

RESEARCH ARTICLE

Experimental evolution of the coral algal endosymbiont, *Cladocopium goreau*: lessons learnt across a decade of stress experiments to enhance coral heat tolerance

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Projected increases in sea surface temperatures will exceed corals' ability to withstand heat stress within this century. Experimental evolution of cultured symbionts (Symbiodiniaceae) at high temperatures followed by reintroduction into corals can enhance coral heat tolerance. Several studies have selected for enhanced tolerance in *Cladocopium goreau* (C1) over multiple time scales and then compared the performance of coral juveniles infected with the heat-tolerant C1 selected strain (SS) to the performance of juveniles infected with the C1 wild type (WT). To derive lessons about host benefits when symbionts are experimentally selected, here we compare the performance of SS- and WT-juveniles after 21 cell generations of heat selection versus longer periods (73–131) in recently published experiments. After 21 generations, we found rapid improvement in heat tolerance of SS through an overall shift in the mean tolerance to temperature. This did not translate to improved growth and survivorship of the coral. Specifically, survival did not differ significantly between juveniles of *Acropora tenuis* hosting WT versus SS at any temperature. Juveniles infected with WT exhibited greater skeletal growth than those infected with SS at 27 and 31°C but not at 32.5°C. SS-juvenile symbiont cell densities increased significantly at 27°C relative to SS-juveniles in the 31 and 32.5°C. Photosynthetic efficiencies in SS-juveniles were higher compared to WT-juveniles at 31°C, equal at 27°C, and lower at 32.5°C. These results suggest that selection over longer generation (>130) times will be needed to confer host benefits and will be dependent on the stability of this association being maintained in nature.

Key words: assisted evolution, coral reefs, heat tolerance, restoration, Symbiodiniaceae

Implications for Practice

- The use of assisted evolution with Symbiodiniaceae results in rapid acquisition of heat tolerance of the symbiont but not necessarily of the host after short periods of experimental selection. To further assess the effectiveness of experimental evolution, a range of different cell generation times across individually cultured strains is needed.
- To overcome limitations due to long cell generation times, culturing of new Symbiodiniaceae taxa that may provide improved host benefits should be initiated urgently. Further studies will then be needed to gauge whether hosts benefit from ongoing symbiont assisted evolution and the tractability of this approach for restoration.
- Thermal performance curves of juveniles associated with a diversity of Symbiodiniaceae strains and exposed to different temperatures are recommended.

(Gleason & Wellington 1993; Takahashi et al. 2009; Krämer et al. 2012; Hughes et al. 2018). Increases in temperatures of 0.4–2.6°C are projected to exceed corals' natural thermal tolerance limits and lead to bleaching, defined as the loss of their symbiotic dinoflagellates (Symbiodiniaceae) (Bhagooli et al. 2008; Hoegh-Guldberg 2011; Krämer et al. 2012). Symbiodiniaceae provide corals with most of their metabolic requirements (Muscatine & Porter 1977). Coral bleaching is often followed by lower fecundity and growth rates, increased susceptibility to disease, and eventual mortality if heat stress

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Introduction

Coral reefs are amongst the most threatened ecosystems on the planet, particularly from rising sea surface temperatures

continues (Szmant & Gassman 1990; Brandt & McManus 2009; Hoegh-Guldberg 2011). If coral ecosystems are to persist into the future, corals will need to either acclimatize or adapt to increasing ocean temperatures and other stressors like rising pCO₂ (Torda et al. 2017). One proposed avenue of acclimatization involves the formation of symbioses with naturally or artificially selected thermally tolerant Symbiodiniaceae (van Oppen et al. 2015).

Interactions between the coral host and their endosymbionts influence the capacity for corals to resist elevated seawater temperatures by increasing the physiological breadth of the coral holobiont (Ware et al. 1996; Day et al. 2008; Parkinson et al. 2015; van Oppen et al. 2015). Photochemical efficiency, which characterizes the efficiency with which electrons are transferred through the photosystem complex of algal symbionts (Werner et al. 2001; Lesser et al. 2013; Roth 2014), is an indicator of photosynthetic health, whereby declines indicate photodamage (Warner et al. 1996; Baker et al. 2008; Warner et al. 2011) or the activation of photoprotective mechanisms (Brown et al. 1999). Symbiodiniaceae are able to adjust their light-harvesting pigments to accommodate increased temperatures by balancing the amount of light absorbed to limit photo-inhibition and damage to PSII (Niyogi 1999; Hennige et al. 2009). Importantly, Symbiodiniaceae vary substantially in their photobiology, resistance to photosynthetic stress, and their influence on host metabolism via carbon transfer (Suggett et al. 2015). Hence, Symbiodiniaceae play a key role in regulating corals' susceptibility to bleaching via their photosynthetic responses.

