



Experimental Evolution in Coral Photosymbionts as a Tool to Increase Thermal Tolerance

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Coral reefs are under major threat from ocean warming. When temperatures become too high corals bleach, expelling their symbiotic, photosynthetic microalgae (*Symbiodinium*), which they depend on for much of their nutritional requirements. Prolonged bleaching has led to widespread coral mortality and the severity and frequency of bleaching events are predicted to increase in the future. Coral bleaching tolerance is influenced by the thermal tolerance of the *Symbiodinium* harboured, and these microbial members of the coral holobiont may be able to evolve more rapidly than the coral host itself. Here, we examined the response of replicate cultures of five genetically distinct *Symbiodinium* strains (A3c, two types of D1, G3, and F1) to increasing temperatures over the course of approximately one year. For three *Symbiodinium* types (types A3c, G3, and F1), we observed a stable adaptive change at the end of this exposure period, which equated to only 41–69 asexual generations. The long-term selected *Symbiodinium* culture replicates (SS) showed faster growth rates under short-term, acute heat stress, and in some cases higher photosynthetic efficiencies, compared to wild-type populations (WT). Our results considerably extend the field of experimental evolution in *Symbiodinium* and with further work into the *Symbiodinium*-coral association and bleaching response, this approach may become a valuable tool in coral reef conservation and restoration initiatives.

Keywords: *Symbiodinium*, adaptation, acclimation, symbiosis, ocean warming, climate change, assisted evolution

INTRODUCTION

Coral reefs are the most biodiverse ecosystem in the marine world, contributing billions of dollars' worth of ecosystem services through tourism, coastal protection and fishing industries, with invaluable cultural and medicinal significance (Moberg and Folke, 1999). However, reef-building corals are under serious threat from rapid ocean warming, driven by global warming, as a consequence of rising anthropogenic carbon emissions. The unprecedented rate of global change is considered too rapid for many marine organisms to keep up, leading to the prediction of mass species extinction by the end of the century (Hoegh-Guldberg and Bruno, 2010; Pereira et al., 2010; Dawson et al., 2011; Pacifici et al., 2015; Urban, 2015). Increases in temperature are one of the main causes of coral bleaching, whereby corals' symbiotic algae, *Symbiodinium* spp., are lost from coral tissue in a stress response driven by the *Symbiodinium* and/or the coral (Pandolfi et al., 2011). Corals meet most of their energy requirements from the *Symbiodinium* living inside their cells

(Falkowski et al., 1984) and although corals can survive without their photosymbionts for a limited period of time, prolonged bleaching leads to coral starvation and will ultimately lead to death. Even if corals can re-establish a symbiosis when temperatures return to normal, they may be left prone to disease (e.g., Kushmaro et al., 1997; Harvell et al., 1999; Howells et al., 2016), show reduced growth and can be reproductively compromised (e.g., Baird and Marshall, 2002; Miller et al., 2009)

Rising ocean temperatures have triggered three global mass bleaching events (1998, 2010, and 2014–2017) since records began in the 1980s. Not only have bleaching events become more extreme and widespread, but the time between successive events has declined by 4.6-fold from the early 1980s (Hughes et al., 2017), giving surviving coral less time to recover. Ocean warming is unlikely to be curbed, with models showing only a 5% chance that the global temperature increase since pre-industrial times will be less than 2°C by 2050 (Raftery et al., 2017). To preserve just $\leq 10\%$ of the world's coral reefs, it has been predicted that warming must be limited to below 1.5°C (Frieler et al., 2013). Some marine species may be able to adapt, acclimate or simply move to cooler places in response to rapid ocean warming (Cheung et al., 2009), however for reef-building corals that have relatively long generation times (4–20 years, Babcock, 1991) are already near their physiological thermal limits and are sessile, such options are limited. Therefore, the need to develop measures that enhance the thermal resilience of corals is pressing.

Assisted evolution is the acceleration of naturally occurring evolutionary processes to enhance certain traits; it involves a set of direct interventions and aims to increase coral climate resilience (van Oppen et al., 2015, 2017). One approach that has recently been pioneered is to make use of the comparatively short generation time of the asexually reproducing coral photosymbionts, *Symbiodinium*, by subjecting them to laboratory thermal selection. *Symbiodinium* generation times *in hospite* correspond to between three and 74 days (Muscatine et al., 1984; Hoegh-Guldberg et al., 1987; Wilkerson et al., 1988) and may be restricted by the host through the amount of nutrients provided, host digestion and expulsion (Muscatine and Pool, 1979; Cook et al., 1988; Titlyanovl et al., 1996; Stat et al., 2008; Cantin et al., 2009), which could limit their rate of adaptation. However, in culture, typical doubling times for *Symbiodinium* are often less than three days (Fitt and Trench, 1983; Kinzie et al., 2001; Taguchi, 2001), and in a laboratory environment, *Symbiodinium* can be cultured *ex-hospite* under strong thermal selection conditions that encourage large population sizes and minimise generation times, thus maximising the rate of genetic adaptation (Bromham, 2009). As coral survival is highly dependent on the thermal performance of their symbionts, such research may hold the key to reducing the extent of coral bleaching under thermal stress (van Oppen et al., 2015; Chakravarti et al., 2017).

The genus *Symbiodinium* currently comprises nine phylogenetic clades (A-I; Pochon and Gates, 2010), and each clade consists of multiple types (e.g., van Oppen et al., 2005b). Corals can host multiple types simultaneously (Mieog et al., 2007; Silverstein et al., 2012; Boulotte et al., 2016) and dominant *Symbiodinium* types *in hospite* can vary in relative

abundance, depending on coral life-stage (e.g., Little et al., 2004) and environmental factors (e.g., Baker et al., 2004; Rowan, 2004). Most importantly, the association of specific *Symbiodinium* types might alter the thermal stress tolerance of the holobiont (Berkelmans and van Oppen, 2006; Sampayo et al., 2008; Abrego et al., 2009; Mieog et al., 2009; Stat and Gates, 2011; Howells et al., 2012). Members of *Symbiodinium* clade D in particular have attracted attention as they have been linked to high bleaching tolerance in corals, where under heat stress, they out-compete other, perhaps more functionally beneficial *Symbiodinium* (reviewed by Stat et al., 2008). However, Chakravarti et al. (2017) demonstrated that for a laboratory evolved strain of *Symbiodinium* C1 that had a widened thermal niche, there was limited transfer of heat tolerance when in symbiosis. Such responses could be strain-specific and thus it is important to examine the response of a wide taxonomic diversity of *Symbiodinium* strains to temperature selection for their potential use in assisting coral evolution.

Here, we carried out a laboratory selection experiment with five different types of *Symbiodinium* isolated from the Great Barrier Reef (GBR, types A3, two strains of D1, F1, and G3), by exposing cultures to increasing temperatures, up to 34°C, for 1 year. Subsequently, we investigated the thermal tolerances of these selected *Symbiodinium* (SS) cultures relative to the unselected wild-type (WT) cultures that had been kept under control temperature conditions of 27°C.

MATERIALS AND METHODS

Symbiodinium Isolation and Culture

Five strains of *Symbiodinium* were isolated from four species of coral and one species of giant clam from the central and southern Great Barrier Reef (Table 1). Types F1 and A3c were obtained from the University of Technology Sydney (Table 1), and are described in (Suggett et al., 2015) while the remaining strains were isolated and cultured at the Australian Institute of Marine Science. This involved air-brushing the coral tissue, centrifuging (5 min, 1,600 g), decanting, and re-suspending the pellet three times in 0.2 μm filtered seawater. Isolated 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 $\mu\text{g mL}^{-1}$), amphotericin (final concentration 2.5 $\mu\text{g mL}^{-1}$), and GeO_2 (final concentration 50 μM , (Beltran et al., 2012)). To minimise bacterial growth, cells were inoculated into fresh IMK+antibiotics monthly, for five months. Each culture was genotyped based on the ITS2 rDNA region and annual sequencing checks confirmed that the cultures did not become contaminated during longer-term laboratory culture. Cultures were maintained at 27°C in an environmental chamber (Steridium, er-rh-500) and $65 \pm 10 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Sylvania FHO24W/T5/865 fluorescent tubes) under a 14:10 light:dark cycle. It is important to note that isolation of symbionts from hosts may not necessarily represent their dominant symbiont type. Additionally, it is possible that the *Symbiodinium* strains cultured could have either represented free-living cells, perhaps associated with the corals surface, or symbiotic cells

TABLE 1 | Symbiont species, type (ITS2), host species and site of origin for each *Symbiodinium* strain used in this study.

