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Photochemical efficiency and antioxidant capacity in relation to *Symbiodinium* genotype and host phenotype in a symbiotic cnidarian

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ABSTRACT: This study analysed the effects of elevated temperature on chlorophyll fluorescence parameters and superoxide dismutase (SOD) activities in relation to *Symbiodinium* genotype, and host pigmentation in 2 distinct colour phenotypes (green and pink) of the sea anemone *Entacmaea quadricolor*. Overall, the phenotypes differed with respect to the relative content of *Symbiodinium* internal transcribed spacer 2 (ITS2) types C25 and C3.25 and their maximum quantum yield of PSII (F_v/F_m) during baseline conditions. However, different PSII photochemical efficiencies were not correlated with symbiont assemblage. Also, the responses to elevated temperatures were phenotype-specific. The PSII photochemical efficiencies had different critical thermal thresholds of <24.5°C in the green phenotype and >24.5°C in the pink phenotype. The highest temperature treatment (27.6°C) resulted in symbiont shuffling towards a higher relative content of C3.25 in the green but not the pink phenotype. However, the observed shuffling of *Symbiodinium* types could not be linked to enhanced algal SOD activity or PSII photochemical efficiency. These results suggest that different photobiological properties and thermal responses of *Symbiodinium* ITS2 consortia might be, at least in part, influenced by host-derived factors, possibly chromophore proteins that also determine host pigmentation. The differential ability to cope with elevated temperatures might have profound impacts on *E. quadricolor* phenotype abundance in response to changing climate.

KEY WORDS: *Entacmaea quadricolor* · ITS2 · Photosynthesis · Superoxide dismutase · *Symbiodinium* shuffling · Thermal stress · Zooxanthellae · Sea anemone

INTRODUCTION

The health and persistence of *Symbiodinium*-associated species are threatened by rapidly changing environmental conditions, such as anthropogenically driven increases in ocean temperatures (Hoegh-Guldberg & Bruno 2010). High temperature, in combination with high light, is the main cause for a collapse of the symbiosis, which is often linked to the loss of *Symbiodinium* cells, their photosynthetic pigments and/or photosynthetic capacity (i.e. bleaching; Brown 1997, Hoegh-Guldberg 1999).

The genus *Symbiodinium* is represented by 9 clades (A to I; Pochon & Gates 2010), each with numerous subclades (hereon referred to as 'types') based on their internal transcribed spacer (ITS) phylogeny (LaJeunesse 2001, 2005, Thornhill et al. 2006). It has been recognized that different physio-

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logical capabilities within clades and types (e.g. Robison & Warner 2006, Hennige et al. 2009, Krämer et al. 2012) facilitate host responses to environmental perturbations (e.g. Sampayo et al. 2008, Fisher et al. 2012). For example, symbiont partner determined the fitness of the reef-building coral *Pocillopora damicornis* during an extreme cold-water event in the Gulf of California; with corals hosting *Symbiodinium* type C1b-c bleaching, and those harbouring *Symbiodinium* type D1 mostly unaffected (LaJeunesse et al. 2010). However, the host itself may be equally important for the bleaching response. There are several host-derived mechanisms that may affect *Symbiodinium* photophysiology (Bhagooli et al. 2008, Baird et al. 2009), such as incorporated fluorescent or non-fluorescent chromophore proteins that absorb, dissipate and/or scatter photosynthetic active radiation (PAR; Salih et al. 2000, Dove et al. 2001) or compounds that screen for ultraviolet (UV) radiation (Shick et al. 1995) which modify the internal light environment (Dove et al. 2006, Hennige et al. 2008a). In the anemone *Condylactis gigantea*, distinct colour phenotypes differ in their UV absorbance and UV acclimatization capacities (Stoletzki & Schierwater 2005). It has been shown that host pigments can act in either a photoprotective (Salih et al. 2000, Dove et al. 2001, Smith et al. 2013, but see Dove 2004) or photoenhancing manner (Schlichter et al. 1994), and that the optical properties of the host tissue influence the microenvironmental light field (Wangpraseurt et al. 2012). In corals with multiple colour phenotypes, an alteration of the internal light climate by host pigments can affect the *Symbiodinium* type present (Frade et al. 2008) and their cellular and physiological properties (Dove 2004, Dove et al. 2006, 2008, Klueter et al. 2006). However, it is not known whether colour phenotype influences the distribution of *Symbiodinium* types or their physiological properties in sea anemones.

It has been hypothesized that the host's potential to associate with multiple symbiont types might be advantageous, as it could facilitate a change in symbiont dominance that creates a more stress-tolerant association during environmental change (Baker 2003). This mechanism could be achieved by the repopulation of a bleached individual with a new *Symbiodinium* partner, or by the shuffling of the relative proportion of pre-existing multiple *Symbiodinium* partners (Buddemeier & Fautin 1993, Baker 2003). To date, there is no evidence for the stable uptake of new *Symbiodinium* types from the environment (but see Coffroth et al. 2010 for transient uptakes); however, symbiont shuffling has been reported in various reef-building corals (Berkelmans & van Oppen 2006, Jones et al. 2008). For example, Jones et al. (2008) demonstrated that the type of symbiont partner influenced the warm-water bleaching susceptibility of *Acropora millepora* in the Keppel Islands (Great Barrier Reef), with the majority of surviving coral colonies that initially hosted ITS1 type C2 as their dominant symbiont changing to ITS1 type C1 or D post bleaching. Shuffling might also have its drawbacks, as corals associating predominantly with clade D have higher energetic costs than those with C1 (Abrego et al. 2008).

Bleaching susceptibility has been partly linked to the capability of the antioxidant defence system of both partners to deal with reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH) (Lesser 2011). In small quantities, ROS are important cell signalling molecules (Apel & Hirt 2004) and may be photoprotective (Asada 2000). However, excessive amounts can adversely affect cellular processes such as photosynthesis (Takahashi & Murata 2008, Lesser 2011) and induce programmed cell death (apoptosis) of *Symbiodinium* or host cells (Franklin et al. 2004, Tchernov et al. 2011). To control levels of ROS, among a number of antioxidant molecules and enzymes, superoxide dismutase (SOD) catalyses the conversion of O_2^- to oxygen and H_2O_2, which in turn is detoxified by the enzymes ascorbate peroxidase or catalase. SOD is the first line of defence, and as such a key enzyme in the antioxidant response (reviewed in Halliwell 2006). Both ROS generation (Suggett et al. 2008) and detoxification (McGinty et al. 2012) are highly variable between *Symbiodinium* types and may therefore affect the fitness of the host. As such, symbiont SOD capacities may be a crucial factor for regulating symbiont assemblages within a host.

While symbiont shuffling has been observed in various reef-building corals, there is no evidence for this mechanism occurring in sea anemones. *Entacmaea quadricolor* (Rüppell & Leuckart, 1828) is the most common and geographically widespread species of sea anemone that hosts *Symbiodinium* and anemonefishes in a 3-way symbiosis (Dunn 1981, Fautin & Allen 1997). Throughout its Indo-Pacific distribution, this species occurs in a range of distinctly pigmented phenotypes, with varying column, oral disc or tentacle colouration (Dunn 1981, Fautin & Allen 1997). The highest published density of this species occurs at North Solitary Island, Solitary Islands Marine Park (SIMP), Australia, where it provides habitat for 3 species of anemonefish (Richardson et al. 1997,

Scott et al. 2011) and harbours *Symbiodinium* ITS2 types C25 and C3.25 simultaneously (Pontasch et al. 2014). This location has been identified as a climate change hotspot, with water temperatures expected to be 2°C higher by 2050 (compared to the 1990–2000 average; Hobday & Lough 2011). Given that *E. quadricolor* has been shown to be living within 1°C of its upper physiological threshold (Hill & Scott 2012), rapid acclimatization or adaptation mechanisms may be necessary to ensure its survival at this high latitude site.