The capacity of corals to adapt or acclimatize to environmental change is a function of characteristics of both the coral host and its symbiotic partners (Symbiodiniaceae and prokaryotic communities). Acclimatization responses of the host may be rapid, in some cases involving rapid changes in plasticity in host gene expression (Kenkel & Matz 2016), and could become fixed during ontogeny through irreversible (developmental) plasticity (Schaefer & Ryan 2006; Hoegh-Guldberg et al. 2007; Mieog et al. 2009). One mechanism of rapid acclimatization involves varying the relative abundance of the community composition of symbionts with changing environmental conditions ("shuffling") (Fabricius et al. 2004; Quigley et al. 2018). Within the family Symbiodiniaceae, shuffling from the dominance in relative abundance of *Cladocopium goreaui* (C1) to increased abundances of *Durudinium* (formerly clade D) increased the thermal tolerance of *Acropora millepora* by 1–1.5°C (Berkelmans & van Oppen 2006). However, the increased heat tolerance provided by some symbiont types comes at a cost, such as decreased growth and carbon translocation (Little et al. 2004; Cantin et al. 2009). In addition, because Symbiodiniaceae have orders of magnitude faster generation times compared to their hosts, they are ideal candidates for rapid adaptation (van Oppen & Medina 2020). The capacity to maximize the rate of adaptation of algal symbionts in culture, given the greater potential to generate beneficial mutations through accelerated growth rates in culture compared to *in hospite* (van Oppen et al. 2011; Chakravarti et al. 2017), makes experimental

evolution of Symbiodiniaceae a potentially viable option for enhancing the thermal tolerance of corals.

The application of assisted evolution interventions in the field has been limited thus far to studies manipulating host genetics via methods like assisted gene flow (van Oppen et al. 2014; Quigley et al. 2016). Manipulations involving selected symbionts have not yet been deployed in the field. Therefore, ecological evidence of the relevance of these methods is needed. However, to prepare for field deployment, information as to the feasibility and safety of these interventions in the lab is a critical first step. To explore the efficacy of artificially selected Symbiodiniaceae to enhance the thermal tolerance of corals, lab experiments after differing artificial selection periods provide important knowledge about the time scales, feasibility (scalability and time effort for implementation), and benefits/costs required to accomplish viable experimental evolution. To date, there have been three experimental studies utilizing strains of *C. goreaui* chosen for their thermal tolerance over recent years (Fig. 1). All of the strains were developed at the Symbiont Culture Facility at the Australian Institute of Marine Science (AIMS). Hence, comparing these three studies provides a baseline for identifying time scales required for viable experimental evolution. In a study by Chakravarti et al., selection experiments involving one strain of *C. goreaui* maintained at 31°C for 73 generations were followed by exposure of *Acropora* juveniles infected with the wild type (WT) strain and the selected strain (SS) to 27 and 31°C treatments (Chakravarti et al. 2017). Overall, coral juveniles infected with either WT or SS showed little to no difference in growth, bleaching, or survival when exposed to the 31°C temperature treatment (Chakravarti et al. 2017). After 130 generations of selection, a study by Buerger et al. evaluating a wider range of individual selected strains (including the strain used in Chakravarti et al.) found that this longer period of selection increased the bleaching tolerance of coral larvae associated with single selected strains in a 31°C experimental treatment (Buerger et al. 2020). This improvement in translated host benefits with experimental selection time suggests that the amount of time the symbiont spends evolving outside the host may be an important factor in determining the feasibility of this intervention method. Specifically, evolution that occurs outside the host is likely to optimize for "selfish" traits of the symbiont, as it is not regulated by its host. These results suggest that optimizing for fast selection of heat tolerance whilst minimizing time outside the host may be critical for the translation of thermal tolerance to the host from the symbiont.