Strain identification	ITS2 type	Species	Host species	Geographic origin	GenBank accession number
CS73/SCF022.01	A3c	<i>S. tridacnidorum</i>	<i>Tridacna maxima</i> (giant clam)	Heron Island (southern GBR)	KR013750
SCF082	D1	Undescribed	<i>Acropora muricata</i> (coral)	Magnetic Island (central GBR)	MH229352
SCF086.01	D1	Undescribed	<i>Porites lobata</i> (coral)	Davies Reef (central GBR)	MH229353
UTSC/SCF089.01	F1	<i>S. kawagutii</i>	<i>Pocillopora damicornis</i> (coral)	Heron Island (southern GBR)	AF427462
SCF097.01	G3	Undescribed	<i>Diploastrea heliopora</i> (coral)	Davies Reef (central GBR)	MH229354

harboured within the hosts' tissues. However, in preliminary trials, all five strains were able to infect the coral species *Acropora tenuis* and *A. millepora* suggesting that they are true symbionts (LJC, unpubl.).

Symbiodinium Thermal Selection Experiment

Experimental Design

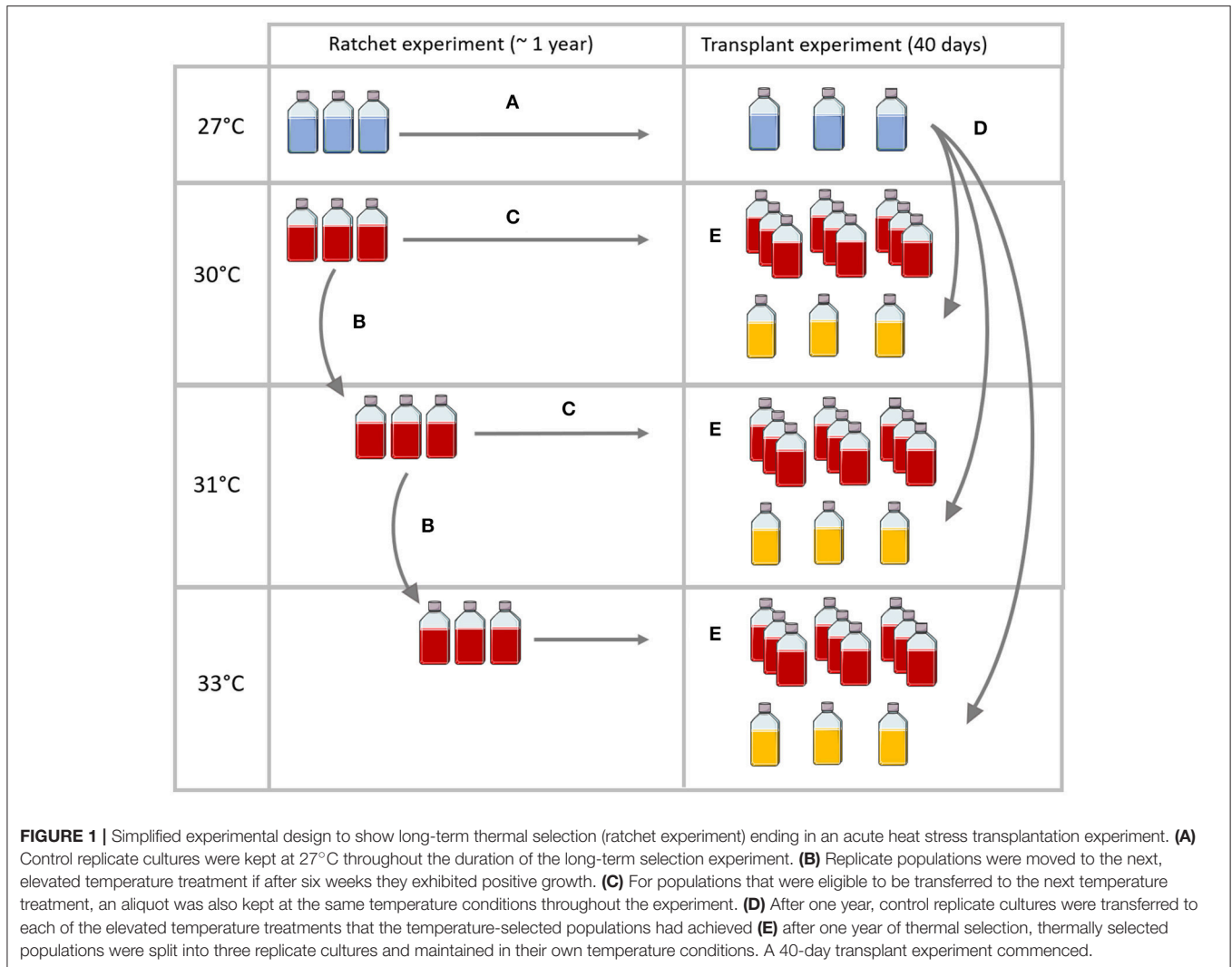
A thermal selection experiment was carried out using a modified ratchet design (Huertas et al., 2011, **Figure 1**). The ratchet design is a method used to maximise the number of spontaneous mutations arising from asexual cell division, by maintaining large population sizes while increasing levels of thermal selection pressure in a step-wise fashion.

Our ratchet experiment involved five temperature treatments starting at control temperature conditions of 27°C (27.04°C ± 0.002, $n = 54275$) and 30°C (30.06°C ± 0.0008, $n = 54347$), with further 1–2°C increments; 31°C (31.17°C ± 0.002, $n = 48123$), 33°C (32.78°C ± 0.006, $n = 35702$) and 34°C (34.04°C ± 0.001, $n = 14045$), under the same light conditions as their prior culture (65 ± 10 μmol photons m⁻² s⁻¹ 14:10 light:dark cycle) and in five temperature-controlled environmental chambers (Steridium, er-rh-500). Temperature measurements were recorded every 10 min, using a data logger (HOBO Pendant®). To begin the ratchet experiment, replicate control cultures ($n = 3$) of each strain were inoculated into fresh media at 100,000 cells mL⁻¹ to give a final volume of 20 mL of culture and kept at 27°C (control) and the first treatment temperature of 30°C (**Figure 1**). This jump in temperature from 27°C to 30°C was chosen based on previous observations showing most *Symbiodinium* strains can survive 30°C. Importantly, once the experiment started, each “replicate” culture at 30°C (or subsequent temperature treatments) was no longer considered a replicate but an independent evolutionary unit in which different random mutations could arise (Huertas et al., 2011). The first ratchet level consisted of populations (both control and treatment) being cultured for a three-week pre-acclimation period before being inoculated into fresh media at the same starting concentration of 100,000 cells mL⁻¹ and being cultured

for a further three weeks before being transferred to fresh media again. Every three weeks, aliquots of each population were taken to determine the growth rate, which was used to calculate the number of generations each replicate had gone through (see section Growth Rate and Number of Cell Generations for calculation of growth rate number of generations).