This is the first study to compare the effects of elevated temperature on photophysiology and antioxidant capacity in relation to host pigmentation and *Symbiodinium* assemblage in 2 distinct colour phenotypes of the sea anemone *E. quadricolor.* The 2 phenotypes analysed differ in the colouration of their tentacle tips and column. Because this differential pigmentation is likely to influence the internal light environment available for the symbionts, we hypothesised that the 2 phenotypes would differ in their relative compositions of *Symbiodinium* types C25 and C3.25, resulting from taxonomic variations in symbiont photobiological characteristics, which are commonly observed amongst *Symbiodinium* types (e.g. Hennige et al. 2009). We then hypothesised that both phenotypes of *E. quadricolo*r would alter the balance of their resident *Symbiodinium* ITS2 composition in response to thermal stress, similar to the change in symbiont dominance towards an apparently heat-tolerant association that has been observed in reef-building corals (Berkelmans & van Oppen 2006, Jones et al. 2008). Further, given the importance of the antioxidant network in determining thermal tolerance and the fact that the antioxidant system is considered to be an important regulatory mechanism during temperature stress (Lesser 2011), we investigated whether symbiont assemblage structure and differences in photophysiology are regulated by the use of the key antioxidant enzyme, SOD.

MATERIALS AND METHODS

Entacmaea quadricolor (n = 36) was collected at approximately 18 m depth from North Solitary Island, SIMP, New South Wales, Australia (29° 55′ S, 153° 23′ E) on 9 August 2012 (southern hemisphere winter). Eighteen individuals had a red column, brown tentacles and green tips (referred to as 'green phenotype'; Fig. 1A), and 18 individuals had an orange column and brown tentacles, with white pigmentation below pink tentacle tips (referred to as 'pink phenotype'; Fig. 1B). Anemones were maintained outdoors in 3000 l tanks supplied with flow-through seawater from the SIMP (10 l min^{-1}, ambient temperature 19 to 20°C) and an irradiance of <50 µmol photons m^{-2} s^{-1} (to ensure light-induced bleaching did not occur) for 32 d before the experiment. Anemones were fed to satiation with prawn meat every 2 wk, with the last feeding occurring 4 d before the start of the experiment in order to provide a heterotrophic food source. Anemones were not fed during the experiment as this may have altered their metabolic response, for example with respect to antioxidant capacity.

Experimental setup

On Day 1 of the experiment, anemones were placed into individual transparent 15 l tubs supplied with flow-through seawater (600 ml min^{-1}, 5 µm filtered with Filtaflo sediment filters) from 3 thermostat controlled (heat pump, Aquahort) 3000 l header tanks.

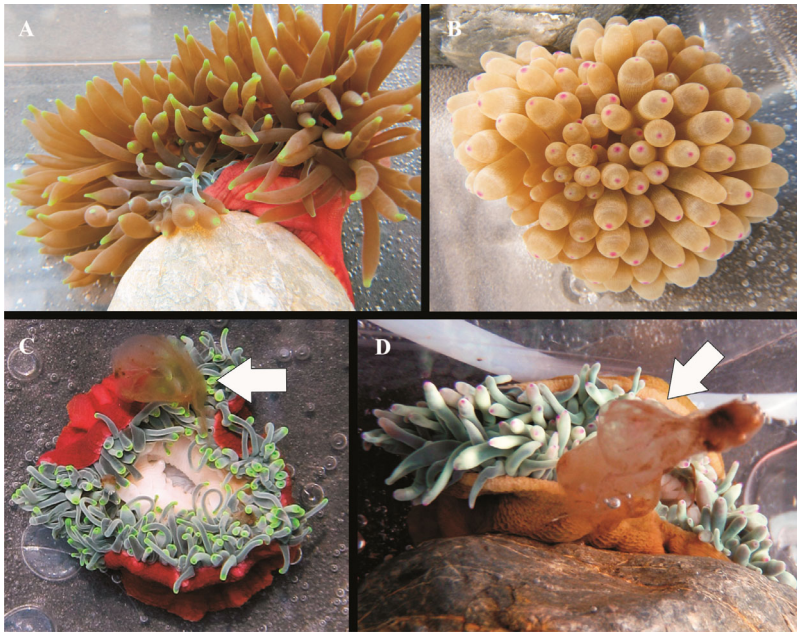

Fig. 1. (A) Green and (B) pink phenotypes of *Entacmaea quadricolor.* (C,D) White arrows indicate expulsion of *Symbiodinium* cells observed on Day 3 in both phenotypes exposed to the highest temperature. Anemones are approximately 15 to 20 cm in diameter

Three 1200 l tanks each contained twelve 15 l tubs (so that in total 36 tubs were used), each containing a single anemone. These individuals were allocated to 3 treatments: 21.3°C (control, C), 24.5°C (medium, M, 1.5°C below maximum summer temperature), and 27.6°C (high, H, 1.6°C above maximum summer temperature; http://data.aims.gov.au, Fig. 2A). Temperature treatments were allocated haphazardly within each 1200 l tank, so that each tank contained 4 tubs of each temperature treatment. Anemone phenotypes were placed haphazardly within each treatment (n = 6, 2 anemones per 1200 l tank). The final temperature was reached by increasing the temperature by small increments starting at 12:00 h on Day 1. The desired temperature in all treatments was attained after 36 h (24:00 h on Day 2) and was maintained until the end of the experiment (12:00 h on Day 9) (Fig. 2A). The heating rate approximated 0.04°C h^{-1} in the control, 0.13°C h^{-1} in the medium, and 0.21°C h^{-1} in the high temperature treatments. Temperature was monitored using 9 Thermochron iButton temperature loggers per treatment (accuracy ±0.5°C; Maxim), which were haphazardly placed into 15 l treatment tubs, and were calibrated against a high precision mercury thermometer. The tanks were covered with 2 layers of white shade cloth to reduce the natural light intensity to approximately 25% of incoming solar radiation. The light intensity was monitored using an underwater Odyssey light logger (Dataflow Systems), calibrated against a Li-1400 photometer with a 2π Li-192SA quantum sensor (Lincoln). Daily maximum light intensities ranged between 421 and 504 µmol photons m^{-2} s^{-1}, daily mean light intensities ranged between 173 and 209 µmol photons m^{-2} s^{-1}, and the daily median light intensities ranged between 154 and 274 µmol photons m^{-2} s^{-1} for the duration of the experiment (Fig. 2B).

