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Rapid thermal adaptation in photosymbionts of reef-building corals

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Abstract

Climate warming is occurring at a rate not experienced by life on Earth for 10 s of millions of years, and it is unknown whether the coral-dinoflagellate (*Symbiodinium* spp.) symbiosis can evolve fast enough to ensure coral reef persistence. Coral thermal tolerance is partly dependent on the *Symbiodinium* hosted. Therefore, directed laboratory evolution in *Symbiodinium* has been proposed as a strategy to enhance coral holobiont thermal tolerance. Using a reciprocal transplant design, we show that the upper temperature tolerance and temperature tolerance range of *Symbiodinium* C1 increased after ~80 asexual generations (2.5 years) of laboratory thermal selection. Relative to wild-type cells, selected cells showed superior photophysiological performance and growth rate at 31°C in vitro, and performed no worse at 27°C; they also had lower levels of extracellular reactive oxygen species (exROS). In contrast, wild-type cells were unable to photosynthesise or grow at 31°C and produced up to 17 times more exROS. In symbiosis, the increased thermal tolerance acquired ex hospite was less apparent. In recruits of two of three species tested, those harbouring selected cells showed no difference in growth between the 27 and 31°C treatments, and a trend of positive growth at both temperatures. Recruits that were inoculated with wild-type cells, however, showed a significant difference in growth rates between the 27 and 31°C treatments, with a negative growth trend at 31°C. There were no significant differences in the rate and severity of bleaching in coral recruits harbouring wild-type or selected cells. Our findings highlight the need for additional *Symbiodinium* genotypes to be tested with this assisted evolution approach. Deciphering the genetic basis of enhanced thermal tolerance in *Symbiodinium* and the cause behind its limited transference to the coral holobiont in this genotype of *Symbiodinium* C1 are important next steps for developing methods that aim to increase coral bleaching tolerance.

KEYWORDS

Acropora cytherea, *Acropora hyacinthus*, *Acropora sarmentosa*, assisted evolution, coral bleaching, experimental evolution, Great Barrier Reef, *Symbiodinium*, thermal stress

1 | INTRODUCTION

Climate warming is occurring at an unprecedented rate as a result of increasing atmospheric CO_2 and other greenhouse gasses (Hönisch et al., 2012; Zeebe, Dickens, Ridgwell, Sluijs, & Thomas, 2014) and has negatively affected terrestrial and marine ecosystems (Parmesan, 2006). On coral reefs, rising seawater temperatures cause the breakdown of the critical association between the coral animal and its intracellular photosymbionts, *Symbiodinium* spp., leading to bleaching and often coral mortality. Extensive bleaching-related loss of coral

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cover has occurred over the past 3–4 decades with the worst mass bleaching event on record having occurred most recently during the 2015–2016 El Niño temperature anomaly (Eakin et al., 2016; Hughes et al., 2017; Normile, 2016) where over 60% of reefs surveyed on the Great Barrier Reef (GBR) experienced extreme bleaching (Hughes et al., 2017). The frequency and severity of such heating events are anticipated to increase in future with climate models predicting up to 99% of the world's coral reefs to experience severe annual bleaching before 2,100 (van Hooidonk et al., 2016).

Increases in temperature can affect *Symbiodinium* and the coral host in a number of ways. The photosynthetic machinery of *Symbiodinium* is sensitive to even moderate temperature increases, causing photoinhibition and photodamage (Warner, Fitt, & Schmidt, 1999) of photosystem II (PSII; Iglesias-Prieto, Matta, Robins, & Trench, 1992) and a disruption of the structural integrity of the chloroplast thylakoid membranes (Tchernov et al., 2004). Such changes limit the fixation of CO_2 (Murata, Takahashi, Nishiyama, & Allakhverdiev, 2007), thus affecting *Symbiodinium* cell growth. The transfer of photosynthate from the symbionts to the coral tissues accounts for up to 95% of the coral's total energy requirements (Bythell, 1988; Falkowski, Dubinsky, Muscatine, & McCloskey, 1993; Yellowlees, Rees, & Leggat, 2008) and a reduction in the amount of photosynthetically fixed carbon produced and translocated from symbiont to host can lead to reduced coral growth (Cantin, van Oppen, Willis, Mieog, & Negri, 2009; Goreau & Macfarlane, 1990) and tissue loss (Szmant & Gassman, 1990). Furthermore, under heat stress, an excess production of reactive oxygen species (ROS) in the chloroplast can cause damage to DNA, proteins and lipids and in turn ROS can cause further detriment to photosynthetic activity by inhibiting PSII repair mechanisms (Murata et al., 2007) and damaging photosynthetic membranes (Lesser, 2006). Importantly, leakage of ROS out of the cell (extracellular ROS or exROS) by *Symbiodinium*, when produced in excess, has been linked to coral bleaching by stimulating the expulsion or digestion of *Symbiodinium* by host cells, or the removal of *Symbiodinium* by apoptotic host cell death (Weis, 2008).

Many coral species live near their upper thermal limits, and it is unlikely that physiological plasticity will enable them to mount a response great enough to cope with further environmental change (Hoegh-Guldberg et al., 2007). When physiological limits are exceeded, survival may depend on genetic adaptation through natural selection (Hoffmann & Sgrò, 2011). However, the generally long sexual generation times of corals (2–20 years) in the face of rapid ocean warming, renders coral species unlikely to undergo significant adaptation in time to persist into the future (Hughes et al., 2003). Coral thermal tolerance is partly dependent on the genetic variant(s) of *Symbiodinium* that they host (Baker, 2003; Baker, Starger, McClanahan, & Glynn, 2004; Berkelmans & van Oppen, 2006; Howells et al., 2012; Mieog et al., 2009; Sampayo, Ridgway, Bongaerts, & Hoegh-Guldberg, 2008). A fast rate of asexual reproduction (3–74 days; Wilkerson, Kobayashi, & Muscatine, 1988) in *Symbiodinium*, in combination with their large population sizes (~10^{10} cells in a branching coral ~30 cm diameter in size; van Oppen, Souter, Howells, Heyward, & Berkelmans, 2011), has the potential to give rise to many spontaneous, random mutations over a short period of time, a small number of which may be beneficial to the changed conditions experienced due to climate change. These properties suggest a high adaptive potential exists in *Symbiodinium*. However, in hospite, host factors may actively retard the rate of asexual division in *Symbiodinium* where the host can regulate growth through carbon allocation (Stat, Morris, & Gates, 2008) and the active digestion or removal of *Symbiodinium* cells (Titlyanov et al., 1996). Such factors could slow *Symbiodinium* generation times and thus rates of evolution in hospite. This has led to the proposal of in vitro laboratory evolution in *Symbiodinium* with subsequent inoculation of the coral host, to be used as a potential conservation or restoration tool in assisting corals in coping with ongoing ocean warming (van Oppen, Oliver, Putnam, & Gates, 2015; van Oppen et al., 2017).