At the end of the six-week ratchet, if an individual population at 30°C showed positive growth, the population was transferred into the next temperature treatment (31°C), into fresh media at 100,000 cells mL⁻¹. If a population did not display positive growth it remained at the same temperature until the next ratchet cycle when growth performance was assessed again. The experiment continued in this way, with populations being transferred to incremental temperature increases every six weeks, where appropriate (**Figure 1**).

Our ratchet experimental design differed from that of Huertas et al. (2011) in three ways. Firstly, we allowed six weeks before assessing the growth of each population and deciding to move a population(s), whereas microalgal growth was assessed after only 15–20 days in Huertas et al. (2011). Six weeks at each treatment temperature was important in our experiment because we observed that for one strain (type D1, SCF086) the full, negative effects of elevated temperature on growth were cumulative and not observed before three weeks, which may have led to an uninformed decision on which populations were suitable to transfer to the next ratchet temperature. Alternatively, we did not want to mistake an initial, positive plastic response for a stable adaptive change. Secondly, we transferred cells into the next selecting temperature if they exhibited any positive growth, whereas in Huertas et al. (2011) only if a population exhibited growth equal to, or greater than, the control population, was it transferred; our decision was made based on a previous experiment (Chakravarti et al., 2017), where a laboratory selected SS strain of *Symbiodinium* (type C1) that had been cultured for approximately 80 generations at 31°C was not able to match the growth rate of its WT counterpart at the control temperature, but exceeded the growth of the WT transferred short-term into elevated temperature. Thirdly, even if



a population was transferred to the next temperature treatment, a copy was also always maintained at the previous temperature treatment(s). Previous temperatures may have been sub-optimal for a population, even if they were able to maintain some growth, thus more generations of culture could have allowed a (further) adaptive response to occur.

Testing for a Stable Adaptive Response

To test whether a stable adaptive response had occurred after ~1 year of thermal selection in the ratchet experiment, we carried out a transplantation experiment. This involved control or wild-type (WT) populations being both kept at 27°C (WT@27, 26.84°C ± 0.005, $n = 4394$) and also transferred to the elevated temperature treatments e.g., WT@30 (29.89°C ± 0.0004, $n = 54347$), WT@31 (31.02°C ± 0.021, $n = 4322$), WT@33 (32.93°C ± 0.016, $n = 4409$) and/or WT@34 (34.08°C ± 0.0009, $n = 4322$). Growth rates and photophysiological measurements of the selected *Symbiodinium* populations (SS) already at elevated temperature (SS@30, SS@31 etc.) were compared to those from WT cultures at control temperature moved to elevated temperatures.

Each WT population was transferred to fresh media at a cell density of 200,000 cells mL⁻¹ and pre-acclimated for two weeks to each temperature treatment that their counterpart SS resided in. SS populations were also transferred to fresh media and cultured for two weeks at their appropriate temperature conditions. The pre-acclimated cells were then transferred into fresh media at the same starting concentration to give three replicate cultures ($n = 3$) for each population in each temperature treatment.

On days 3, 10, 17, and 26 after inoculation, 100 μL aliquots of homogenised culture from each WT and SS replicate was transferred into a black, clear-bottom 96-well culture plate (Costar, Corning; Sigma-Aldrich) for photophysiology and growth rate measurements.

Photophysiology

Culture aliquots were transferred to multi-well plates during the end of the dark period and were dark-adapted for a minimum of a further 30 min at their appropriate temperature treatments. The maximum quantum yield of PSII ($F_v/F_m = (F_m - F_o)/F_m$)

was measured on dark-adapted samples using an imaging PAM (iPAM) with the measuring light set at 10 and gain and damping at two. The cultures were then subjected to 6 min of actinic light of two ($\text{PAR} = 20 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) before measuring the effective quantum yield of PSII ($\Delta F/F_m' = ((F_m' - F)/F_m')$). Cells were then fixed in the well-plates by adding $3.4 \mu\text{L}$ of 25% glutaraldehyde for later cell counts and calculation of growth rate.

Growth Rate and Number of Cell Generations

To measure growth rate, cell density was determined on day 17 post-acclimation in each replicate by triplicate haemocytometer counts. The specific growth rate (μ , doubling/day) was calculated as

$$u = \frac{\ln N_1 - \ln N_0}{\Delta t} \quad (1)$$

where N_0 is the cell density at the start (200,000 cells/mL) of the experiment and N_1 is the cell density at day 17 and t is the duration of culture (17) in days. To estimate the number of generations each population had been through during long-term selection, the doubling time (or generation time) was calculated, according to the equation

$$T = dx \left(\frac{\log(2)}{\log\left(\frac{N_1}{N_0}\right)} \right) \quad (2)$$

Where d is the number of days of growth and N_0 is the initial inoculation cell density (i.e., 100,000 cells mL^{-1} during the ratchet experiment and 200,000 cells mL^{-1} during the transplant experiment). Subsequently, the number of generations was calculated as

$$\text{Generations} = N/T \quad (3)$$

Where N is the number of days of culture and T is the doubling time.

Statistical Analyses

To test whether the selected *Symbiodinium* (SS) populations had mounted an adaptive response as a result of long-term thermal selection, we compared the growth and photosynthetic performance of the three SS populations (a-c) at each temperature treatment that they had reached (e.g., SS@30a/b/c, 31, 33, and/or 34) with the performance of the control population, maintained at 27°C long-term (e.g., WT@27) and the WT population transferred into the elevated temperature treatment (e.g., WT@30, WT@31, WT@33 and/or WT@34). This was done for each *Symbiodinium* type using linear models with “population” (i.e., WT@27, WT@30, SS@30a, SS@30b, SS@30c) as a fixed factor for both growth rate and photosynthetic efficiencies and with “time” as an additional fixed factor for the latter. All analyses were conducted in R v. 3.4.1 (R Core Team, 2016). Data Sheets 1 and 2 include all raw data.

RESULTS

Long-Term Culture

One strain of *Symbiodinium*, D1 (SCF082), was not able to grow at the first ratchet temperature of 30°C, despite two, six-week

attempts of culture and was therefore could not be included in the subsequent transplant experiment. *Symbiodinium* A3c, D1 (SCF086.01), F1 and G3 all exhibited positive growth at 30°C and were cultured for between 25 and 71 generations compared with 47–73 generations at 27°C (Table 2). Only two *Symbiodinium* types, F1 and G3, were able to grow at the next ratchet temperature of 31°C with all three SS populations of both types cultured at 31°C for up to 69 and 53 generations, respectively. At 33°C and 34°C only *Symbiodinium* F1 was able to grow, with the three SS populations having gone through 41–46 generations at this temperature by the end of the ratchet experiment. At the highest ratchet temperature, F1 was cultured for between 16 and 17 generations, before the transplant experiment was carried out.

Testing for a Stable Adaptive Response (Transplantation Experiment)

Symbiodinium A3c

Two of the A3c SS populations (SS@30a and SS@30c) were able to grow significantly faster than the WT@30 population (max $t_2 = 5.28$, $P < 0.01$, Table S1) by up to 49% ($\pm 8.80\%$) after long-term culture at 30°C, with the growth rate of population SS@30a being significantly faster than the WT@27 ($t_2 = 3.51$, $P = 0.04$, Table S1), by 28% ($\pm 7.53\%$ Figure 2A). Mean effective yields for the three SS@30 populations, were between 10.68% ($\pm 1.45\%$) and 21.75% ($\pm 2.61\%$) lower than the controls throughout the experiment, however they remained stable over the 26 days period (min $t = -1.79$, $P = 0.71$, Figure 2B). In contrast, the WT@30 population, showed a significant decline of 17.19% ($\pm 4.75\%$) in mean effective quantum yield by the end of the experiment with a final mean value that was significantly lower than that of the three SS@30 populations (max $t = 5.56$, $P < 0.01$, Table S1) by up to 23.92% ($\pm 4.75\%$, Figure 2B). The maximum quantum yield values followed a similar trend (Figure 2C), although the drop in WT@30 mean yield value by the end of the experiment was not as marked.