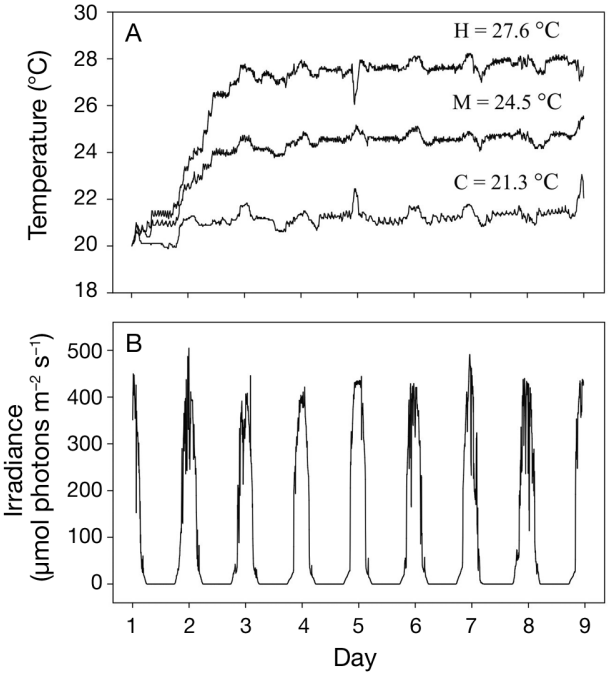

Fig. 2. (A) Average temperature and (B) irradiance over the 9 d period. H = high, M = medium, C = control temperatures

Quantification of ITS2 copies

At 12:00 h on Days 1 and 9 of the experiment, 1 tentacle per anemone was preserved in 95% ethanol. DNA was extracted from all of the individuals, the ITS2 region of the ribosomal DNA was amplified, and the denaturing gradient gel electrophoresis profiles of amplicons were compared to those described in Pontasch et al. (2014). Furthermore, ITS2 amplicons obtained from one *E. quadricolor* individual were ligated with pCR®4-TOPO® TA vector and 10 clones were sequenced following Pontasch et al. (2014) (Genbank accession numbers KF982278 to KF-982286). Plasmid DNA concentrations were generated by 10-fold serial dilutions. The absolute copy number of the ITS2 sequences within C3.25 plasmid DNA standards ranged from 2.39×10^3 to 2.39×10^9 copies μl^{-1} and the ITS2 copy numbers in C25 plasmid DNA standards ranged from 2.55×10^4 to 2.55×10^9 copies μl^{-1}, as calculated according to the PCR-guide available from Qiagen (www.qiagen.com). Primers amplifying a 120 bp-long ITS2 fragment specific for C25 and C3.25 were designed for quantitative PCR (Table 1), which was run on a Step One Real-time PCR system thermal cycler (AB Applied Bioscience) using SYBR® Green Real-Time PCR Master Mix (Invitrogen) containing 10 µl Master Mix, 0.5 µl primer (10 mM) forward, 0.5 µl primer (10 mM) reverse, 1 µl sample DNA and 8 µl water in a total reaction volume of 20 µl. Thermal cycling conditions were specified as: initial start at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and annealing temperature (T_a) (ESM 1) for 60 s. The threshold cycle (C_T) was set by default to 0.35, and kept at this level throughout the standard and experimental runs. The efficiency of amplification was 93.7% for C3.25 and 90.5% for C25, and the correlation coefficient (R^2) was 0.997 for both ITS2 types. The potential of cross-amplification (i.e. the amplification of C3.25 ITS2 amplicons using C25 primer-pair and vice versa) was tested across the whole range of plasmid DNA dilutions. Here, the C3.25

Table 1. Sequence and properties of primer pairs specific for the amplification of C25 and C3.25. T_a = annealing and extension temperature

ITS2 type	Primer	Primer sequence	T_a (°C)
C25	ITS2-C25-FW	5′-TCA ATG GCC TCC TGA ACG TTC-3′	67
	ITS2-C25-REV	5′-GCA ATG ACT CAT AAG AGC GC-3′	67
C3.25	ITS2-C3-FW	5′-CCA ATG GCC TCC TGA ACG TGC-3′	68
	ITS2-C3-REV	5′-GGG CAA TAG CTC ATA AGA ACG C-3′	68

primer-pair amplified <0.04% of C25 copies and the C25 primer-pair amplified <0.001% of C3.25 copies. Moreover, the specificity of each primer-pair was tested over a range of C25:C3.25 dilutions (0:1, 1:3, 1:1, 3:1, 1:0). The ratio of ITS2 assemblages was calculated by dividing the C25 copy number by the C3.25 copy number (C25:C3.25).

Chlorophyll fluorescence

Chlorophyll fluorescence was measured using a Diving Pulse Amplitude Modulated (PAM) fluorometer (Walz). Maximum (F_v/F_m) and effective ($\Delta F/F_m'$) quantum yield were measured daily. F_v/F_m was measured before transfer of the anemones from the holding tanks to the treatment tanks, and then daily at 19:00 h after natural dark acclimation (>80 min after sunset). $\Delta F/F_m'$ was measured daily at 12:00 h. The PAM settings were as follows: measuring intensity 3 (<0.15 µmol photons m^{-2} s^{-1}); saturation intensity 12 (>4500 µmol photons m^{-2} s^{-1}); saturation width 0.8 s; and gain 1. The light pressure over PSII (Q_m) (Iglesias-Prieto et al. 2004) was calculated using the equation: $Q_m = 1 - [(\Delta F/F_m') / (\text{initial } F_v/F_m \text{ on Day 1})]$.

Sampling and sample processing

At 12:00 h on Days 1, 3, 5, and 9, 3 tentacles per anemone were snap-frozen in liquid nitrogen, and then processed while on ice. Frozen tentacles were homogenized in 6 ml of 75 mM sodium phosphate buffer, pH 7.4 for 10 s using an Ultra-Turrax homogenizer (IKA). Homogenates were centrifuged at 4500× *g* for 10 min to separate the algal symbionts from the host. Pelleted algal cells were re-suspended in 6 ml sodium phosphate buffer and the host supernatant was centrifuged 2 more times at 4500× *g* for 5 min to remove any remaining symbiont cells. The anemone host and *Symbiodinium* cell fractions were each split into aliquots of 1 ml and sonicated for 5 min in a chilled sonicating bath, snap-frozen in liquid nitrogen and kept at −20°C for the remainder of the experiment, and then stored at −80°C until analysis. To increase the SOD detection signal in the *Symbiodinium* cell fraction, one 1 ml aliquot of the algal suspension was centrifuged at 7000× *g* for 5 min, the supernatant discarded and the pellet re-suspended in 300 µl sodium phosphate buffer; it was then sonicated and processed as described above.

SOD assay

The activities of SOD in anemone host (SOD_A) and *Symbiodinium* fractions (SOD_S) were measured using the riboflavin/nitroblue tetrazolium (RF/NBT) assay (Beauchamp & Fridovich 1971) as performed in Krueger et al. (2014). Standards were prepared from bovine SOD and samples were diluted in 75 mM potassium phosphate buffer, pH 7.4. The assay was conducted in 96-well microtiter plates using 20 µl cell lysate or SOD standard in a final volume of 300 µl potassium phosphate buffer (50 mM, pH 7.8) containing EDTA (0.1 mM), riboflavin (1.3 µM), L-methionine (10 mM), nitroblue tetrazolium chloride (57 µM) and 0.025% (v/v) triton X100. The method is based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium chloride (NBT) by O_2^- generated by photooxidized riboflavin. The reduction of NBT was monitored spectrophotometrically at 560 nm at 25°C. A measurement was taken at the start, and after 10 min incubation at a light intensity of 130 µmol photons m^{-2} s^{-1}. At this light intensity, a blank absorbance reading (maximal absorbance when there is maximal reduction of NBT) of approximately 0.5 allowed for a sufficient resolution for the standard and experimental measurements. One unit of SOD was defined as the amount of SOD inhibiting 50% of the reduction and was determined by comparison to a sigmoidal 5-parameter semi-logarithmic standard curve. The activity of SOD in samples is expressed per mg host or *Symbiodinium* soluble protein, as quantified using the technique of Bradford (1976).

