=0.020$; Fig. 2E).

Photosynthesis and respiration

At 26°C, there was no significant difference in the rates of areal net photosynthesis or respiration in C-26 and N-26 samples (ANOVA, $P>0.05$). Rates were also not different when they were normalized to the chlorophyll content. Seven days of thermal stress induced a significant increase in the respiration rates and a significant decrease in the photosynthetic rates of nubbins from both treatments, compared with 26°C (ANOVA, $P<0.05$; Fig. 3A). However, while respiration rates were not different between C-31 and N-31 nubbins, N-31 nubbins kept a significantly higher areal net photosynthesis compared with C-31 samples, for which net photosynthesis was slightly negative (Tukey test, $P<0.05$). As a consequence, the CZAR (percent contribution of autotrophically acquired carbon to daily animal respiration) decreased from *ca.* 100–130% at 26°C to 52−75% at 31°C in non-enriched and nitrogen-enriched corals, respectively (Fig. 3B). There was also a linear relationship between the chlorophyll *a*/*c* ratio, which is a stress marker, and oxygen production ($R^2=0.81$, Fig. 3C). Indeed, the decrease in oxygen production following the thermal stress was accompanied by a parallel decrease in the chlorophyll ratio (Tukey test, $P=0.020$). Also, at 31°C, ammonium addition significantly increased this ratio (Tukey test, $P=0.002$) as well as the oxygen production.

Photochemistry

PCA was used to determine which of the measured fluorescence and absorbance parameters best captured variation in PSII and PSI photophysiology under the set of experimental conditions. Two main principal components were identified that explained more

Table 1. Results of the principal component analysis on the effect of temperature and nitrogen enrichment on PSI and PSII photochemistry

	PC1	PC2	PC3	PC4	PC5	PC6
Y_{maxI}	**0.784266**	–0.165256	–0.415845	0.429673	0.006601	0.005261
$rETR_{maxI}$	**0.787274**	0.130158	–0.417510	0.424164	0.093859	0.008078
Y_{ND}	**–0.792926**	0.144222	0.357133	0.472109	–0.005905	0.001387
Y_{maxII}	**0.752484**	0.615862	0.199912	0.001959	0.117157	0.028061
$rETR_{maxII}$	**0.729466**	0.614662	0.023154	0.211100	–0.040385	0.175825
Y_{NO}	**–0.824896**	0.476610	–0.294824	–0.037981	–0.025790	0.057974
Y_{NPQ}	0.154615	**–0.969193**	0.183418	–0.017445	0.009889	0.052099
NPQ	0.331320	**–0.919248**	0.030303	–0.198131	–0.029900	0.026780
qN	0.425336	**–0.887063**	0.056722	–0.154920	–0.001917	0.004956
qP	**0.899314**	0.359764	0.208297	0.008076	–0.134348	0.017374
qL	**0.893008**	0.317599	0.101086	0.236922	–0.019450	0.181261
*Temperature	**–0.432606**	**–0.509554**	–0.219290	–0.051358	–0.040536	0.057005
*NH^{4+}	**0.237643**	0.058118	0.053526	–0.234019	–0.299416	–0.300955
% of variance explained	**55.38604**	**28.72197**	8.40644	6.82203	0.54361	0.11990

Asterisks represent factors. Bold indicates the variables with the highest contribution to the observed variance in photochemistry for PC1 and PC2.