Despite the potentially high adaptive capacity of *Symbiodinium*, most warming perturbation studies on these coral photosymbionts have been carried out over short timescales with treatment exposure lasting few generations. Although such experiments show that thermal acclimation, equating to temperature changes on a seasonal scale, is possible in *Symbiodinium* (Takahashi, Yoshioka-Nishimura, Nanba, & Badger, 2013), little is known about the longer term, evolutionary consequences of ongoing ocean warming. Only one study has examined the longer-term response of *Symbiodinium* to ocean warming, reporting that *Symbiodinium* strains CCMP 2429 and CCMP 2433 (isolated from *Heliofungia actiformis* and *Pocillopora damicornis*, respectively) from the GBR were able to grow at 30°C after 55–70 generations of temperature selection, where previously they could not (Huertas, Rouco, Lopez-Rodas, & Costas, 2011). Long-term, evolutionary experiments involving other microalgae have mainly looked at their response to elevated pCO_2 and not temperature (Collins & Bell, 2004, 2006; Flores-Moya et al., 2012; Jin, Gao, & Beardall, 2013; Lohbeck, Riebesell, & Reusch, 2012); but see Flores-Moya et al., 2012; Schlüter et al., 2014). Some of these experiments have provided evidence for the evolution of key fitness traits across multiple generations in the laboratory (Flores-Moya et al., 2012; Jin et al., 2013; Lohbeck et al., 2012), most commonly using reciprocal transplant experiments (Jin et al., 2013; Lohbeck et al., 2012; Schlüter et al., 2014) to infer adaptation.

To examine whether rapid thermal adaptation of *Symbiodinium* is possible, we conducted a laboratory selection experiment using a monoclonal culture of *Symbiodinium* (type C1) that is common in reef-building corals and widespread on coral reefs around the world (Fabina et al., 2012; Franklin, Stat, Pochon, Putnam, & Gates, 2012; Thornhill, Lewis, Wham, & LaJeunesse, 2014). In our study, *Symbiodinium* C1 was isolated from a colony of *Acropora tenuis* on the Great Barrier Reef (GBR) and 16 culture replicates, each representing an independent evolutionary unit accumulating independent mutations, underwent a ratchet experiment (Huertas et al., 2011) designed to select for increased temperature tolerance. After the ratchet experiment (lasting ~5 generations), the selected *Symbiodinium* cells were cultured for a further ~73 generations (approximately 2.5 years) at 31°C, while the ancestral monoclonal culture from which it was derived (the wild type [WT]) remained at 27°C for

TABLE 1 In vitro culture temperature conditions for the wild-type (WT) and selected *Symbiodinium* (SS) cells

Culture	Treatment	Temperature (°C)
Long term	WT@27	26.63 ± 0.001 (76,599)
	SS@31	31.15 ± 0.002 (77,730)
Experimental	WT@27	26.85 ± 0.005 (5,032)
	SS@27	
	SS@31	31.05 ± 0.002 (5,032)
	WT@31	

Pre-experimental temperature refers to the long-term culture history (210 weeks) of the WT and SS cells in control and elevated temperatures, respectively. Experimental phase temperatures are those experienced during the reciprocal transplant experiment (5 weeks) by both the WT and SS cells during 2 weeks of pre-acclimation and 3 weeks of experiment. Values are mean ± *SEM*. Number of replicate measurements are provided in parentheses.

the same period of time (Table 1). To determine whether the selected *Symbiodinium* (SS) had adapted rather than (reversibly) acclimated to 31°C, replicate cultures of the SS and WT were subjected to a reciprocal transplant experiment (i.e., WT@27, SS@27, WT@31 and SS@31), the most direct approach for conferring adaptive change (Jin et al., 2013; Lohbeck et al., 2012; Merilä & Hendry, 2014; Schlüter et al., 2014).

The mean fitness of the WT and SS was assessed based on a number of key traits. Six photophysiological traits were measured; the photochemical efficiency of PSII in the dark (F_v/F_m) and the light ($\Delta F_v/F'_m$; indicators of the maximum and effective quantum yield of PSII, respectively), the maximum excitation pressure over PSII (Q_m; Iglesias-Prieto, Beltrán, Lajeunesse, Reyes-Bonilla, & Thomé, 2004), the maximum electron transport rate (rETRm; an indicator of maximum photosynthetic capacity; Schreiber, 2004), the minimum saturating irradiance of photosynthesis (E_k; Henley, 1993) and maximum nonphotochemical quenching (NPQ_m; a photoprotective mechanism involving the dissipation of excess light energy as heat; Ralph, Polk, Moore, Orth, & Smith, 2002). Two additional traits, the specific growth rate and the amount of exROS *per* cell were also compared between WT and SS cells at ambient and elevated temperature.

To understand the impact of in vitro thermal selection of *Symbiodinium* on coral bleaching susceptibility, we inoculated aposymbiotic recruits of three GBR coral species, *Acropora cytherea*, *Acropora hyacinthus* and *Acropora sarmentosa*, with the WT and SS cells and subjected them to heat stress. For this experiment, we used the same environmental conditions that were used in the in vitro reciprocal transplant experiment. After inoculation with the WT and SS *Symbiodinium*, recruits were reared for 5 weeks at 27°C before being subjected to the different treatments for 28 days, during which their thermal tolerance was assessed based on growth (increase in recruit size) and the extent of bleaching (proportion of recruit surface that was pigmented).

Our study tests the evolutionary consequences of culturing *Symbiodinium* under thermal stress, adding to the important field of microalgal adaptation through experimental evolution. Furthermore, our results provide insights into the use of experimentally evolved *Symbiodinium* as a potential conservation or restoration tool that aims to increase coral thermal tolerance.

2 | MATERIALS AND METHODS

2.1 | *Symbiodinium* isolation

Symbiodinium cells were extracted from the coral *A. tenuis*, Nelly Bay, Magnetic Island, Australia (19°10′6″S, 146°50′60″E) in 2010. Extraction occurred by air-brushing the coral tissue from the coral skeleton, centrifuging (5 min, 1,600 *g*), decanting and resuspending the pellet three times in 0.2 μm filtered seawater. Extracted cells were transferred into sterile culture media, Daigo's IMK for Marine Microalgae (Nihon Pharmaceutical Co., Ltd) containing antibiotics (penicillin, neomycin, streptomycin, nystatin, final concentration [100 μg/ml] each, amphotericin final [2.5 μg/ml], plus GeO_2 final [50 μM]). Cells were inoculated into fresh IMK + antibiotics monthly, for 5 months to minimize bacterial contamination. This heterogeneous culture was previously used by Howells et al. (2011) and Levin et al. (2016). Subsequently, cells were plated onto IMK + antibiotics and 1% agar. A monoclonal culture was created by picking cells from a single colony forming unit. Monoclonal cultures were grown in IMK without antibiotics. The monoclonal strain was confirmed as belonging to the subclade C1 based on the ITS2 rDNA region (GenBank accession number AB778664.1) and named SCF055-1. Annual sequencing checks confirmed that the culture did not become contaminated during longer term laboratory culture.

2.2 | *Symbiodinium* thermal selection and long-term culture

The monoclonal culture was maintained at 27°C and 65 ± 10 μmol photons m^{-2} s^{-1} (Sylvania FHO24W/T5/865 fluorescent tubes) under a 14:10 light:dark cycle for ~6 months before undergoing a thermal selection experiment. Thermal selection was performed using a ratchet design (Huertas et al., 2011) lasting 2 months (~5 generations). The ratchet design is a method that maintains large population sizes by increasing the temperature in a stepwise fashion. With this approach, a population is only subjected to increased temperature (i.e., the next ratchet) when it shows positive growth. By maintaining population growth, this method maximizes the number of spontaneous mutations arising from asexual cell division. With increasing levels of thermal selection pressure, natural selection can then act upon beneficial new mutations. This design allows the selection of best performers (assumed to be mutants as genetically homogenous monoclonal cultures are used) at each temperature ratchet.