Statistical analysis

Repeated measures analysis of variance (rm-ANOVA) and post hoc pairwise comparison with Bonferroni adjustment were used to analyse the effects of temperature. If necessary, data were arcsine or log

transformed to meet assumptions of normality, which were ascertained by the Kolmogorov-Smirnov test. Data were evaluated for assumptions of sphericity using Mauchly's test and, if violated, the Greenhouse-Geisser correction was applied. Univariate ANOVA was used to analyse photophysiological parameters between phenotypes at the beginning of the experiment. Because symbiont ratio data did not meet assumptions of normality, they were analysed using a non-parametric Friedman test (the non-parametric alternative to rmANOVA) with post hoc Wilcoxon rank comparisons to examine the null hypothesis that symbiont ratios were the same across days at a particular temperature, while the non-parametric Mann-Whitney *U* test was used to test the null hypothesis that symbiont ratios were equal across phenotypes at the beginning of the experiment.

To test whether F_v/F_m, Q_m and SOD activity in *Symbiodinium* and anemone host are a function of *Symbiodinium*-type specific differences, we correlated symbiont ratio with F_v/F_m, Q_m, SOD_A and SOD_S, on Day 1 (independent of temperature) or on Day 9 (under thermal treatment), using independent bivariate analysis (Pearson correlation) of log-transformed parameters. A discriminant function analysis (DFA) was applied to test if the constitutive set of log-transformed parameters (F_v/F_m, SOD_A, SOD_S on Day 1; Q_m on Day 2) could predict *Symbiodinium* ratio or anemone colour phenotype. For this analysis, symbiont ratios were ranked from 1 to 4, coding symbiont ratios (C25:C3.25) of <5, 5.01–10, 10.01–15 and >15, respectively. In 2 separate analyses, ranked symbiont ratio or colour phenotype were used as the grouping variables, and F_v/F_m, Q_m, SOD_A and SOD_S were used as independent variables. DFA was also used to determine whether the set of parameters allowed for a discrimination between the 3 temperature treatments within colour phenotypes. For this analysis, values at Day 1 for each of the parameters (except values at Day 2 for Q_m) were subtracted from those at Day 9 (except Day 8 for F_v/F_m). DFA was run separately for both phenotypes using temperature as the grouping variable and F_v/F_m, Q_m, SOD_A and SOD_S as independent variables. The identification of the most important variables that predicted a dimension was based on significant differences of group means and structure matrix. Because DFA is very sensitive to outliers, data were checked for univariate outliers visually by scatter plots and mathematically by conversion to standard *Z*-scores. Two outliers (defined as those cases with a standard *Z*-score ± 3.0; Shiffler 1988) were identified and were removed and replaced with the adjacent values of the remaining data. The highest/lowest data point at the opposite end of the ranked data was also replaced with the adjacent value, thereby computing a Winsorized mean (Barnett & Lewis 1994). This method is considered a robust estimation method for univariate distributions (Osborne & Overbay 2004). Data were also checked for multivariate outliers using the Mahalanobis D^2 test, and no multivariate outliers were identified based on Mahalanobis $D^2 \leq 0.001$ (Hadi 1992). Data were analysed using the IBM SPSS statistics 20.0 software.

RESULTS

Visual observations of symbiont expulsion

At noon on Day 3, all anemones at 27.6°C expelled mucus masses containing *Symbiodinium* cells through their mouths (Fig. 1C,D). By Day 5, 3 of 6 pink individuals and 1 of 6 green individuals appeared pale at 27.6°C. By Day 6 all anemones in this treatment had paled. Symbiont expulsion was also sporadically observed in both phenotypes at 24.5°C (4.5 to 5.5°C above ambient temperature) but not in the control during the observation period.

Symbiodinium ITS2 shuffling

All anemones used in the study simultaneously hosted a mixed ITS2 assemblage of *Symbiodinium* C25 and C3.25. At Day 1, the ratio of ITS2 types C25:C3.25 was higher in the green (8.6 ± 1.2, mean ± SE) than the pink phenotype (5.1 ± 0.6; Table 2, Fig. 3).The high baseline variation in symbiont assemblages within and between colour phenotypes was not reflected in the signatures of their baseline F_v/F_m (Day 1; Pearson Correlation [PC], r = 0.013, p = 0.938, n = 36) or baseline Q_m (Day 2; PC, r = –0.040, p = 0.816, n = 36). Over time, the C25:C3.25 ratio declined by 47.2% in the green phenotype at 27.6°C (Fig. 3A; Friedman test: $\chi^2_{(1)} = 5.6$, p = 0.018; Wilcoxon post hoc: $Z = -2.2$, p = 0.028) resulting in a symbiont ratio of 5.7 ± 1.3 on Day 9. Although this ratio was significantly lower than on Day 1, it was similar to the symbiont ratio in the pink phenotype under the same treatment (6.4 ± 0.8) and similar to the symbiont ratio in the green phenotype under the other temperature treatments (5.6 ± 0.6 at 21.3°C and 4.6 ± 1.2 at 24.5°C). In the pink phenotype, the symbiont ratio was stable over time in all temperature treatments (Fig. 3B). As at the start of the experiment,

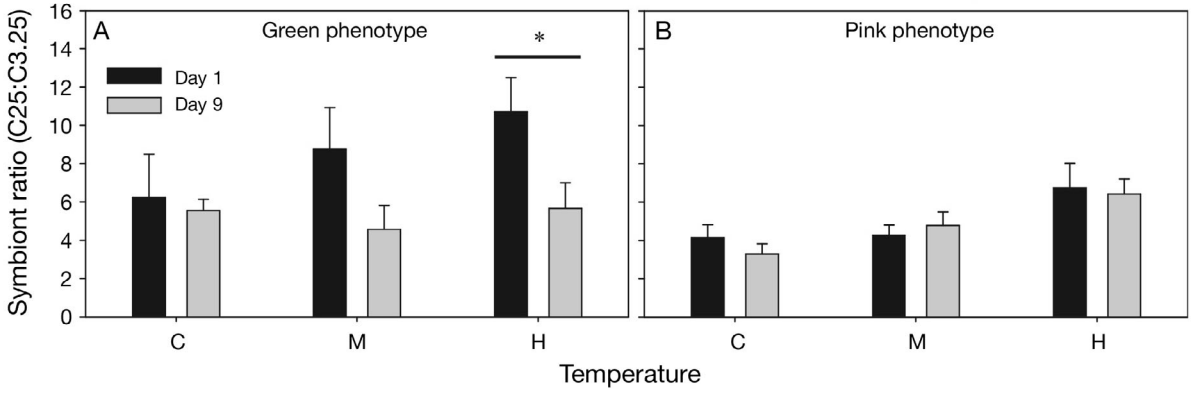

Fig. 3. Change in *Symbiodinium* C25:C3.25 ratio between Days 1 and 9, when (A) green and (B) pink phenotypes of *Entacmaea quadricolor* were exposed to control (C; 21.3°C), medium (M; 24.5°C) and high (H; 27.6°C) temperatures for 9 d. Asterisk indicates significant difference at the level of $p \leq 0.05$ (Friedman test with post hoc Wilcoxon rank comparison); results presented as mean ± SE (n = 6)