Here, we report on an experiment carried out with the same WT and SS strains of *C. goreaui* used in the Chakravarti et al. (2017) and Buerger et al. (2020) studies, but conducted after only 21 generations of experimental evolution. Our study provides relevant insight into the possible rates and mechanisms involved in the evolution of enhanced thermal tolerance of coral-Symbiodiniaceae holobionts and enhanced understanding of potential consequences of shorter time periods of symbiont adaptation for the coral host. As in methods used in these two recent studies, we provided coral juveniles with

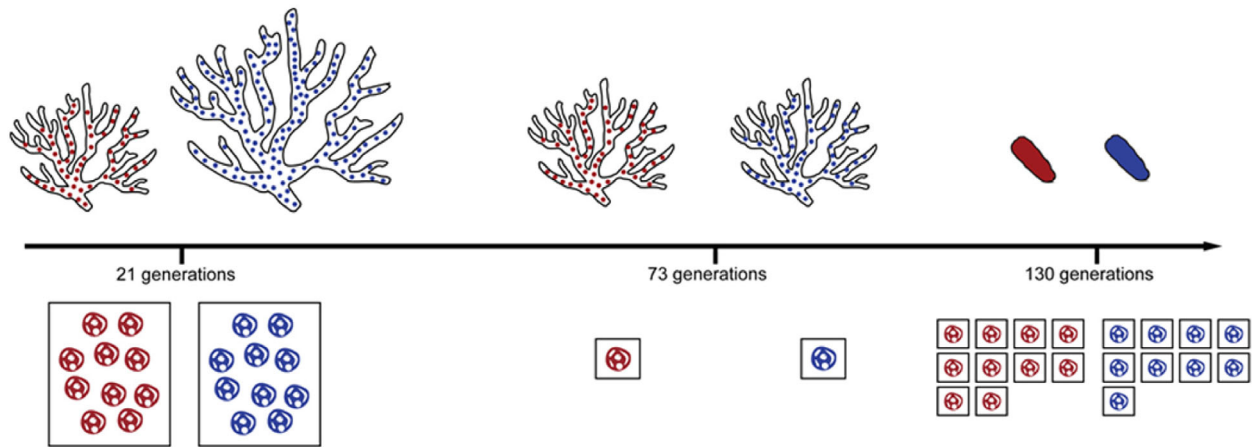


Figure 1. Timeline of experiments carried out with the wild type (WT, blue) and selected strains (SS, red) of *Cladocopium goreaui* over different generation times. At 21 generations, juveniles infected with a mix of wild type symbionts recorded higher growth than those infected with a mix of selected symbionts. At 73 generations (Chakravarti et al. 2017), no differences in growth were recorded between juveniles infected with either strain of symbionts. At 130 generations (Buerger et al. 2020), a single strain of SS provided coral larvae with higher bleaching tolerance than those infected with a WT strain. The difference in red and blue symbiont panels at 130 generations symbolizes that the experiment with the larvae was carried out with a different number of separate cultures (10 SS cultures and 9 WT cultures).

a mix of WT strains (10 strains) or a separate mix of SS (10 strains) and compared survival and growth of juveniles, and cell density and photosynthetic function of symbionts at three temperatures (27, 31, and 32.5°C) over short (28 days) and longer (72 days) periods of heat stress. We then compared the results of this early experiment using symbionts selected after 21 generations with later studies that used symbionts after 73 or 130 generations of selection, enabling us to test host costs or benefits over varying periods of time. Finally, we summarize important lessons and caveats learnt for practitioners interested in taking-up artificial selection for thermally resistant Symbiodiniaceae as an intervention strategy for reef corals. Further work is needed to ground-truth the ecological relevance of these laboratory-based results if this intervention is to be deployed at-scale in the wild.

Methods

Isolation of *Cladocopium goreaui* and Selection of Heat-Tolerant Strains

In 2010, symbiotic cells were extracted from colonies of the coral *Acropora tenuis* collected from Nelly Bay, Magnetic Island, Australia (19°10'6"S, 146°50'60"E, permit number G10-33440.1). Cells were isolated, cultured, and maintained as described in earlier studies (Beltran et al. 2012; Howells et al. 2012; Chakravarti et al. 2017). The culture was named SCF055 (historically named as aims-aten-C1-MI) The strain was split into 16 independent replicates that underwent a 2 month ratchet experiment (Huertas et al. 2011) to select for thermally tolerant Symbiodiniaceae (SS) and another 16 independent replicates that remained at ambient temperature (27°C, WT). After 1 year and approximately 21 generations, 4,000,000 cells from each of the 10 selected strains (specifically SCF055-01.01 to SCF055-01.09, and SCF055-01.19)