Symbiodinium D1

Long-term culture at 30°C did not improve the mean growth of any D1 SS population. In fact, one population, SS@30b grew 43.8% ($\pm 15.2\%$) slower than the WT@30 ($t_2 = -3.84$, $P = 0.02$, Figure 3A, Table S2). There was some variation in growth rates among the three SS populations, with SS@30b growing 43.1% ($\pm 15.4\%$) and significantly slower than SS@30c ($t_2 = 3.73$, $P = 0.03$, Table S2). In general, the SS@30 populations displayed a trend of both lower effective quantum yield values and maximum quantum yield values compared to both the WT@27 and WT@30 populations during the transplant experiment (Figures 3B,C), despite long-term thermal exposure.

Symbiodinium F1

After long-term thermal selection, one population, SS@30b, grew 24.84% ($\pm 0.95\%$) slower compared to the WT@27 ($t_2 = -3.810$, $P = 0.02$, Table S3) and 23.80% ($\pm 0.96\%$) slower compared to the WT@30 ($t_2 = -3.60$, $P = 0.03$, Figure 4A, Table S3), while the remaining two SS@30 populations (a and c), maintained mean growth rates that were similar to the WT@27 and WT@30 (min $t_2 = -2.37$, $P = 0.20$). By day 17 and 26, WT@27 and

TABLE 2 | Number of generations that each population spent at each temperature condition during the ~1-year ratchet experiment.

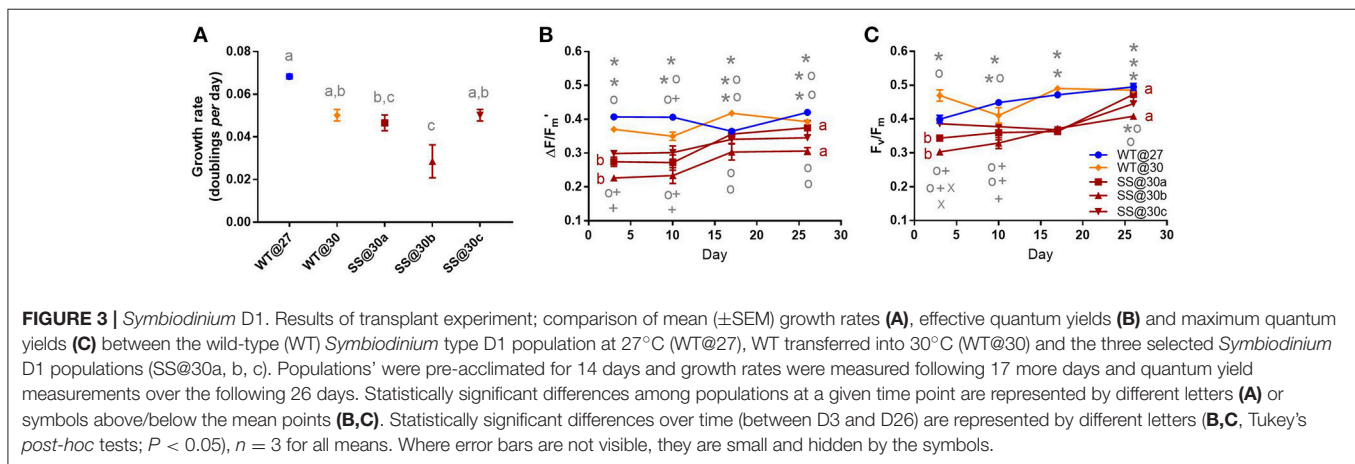
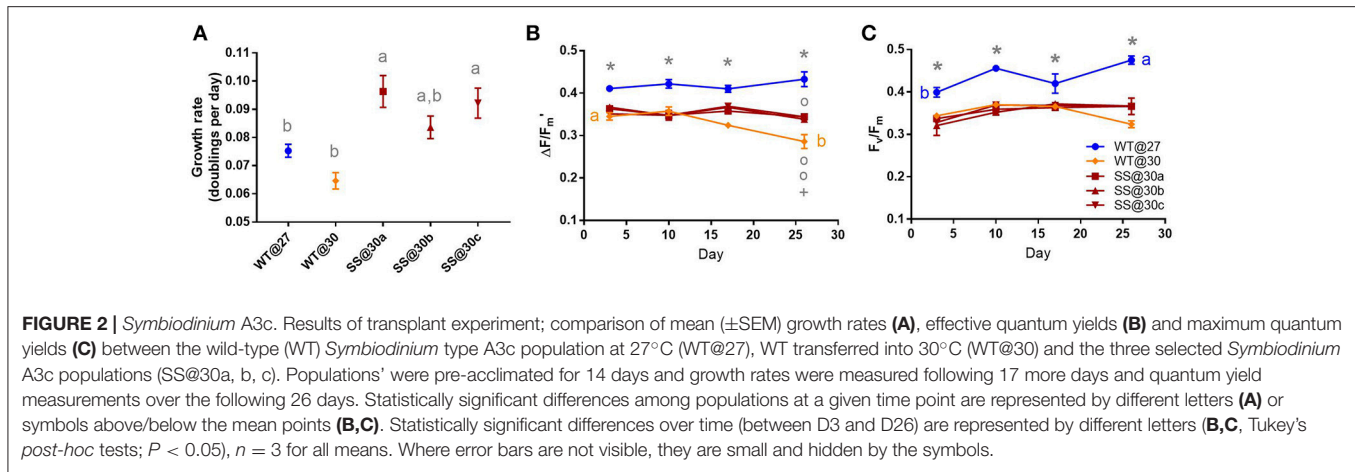
Symbiodinium type	Population	Generations at each temperature					Cumulative generations
		27°C	30°C	31°C	33°C	34°C	
A3c	WT@27	50					50
	WT@27	52					52
	WT@27	51					51
A3c	SS@30a		46				46
	SS@30b		44				44
	SS@30c		41				41
D1 (SCF086.01)	WT@27	47					47
	WT@27	48					48
	WT@27	49					49
D1 (SCF086.01)	SS@30a		26				26
	SS@30b		26				26
	SS@30c		25				25
F1	WT@27	69					69
	WT@27	72					72
	WT@27	70					70
F1	SS@30a		69				69
	SS@30b		66				66
	SS@30c		67				67
F1	SS@31a		9	62			70
	SS@31b		9	59			68
	SS@31c		9	61			70
F1	SS@33a		9	18	41		68
	SS@33b		9	18	42		69
	SS@33c		9	18	43		70
F1	SS@34a		9	18	26	17	69
	SS@34b		9	18	26	16	69
	SS@34c		9	18	25	17	68
G3	WT@27	73					73
	WT@27	75					75
	WT@27	71					71
G3	SS@30a		71				71
	SS@30b		69				69
	SS@30c		70				70
G3	SS@31a		6	52			58
	SS@31b		6	52			57
	SS@31c		6	51			57

WT@30 effective quantum yield values were reduced by as much as 24.74% ($\pm 1.25\%$), compared to all three SS populations, which had statistically similar yields (min. $t = -1.62$, $P = 0.98$, **Figure 4B**). Similarly, maximum quantum yield values became reduced for the WT@27 and WT@30 populations at day 26; by up to 19.56% ($\pm 0.40\%$) compared with the SS populations ($\pm 0.14\%$, min $t = -8.12$, $P < 0.01$, **Figure 4C**, Table S3).