Table 2. Results of independent sample *t*-tests analyzing the similarity of parameters between green and pink phenotypes of *Entacmaea quadricolor* on Day 1 (Day 2 for Q_m). Parameters are: symbiont ratio; F_v/F_m (maximum quantum yield at 19:00 h); Q_m (excitation pressure over PSII); superoxide dismutase activity in the anemone host (SOD_A) and in *Symbiodinium* (SOD_S). Significant differences (2-tailed; $p \leq 0.05$) are highlighted in **bold**

Parameter	*t*	df	p	
Symbiont ratio	−2.6	34	**0.013**	**Green > Pink**
F_v/F_m	2.7	20	**0.015**	**Green > Pink**
Q_m	0.1	34	0.911	
SOD_A	1.6	34	0.110	
SOD_S	0.6	34	0.568	

the various symbiont assemblages identified after thermal treatment had no impact on the signatures of F_v/F_m (PC, r = 0.033, p = 0.849, n = 36) or Q_m (PC, r = 0.082, p = 0.633, n = 36).

Chlorophyll fluorescence

F_v/F_m

The initial F_v/F_m value was higher in the green than the pink phenotype (Table 2). In both colour phenotypes, F_v/F_m differed among temperature treatments (significant time × temp interaction; see Tables 3 & 4). At 21.3°C, F_v/F_m was stable in both phenotypes throughout the experiment (Fig. 4A,B). At 24.5°C, F_v/F_m declined from Day 3 onwards in the green phenotype (Bonferroni: $p \leq 0.048$ for Days 3 to 8 vs. Day 1) resulting in an overall decline of 22.3% by Day 8. In contrast, it was stable in the pink phenotype. In both phenotypes, F_v/F_m declined markedly at 27.6°C, with reductions of 27 and 23% in the green and pink phenotypes, respectively. At this temperature, the green phenotypes had significantly lower F_v/F_m values from Day 5 onwards ($p \leq 0.017$ for Days 5 to 8 vs. Day 1), while the pink phenotype had significantly lower F_v/F_m values from Day 4 onwards ($p \leq 0.007$ for Days 4 to 8 vs. Day 1).

When compared to 21.3°C, exposure to 24.5°C resulted in a lower F_v/F_m value on Day 5 in the green phenotype (Bonferroni: p = 0.007), whereas the F_v/F_m value in the pink phenotype did not differ at any time point. Furthermore, compared to 21.3°C, exposure to 27.6°C resulted in a lower F_v/F_m value on all days except for Day 3 in the green phenotype ($p \leq 0.047$ for Days 1, 2, 4 and p = 0.007 from Day 5 onwards) and a lower F_v/F_m value from Day 5 onwards in the pink phenotype ($p \leq 0.004$ for Days 5 to 8).

Q_m

The initial Q_m value was similar between colour phenotypes (Table 2). In both phenotypes, Q_m differed significantly between temperature treatments (significant time × temp interaction; see Tables 3 & 4). Over time, at 21.3°C, Q_m showed no significant variation in either phenotype (Fig. 4C,D). By Day 9 at 24.5°C, Q_m had increased ~1.8 fold in the green (Bonferroni: p = 0.016), and ~1.6 fold in the pink phenotype (p = 0.060). By Day 9 at 27.6°C, Q_m had increased ~1.4 fold (p = 0.045) in the pink phenotype and to a similar extent in the green phenotype, though this increase was not significant.

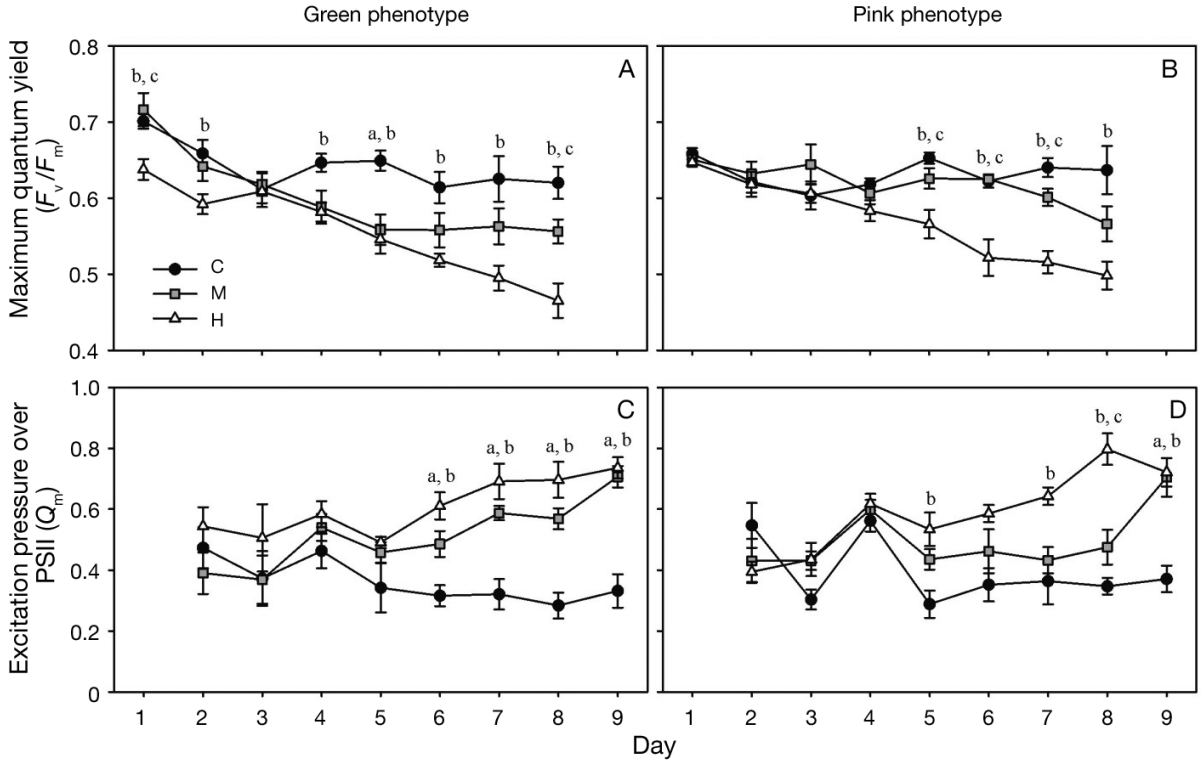

Fig. 4. Effect of temperature on (A,B) maximum quantum yield of PSII (F_v/F_m) and (C,D) excitation pressure over PSII (Q_m) in the (A,C) green and (B,D) pink phenotypes of *Entacmaea quadricolor* exposed to control (C; 21.3°C), medium (M; 24.5°C) and high (H; 27.6°C) temperature for 9 d. Significant differences (indicated by lowercase letters) are reported for (a) C vs. M, (b) C vs. H, and (c) M vs. H at the level of $p \leq 0.05$ (repeated-measures ANOVA and pairwise comparison with Bonferroni correction); results are presented as mean ± SE (n = 6)

In comparison to 21.3°C, exposure to 24.5°C resulted in a significantly higher Q_m value from Day 6 onwards in the green phenotype (Days 6, 7: $p < 0.015$; Days 8, 9: $p \leq 0.001$), while in the pink phenotype Q_m at 24.5°C was significantly higher only on the last day of the experiment ($p = 0.002$). When exposed to 27.6°C, Q_m was higher from Day 6 onwards in the green (Days 6 to 9: $p \leq 0.002$) and from Day 5 onwards in the pink phenotype (Day 5: $p = 0.008$, Days 7 to 9: $p < 0.009$).