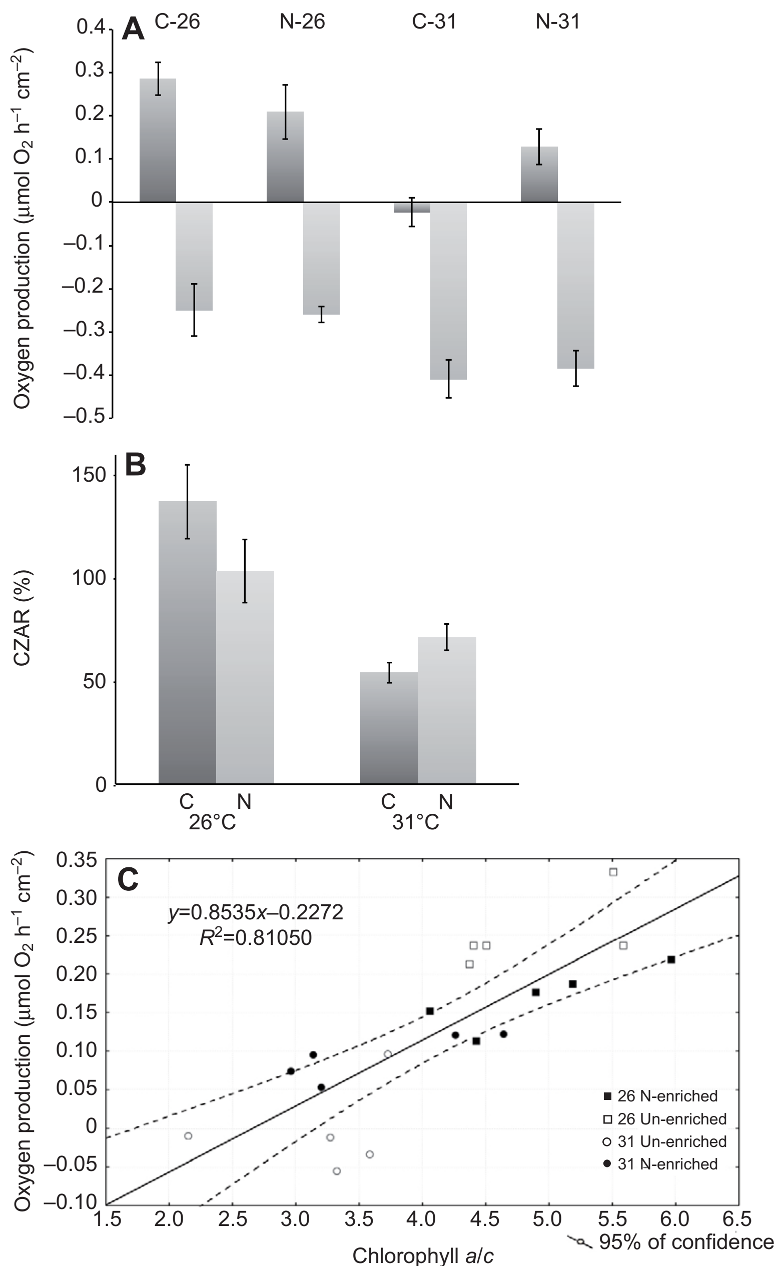

Fig. 3. (A) Oxygen production at 200 μmol photons $m^{-2} s^{-1}$ and (B) percentage contribution of symbiont-acquired carbon to daily animal respiration (CZAR) in the different treatments: 26°C in the absence or presence of nitrogen enrichment (C-26 and N-26), and 31°C in the absence or presence of nitrogen enrichment (C-31 and N-31). (C) Oxygen production *versus* chlorophyll *a*/*c* ratio in the different treatments. Data are means ± s.e.m. of five samples.

than 84% of the variance (Table 1) and were highly correlated with temperature. Y_{maxII}, $rETR_{maxII}$, qP and qL were negatively correlated with temperature and significantly decreased during the thermal stress (ANOVA, Tukey test, P=0.042; Fig. 4). However, Y_{NPQ} was positively correlated with temperature. Nitrogen enrichment did not contribute significantly to the explanation in the changes of PSI and PSII parameters. It was, however, slightly correlated with electron transport rates Y_{maxI} and Y_{maxII}. Therefore, $rETR_{maxII}$ was significantly higher in the presence than in the absence of ammonium, independent of the seawater temperature considered (Tukey test, P=0.042; Fig. 4B). Moreover, after recovery in the dark, Y_{NPQ} of enriched nubbins went back to its initial value (0), whereas it remained 20% higher in un-enriched nubbins (Tukey test, P=0.022).

Total calcification

C-26 and N-26 corals presented an equivalent calcification rate of ca. 450±50 nmol $CaCO_3\,cm^{-2}\,h^{-1}$. Thermal stress induced a significant decrease in the calcification rate of C-31 corals (Tukey test, P=0.006; Fig. 5A), but not of N-31 corals. As a consequence, at 31°C, calcification of N-31 nubbins was significantly higher than for C-31 nubbins (Tukey test, P=0.022; Fig. 5A).

Total organic carbon

TOC fluxes were positive in all treatments, showing an overall release of carbon by corals into seawater. There was a significant and interactive effect of both temperature and ammonium enrichment on TOC fluxes (ANOVA, P=0.015). No significant difference in carbon release between C-26, N-31 and C-31 nubbins was detected. However, carbon release in nitrogen-enriched nubbins doubled between 26 and 31°C (Tukey test, P=0.007; Fig. 5B).