Replicates (n = 16) of the monoclonal culture were inoculated into fresh media at 300,000 cells/ml and placed at 26°C. High replication was used initially to increase the chances of a spontaneous, beneficial new mutation occurring in at least one population. Importantly, each "replicate" population was no longer considered a

replicate once the experiment commenced, but an independent population representing a different random chance for a beneficial mutation to arise (Huertas et al., 2011). After 30 days, the four populations displaying the highest growth at 28°C, or the greatest cell densities, were transferred to the next ratchet temperature (2°C higher than the previous) and split between four more vessels containing fresh media, at the same starting cell density, resulting in $n = 16$ populations at both 28 and 30°C, respectively. Those kept at the next ratchet temperature of 32°C, however, did not exhibit any net growth, and 30°C was considered their maximum adaptive capacity. The cells able to survive and grow at an elevated temperature are hereafter named the selected *Symbiodinium* (SS) and their counterpart cells, that remained in control temperatures, named the wild type (WT). Following the ratchet experiment, ten randomly selected WT populations were kept at 26.63 ± 0.001°C and ten of the fastest growing SS populations, resulting from the selection experiment, were kept at 31.16 ± 0.002°C and cultured separately for approximately 30 months (ca. 142 and 73 generations, respectively, see below for description on estimation of generation number). The ten WT populations were named SCF055-1-1 to SCF055-1-10, and ten SS populations were named SCF055-1-11 to SCF055-1-20. Temperature measurements were recorded every 10 min, using a data logger (HOBO Pendant®; Table 1). Monthly, each WT and SS population was subcultured into fresh culture media (IMK).

2.3 | Experiment 1: in vitro reciprocal transplant

2.3.1 | Experimental design

We chose the SS population that had the greatest cell density at 31°C (SCF055-1-18), 2 weeks after subculturing, and a randomly chosen WT population (SCF055-1-3). To test whether adaptation in the SS cells had arisen from long-term acclimation or from genetic selection on beneficial mutations arisen through cell division, the SS and WT cells then underwent a reciprocal transplant experiment. SS and WT cells were pre-acclimated for 2 weeks in their own (WT@27 and SS@31) or transplanted into reciprocal (WT@31 and SS@27) temperature conditions at a starting cell density of 200,000 cells/ml. The pre-acclimated cells were then transferred to culture vessels (25 cm^2, Corning®; Sigma-Aldrich) containing 10 ml of fresh media at a density of 200,000 cells/ml to give 12 replicate cultures for each of the four treatments. This replicate number was chosen so that at least three replicates could be used for the quantification of different physiological traits.

During a previous study, using the heterogeneous population from which our WT strain was derived, heating for 15 days at 32°C revealed no difference in performance traits under elevated compared to ambient temperature conditions (Levin et al., 2016). Therefore, we did not carry out performance measurements until day 3, postacclimation (representing 17 days in temperature treatment conditions). On days 3, 7, 10, 14, 17 and 21 (D3–D21) postacclimation, measurements for photosynthetic performance and growth were taken in six replicates (replicates seven to 12). On D7, D14 and D21 postinoculation, three replicates from each transplant (D7—replicates one to three, D14—four to six and D21—seven to nine) were sacrificed for the measurement of extracellular ROS (exROS) production. Cells therefore spent a total of 5 weeks in experimental conditions, corresponding to up to eight asexual generations. For bacteria, seven generations are enough to attribute whether adaptation has occurred through genetic mechanisms or through acclimation (Cooper, 1991), where genetic adaptation is shown by the difference in growth rate of selected cells under the selective conditions (SS@31) with the growth rate of the wild-type cells that have been transferred to the selective conditions (WT@31).

2.3.2 | Photosynthetic performance

The maximum quantum yield of PSII fluorescence ($F_v/F_m = F_m - (F_0/F_m)$) was measured one hour before the end of the dark cycle, while the effective quantum yield ($\Delta F/F'_m$) of PSII was measured after 6 hrs of light exposure. Both maximum and effective quantum yield measurements were carried out using an imaging pulse amplitude fluorometer (iPAM; Walz), with a Measuring Intensity of four, Saturating Intensity of seven, and Gain and Damping of two. PAM settings were chosen based on preliminary experiments as a compromise between fluorescence detection of low cell densities and signal saturation for high cell densities. The maximum excitation pressure over PSII (Q_m) was calculated using measurements for the maximum and effective quantum yields of PSII (Iglesias-Prieto et al., 2004) using the equation

$$Q_m = 1 - [(\Delta F/F'_m)/(F_v/F_m)]$$

Rapid light curves (RLCs) were carried out following $\Delta F/F'_m$ measurements by exposing each replicate culture to 10 steps of increasing actinic light (0–461 μmol photons m^{-2} s^{-1} PAR) each lasting 10 s. The RLCs for each replicate were fitted to the model by Platt, Gallegos, and Harrison (1980). The parameters of $rETR_m$ and E_k were calculated using SIGMAPLOT (Hill et al., 2004). By D17 and D21, the WT@31 replicates were no longer able to respond to the increases in the light induced by the RLCs and data could not be fitted to the model. Values for E_k could therefore not be calculated at these time points. Nonphotochemical quenching (NPQ) was calculated for each step of the RLCs using the equation

$$NPQ = \frac{F_m - F'_m}{F_m}$$

Values for the maximum NPQ (NPQ_m) were extracted from the highest PAR exposure during the RLCs.

2.3.3 | Growth rate and estimation of cell generation number

For cell density measurements, aliquots of 50 μl from replicates 6–12 ($n = 6$) were fixed by adding 4 μl of 25% glutaraldehyde and stored at 4°C for later measurements of cell density. Cell density was determined by triplicate haemocytometer counts and specific growth rate (μ, doubling/day) was calculated as follows:

$$\mu = \frac{\ln N_1 - \ln N_0}{\Delta t}$$

where N_0 is the cell density at D3, N_1 is the cell density at D17 and t is the duration (17-3) of culture in days. Cell density values for D3 and D17 were chosen as they represented the fastest growth rates during the experiment.

To estimate the number of generations that both the WT@27 had SS@31 had been through, both during their long-term culture and the reciprocal transplant experiment, we used cell density values from the 3 week experimental phase of the reciprocal transplant experiment. First, the doubling time (or generation time) was calculated according to the equation

$$T = 21 \times \left(\frac{\log(2)}{\log\left(\frac{N_1}{N_0}\right)} \right)$$

where N_1 is the end cell density after 21 days of growth and N_0 is the cell density at the start of the experiment (200,000 cells/ml). Next the number of generations was calculated as follows:

$$\text{Generations} = N/T$$

where N is the number of days of long-term culture (e.g., 840 days post-ratchet experiment) and T is the doubling time. Although doubling times could have varied during the long-term culture, to provide an estimate of the generation number, we assume that they were constant.

2.3.4 | Extracellular ROS production

For extracellular reactive oxygen species (exROS) determination, 1 ml of culture from each sacrificial replicate ($n = 3$) was pelleted (3000 *g*, 5 min) and 250 μl of the supernatant from each replicate was plated three times into a black, clear-bottom 96-well culture plate (Costar, Corning®; Sigma-Aldrich). The supernatant was incubated with 0.5 μl of CellROX Orange (Levin et al., 2016) (final concentration 5 μm, 25 min at 37°C), a fluorogenic probe that exhibits orange fluorescence upon oxidation by ROS (545 nm absorption, 565 nm emission). Fluorescence measurements were taken using a microplate reader (Synergy™ H4 Hybrid, Biotek®) immediately after incubation. Prior to pelleting, an aliquot of 50 μl from each sacrificial replicate was taken for the determination of cell density. The three fluorescence values for each replicate were then standardized to cell number to give relative exROS production *per* cell in arbitrary fluorescent units.