SOD

SOD_A and SOD_S activities were similar between phenotypes, with the latter being higher in both phenotypes (Mann-Whitney *U* test: $p < 0.001$ for both comparisons, Table 2, Fig. 5A,B). Neither SOD_A nor SOD_S were correlated with symbiont assemblage (Day 1; PC, SOD_A: $r = 0.201$, $p = 0.240$, $n = 36$, SOD_S: $r = 0.099$, $p = 0.564$, $n = 36$).

SOD_A activities over time were similar among temperature treatments (no significant time × temp × phenotype interaction; Table 3). However, when temperatures were pooled, clear differences between the 2 phenotypes were found (significant effect of phenotype; Table 3). This effect was driven by the green phenotype, which showed temperature independent changes in SOD_A activity over time (Table 4, Fig. 5A). Here, SOD_A activity was higher on Day 5 than Day 3 (Bonferroni: $p = 0.002$), and higher on Day 9 than Days 1 and 3 ($p = 0.036$ and $p = 0.002$, respectively). In contrast, the pink phenotype showed no changes in SOD_A activity over time (Table 4, Fig. 5B). Furthermore, SOD_A activity over Days 1 to 9 was significantly lower in the host tissue of the green phenotype (14.2 ± 2.51 units mg^{-1}) than the pink phenotype (20.9 ± 3.57 units mg^{-1}; Table 3). Under temperature treatment, SOD_A was not correlated with symbiont assemblage (Day 9; PC, SOD_A: $r = -0.057$, $p = 0.741$, $n = 36$).

SOD_S activity was similar among temperature treatments in both phenotypes (no significant time × temp × phenotype interaction; Fig. 5C,D, Table 3). However, when temperatures were pooled, the overall response over time was different between pheno-

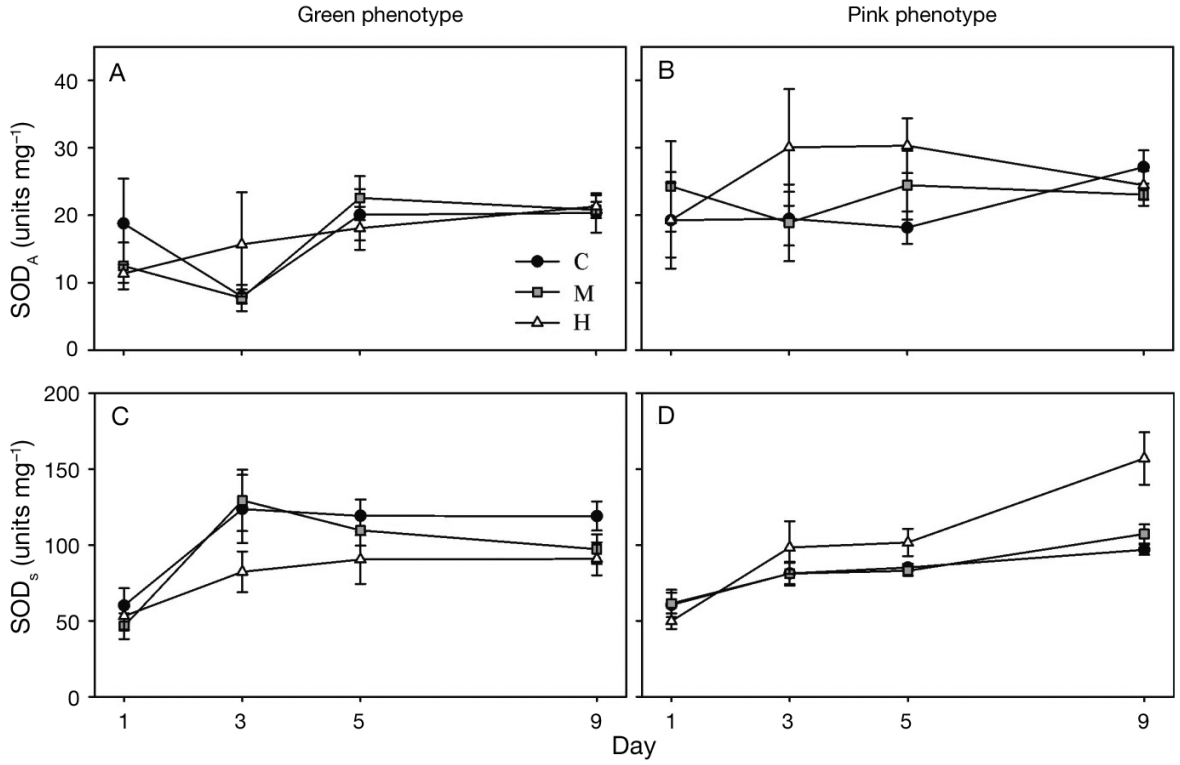

Fig. 5. Effect of temperature over a 9-d period on (A,B) superoxide dismutase activity in the host (SOD_A) and (C,D) *Symbiodinium* fractions (SOD_S) of *Entacmaea quadricolor* phenotypes exposed to control (C; 21.3°C), medium (M; 24.5°C) and high (H; 27.6°C) temperature. Results are presented as mean ± SE (n = 6)

Table 3. Results of repeated-measures ANOVA for the parameters: F_v/F_m (maximum quantum yield at 19:00 h), Q_m (excitation pressure over PSII), and superoxide dismutase activity in the anemone host (SOD_A) and in *Symbiodinium* (SOD_S) when both anemone colour phenotypes are included in the analysis. The Greenhaus-Geisser correction is presented for all parameters. Significant effects ($p \leq 0.05$) are highlighted in **bold**. Temp = Temperature