$\delta^{15}N$ and $\delta^{13}C$ determination

In C-26 nubbins (Fig. 6), $\delta^{15}N$ and $\delta^{13}C$ signatures of the symbionts were 1 and 0.6‰ higher, respectively, than those of the host. Ammonium enrichment induced, independent of seawater

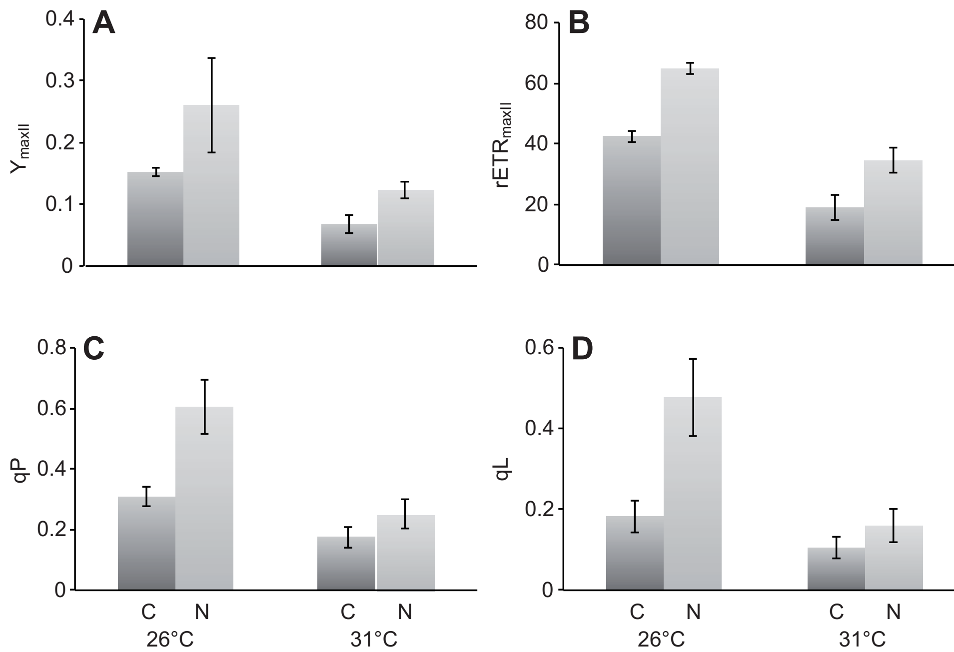

Fig. 4. (A) Fluorescence-based maximum quantum yield (Y_{maxII}), (B) relative maximum electron transport rate ($rETR_{maxII}$), (C) minimum photochemical quenching (qP) and (D) minimum fraction of overall PSII reaction centers open (qL) in colonies incubated at 26 and 31°C in the presence (N) or absence (C) of nitrogen. Data are means ± s.e.m. of five samples.

temperature, a significant shift in the $\delta^{15}N$ signature of both the symbionts and the host compared with C-26 or C-31 corals (Tukey test, $P<0.001$). $\delta^{15}N$ of the nitrogen-enriched symbionts and host was therefore depleted by *ca.* 4–5‰ and 1–2‰, respectively. As a consequence, under ammonium enrichment, the $\delta^{15}N$ signature of the host was 2‰ higher compared with that of the symbionts (Tukey test, $P<0.001$). The larger shift in the signature of the symbionts compared with the host tissue indicates that symbionts are the primary site of nitrogen assimilation. Nitrogen enrichment also induced a significant increase in the $\delta^{13}C$ of the symbionts compared with the host tissue (Fig. 6, $P=0.015$).

Thermal stress did not significantly change the isotopic signature compared with 26°C (ANOVA, $P>0.05$). Therefore, the $\delta^{13}C$ signature of the enriched symbionts remained 1.3‰ higher than the signature of the host (Tukey test, $P=0.015$, respectively).

DISCUSSION

This study has shown that under thermal stress, nitrogen availability can be important in determining the capacity for photoprotection in some scleractinian coral species, such as *T. reniformis*. Moderate nitrogen enrichment increased the photosynthetic and photoprotective pigment contents in coral tissue during a thermal stress, sustaining rates of photosynthesis and calcification.