2.4 | Experiment 2: in hospite reciprocal transplant experiment

2.4.1 | Coral settlement and inoculation with *Symbiodinium*

To determine whether thermal selection in *Symbiodinium* could enhance the thermal tolerance of the holobiont, we chose three species of coral: *A. cytherea*, *A. hyacinthus* and *A. sarmentosa*. Coral colonies of each species were collected from Trunk Reef, Great Barrier Reef, Australia (18°18′101″S, 146°52′226″E) and kept in the National Sea Simulator (SeaSim) at the Australian Institute of Marine Science (Townsville, Australia) for 3 days before the full moon. Following spawning, gametes from four colonies of *A. cytherea* and five of *A. hyacinthus* and *A. sarmentosa* were each mixed in equal quantities (sperm concentration: ~10^6/ml), within a species, for fertilization. Resulting larvae were kept in aerated 0.4 μm filtered seawater for ca. 3 weeks.

Six-well, multiwell plates (well volume 16.8 ml, Corning®, Sigma-Aldrich®) were prepared with a settlement cue- the peptide Hym-248 (Iwao, Fujisawa, & Hatta, 2002), by placing three, 3 μl droplets of 60 μM in each well, and leaving to dry. These droplets were placed at equal distance from the edge of each well using a template placed underneath the plate. Planulae were taken from the SeaSim and washed three times in 0.2 μm filtered seawater (FSW). Three, 90 μl droplets of FSW, each containing one planula, were plated into each well on top of the dried peptide dots. Planulae were left for 12 hr in their droplets, in the dark, at 27°C to metamorphose and settle. Subsequently, each well was filled with 9 ml of FSW and planulae that had not metamorphosed or settled were removed. This method resulted in 134 individual recruits of *A. cytherea* across 14 plates, 136 of *A. hyacinthus* across 14 plates and 125 of *A. sarmentosa* across 15 plates. Coral recruits were reared at 27°C, 65 ± 10 μmol photons m^{-2} s^{-1} under a 14:10 light:dark cycle and were supplemented twice weekly with a 0.2 μm, filter-sterilized cocktail of nutrients, glycerol, vitamins and amino acid as in Wang, Chen, Tew, Meng, and Chen (2012). Plates were placed on a slow moving, rotating shaker plate to allow the homogenization of food. Water changes were carried out the day after feeding by removing the water in each well and replacing to total of 9 ml *per* well with fresh FSW.

2.4.2 | Experimental design

Nine days postsettlement, half of the plates containing recruits were inoculated with WT cells and half with SS cells (pre-acclimated to 27°C for 2 weeks) to give a cell density of 30,000 cells/ml and left for 2 days before replacing each well with fresh FSW. Inoculation was repeated 12 days postsettlement to maximize the number of symbionts taken up by the recruits. Five days after the initial inoculation, recruits had visually taken up symbionts and they were subsequently reared for 5 weeks at 27°C to ensure the symbiosis was stable and recruits were sufficiently pigmented for further analyses. Subsequently, half of the plates containing corals infected with the WT and half of those containing corals infected with the SS were transplanted into 31°C (D0) while the remaining recruits were kept at 27°C. This resulted in four experimental treatments, which mirrored those of the in vitro experiment (i.e., WT@27, SS@27, SS@31, and WT@31). For each coral species, this resulted in three to five replicate plates and between 27 and 38 individual recruits *per* treatment. Replicate number was considered sufficient to take into account potential variation in recruit physiology as a result of genetic variation.

2.4.3 | Recruit size and extent of pigmentation

Images of each recruit were taken using a camera-stereomicroscope set-up (SMZ800N; Nikon) on the day of transplantation (D0), 2 weeks (D14) and 4 weeks (D28) post-transplantation. Images were taken while the polyp's tentacles were retracted. The same camera settings were globally applied to each image. Recruit size was determined as the base area of each recruit using imaging software (NIS-Elements BR v430; Nikon). This was performed by detecting the outline of each recruit base and auto-calculating the area within (region of interest (ROI), Figure 1a). Growth was expressed as the percentage change in size of each recruit at D28 from the starting size at D0.

As a proxy for bleaching, we tracked any changes in percentage pigmentation of the surface area of each recruit during the experiment. To do this, we used the images from D0, D14 and D28 with the recruit set as the ROI. The pigmented areas within the ROI were detected by setting red, green, blue (RGB) threshold limits for each colour channel (Red: 90–155, Green: 70–100, Blue: 0–105). The same RGB threshold limits were applied across all images, and values of area pigmentation were expressed as a percentage of the total area of each recruit (Figure 1b).

2.5 | Experimental water parameters

Throughout both the in vitro and in hospite experiments, temperature conditions were measured every 10 min with a HOBO data logger (Tables 1 and 2). Light measurements were checked daily during the middle of the light cycle and were always 65 ± 10 μmol photons m^{-2} s^{-1}. For the in hospite experiment, pH_{NBS}, salinity and oxygen saturation measurements were taken from one random well *per* plate, twice weekly, coinciding with water changes (Table 2) to make sure a high water quality was maintained.

2.6 | Statistical analyses

2.6.1 | In vitro

To analyse the effects of temperature on the WT and SS cells, we compared the physiological responses of WT and SS cells in their own (WT@27, SS@31) and reciprocal (SS@27 and WT@31) temperature treatments across time, where relevant. To test the effects of treatment on in vitro specific growth rate and photophysiological traits, we used a linear model with "treatment" (i.e., WT@27, SS@31, SS@27 and WT@31) as a fixed factor and with "time" as an additional fixed factor for all photophysiological traits. The effect of treatment and time on exROS production was tested using a generalized linear mixed effects model fitted for a Poisson distribution, again with "treatment" and "time"' as fixed factors.

2.6.2 | In hospite

Similarly, to analyse the effects of temperature on the coral recruits infected with the WT and SS cells, we compared the physiological responses of corals infected with WT and SS cells in their own (WT@27, SS@31) and reciprocal (SS@27, WT@31) treatments across time, where relevant. Linear mixed effects models were used to test the effects of treatment and time on the % change in growth for each coral species, while generalized linear mixed effects models fitted for a negative binomial distribution were used to test the effects of treatment and time on area pigmentation (%) of recruits for each coral species. In both cases, "treatment" and "time" were fixed factors and "plate" was included as a random fixed factor.