Long-term culture at 31°C did not significantly affect the growth rates of the F1 SS populations nor did the short-term transfer of WT cells into 31°C (WT@31, max $t_2 = 2.73$, $P = 0.12$, **Figure 4D**). During the latter half of the experiment, the three SS@31 populations and the WT@31 population displayed mean effective quantum yield values that were up

to 17.26% ($\pm 1.93\%$) greater than the mean values for the WT@27 control (max $t = 5.90$, $P < 0.01$, **Figure 4E**, Table S4). Similar trends were apparent for the mean maximum quantum yield values (**Figure 4F**), with the WT@27 having significantly lower values than the remaining treatments by up to 13.50% ($\pm 0.94\%$) at day 26 (max $t = 5.98$, $P < 0.01$, Table S4).

Similarly to the 31°C experiment, no significant differences in growth rate existed between any F1 population in the 33°C transplant experiment (max $t_2 = 2.00$, $P = 0.33$, **Figure 4G**). Furthermore, differences in both mean effective quantum yields and maximum quantum yields by the end of the experiment, were minimal (**Figures 4H,I**, Table S5).



After long-term selection for F1 populations at 34°C there were no significant differences in mean growth rates between the three populations (max $t = -1.65$, $P = 0.50$), however, the SS@34b population exhibited a mean growth rate that was 22% ($\pm 2.10\%$) faster than the WT@34 population ($t_2 = 3.81$, $P = 0.02$, **Figure 4J**, Table S6). Effective quantum yield values between all F1@34 populations, by the end of the experiment, were similar (max $t = 0.20$, $P = 1.00$, **Figure 4K**). Further, maximum quantum yield values were not significantly different to each other between the populations by the end of the experiment (max $t = -2.14$, $P = 0.82$ **Figure 4L**).

Symbiodinium G3

There were no significant differences in growth rate between any of the G3 populations in the 30°C transplant experiment (max $t_2 = 1.71$, $P = 0.47$, **Figure 5A**). There were also no notable differences in effective quantum yield values by the end of the experiment (max $t_2 = 0.03$, $P = 1.00$, **Figure 5B**, Table S7) or maximum quantum yield values (max $t_2 = 1.445$, $P = 0.10$, **Figure 5C**, Table S7).

Wild-type (WT) G3 cells transferred into 31°C grew 27.9% ($\pm 1.78\%$) slower, compared to the WT@27 controls ($t_2 = -4.23$, $P = 0.01$, Table S8). For two of the SS populations (b and c) growth rates were intermediate to that of WT@31 and WT@27,

and not significantly different (min $t_2 = -2.26$, $P = 0.24$). However, for the remaining SS@31a population, the mean growth rate was similar to the WT@27 cells ($t_2 = -2.260$, $P = 0.999$) and significantly greater, by 36% ($\pm 9.82\%$), than the WT@31 cells ($t_2 = 3.97$, $P = 0.02$, **Figure 5D**, Table S8). The mean effective quantum yield for the WT@31 population was significantly lower by up to 13.47% ($\pm 3.74\%$) compared to the WT@27, SS@31a and SS@31b populations at three days post-acclimation (max $t = -3.92$, $P = 0.04$, Table S8). By the end of the experiment, however, mean effective quantum yields were similar among all populations (min $t = -0.57$, $P = 1.00$, **Figure 5E**). Again, at three days post-acclimation the WT@31 population had the lowest maximum quantum yield values compared to the WT@27, SS@31a and SS@31b populations (max $t = -4.06$, $P = 0.02$, **Figure 5F**, Table S2), by up to 17.27% ($\pm 4.62\%$), but by the end of the mean values were similar among all populations (min. $t = -0.57$, $P = 1.00$, **Figure 5F**).

DISCUSSION

The thermal tolerance response to long-term exposure to increasing temperatures of five *Symbiodinium* wild-types (WT) representing four distinct phylogenetic clades exhibited considerable variation. After one year of culture at step-wise