In non-stressed healthy corals, it has been shown that nutrient addition (nitrogen alone or in combination with phosphorus) may negatively impact coral metabolic functions, such as calcification. Indeed, symbiont and/or chlorophyll concentrations as well as areal rates of photosynthesis are often increased under eutrophication (Snidvongs and Kinzie, 1994; Marubini and Davies, 1996; Fagoonee et al., 1999). This leads to a limitation in dissolved inorganic carbon for calcification (instead used for symbiont photosynthesis) or to a decrease in the translocation of photosynthetic products to the coral host (Kinsey and Davies, 1979; Stambler et al., 1991; Marubini and Davies, 1996; Ferrier-Pagès et al., 2000; Koop et al., 2001). Coral mortality was also observed in long-term enrichment treatments (Koop et al., 2001). However, in most of the above studies, levels of nitrogen were relatively high (above 5 $\mu mol\,l^{-1}$), especially when nitrate was used as the nitrogen source. Also, the seawater chemistry was not controlled, and a limitation in inorganic carbon might have occurred in the experimental tanks following a limited seawater renewal or mixing.

In a few experiments involving a moderate increase in nitrogen and/or phosphorus levels, an enhancement of coral metabolism was instead observed (Meyer and Schultz, 1985; Tanaka et al., 2007; Godinot et al., 2011). Our results with non-stressed nubbins of *T. reniformis* (C-26 and N-26 samples) add to the growing body of evidence that a slight increase in nutrient concentration in seawater

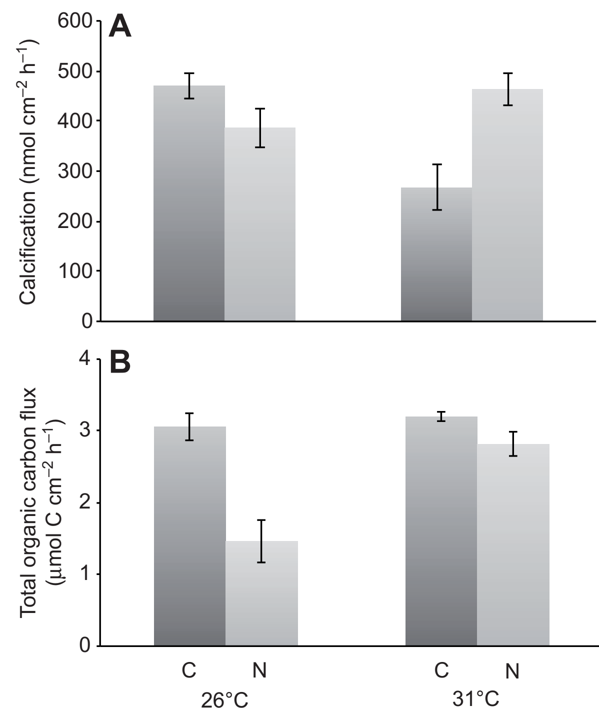

Fig. 5. (A) Calcification and (B) total organic carbon flux in colonies incubated at 26 and 31°C in the presence (N) or absence (C) of nitrogen. Data are means ± s.e.m. of five samples.

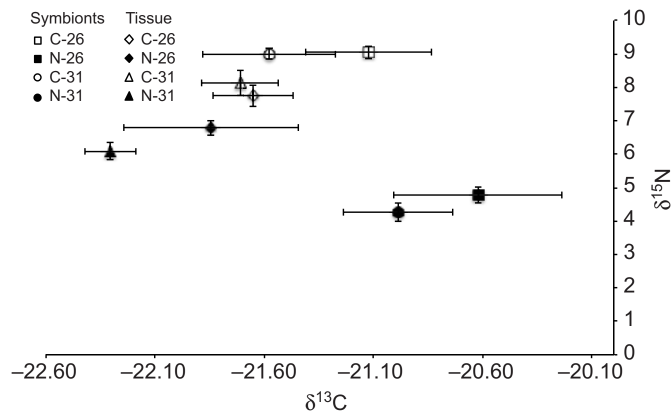

Fig. 6. Relationship between the $\delta^{15}N$ and $\delta^{13}C$ signature of the symbionts and host tissue in colonies incubated at 26 and 31°C in the presence (N) or absence (C) of nitrogen. Data are means ± s.e.m. of five samples.