All analyses were performed in R (v. 3.3.2; R Core Team, 2016), with annotated scripts and raw data included in the Supporting Information (Data S1 and S2, respectively). Linear models and linear mixed effects models were carried out using the package "NLME" (Pinheiro, Bates, DebRoy, & Sarkar, 2017), while generalized linear mixed

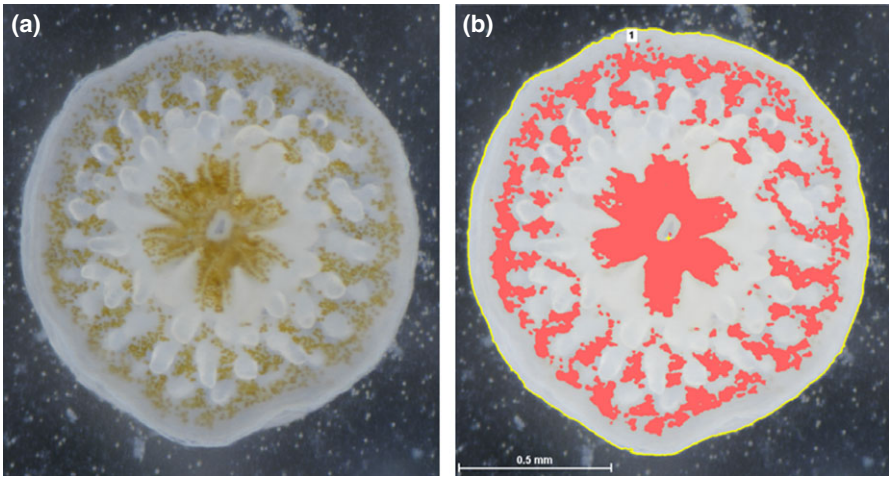

FIGURE 1 Determination of recruit base area and area pigmentation (%) of a coral recruit. (a) Raw image of a coral recruit, (b) same recruit image with the region of interest (ROI) selected (recruit base area) and the area pigmented highlighted after applying red, green blue (RGB) thresholds. The area pigmented is expressed as a percentage of the total base area of the recruit

TABLE 2 In hospite rearing conditions for coral recruits infected with either the wild-type (WT) or selected *Symbiodinium* (SS) cells in two temperature conditions

Phase	Treatment	Temperature (°C)	Salinity (‰)	Dissolved oxygen (%)	pH_{NBS}
Pre-experimental	WT@27		33.13 ± 0.35 (127)	97.89 ± 0.12 (127)	8.06 ± 0.005 (127)
	SS@27	26.81 ± 0.005 (5,183)	33.6 ± 0.06 (138)	97.87 ± 0.10 (138)	8.06 ± 0.005 (138)
Experimental	WT@27		33.36 ± 0.07 (66)	96.83 ± 0.18 (66)	8.09 ± 0.008 (66)
	SS@27	26.81 ± 0.007 (2,971)	33.49 ± 0.09 (66)	96.12 ± 0.53 (66)	8.10 ± 0.008 (66)
	SS@31		33.53 ± 0.09 (66)	95.59 ± 1.15 (66)	8.10 ± 0.007 (66)
	WT@31	31.09 ± 0.002 (3,075)	33.53 ± 0.09 (78)	95.92 ± 0.02 (78)	8.10 ± 0.007 (78)

Pre-experimental conditions refer to the ambient temperature rearing conditions of recruits infected with both the WT and SS cells (5 weeks) before half being moved into elevated temperature treatment during the experimental phase (3 weeks). Values are mean ± *SEM*. Number of replicates are provided in parentheses.

effects models were carried out using the package "LME4" (Bates, Maechler, Bolker, & Walker, 2015). For both in vitro and in hospite traits, all pairwise comparisons were carried out post hoc with Tukey's tests using the package "MULTCOMP" (Hothorn, Bretz, & Westfall, 2008). Heterogeneity and normality were assessed using Residual and Q-Q plots. Where "time" was a fixed factor, we checked for correlation within the model and in all cases, none was detected.

3 | RESULTS

3.1 | Heat stress responses of *Symbiodinium* in vitro

3.1.1 | Photosynthetic performance

Maximum and effective quantum yield values (Figure 2a,b) for the SS@31 were not significantly different to those for the WT@27 and SS@27 throughout most of the experiment, but were higher than mean values for the WT@31 (max. $p < .05$). The WT@31 values were always significantly lower (max. $p < .05$) compared to those in the remaining treatments, with the mean F_v/F_m significantly decreasing over time from 0.46 (±*SEM*, ±0.007) to 0.37 (±0.004) and mean $\Delta F/F'_m$ from 0.41 (±0.002) to 0.22 (±0.036) from day 3 (D3) to day 21 (D21, $p < .05$). The mean maximum excitation pressure over PSII (Q_m, Figure 2c) for the WT@31 was significantly elevated over five of the six time points, compared to the other treatments (max. $p < .05$), and by D21, the mean Q_m had significantly increased to 0.42 (±0.054) from 0.11 (±0.017) at D0 ($p < .05$). The mean irradiance at onset of light saturation (E_k), maximum relative electron transport rate ($rETR_m$) and maximum NPQ (NPQ_m) for the WT@31 were significantly lower than for the remaining experimental groups (max. $p < .05$) across the duration of the experiment (Figure 2d–f) with the mean $rETR_m$ significantly decreasing over time ($p < .05$). One exception was at D7, when the mean E_k value was not statistically different from that in the SS@27 and WT@27 treatments (min. $p > .05$).

3.1.2 | Specific growth rate

The mean growth rate of SS@31 was positive at 0.07 μ ± 0.005. Although this was significantly slower than the growth rates of the WT@27 (0.16 ± 0.007) and SS@27 (0.14 ± 0.008, max. $p < .05$), all three had significantly greater mean growth rates compared to the WT@31 (max. $p < .05$), which exhibited negative growth at 0.05 μ (±0.010, Figure 3a).

3.1.3 | Oxidative stress

ExROS of the WT@31 had mean values that were always significantly greater than those observed for the other experimental groups (max. $p < .05$) and that significantly increased over time from 46.2 (±2.04) at D7 to 105.62 (±22.25) by D21 ($p < .05$, Figure 3b). By comparison, the highest mean value of SS@31 throughout the experiment was 22.19 (±4.10). The amount of ExROS produced in the SS@31 treatment was not significantly different to that of the SS@27 or WT@27 at any time point ($p > .05$) and the production of exROS by SS@31, SS@37 or SS@27 did not significantly change over time ($p > .05$).

3.2 | Heat stress responses of the juvenile coral holobiont

3.2.1 | Growth

Measurements of percentage change in size over the 28-day experiment (Figure 4a–c) revealed no significant differences in growth between the WT@27, SS@27 and SS@31 recruits for each coral species (min. $p \geq .05$), which generally showed a trend of positive mean growth with increases up to 1.29 ± 0.52% over the four-week experiment. In contrast, WT@31 recruits displayed a trend of negative mean growth for all three coral species. Despite this, the mean growth of the WT@31 recruits was not significantly different from that of the SS@31 recruits for all three coral species (min. $p > .05$), although it was significantly lower compared to that of the WT@27 recruits for *A. cytherea* and *A. hyacinthus* (max. $p < .05$). The WT@31 mean growth was significantly lower compared to that of the SS@27 recruits for *A. hyacinthus* and *A. cytherea* ($p < .05$).