that showed the highest effective quantum efficiencies at elevated temperatures were mixed in a single strain. The same procedure was carried out with 10 WT strains to obtain a mix of WT strains (JCU308–310, 312–315, 317, 319, 328). The results presented here represent one of the first in a series of experiments carried out with the same symbionts at different evolutionary stages, including: 1 year of thermal conditioning of the selected strain (approximately 21 generations and a mix of 10 strains for each of the SS and WT mixed strains; this experiment); 2.5 years (approximately 73 generations and one SS and one WT strain each; Chakravarti et al. 2017); 4 years (approximately 130 generations, 10 and 9 individual SS and WT strains, respectively, maintained separately as single strains; Buerger et al. 2020).

Coral Spawning and Larval Rearing

Gravid colonies of *A. tenuis* were stockpiled in Geoffrey Bay, Magnetic Island (19°09.326' S, 146°51.861'E) on the full moon in November 2014 (permit number: G13/36318.1), and then collected the next morning and transported to the National Sea Simulator Facility (SeaSim) at AIMS (full details in Supplement S1).

Larval Settlement

Larvae raised from *A. tenuis* are competent to settle 6 days post-fertilization. Aragonite plugs, which had been pre-washed and autoclaved (15 minutes at 121°C and 211 kPa), were used as settlement substrata. In total, 1,628 plugs fitted with tubing were placed into 11 PVC plastic trays and kept in water baths (300 L acrylic tanks) supplied with 27°C, 0.5 μm fsw for 2 days before inducing larval settlement. GLW-amide neuropeptide Hym-248 was used to induce larval settlement (full details in on larval settlement in Supplement S1).

Temperature Ramping, Symbiont Infection, and Husbandry

Juvenile husbandry and symbiont infection followed established methods (full details in Supplement S1). At 95 days post-fertilization, infected juveniles were randomly distributed across 18 tanks, whereby nine tanks contained juveniles infected with WT symbionts and nine tanks contained juveniles infected with SS symbionts. For each symbiont treatment, juveniles were randomly distributed across three replicate tanks in each of three temperature treatments (27, 31, and 32.5°C), such that an equal number of juveniles were represented within each replicate temperature by symbiont combination. In summary, there were three tanks for each of three temperature treatments per symbiont strain. To reach experimental temperatures in the 31 and 32.5°C treatments, seawater temperatures were ramped up 1°C/day, with both treatments reaching their target temperature on the same day. Experimental groups are denoted by a two-letter code identifying the symbiont type (SS or WT), followed by the character “@” and the temperature of the treatment (27, 31 or 32.5°C).

Pre-experimental Acclimatization

To facilitate equivalent symbiont infection densities across all treatments, a grow-out period at ambient temperatures was required. During this time, juveniles were placed into tanks initially for pre-acclimatization holding. Juveniles were redistributed into plug trays and then subsequently transferred into new tanks that had been bleached and cleaned to assure the removal of any potential contamination from other sources of Symbiodiniaceae. It should be noted that photosynthetic efficiencies of symbiont communities measured in juveniles decreased during the pre-experimental ambient temperature treatment for juveniles infected with both strains, potentially due to handling stress. Given this slight but non-significant change in photosynthetic efficiencies, the other measured traits were also examined to see if they differed statistically across treatments.

Symbiont Density

Seven juveniles were sampled at the beginning of the experiment (t_0 : 104 days post fertilization), and at days 28 (t_{28}) and 72 (t_{72}) and fixed in 5% seawater formalin. Samples were centrifuged at 11,000 rcf for 5 minutes to remove formalin, incubated in 50 μ L of 4% formic acid for 1 hour, by which time skeletons were fully dissolved, and centrifuged at 11,000 rcf for 5 minutes to pellet symbiont cells. Supernatants were discarded and 80 μ L of fsw was added to each sample. Cell counts were carried out using a Neubauer chamber; two replicate chambers were counted per juvenile. Symbiont densities were normalized to percentage change between t_0 and time points t_{28} and t_{72} to account for differences in juvenile size between those infected with WT versus those infected with SS. Densities of symbiont cells within individual juveniles were standardized by dividing the total number of cells counted by the area (mm^2) of the respective juvenile. Changes in cell density through time were calculated for each juvenile by comparing its mean cell density at t_{28} and t_{72} with mean cell density at t_0 .