	F_v/F_m			Q_m			SOD_A			SOD_S		
	df	*F*	p	df	*F*	p	df	*F*	p	df	*F*	p
Time	5.1, 154.2	26.1	**<0.001**	4.9, 148	673.3	**<0.001**	2.1, 64	9.1	**<0.001**	1.8, 54.1	16.1	**<0.001**
Time × Temp	10.3, 154.2	4.8	**<0.001**	9.9, 148	5.1	**<0.001**	4.3, 64	0.8	0.533	3.6, 54.1	1.0	0.781
Time × Phenotype	5.2, 154.2	2.6	**0.029**	4.9, 148	0.4	0.857	2.1, 64	3.0	0.055	1.8, 54.1	4.1	**0.026**
Time × Temp × Phenotype	10.3, 154.2	1.1	0.362	9.9, 148	0.8	0.626	4.3, 64	4.3	0.626	3.6, 54.1	1.0	0.407
Temp	2, 30	56.3	**<0.001**	2, 30	38.8	**<0.001**	2, 30	0.9	0.401	2, 30	0.3	0.734
Phenotype	1, 30	1.7	0.205	1, 30	0.1	0.799	1, 30	25.2	**<0.001**	1, 30	1.4	0.241
Temp × Phenotype	2, 30	2.3	0.120	2, 30	0.7	0.484	2, 30	0.7	0.524	2, 30	1.8	0.186

types (significant time × phenotype interaction; Table 3). Here, the green phenotype significantly increased its SOD_S activity by Day 3 (Bonferroni: p = 0.021) and maintained higher SOD activities for the remainder of the experiment (Bonferroni: $p < 0.001$ for both comparisons, Days 5 and 9 vs. 1). In the green phenotype, there was a similar 1.7- to 2.0-fold increment in SOD activity at all temperatures by Day 9 (Fig. 5C). Symbionts in the pink phenotype significantly increased their SOD activity by Day 5 (Bonferroni: $p < 0.001$), and by Day 9 SOD activity was higher than on Day 1 ($p < 0.001$) and Day 5 (p = 0.034). By Day 9, there was a 1.6- to 1.7-fold increment in SOD activity at 21.3 and 24.5°C, and a

Table 4. Results of repeated-measures ANOVA for the parameters: F_v/F_m (maximum quantum yield at 19:00 h), Q_m (excitation pressure over PSII), and superoxide dismutase activity in the anemone host (SOD_A) and in *Symbiodinium* (SOD_S) when anemone colour phenotypes are analysed separately. Significant effects ($p \leq 0.05$) are highlighted in **bold**. Temp = Temperature

Parameter	F_v/F_m			Q_m			SOD_A			SOD_S		
	df	*F*	p	df	*F*	p	df	*F*	p	df	*F*	p
Green phenotype												
Time	7, 9	22.6	**<0.001**	3.6, 54.4[a]	283.4[a]	**<0.001**[a]	2, 30.1[a]	10.1[a]	**<0.001**[a]	1.5, 23[a]	8.3[a]	**0.004**[a]
Temp	2, 15	26.4	**<0.001**	2, 15	22.8	**<0.001**	2, 15	0.1	0.925	2, 15	0.4	0.657
Time × Temp	14, 18	3.9	**0.004**	7.2, 54.4[a]	2.5[a]	**0.024**[a]	4, 30.1[a]	0.7[a]	0.623[a]	3.1, 23[a]	0.3[a]	0.847[a]
Pink phenotype												
Time	3.5, 52.5[a]	7.9[a]	**<0.001**[a]	4.2, 62.4[a]	414.0[a]	**<0.001**[a]	3, 13	2.3	0.122	3, 13	19.3	**<0.001**
Temp	2, 15	35.9	**<0.001**	2, 15	16.2	**<0.001**	2, 15	1.4	0.274	2, 15	2.3	0.133
Time × Temp	7, 52.5[a]	3.6[a]	**0.003**[a]	8.3, 62.4[a]	3.6[a]	**0.002**[a]	6, 26	1.1	0.395	6, 26	1.8	0.134

[a]Greenhaus-Geisser correction is presented

3.2-fold increment at 27.6°C in this colour phenotype (Fig. 5D).

On Day 9, SOD_S activity correlated with symbiont assemblage (PC, r = 0.357, p = 0.032, n = 36). The significant positive correlation translated to higher SOD activities across all temperature treatments in *Symbiodinium* consortia with higher proportions of C25.

Predictors of thermal response

The set of photophysiological parameters (F_v/F_m, Q_m) and SOD parameters (SOD_A, SOD_S) during baseline conditions were not sufficiently distinct between *Symbiodinium* ITS2 ratios to reliably discriminate between them (DFA: Wilks' lambda = 0.692, $\chi^2_{(12)}$ = 11.4, p = 0.495). In contrast, based on the same parameters, DFA reliably predicted colour phenotype (Wilks' lambda = 0.741, $\chi^2_{(4)}$ = 9.6, p = 0.048).

In both phenotypes, the response of the suite of parameters measured reliably differed between temperature treatments and could be reduced to 2 dimensions (Functions 1 and 2; Fig. 6). However, not all of the most important variables explaining the discrimination were identical between phenotypes. In the green phenotype, Discriminant Functions (DF) 1 and 2 explained 94 and 6% of the variation, respectively (Wilks' lambda = 0.182, $\chi^2_{(8)}$ = 23.0, p = 0.003; Fig. 6A). Q_m was the most important predictor for DF 1, and Q_m, F_v/F_m and SOD_S contributed for the delineation of DF 2. In the pink phenotype, DF 1 and 2 explained 76 and 24% of the variation, respectively (Wilks' lambda = 0.230, $\chi^2_{(8)}$ = 19.8, p = 0.011; Fig. 6B). Here, F_v/F_m, Q_m and SOD_S defined DF 1, and Q_m and SOD_S defined DF 2.

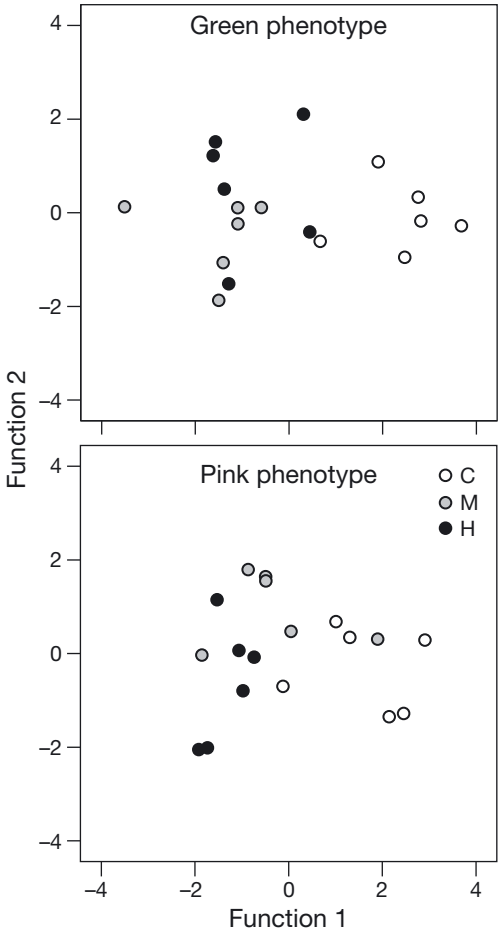

Fig. 6. Canonical discriminant function scatterplot showing the discrimination of temperature effects in the phenotypes of *Entacmaea quadricolor* based on the integration of the parameters F_v/F_m (maximum quantum yield), Q_m (excitation pressure over PSII), SOD_A (superoxide dismutase activity in the anemone host), and SOD_S (superoxide dismutase activity in *Symbiodinium*). C = control temperature (21.3°C); M = medium temperature (24.5°C); H = high temperature (27.6°C)