does not necessarily impact coral health. Indeed, although there was an increase in cellular chlorophyll levels, this increase was counterbalanced by a decrease in symbiont density. Therefore, no main physiological changes in terms of calcification, photosynthesis and respiration were observed in these non-stressed enriched nubbins. Stambler (Stambler, 1998) and Sakami (Sakami, 2000) also concluded that there was a lack of ammonium effect on the photosynthesis of *Stylophora pistillata*, and of symbionts extracted from the coral *Pocillopora damicornis*. Nitrogen-enriched corals, however, presented a decrease in organic carbon release, likely due, in the present experiment, to a higher utilization of photosynthetically acquired carbon, in combination with nitrogen, to form molecules of interest such as proteins. Three other studies have investigated the release of organic carbon by scleractinian corals in the presence of elevated nutrient concentrations (Tanaka et al., 2007; Naumann et al., 2010; Bednarz et al., 2012), and have also observed a reduced release or an increased uptake of organic carbon. In Bednarz et al. (Bednarz et al., 2012), it was hypothesized that the enhanced uptake of dissolved organic carbon by the soft coral *Xenia* under nitrogen enrichment was due to a need for additional carbon compounds following the decrease in photosynthetic rates and carbon translocation of the symbionts. In the present study, however, rates of photosynthesis, normalized either to the surface area or to the total chlorophyll content, were not different between nitrogen-enriched and control corals, suggesting that the photosynthesized carbon, instead of being released as junk food, was combined with nitrogen and kept for the coral metabolism.

Comparison of the isotopic signature of the C-26 and N-26 nubbins shows that large amounts of light nitrogen (^{14}N) were indeed taken up and assimilated both by the host and the symbionts of *T. reniformis*, as their $\delta^{15}N$ signature significantly decreased after 4 weeks enrichment. Changes in $\delta^{15}N$ of anthozoan and algal tissue are regularly used to track nitrogen sources in these organisms [see discussion by Swart et al. (Swart et al., 2005)]. In the present study, the largest change was observed in the symbionts (−4‰), which are the main site of ammonium assimilation (Grover et al., 2002; Pernice et al., 2012). Moreover, the observation that the $\delta^{15}N$ of the host tissue was ~2‰ more positive than the symbionts supports the notion of isotopic enrichment associated with changes in trophic levels and photosynthate translocation (Muscatine et al., 1989; Swart et al., 2005). Although the coral host has also the capacity to directly assimilate ammonium from seawater (Wang and Douglas, 1998; Lipschulz and Cook, 2002; Pernice et al., 2012), this assimilation has been shown to be eight to 13 times smaller than assimilation by the symbionts (Pernice et al., 2012), suggesting that nitrogen-enriched photosynthates are actually the main inorganic nitrogen source of the coral host. Nitrogen enrichment also led to a slight increase in the carbon isotopic signature of the symbionts, suggesting that they are more enriched in ^{13}C. This enrichment is usually related to carbon limitation during high rates of photosynthesis and increased fractionation of the inorganic carbon pool (Swart et al., 2005). As rates of net photosynthesis were not significantly different between nitrogen-enriched and control corals, this result might be linked to the greater rETR in nitrogen-enriched corals. This greater rETR can be explained by the same process as that occurring in the free-living dinoflagellate *Heterocapsa* sp. (Korbee et al., 2010). Compared with nitrogen-limited *Heterocapsa*, nitrogen-enriched cells likely allocate supplemental nitrogen for the maintenance of the ribulose bisphosphate carboxylase (RuBisCo), the enzyme of the Calvin cycle, which catalyses the reaction in which carbon dioxide combines with ribulose bisphosphate to create carbon molecules. This enhanced level of RuBisCo ensures a more efficient downstream removal of electrons to release pressure on PSII. Also, in higher plants, more than half of the total nitrogen is allocated to the photosynthetic apparatus (Makino and Osmond, 1991).