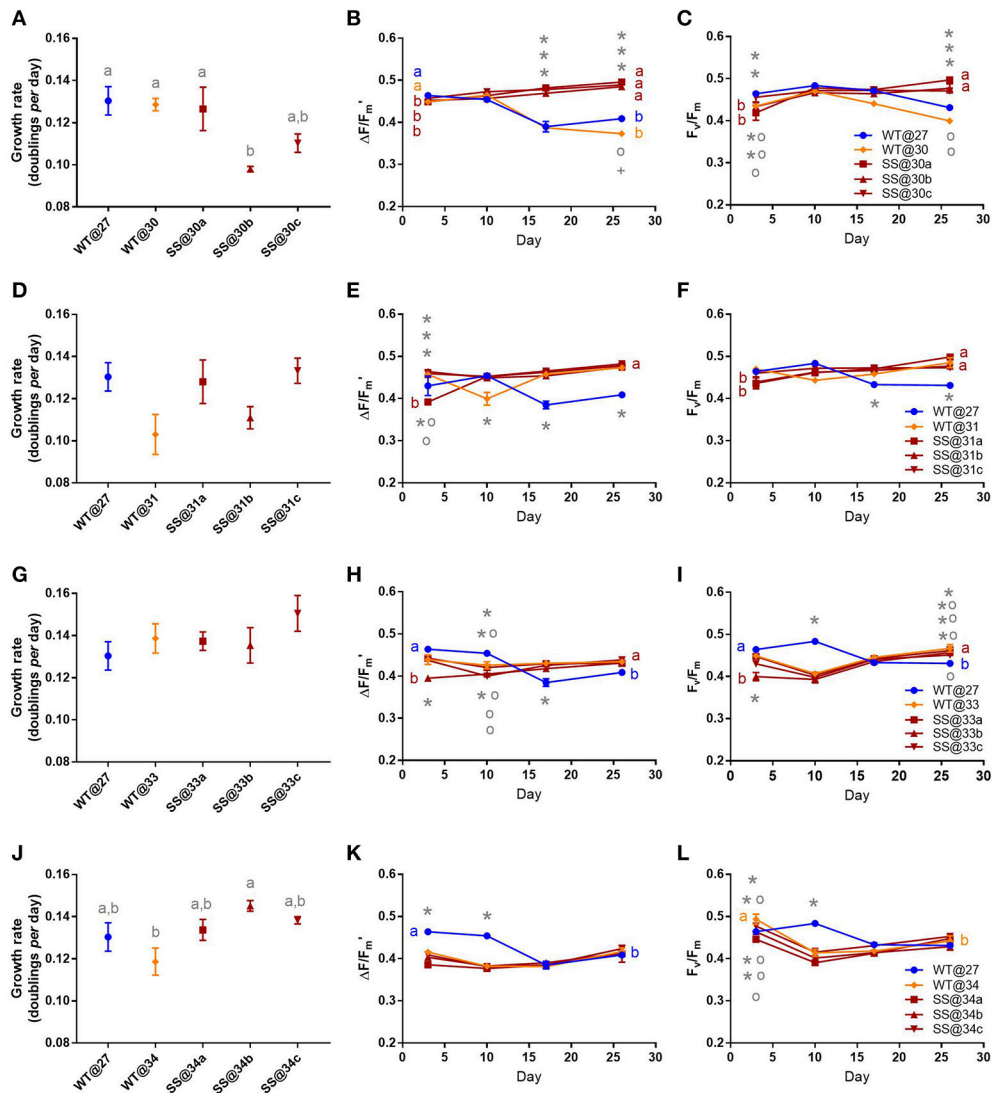


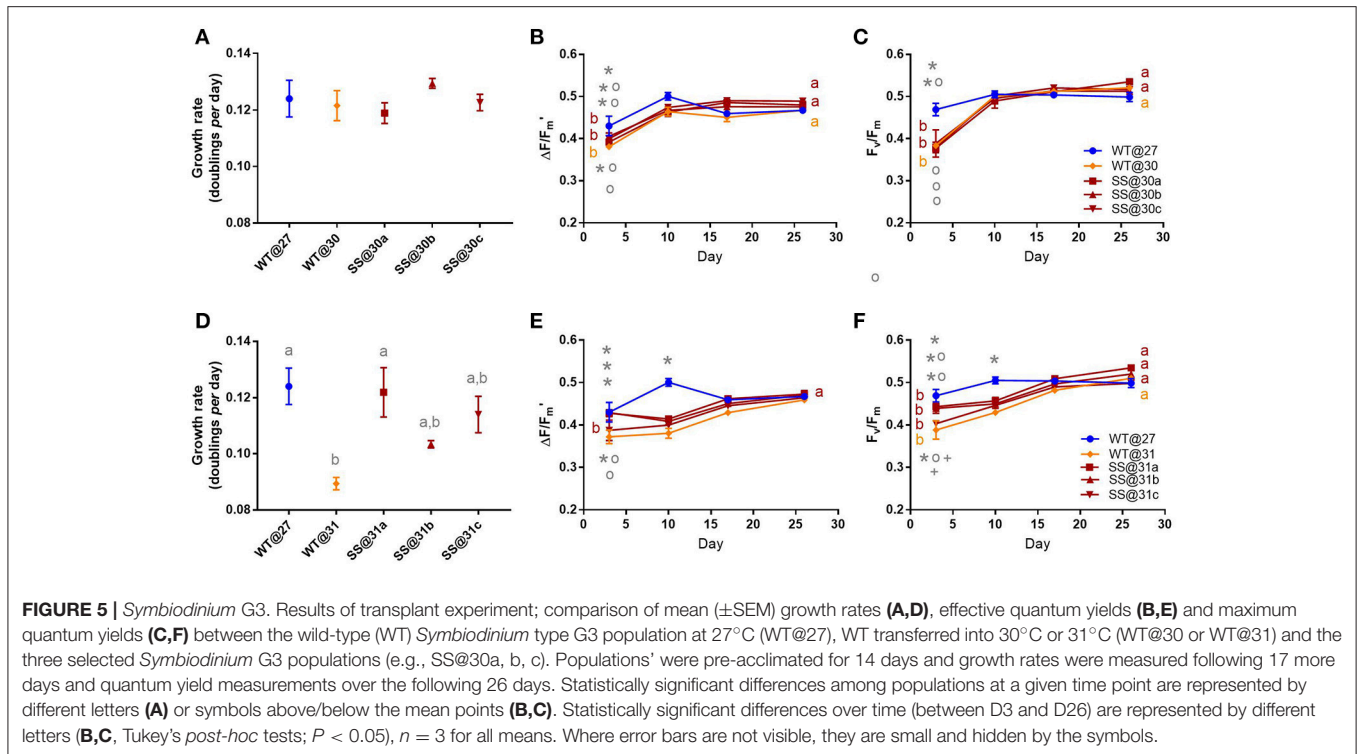
FIGURE 4 | *Symbiodinium* F1. Results of transplant experiment; comparison of mean (\pm SEM) growth rates (**A,D,G,J**), effective quantum yields (**B,E,H,K**) and maximum quantum yields (**C,F,I,L**) between the wild-type (WT) *Symbiodinium* type F1 population at 27°C (WT@27), WT transferred into elevated temperature (WT@30, WT@31, WT@33, or WT@34) and the three selected *Symbiodinium* F1 populations (e.g., SS@34a, b, c). Populations were pre-acclimated for 14 days and growth rates were measured following 17 more days and quantum yield measurements over the following 26 days. Statistically significant differences among populations at a given time point are represented by different letters (**A**) or symbols above/below the mean points (**B,C**). Statistically significant differences over time (between D3 and D26) are represented by different letters (**B,C**, Tukey's *post-hoc* tests; $P < 0.05$), $n = 3$ for all means. Where error bars are not visible, they are small and hidden by the symbols.

increases in temperature, three of the five *Symbiodinium* types showed signs of a stable adaptive change; two thermally-derived populations of type A3c at 30°C, one of type G3 at 31°C and one of type F1 at 34°C after only 41–69 generations at elevated temperature showed increased performance at elevated temperatures compared to the respective WT cultures.

***Symbiodinium* A3c**

The acute heat stress (i.e., transplant) experiment of the WT A3c cells into the first elevated temperature treatment of 30°C resulted in a 17% decrease in growth rate and declines in

photosynthetic efficiencies, suggesting a relatively low natural thermal tolerance for this strain. This is perhaps surprising as the literature describes most *Symbiodinium* clade A strains as thermally tolerant (Robison and Warner, 2006; Reynolds et al., 2008; Kemp et al., 2014) and typically prevalent in shallow-water cnidarians, where high light and temperature co-occur (LaJeunesse, 2002). This has generally been attributed to enhanced and alternative photosynthetic electron pathways such as cyclic electron transport and light harvesting complex dissociation mechanisms as well as photoprotection processes (Reynolds et al., 2008). In contrast, two of the SS A3c



populations in our study that were cultured for up to 46 generations at 30°C, were able to grow 43–49% faster than the WT@30 and up to 28% faster than the WT at the control temperature. Furthermore, throughout the transplant experiment, the photosynthetic efficiencies of the WT@30 significantly decreased over time, while the SS populations were able to maintain high photosynthetic efficiencies. These results suggest a stable, adaptive change having occurred after only up to 46 generations at 30°C.

Current evidence on the value of members of clade A to the coral host is varied. Clade A *Symbiodinium* have been associated with the fast colonisation of health-compromised corals (Toller et al., 2001), low transfer of photosynthate compared to C1 (Stat et al., 2008) and high competitive ability in mixed *in vitro* cultures (Rowan, 1998). Furthermore, there have been reports of wide phenotypic and physiological diversity of clade A (Robison and Warner, 2006; Takahashi et al., 2009; Ragni et al., 2010; Krämer et al., 2012). These factors have led to the suggestion that members of clade A are more parasitic in nature and suboptimal as symbionts (Stat et al., 2008; Mieog et al., 2009). However, other studies have suggested their ecological value; type A3c was associated with juvenile GBR *Acropora tenuis* that had lower mortality than when harbouring other types of *Symbiodinium* (Quigley et al., 2016) and early larval uptake of clade A in *Acropora yongei* was associated with a higher post-settlement survival rate (Suzuki et al., 2013). These studies suggest that some members of clade A could be valuable in experimental evolution studies aimed at enhancing the tolerances of the early life stages of coral. Thus, the stable enhanced thermal tolerance we observed here is promising,

particularly because we observed this in two of three SS populations.

Symbiodinium D1

We experimented with two clade D strains of *Symbiodinium* that are genetically distinct based on ITS2 sequences. Surprisingly, neither were particularly thermally tolerant, considering members of clade D are widely described as being heat resistant in the literature. The D1 strains, SCF082 and SCF086.01, were not able to grow at, or above 30°C, respectively; for *Symbiodinium* type D1 (SCF086.01), SS populations managed to grow for only 25–26 generations at 30°C over a one-yr period, compared to the 47–49 generations of the WT@27. Furthermore, long-term culture at 30°C had a negative effect on the photosynthetic efficiencies of the SS populations and a 44% decrease in growth of one population. In contrast, *Symbiodinium* clade D has often been found to dominate corals on reefs that are exposed to heat stress or have had a history of bleaching (Glynn et al., 2001; Toller et al., 2001; Baker et al., 2004; Rowan, 2004; van Oppen et al., 2005a; Jones et al., 2008; LaJeunesse et al., 2009; Pettay et al., 2015) and has been shown to enhance the thermal tolerance of the coral *Acropora millepora*, a common Indo-Pacific species, by 1.0–1.5°C through the shuffling of symbiont types (Berkelmans and van Oppen, 2006). Other studies have shown high abundances of *Symbiodinium* clade D in corals at 32°C in Thailand (LaJeunesse et al., 2010), 31.8°C in Palau (Fabricius et al., 2004) and >33°C in the Persian Gulf (Baker et al., 2004; Mostafavi et al., 2007). This suggests that there is either wide variation in thermal tolerance between clade D types and/or thermal tolerances are different

in free-living *Symbiodinium* cultures compared to *in hospite* populations.