DISCUSSION

Our results revealed phenotype-specific responses to short-term heat stress with distinct critical thermal thresholds of PSII photochemical efficiency in 2 colour phenotypes of *Entacmaea quadricolor*, corresponding to <24.5°C in the green phenotype and >24.5°C in the pink phenotype. Hill & Scott (2012) showed that thermal bleaching (exposure to 25, 27 or 29°C) in a range of *E. quadricolor* phenotypes was accompanied by declining photosynthetic efficiency (i.e. F_v/F_m). In the present study, the highest temperature resulted in substantial declines in F_v/F_m in both phenotypes, whereas exposure to 24.5°C caused significant declines in F_v/F_m in the green but not the pink phenotype. Decreases in F_v/F_m may have resulted from sustained non-photochemical quenching (NPQ) mechanisms (Middlebrook et al. 2010), and/or inactivation of functional reaction centres which can be dynamic or sustained (Warner et al. 1999). Furthermore, at 24.5°C, Q_m was higher in the green phenotype from Day 6 onwards, while in the pink phenotype, Q_m only increased after 9 d of exposure. Higher Q_m values indicate that a higher proportion of reaction centres associated with PSII are closed (Iglesias-Prieto et al. 2004). The accumulation of non-functional PSII reaction centres might be associated with ROS, such as $^1O_2$, O_2^-, H_2O_2 and •OH, which can damage cellular components (Halliwell 2006) and/or inhibit protein translation, and as such decelerate PSII repair rates (Takahashi & Murata 2008). F_v/F_m and Q_m after 9 d of temperature treatment were not correlated with the composition of *Symbiodinium* ITS2 types or SOD_S activity (the first-in-line antioxidant enzyme which detoxifies O_2^- in *Symbiodinium*), suggesting that neither *Symbiodinium* type alone, nor the capacity to activate SOD, is the primary determinant for the thermal response of PSII.

Shuffling of *Symbiodinium* ITS2 composition towards a lower C25:C3.25 ratio was observed in the highest temperature treatment in the green but not the pink phenotype, showing that the *Symbiodinium* association within *E. quadricolor* can be highly flexible in response to changing environments, as has been demonstrated for scleractinian corals (Berkelmans & van Oppen 2006, Jones et al. 2008, LaJeunesse et al. 2009). However, this flexibility did not explain the observed variability in photochemical efficiency under thermal stress. In *Symbiodinium*, the activity of SOD increased in both host phenotypes regardless of temperature. A positive correlation between light and SOD activity is well described (Shick et al. 1995, Richier et al. 2008), and therefore suggests that the antioxidant defense was primarily induced by experimental light intensities which were higher than the acclimation light intensities. After 9 d of heat stress, *Symbiodinium* ratio correlated with SOD_S activity, with a higher SOD activity detectable in the presence of more *Symbiodinium* C25. While C25 apparently activated more SOD under thermal stress, differential degrees of O_2^- scavenging were not reflected in the signatures of F_v/F_m or Q_m. In anemone tissues, ROS are produced during aerobic pathways in mitochondria (Nii & Muscatine 1997). Furthermore, ROS, particularly H_2O_2 — the conversion product of SOD — are believed to penetrate cell membranes and walls, and may leak from *Symbiodinium* cells to host tissues (Lesser 2011). The green phenotype increased its SOD_A activity over time at all temperatures, suggesting that this phenotype experiences higher levels of oxidative stress. In contrast, the pink phenotype maintained a constant SOD_A activity over time at all temperatures, suggesting oxidative homeostasis, even though SOD_S activity significantly increased with time. These data highlight different SOD scavenging capacities amongst the hosts that are independent of the resident symbionts, and may reflect different inherent antioxidant capacities in response to thermal stress. The lower SOD_A activity in the pink morph could be due to the use of alternative O_2^- quenching molecules, such as fluorescent proteins (Bou-Abdallah et al. 2006), which were not measured in the present study. While both symbiont ratio and SOD activity were not correlated with photobiological properties under thermal stress, anemone colour phenotype was reliably predicted using these variables. Taken together, our findings present intraspecific variation in the thermal tolerance of *E. quadricolor* that is independent of the resident symbionts and highlights the importance of the host in influencing the thermal response, and consequently the long-term health and survivorship of the symbiosis in response to increasing seawater temperature.

Baseline *Symbiodinium* ITS2 composition and F_v/F_m signatures also differed markedly in the 2 distinct colour phenotypes (that were notably collected from the same depth and acclimated to the same light regime for 32 d). Compared to the pink phenotype, the green phenotype had a higher ratio of *Symbiodinium* C25:C3.25 and higher F_v/F_m values. *Symbiodinium* type-specific differences in photobiology have been demonstrated repeatedly (e.g. Hennige et al. 2009, Krämer et al. 2012). However, in the present study, baseline F_v/F_m values were not correlated with symbiont composition, suggesting that distinct PSII

photochemical efficiencies resulted from acclimation to different internal environments created by the host. Higher F_v/F_m values in the green phenotype indicate acclimation to lower light, to increase light harvesting and optimise photosynthesis (Hennige et al. 2008b). Internal light intensity and quality may be altered by factors including tissue thickness (Loya et al. 2001), symbiont concentration (Stimson et al. 2002), behavioural changes in tissue contraction (Brown et al. 2002) and optical properties of the host, where mycosporine-like amino acids (MAAs) absorb high-energy UV light (Shick et al. 1995), and incorporated fluorescent or non-fluorescent chromophore proteins absorb PAR or UV (Salih et al. 2000, Dove et al. 2001). Recently, Roth & Deheyn (2013) showed that F_v/F_m positively correlates with green fluorescent protein (GFP) concentrations in healthy individuals of the reef-building coral *Acropora yongei*. In *E. quadricolor*, a far-red fluorescent protein (eqFP611) has been identified (Wiedenmann et al. 2002), but it remains to be shown how eqFP611 influences the photobiology of resident *Symbiodinium* cells under optimal and bleaching conditions. The distinct colouration of tentacle tips and body column is probably due to chromophore proteins with different reflectance and absorption properties. Green chromophores reflect shorter wavelengths of the high-energy blue–green light, suggesting that resident symbionts experienced lower-energy yellow–red light. In contrast, pink chromophores reflect red light, suggesting that resident symbionts would have experienced a blue light spectral environment. Fitt & Warner (1995) demonstrated that UV-A and blue-enhanced light resulted in lower maximum quantum yields in the reef-building coral *Montastraea annularis* under bleaching conditions. Furthermore, more recent studies have demonstrated that blue light can promote coral and *Symbiodinium* growth and photosynthetic rates (Mass et al. 2010, Wijgerde et al. 2014). The exact role of differential pigmentation in regulating *Symbiodinium* photobiology remains to be investigated in *E. quadricolor*.

The findings of this study show, for the first time, intraspecific variation in thermal tolerance within *E. quadricolor*. Expected temperature increases (Hobday & Lough 2011) render the green phenotype more vulnerable than the pink phenotype and might, in the long term, alter the community structure of *E. quadricolor* at North Solitary Island. The presence of a mixed *Symbiodinium* assemblage in both phenotypes does not seem to provide an advantage when temperatures change, because shuffling of the symbiont ITS2 composition did not enhance thermal tolerance during the time frame of our experiment. Long-term studies are needed to verify this hypothesis. Our results suggest that thermal tolerance in *E. quadricolor* phenotypes is regulated by distinct spectral properties of the host that regulate the internal light environment, as has been shown for scleractinian corals (Dove et al. 2006, Hennige et al. 2008a). Although anemone survivorship was not affected in this study, distinct thermal thresholds of PSII photochemical efficiency might ultimately have profound impacts on holobiont survivorship and phenotype abundance if periods of elevated temperature occur more frequently in the future.

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