Conversely to non-stressed corals, a moderate increase in ammonium helped heat-stressed nubbins to maintain photosynthetic pigment levels close to their optimal concentration, and to prevent a large decrease in the rates of photosynthesis. As calcification is tightly linked to photosynthesis in symbiotic corals (Gattuso et al., 1999), it was also sustained under nutrient-enriched conditions. Without ammonium supplementation, thermal stress induced a significant bleaching, as often observed (Hoegh-Guldberg, 1999; Dove et al., 2006). However, there was not only a 70% loss in total chlorophyll content, but also a loss in other pigments such as the peridinin and xanthophyll-cyclic carotenoids, a decrease previously observed in *Montipora monasteriata* heated to 32°C (Dove et al., 2006). Xanthophyll cycling pigments (diadinoxanthin and diatoxanthin) and carotenoids in general contribute to photoprotection in dinoflagellates (Havaux and Niyogi, 1999) through three mechanisms: (1) xanthophyll pigments directly prevent formation of triplet chlorophyll, which leads to (2) the production of reactive oxygen species (Szabó et al., 2005), whereby there is a singlet-singlet energy transfer from chlorophyll to carotenoids; and (3) the xanthophyll cycle, measured through the de-epoxidation ratio (the reversible de-epoxidation of diadinoxanthin to diatoxanthin), mediates structural-organizational changes in those light-harvesting complexes that promote energy dissipation (Szabó et al., 2005). All these pigments have also a role in membrane protection and in the inhibition of lipid peroxidation (Havaux and Niyogi, 1999; Tardy and Havaux, 1997). As a result of thermal stress, photosynthetic activity was completely impaired in heat-stressed corals (100% decrease in net photosynthesis) through the loss of photoprotective pigments and the lack of xanthophyll cycle induction, due to either a lack of sufficient pigment concentration or a disruption of the thylakoid membrane integrity (Iglesias-Prieto et al., 1992; Tchernov et al., 2004). Indeed, the stimulation of the diadinoxanthin epoxidase activity requires an increased ΔpH across the thylakoid membrane that cannot occur if the membrane integrity is disrupted.

Ammonium supplementation during thermal stress significantly increased the accumulation of photoprotective pigments. The effect of nitrogen is not direct because these pigments are not nitrogenous

compounds. This process can be explained by an enhancement in nucleic acid synthesis and protein turnover, contributing to an increase in the rate of molecule repair. Such nitrogen involvement in pigment synthesis has been demonstrated in free-living dinoflagellates (Korbee et al., 2010; Barufi et al., 2011). For these dinoflagellates, ammonium addition also limited photoinhibition of photosynthesis under stress conditions (high levels of ultraviolet radiation) and therefore improved photosynthesis (Korbee et al., 2010; Barufi et al., 2011) and maximal photosynthetic efficiency (Parkhill et al., 2001). The same observation was made here with dinoflagellates in symbiosis with heat-stressed *T. reniformis* nubbins. This photoprotection went through an enhancement of the diadinoxanthin de-epoxidation state, induced by a higher synthesis of xanthophyll cyclic pigments. The importance of nitrogen addition in the maintenance of high rates of photosynthesis under thermal stress is comparable to that observed during an elevation in P_{CO_2} level (Atkinson et al., 1995; Cohen and Holcomb, 2009; Holcomb et al., 2010; Chauvin et al., 2011). The explanation given in this latter case was that nutrient-enriched corals experienced dissolved inorganic carbon limitation due to high symbiont densities and therefore benefited from higher dissolved inorganic carbon levels (Holcomb et al., 2010). The maintenance of high rates of photosynthesis in nitrogen-enriched and heat-stressed corals also allowed them to maintain higher levels of lipids in their tissue compared with non-enriched corals, as well as higher rates of calcification. Inorganic nitrogen had therefore the same function as feeding concerning the maintenance of coral metabolism under stress conditions (Grottoli et al., 2006; Borell and Bischof, 2008; Ferrier-Pages et al., 2010; Godinot et al., 2011; Tolosa et al., 2011; Hoogenboom et al., 2012).

Large bleaching events in nature often occur during periods of high irradiance and seawater temperature levels. These conditions also favour the formation of a thermocline (warm surface seawater layer isolated from the colder deeper waters). In such conditions, this surface layer becomes also rapidly nutrient-depleted, affecting coral's capacity to resist to thermal stress. Addition of small amounts of nutrients, and especially nitrogen, may sustain the equilibrium of the symbiotic association in some scleractinian coral species during thermal stress conditions. In particular, nitrogen addition maintains high levels of photosynthetic and photoprotective pigments in the symbionts, and allows for maximal rates of photosynthesis.

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AUTHOR CONTRIBUTIONS

E.B. and C.F.-P. contributed to the conception, design and execution of the study, the interpretation of the findings, and the drafting and revising of the article. F.G. performed the pigment analysis, and contributed to the interpretation of the findings. C.R. contributed to the design and execution of the study.

COMPETING INTERESTS

No competing interests declared.

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