Symbiodinium F1

Symbiodinium type F1 was the most thermally tolerant strain in this study. In the transplant experiments, growth rates of the WT transferred to 30, 31, 33, and 34°C did not significantly change, while photosynthetic efficiencies remained similar. This agrees with previous *in vitro* studies on other members in this clade; between 25°C and 33°C, a different strain of *Symbiodinium* F1 did not show any differences in growth or photosynthetic efficiencies over two weeks, while expressing significantly higher levels of antioxidant defense enzymes at 33°C aimed to scavenge reactive oxygen species (ROS) produced as a by-product of photosynthesis and cellular respiration (Krueger et al., 2014). The ability to mitigate damage caused by excess ROS was also shown by *Symbiodinium* F2 after 12 days at 29–31°C (McGinty et al., 2012), while the same F2 isolate showed the greatest capacity for both photo-acclimation and growth at 32°C compared with isolates belonging to clades A and B (Robison and Warner, 2006).

Long-term culture in this study did not significantly affect the ability of F1 to grow or its photosynthetic efficiency under elevated temperature, apart from two cases. Firstly, 66–67 generations at 30°C resulted in a significant drop in growth rate for two SS populations (SS@30b and c), with SS@30b being statistically lower than the WT@30 transplant by 23.80% and WT@27 control by 24.84%. Despite this, the photosynthetic efficiencies of all three SS populations were greater than the WT@27 and WT@30 across much of the transplant experiment and remained stable. Secondly, long-term culture at 34°C enhanced the growth rate of one SS population (SS@34b) such that it grew 18% faster than the WT@34.

Symbiodinium type F1 is rarely described in the literature, in symbiosis or as free-living populations. Clade F made up only 0.9% of *Symbiodinium* sequences recovered from 10 coral species, sediments and water samples across contrasting thermal environments in American Samoa (Cunning et al., 2015). In the Indian Ocean, 47 ITS2 *Symbiodinium* types were characterised from over 600 samples that ranged over 50 host genera and only one was found to harbour a member of clade F, an anemone species (LaJeunesse et al., 2010). Only one of 16 coral species, *Goniastrea* sp., around the Arabian Peninsula harboured clade F (F4.3) and at >5% abundance (Ziegler et al., 2017). In the GBR, eastern Pacific and Caribbean 114 coral species revealed no *Symbiodinium* clade F (van Oppen et al., 2005b). Such a lack of biogeographic prevalence and abundance is surprising given this clade's ability to cope with heat stress. This calls for the need to study clade F's efficacy as a symbiont.

Symbiodinium G3

After 57 generations at 31°C, a population of *Symbiodinium* type G3 was able to grow 36% faster than the WT@31, with small but apparent improvements in photosynthetic efficiencies. These results suggest a stable adaptive change had occurred after only one year at elevated temperature for at least one of the SS G3 populations.

Clade G has been found to associate with a wide variety of hosts and environments including sponges (Schönberg and Loh, 2005; Granados et al., 2008; Schönberg et al., 2008; Hill et al., 2011), soft corals (van Oppen et al., 2005a) and foraminiferans (Pochon et al., 2001), and is also known to occur in the water column and in benthic sediments (Takabayashi et al., 2012). Occasionally, members of clade G have been found to associate with scleractinian corals including *Porites lobata* in Hawaii (Stat et al., 2013) and low frequencies (>0.09% of all sequences) have been observed in *Orbicella annularis* in US Virgin Islands (Edmunds et al., 2014; Pochon et al., 2014) and *Acropora* in Western Australia (Thomas et al., 2014).

Although members in this clade do not seem to be common symbionts of reef-building corals, our study strain, extracted from a scleractinian coral species *Diploastrea heliophora* in the GBR, adds to the growing body of evidence of clade G being able to form a symbiosis with scleractinian corals. Its ability to exhibit a stable adaptive change after one year of culture at 31°C, is promising for evolutionary experiments aimed to develop heat resistant *Symbiodinium*.

Experimental Evolution in Microalgae and Mechanisms Underlying Adaptive Changes

To our knowledge, there have been five experimental evolution studies involving other species of marine microalgae that have subjected cells to long-term elevated temperature (Table 3). Growth rate is used as the universal indicator of fitness, allowing for comparisons between experiments. These studies indicate that after 20–460 generations (from 45 days to two years) there have been stable increases in growth of between 16 and 60% for elevated-temperature-derived strains compared to ancestral populations placed at acute elevated temperature; results comparable with our findings of increases in growth rate of 22–49%. Evolutionary experiments involving *Symbiodinium* have been carried out on only three different strains, which are reported in two studies (Table 3). After only 55 generations (Huertas et al., 2011) to 80 generations (Chakravarti et al., 2017) *Symbiodinium* were able to grow at elevated temperatures of 30°C and 31°C, respectively, where previously there had been no growth at these temperatures.

The current study adds three more strains of *Symbiodinium* that provide evidence of a stable adaptive change to increased temperature. These stable, physiological improvements could have resulted from either genetic adaptation through selection on random, beneficial mutations and/or acclimatisation processes that can be heritable across generations, *via* epigenetic modifications of gene expression *via*, for example, DNA methylation, small RNAs and chromatin modifications (Bird, 2002; Greer and Shi, 2012; Castel and Martienssen, 2013). Under experimental heat stress a warmer water population of *Symbiodinium* C1 significantly up-regulated ROS scavenging and molecular chaperone gene expression, vital for repairing damaged proteins, compared to a cooler water population of *Symbiodinium* C1, despite both being identical in ITS1 and ITS2 sequences and cultured under “ambient” conditions for four years prior to experiments

TABLE 3 | Experimental evolution studies on marine microalgae that show an adaptive response to elevated temperature.

Microalgal species	Taxa	Selective condition(s)	Temperature increase	Growth rate increase	Approx. generations	Approx. duration	Study
<i>Procentrum triestinum</i>	dinoflagellate	25°C + elevated nutrients	5°C	12%	400	2 years	Flores-Moya et al., 2008
<i>Isochrysis galbana</i>	haptophyte	35°C	5°C	*	40–50	unknown	Huertas et al., 2011
<i>Tetraselmis suecica</i>	green alga	35°C	5°C	*	90–120	unknown	Huertas et al., 2011
<i>Procentrum triestinum</i>	dinoflagellate	30°C	8°C	*	25–30	unknown	Huertas et al., 2011
<i>Nitzschia closterium</i>	diatom	30°C	8°C	*	20–30	unknown	Huertas et al., 2011
<i>Navicula sp.</i>	diatom	30°C	8°C	*	20–34	unknown	Huertas et al., 2011
<i>Symbiodinium</i>	dinoflagellate	30°C	8°C	*	55–65	unknown	Huertas et al., 2011
<i>Symbiodinium</i>	dinoflagellate	30°C	8°C	*	60–70	unknown	Huertas et al., 2011
<i>Procentrum triestinum</i>	dinoflagellate	25°C + reduced pH	5°C	50–60%	250	2 years	Flores-Moya et al., 2012
<i>Emiliana huxleyi</i>	coccolithophore	26°C + elevated CO ₂	11°C	16%	460	1 year	Schlüter et al., 2014
<i>Symbiodinium C1</i>	dinoflagellate	31°C	4°C	*	80	2.5 years	Chakravarti et al., 2017
<i>Symbiodinium A3c</i>	dinoflagellate	30°C	3°C	43–49%	41–46	1 year	This study
<i>Symbiodinium F1</i>	dinoflagellate	34°C	7°C	22%	69	1 year	This study
<i>Symbiodinium G3</i>	dinoflagellate	31°C	4°C	36%	58	1 year	This study

*Temperature increase" represents the increase in temperature from experimental ambient to derived experimental conditions. "Growth rate increase" is that of derived cells compared to the growth of ancestral cells transferred to derived conditions. *For some strains there was no growth when ancestral cells were transplanted into derived conditions.