3.2.2 | Area pigmented

There were no significant differences in mean percentage pigmentation at D0 for all coral species harbouring either the SS or WT cells

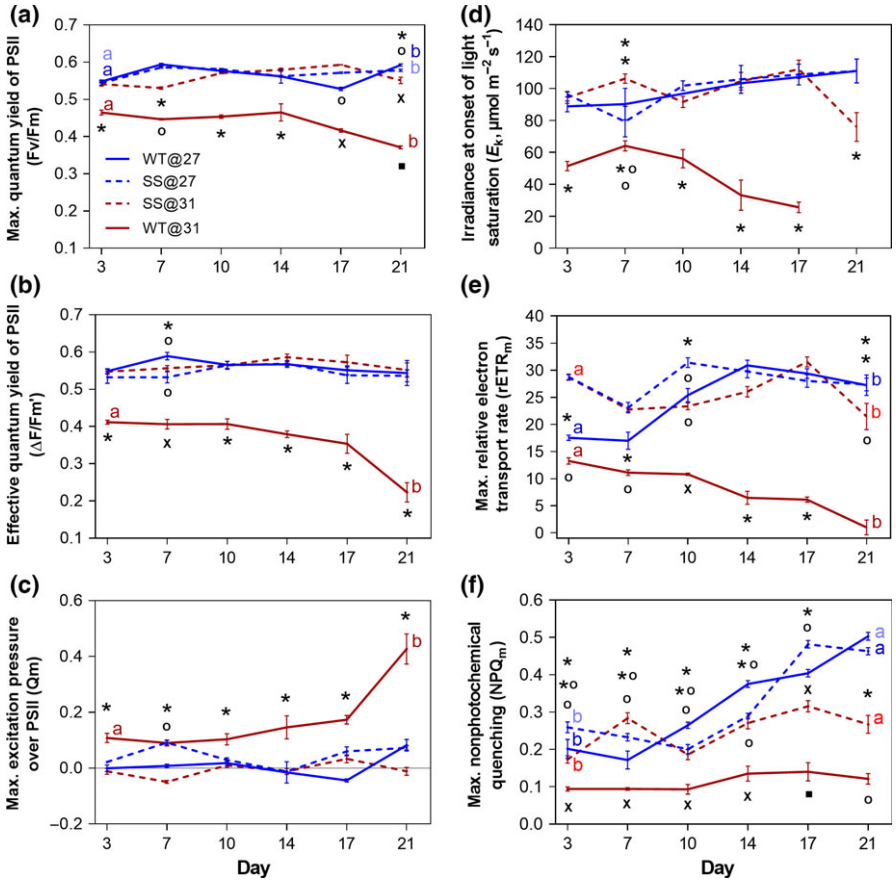

FIGURE 2 Comparison of mean (±*SEM*) photochemical performance traits in wild-type (WT) and selected (SS) *Symbiodinium* C1 at 27°C (WT@27, SS@27) and 31°C (SS@31, WT@31) over 21 days after 14 days of pre-acclimation. (a) Maximum quantum yield of photosystem II. (b) Effective quantum yield of photosystem II. (c) Maximum excitation pressure over photosystem II. (d), Irradiance at onset of light saturation. (e) Maximum relative electron transport rate. (f) Maximum nonphotochemical quenching. Statistically significant differences among "treatments" (i.e., WT@27, SS@27, SS@31, WT@31) within a time point are represented by different symbols above/below the line plots, while statistically significant differences over time (between D3 and D21) for each treatment are represented by different letters (Tukey's post hoc tests; $p < .05$). $n = 6$ for all means. Where error bars are not visible, they are small and hidden by the symbols

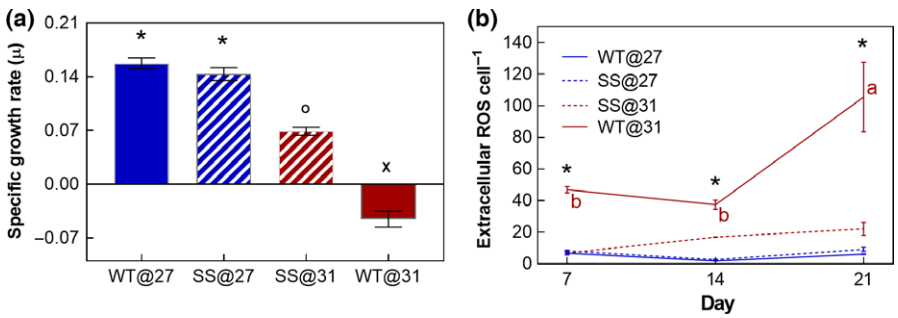

FIGURE 3 Comparison of mean (±*SEM*) growth and oxidative stress of wild-type (WT) and selected (SS) *Symbiodinium* C1 at 27°C (WT@27, SS@27) and 31°C (SS@31, WT@31) after 14 days of pre-acclimation. (a) Specific growth rate ($n = 6$) calculated from cell densities at D3 and D17. (b) Extracellular reactive oxygen species production per cell ($n = 3$). Statistically significant differences among "treatments" (i.e., WT@27, SS@27, SS@31, WT@31) are represented by different symbols above/below the bar/line plots (Tukey's post hoc tests; $p < .05$) (a, b). Statistically significant differences over time for each treatment are represented by different letters (b). Where error bars are not visible, they are small and hidden by the symbols

($p > .05$, Figure 4d–f). By D14, the mean percentage pigmentation of all three coral species harbouring the SS and WT cells had not significantly changed in the 31°C treatment ($p > .05$), while the majority of those harbouring the WT and SS at 27°C had increased ($p < .05$). While the recruits harbouring WT cells were significantly less pigmented at 31°C than those at 27°C across the duration of the