(Levin et al., 2016). Gene expression responses such as these can result from genetic adaptation or epigenetic processes where historical stress induced modifications that are inherited across generations (Bonduriansky et al., 2012); either (or both) could be mechanisms for observed enhanced thermal tolerances in our study. It is possible that the historical, laboratory conditions, particularly the comparatively low light conditions that the strains were cultured under could have had some influence on their physiological responses to elevated temperature.

It is important to note that most long-term evolutionary studies on microalgae have inoculated experimental replicates with a single cell to ensure each replicate started with only one genotype (e.g., Huertas et al., 2011; Lohbeck et al., 2012). In our study, cultures A3c and F1 originated from a single cell, however G3 and clades D did not. It is therefore possible that either selection on standing genetic variation and/or selection on random, beneficial mutations could have occurred in our ratchet experiment. Additionally, it is possible that for those originally monoclonal isolates, genetic diversity arising from random somatic mutations before carrying out our study may have been enough genetic variation for selection to act upon when our experiment commenced. Furthermore, our study and other evolutionary experiments on microalgae assume mitotic, asexual cell divisions produce genetic adaptations or induce heritable acclimatisation responses across generations. However, the discovery of meiosis-specific genes in the *Symbiodinium* genome indicates that sexual reproduction is possible in *Symbiodinium* (Chi et al., 2014; Rosic et al., 2015; Levin et al., 2016). This has led to the hypothesis that a switch from asexual to sexual reproduction (i.e., inducing recombination) may be a response mechanism to environmental stress in *Symbiodinium* (Chi et al.,

2014; Wilkinson et al., 2015), although it has not yet been observed.

Considerations for *Symbiodinium* Experimental Evolution as a Tool for Coral Assisted Evolution

We show that *Symbiodinium* types F1 and G3 are the most naturally thermally tolerant strains in this study, able to survive and grow at 34 and 31°C, respectively, even without long-term thermal selection. Despite their heat resistance, members of clades F and G are some of the least prevalent in corals, with clades A, B, C, and D most commonly described as being associated with scleractinians (van Oppen et al., 2009). *Symbiodinium* types in clade D, the least thermally tolerant strains in this study, with one strain unable to grow at the lowest selecting temperature and the other exhibiting a suppressed photosynthetic response after thermal selection, are the most prevalent in corals after bleaching events. Our findings highlight the complex relationship between symbiont identity, symbiont thermal tolerance *ex hospite* and holobiont bleaching tolerance (Bhagooli and Hidaka, 2003; Goulet et al., 2005; Chakravarti et al., 2017; Gabay et al., 2018). Here, the continuum of parasitism to mutualism of photosymbionts (Baker et al., 2018) is an important consideration in *Symbiodinium* thermal selection experiments whereby the relative contribution of a symbiont to the host should be considered, for example, the amount of photosynthate produced and released, in addition to a strains ability to be taken up by the host and retained under heat stress.

It is important to consider the traits that are assessed during *Symbiodinium* evolutionary experiments. Here we use growth rate as well as photosynthetic efficiencies as key traits.

However, additional, or alternative traits may be required to better predict the fitness of corals inoculated with laboratory-evolved *Symbiodinium* strains. For example, fast growth rate, although an important indicator of fitness, may not be advantageous to a symbiont, or its host, *in hospite*. Additionally, in terms of photophysiology, a recent study showed that corals containing type D1a were able to retain a symbiosis at 33°C despite significant losses in photosynthetic function, while those harbouring type C3 also experienced photodamage but in contrast, they lost 99% of their symbionts (Silverstein et al., 2017).

It is also important that for temperature-selected *Symbiodinium* in evolutionary experiments, we test their contribution to the host under heat stress, as well as their symbiotic performance at ambient temperatures—not just their ability to infect and be retained. For instance, following a bleaching event on the GBR in 2006, bleached *Acropora millepora* colonies harbouring clade C were able to recover lipid levels to those of unbleached C colonies, however clade D colonies that had remained unbleached had 26% lower stored lipids compared to C colonies with 28% smaller eggs and did not recover pre-bleaching lipid levels for nine months (Jones and Berkelmans, 2011). In such cases, perhaps factors such as the extracellular production of ROS by *Symbiodinium* or lack of photosynthate release contributed the fitness of the holobiont. Studies investigating *Symbiodinium* carbon fixation, release and incorporation into host tissue through radioactive ¹⁴C labelling and stable isotopic ¹³C and ¹⁵N labelling (Biel et al., 2007; Stat et al., 2008; Cantin et al., 2009; Baker et al., 2013, 2018; Pernice et al., 2015; Matthews et al., 2017) together with newer technologies such as nanoscale secondary ion mass spectrometry (NanoSIMS) (e.g., Wangpraseurt et al., 2016) will be crucial for understanding the relative function of the symbiont within the host. This, along with measuring other physiological parameters relating to coral fitness response when harbouring laboratory-evolved *Symbiodinium* as well as gene expression studies (e.g., DeSalvo et al., 2010; Pinzon et al., 2015; Traylor-Knowles et al., 2017), will thus be informative.

Finally, inoculation of aposymbiotic corals with a mix of experimentally evolved *Symbiodinium* types remains an important consideration. For example, it is possible that the host environment and/or nutritional needs of corals change throughout their life history such that different *Symbiodinium* confer different advantages depending on its hosts' life stage (Abrego et al., 2008; Jones et al., 2008; Quigley et al., 2016). Furthermore, absolute specificity is rare in corals (e.g., Silverstein et al., 2012; Quigley et al., 2014) and thus infecting corals with a cocktail of thermally selected strains could allow the host to retain those most beneficial under thermal stress through symbiont community shuffling (see Chen et al., 2005; Berkelmans and van Oppen, 2006; Thornhill et al., 2006; Mieog et al., 2007), aiding in a flexible response to environmental change. Additionally, it is important that thermal tolerance of *ex hospite* *Symbiodinium* is not overlooked. Eighty five per cent of scleractinian coral species are broadcast spawners, of which 80% acquire symbionts horizontally, from free-living populations (Baird et al., 2009). It is therefore critical that the

free-living *Symbiodinium* pool remains thermally tolerant, as an exogenous source for coral larvae and recruits as well as bleached colonies (Boulotte et al., 2016). Our results show that rapid adaptation is indeed possible in free-living *Symbiodinium* populations undergoing directed selection and we show that this is possible across multiple genetically distinct strains of *Symbiodinium*.

CONCLUSIONS AND NEXT-STEPS

After only one year of thermal selection and as few as 41 generations, we were already able to observe stable adaptive responses to elevated temperature in three of the five strains of *Symbiodinium* included in this study. The observed increases in growth rates are comparable with evolutionary experiments in other microalgae, where selected populations have been exposed to elevated temperatures for up to 460 generations. A further year of thermal selection and we would expect to see the divergence in thermal tolerance of selected populations compared with ancestral ones to expand.

Important next-steps are to thoroughly investigate the infectivity of these strains under different temperature conditions, their stability *in hospite*, their effect on holobiont bleaching tolerance and health across different coral life stages, and to understand if and why thermal tolerance differs *ex-* and *in hospite*. Such experiments will provide insight into which clades and types of *Symbiodinium* will be better sources for evolutionary experiments and which traits should be targeted in directed selection experiments that aim to develop symbionts with the ability to increase coral bleaching tolerance.

AUTHOR CONTRIBUTIONS

LC and MvO contributed to conception and design of the study; LC performed the experiments and analyses. LC wrote the first draft of the manuscript with input from MvO.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2018.00227/full#supplementary-material>

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