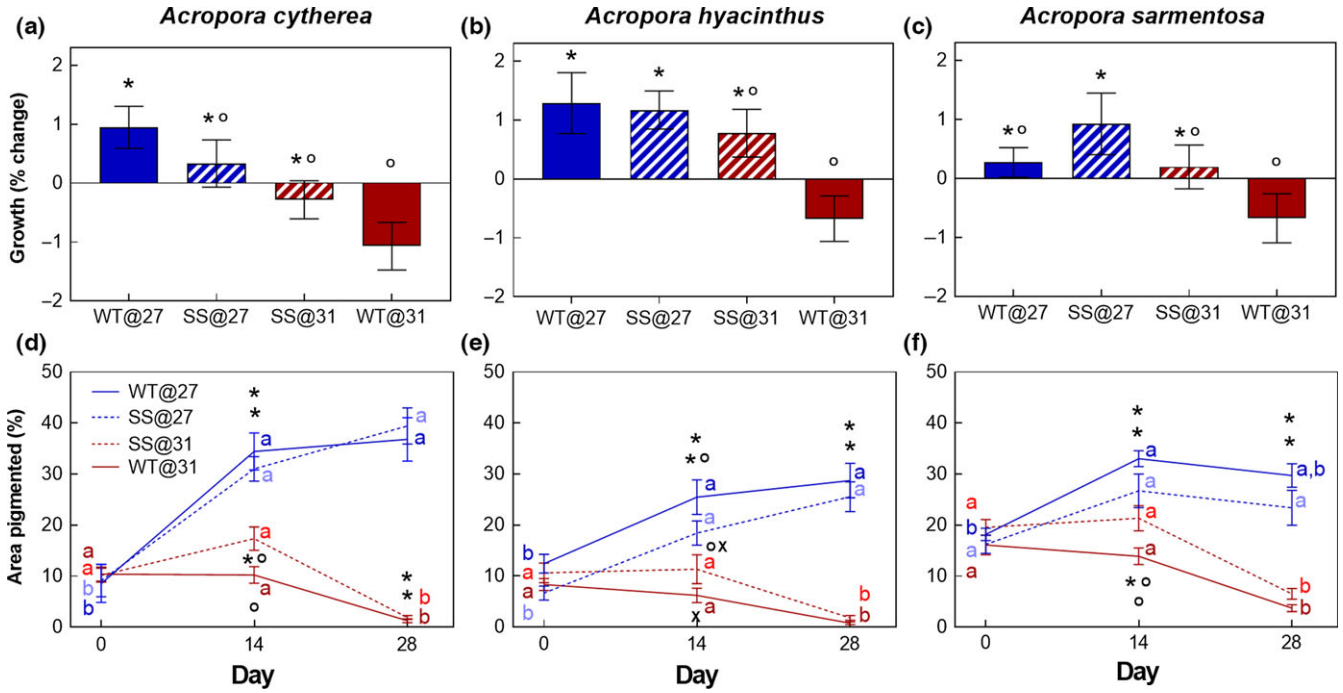

FIGURE 4 Comparison of mean (±*SEM*) growth, and bleaching of coral recruits harbouring wild-type (WT) and selected (SS) *Symbiodinium* C1 at 27°C (WT@27, SS@27) and 31°C (SS@31, WT@31). (a–c) % Change in size between D0 and D28 for three coral species (n = 25–34). (d–f) Proportion of recruit surface area pigmented (%) for each coral species (n = 20–37). Statistically significant differences among "treatments" (i.e., WT@27, SS@27, SS@31, WT@31) within a time point are represented by different symbols above/below the line plots (Tukey's post hoc tests; $p < .05$, a–f). Statistically significant differences over time (d–f) for each treatment are represented by different letters (Tukey's post hoc tests; $p < .05$). Where error bars are not visible, they are small and hidden by the symbols

experiment (max. $p > .05$), the mean percentage pigmentation for recruits in the SS@31 treatment across all three species was not significantly different to the recruits harbouring the WT or SS cells at 27°C (min. $p > .05$). The only exception was *A. hyacinthus*, where the mean percentage pigmentation for SS recruits at 31°C was significantly lower than the for WT recruits at 27°C ($p < .05$, Figure 4e). By D28, WT and SS recruits of all three species had bleached at 31°C (0.75%–6.51% mean pigmentation), with mean pigmentation values not significantly different between the WT and SS recruits (min. $p > .05$) as well as being significantly less pigmented compared to the recruits at D0 (min. $p > .05$).

4 | DISCUSSION

The stable phenotypic changes observed in the selected *Symbiodinium* (SS) cells after ~80 generations (2.5 years) of in vitro selection provides compelling evidence of rapid thermal adaptation in this *Symbiodinium* type C1 genotype. Our study is one of only a few microalgal studies that show thermal adaptation through experimental evolution. SS cells significantly outperformed the WT cells at elevated temperature for all traits measured while they maintained performance levels similar to those of the WT cells at ambient temperature, demonstrating that laboratory selection has widened rather than shifted the SS temperature tolerance range for this strain. This is an important characteristic considering the often large natural daily and seasonal temperature fluctuations experienced on coral reefs (Kline et al., 2015). Contrary to expectations, we found the positive effects of thermal adaptation of the SS cells to be considerably reduced in hospite, although a trend of positive growth was observed in two of three coral species tested under heat stress when inoculated with SS cells, but not with WT cells.

4.1 | Thermal adaptation and broadening of thermal range in algal endosymbionts ex hospite

All photophysiological traits measured showed a significantly lowered performance of the WT@31 compared to the other experimental groups over the 21-day study period. In contrast, the SS@31 could maintain photosynthetic function similar to the WT@27 and SS@27 throughout the experiment. The specific growth rate and exROS data confirm an increase in temperature tolerance for the SS relative to the WT cells. The mean growth rate of SS@31, while up to 44% slower than that of the WT@27 and SS@27, exhibited positive growth (0.07 doublings/day), while the WT@31 exhibited mean negative growth of −0.05 doublings/day. Elevated temperature inevitably requires increases in metabolic activity (Beardall & Raven, 2004). Selection on more efficient metabolic pathways for the SS may have been enough to maintain energetically costly photosynthetic processes (and/or photosystem and cellular repair mechanisms) as well as positive growth at 31°C, but not enough to sustain growth rates to match those at 27°C. Reduced growth rates as a

result of greater thermal tolerance have been described previously through both short-term acclimatory and adaptive responses. A thermally tolerant isolate of *Symbiodinium* A1 cultured for 10 days at 32°C exhibited only a slight decline in photosynthetic activity but a considerable decline in growth, compared to control temperature conditions (Robison & Warner, 2006). In another example, evolution of thermal tolerance in the phytoplankton *Chlorella vulgaris* resulted in a downregulation in respiration relative to photosynthesis through changes in energy allocation (Padfield, Yvon-Durocher, Buckling, Jennings, & Yvon-Durocher, 2016).

Extracellular reactive oxygen species (exROS) production by the WT@31 had mean values that were always higher than those for the remaining experimental groups and up to 17 times greater by D21. Conversely, the SS@31 was able to maintain lower exROS levels similar to the WT@27 and SS@27 throughout most of the experiment. Reactive oxygen species are molecules produced as a by-product of photosynthesis and respiration. If produced in excess and not neutralized by antioxidant molecules and enzymes, ROS are secreted extracellularly into their surrounding medium. Excessive ROS production in hospite has been linked to triggering the bleaching response of corals (Downs et al., 2002; Lesser, 2006); thus, their production is an important trait to consider in *Symbiodinium* thermal selection experiments. Cellular ROS production can occur as a result of uncoupling of the photosystems (PSII and PSI) during heat stress (Iglesias-Prieto et al., 1992), and in excess can cause damage to DNA, proteins, lipids and cell membranes (Lesser, 2006; Tchernov et al., 2004; Venn, Loram, & Douglas, 2008). The high exROS produced by the WT@31 likely explains the inability for growth and severe photoinhibition at an elevated temperature.

Two previous studies have experimented with the heterogeneous population from which our WT strain was derived. Howells et al. (2011) showed that, after 11 days of heating, *Symbiodinium* C1 showed no signs of stress at 32°C and displayed maximum quantum yields 16% higher than at the control temperature of 27°C. In a more recent study, heating of the same *Symbiodinium* C1 for 15 days at 32°C revealed no difference in photosynthetic performance, or amount of exROS compared to culture replicates maintained under ambient temperature conditions. Heat-exposed cells were able to acclimate via upregulation of a number of antioxidant and molecular chaperone genes (Levin et al., 2016), preventing damage to the photosynthetic apparatus and allowing cell growth to be maintained. In our study, D3 already represented 17 days of exposure to 31°C for the WT@31 as a result of 2 weeks pre-acclimation to experimental conditions. The reduced performance of our WT@31, and subsequent decline in fitness, indicates that more than 15 days are needed before thermal stress significantly impacts the WT's physiological performance. The energetic costs of upregulating pathways to combat the effects of thermal stress could have become too high for thermal tolerance to be maintained (DeWitt, Sih, & Wilson, 1998). Maintenance of low exROS levels by the SS@31 could be a result of a greater antioxidant capacity under heat stress (McGinty, Pieczonka, & Mydlarz, 2012) compared to the WT, perhaps through the selection of cells with a higher metabolic capacity able to support such costly antioxidant responses. Alternatively, a more stable photosynthetic apparatus, through alterations in the fatty acid composition of thylakoid membranes (Tchernov et al., 2004) for example, could limit cellular ROS production in the SS cells.

Climate change-relevant evolutionary experiments have been carried out on other microalgae, although none of these previously studied algae have a symbiotic life stage. These microalgal studies have mainly focussed on the effects of selection to an elevated pCO_2 regime and often used growth rate as the only fitness parameter measured. Some have failed to show adaptation (Crawfurd, Raven, Wheeler, Baxter, & Joint, 2011; Tatters et al., 2013), while others have provided evidence for adaptation to elevated pCO_2 (Collins & Bell, 2004; Jin et al., 2013; Lohbeck et al., 2012). A limited number of studies have investigated the evolutionary consequences of selection to elevated temperature; the dinoflagellate *Alexandrium minutum* adapted to a combination of elevated temperature and pCO_2 after ~250 generations (2 years) of selection (Flores-Moya, Costas, & López-Rodas, 2008), a number of phytoplankton strains showed interspecific differences in thermal adaptive capacity (Huertas et al., 2011) and the coccolithophore *Emiliania huxleyi* showed a 16% higher growth rate in thermally adapted populations after 460 generations (1 year) of selection (Schlüter et al., 2014). Our results show that thermal adaptation is possible after only ~80 generations (2.5 years) of selection in *Symbiodinium* C1. Such rapid adaptation may be in part be due to the nature of our initial experimental design (ratchet technique; Huertas et al., 2011), where sequential increases in temperature, only to levels that maintained population growth, meant maximizing the potential for beneficial, random mutations to occur and thus maximizing the rate of adaptation. Additionally, a further culture of ~73 generations (more than 2 years) at elevated temperature after the ratchet experiment could have allowed any such mutations to become fixed in the population.

4.2 | Thermal adaptation of algal endosymbionts shows limited expression in symbiosis

While our in vitro results provide compelling evidence for an adaptive response to temperature selection in *Symbiodinium* C1, the benefits of temperature adaptation showed limited expression in symbiosis with juvenile *Acropora* corals. There were no significant differences in tissue pigmentation between treatment groups and all were equally bleached at the end of the experiment when under heat stress. At the higher experimental temperature of 31°C, there was no significant difference in growth rates between juveniles that harboured SS or WT cells but two of the three coral species harbouring the SS cells exhibited a trend of positive growth, while all species harbouring the WT displayed a trend of negative growth. Recruit growth at 27°C was independent of infection with WT or SS cells, indicating no negative impact of thermal adaptation of the SS on holobiont growth at ambient temperature in this experiment. This contrasts with other studies that have shown that higher thermal tolerance in *Symbiodinium* type D comes at the cost of reduced growth for *Acropora millepora* juvenile and adult corals at ambient

temperatures, as well as *P. damicornis*, compared to corals harbouring more thermosensitive C1 (Little, van Oppen, & Willis, 2004), C2 (Jones & Berkelmans, 2010) and (Cunning, Gillette, Capo, Galvez, & Baker, 2015) *Symbiodinium*, respectively.

Differences between the in vitro and in hospite responses under heat stress have previously been reported for other *Symbiodinium* strains (Bhagooli & Hidaka, 2003; Goulet, Cook, & Goulet, 2005). Our results, in conjunction with the observed differences in bleaching responses of different coral species that harbour the same *Symbiodinium* type (Abrego, Ulstrup, Willis, & van Oppen, 2008; Berkelmans & van Oppen, 2006; Fisher, Malme, & Dove, 2012), confirm that the coral host contributes considerably to the bleaching tolerance level of the coral-*Symbiodinium* holobiont (Baird, Bhagooli, Ralph, & Takahashi, 2008). Little is known about potential host-factors influencing the holobiont response, but a number of species-specific variables have been suggested whereby the coral host can alter the algal microenvironment through differences in the amount and type of light reaching symbionts in hospite, host-based pigments (Dove, 2004), host skeletal morphology (Enríquez, Méndez, & Iglesias-Prieto, 2005; Kaniewska, Anthony, & Hoegh-Guldberg, 2008) and tissue thickness (Loya et al., 2001). Further, a potentially dissolved inorganic carbon (DIC) limited environment in hospite (Jarrold et al., 2013; Leggat, Rees, & Yellowlees, 2000; Marubini, Ferrier-Pagès, Furla, & Allemand, 2008) could drive differences in photosynthesis between in vitro cultures that may be DIC replete. Alternatively, either the production of ROS by the host itself under heat stress or host antioxidant capacity could have been so high that the increase in ROS produced by WT symbionts at 31°C was relatively insignificant. Furthermore, a breakdown of symbiont-host communication under stress has been suggested to affect the ability of a coral to discriminate between healthy and unhealthy *Symbiodinium* cells (Baird et al., 2008), which could explain factors such as host-digestion of *Symbiodinium*, symbiont expulsion or apoptotic host cell death (Dunn, Bythell, Le Tissier, Burnett, & Thomason, 2002; Weis, 2008) as a resulting host response, regardless of *Symbiodinium* health (Baird et al., 2008).

4.3 | Assisted *Symbiodinium* evolution to enhance coral bleaching tolerance

Our results highlight the ability of *Symbiodinium* to evolve greater thermal tolerance over ecological time scales relevant to the pace of climate change. A previous directed evolution experiment on *Symbiodinium* showed positive growth after 55–70 generations of culturing at 30°C, while no growth was observed at this temperature prior to selection (Huertas et al., 2011). These findings indicate that the ability for the rapid temperature adaptation is not limited to the *Symbiodinium* C1 used in our study.

We show that the positive effect of laboratory selection on our particular *Symbiodinium* strain was reduced in symbiosis. Many *Symbiodinium* types are known to occur both free living in coral reef sediments and the water column and in symbiosis with reef-building corals and/or other reef organisms (Huang et al., 2013; Littman, van Oppen, & Willis, 2008), such as the C1 type studied here. Our findings suggest that fast growth may be an important trait to artificially select for when the goal is to enhance thermal tolerance in free-living *Symbiodinium*, but may not be an appropriate trait to select for *in vitro* if the aim is to augment thermal tolerance in hospite (i.e., coral bleaching tolerance) (van Oppen et al., 2015). Additional laboratory selection experiments using several selection pressures and targeted traits are required to resolve this issue. Furthermore, *Symbiodinium* is a highly diverse genus comprising many evolutionary lineages and types (i.e., putative species) (Baker, 2003; Manning & Gates, 2008) and it is possible that other *Symbiodinium* strains belonging to different clades and types may respond to laboratory thermal selection in different ways with varying effects on the holobiont. Finally, it is important to acknowledge the numerous other microbes (e.g., bacteria, fungi, archaea) that are present in corals. These microbes can have vastly different physiologies and can play important, functional roles (Ainsworth, Thurber, & Gates, 2010). Their interaction with *Symbiodinium* could therefore have implications in the overall phenotype of both the *Symbiodinium* and the coral holobiont.

Models of reef futures under climate change have taken into account existing variation in symbiont thermal tolerance (Baskett, Gaines, & Nisbet, 2009) and coral resilience (Baskett, Fabina, & Gross, 2014). The results presented here on the rate of evolution in *Symbiodinium* to rising seawater temperatures, and the potentially different selective forces in the free living vs. the symbiotic life stage (Day, Nagel, van Oppen, & Caley, 2008), are critical to refine such models. Examination of cellular pathways (e.g., *via* transcriptome analysis) underpinning the SS and WT *Symbiodinium* thermal stress response in vitro and in hospite will likely provide insights into the distinct phenotypic responses observed here and may reveal why the positive effects of thermal selection were reduced in hospite in our experiment. Such studies will inform and improve experimental evolution studies in *Symbiodinium* for augmenting coral bleaching tolerance and their ultimate use in coral reef conservation and restoration initiatives.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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