Symbiont Photophysiology

Pulse amplitude modulation fluorometry (PAM) was used to assess the physiological health of symbiont strains *in hospite*. An Imaging PAM (Heinz Walz, Germany) was used to measure maximum quantum efficiency of photosystem II (Fv/Fm) using the following parameters: Intensity = 1, Gain = 1, Damping = 1. Fv/Fm measurements were taken twice per week after an initial 20-minute dark adaptation. Due to juvenile mortality over time, the numbers of replicate juveniles per temperature and symbiont treatment varied over time. The numbers of replicate juveniles measured in the three temperature treatments were as follows: 27°C ($n = 88$ juveniles at the last timepoint—117 juveniles at the first timepoint), 31°C ($n = 62$ –96 juveniles), and 32.5°C ($n = 63$ –83 juveniles).

Coral Growth and Survival

Coral growth (basal area), number of polyps, and survivorship were monitored and measured in photos taken with a NIKON D800-E photomicroscope, using a 100 mm lens set at a distance of 650 mm above the juvenile tray and the following settings: aperture F22, shutter speed 1/100, ISO 100, and 1/8 flash. The basal area of each juvenile (mm^2) and number of polyps were measured or counted from micrographs using ImageJ (v. 2.0.0-rc-43/1.51d). Juvenile growth was normalized to percentage change in area between t_0 and t_{28} or t_{72} to enable size (area) comparisons between juveniles infected with WT and the SS, which differed in size at t_0 . $N = 3$ replicate tanks at each of t_{28} and t_{72} . The number of replicate juveniles measured in each temperature treatment were: 27°C ($n = 117$ –313 juveniles), 31°C ($n = 108$ –303 juveniles), and 32.5°C ($n = 194$ –286 juveniles).

Statistical Analyses

Statistical models, detailed in more depth below, were used to study the effects of temperature treatments on symbiont density, maximum quantum efficiency, and juvenile growth and survivorship over time. A combination of separate generalized linear models (GLM) and linear models (LM) was used to analyze individual time points. The packages “contrast” (Kuhn et al. 2016) and “multcomp” (Hothorn et al. 2008) were used to extract specific comparisons from the different models. “Grid” (R Core Team 2016), “vcd” (Meyer et al. 2015), and “AER” (Kleiber & Zeileis 2008) were used to check GLM assumptions, and “MuMIn” (Bartoń 2016) was used to compare model ranks via AICc. All statistical analyses were performed in R (v. 3.2.3).

Comparing Maximum Quantum Efficiencies, Growth, and Cell Densities Between SS and WT Symbionts

LM and GLMs were applied to determine if Fv/Fm, growth, and cell densities differed significantly between WT and SS. Linear mixed-effects models were run using the packages “nlme” (Pinheiro et al. 2016) and “lme4” (Bates et al. 2015) to determine if Fv/Fm, growth, and cell densities *in hospite* varied

significantly among temperature treatments, between the selected and wild type strains, or through time.

Juvenile Survival

Time-to-event models were used to identify differences in survivorship probabilities between WT- and SS-infected juveniles exposed to 27, 31, and 32.5°C treatments. These models take into account both censoring and time elapsed. The data were considered right-censored, as some individuals were still alive at the end of the experiment. Models were run with “temperature,” “symbiont,” “temperature by symbiont,” and the random effect of “tank” as covariates. The censoring variable was defined as the first day the juvenile was not seen, this day being defined as the time-at-event, where the event is mortality. Different parametric distributions were used to describe survival (exponential, Weibull, log-normal, log-logistic), as well as nonparametric methods (nonparametric: Kaplan–Meier, semi-parametric: Cox-proportional hazards models). Parametric methods were selected due to their significantly lower AIC values using the “survreg” function in the “survival” package (Therneau 2015). None of the time-dependent covariates were significant, indicating that there was no violation of proportionality for these predictors. Survivorship probability plots were constructed using the R package “flexsurv” (Jackson 2016), which constructs maximum likelihood estimates from the “survreg” function. Confidence intervals from “survflexreg” estimates are calculated through the “normboot” function in the same package and were simulated from the asymptotic normal distribution of parameter estimates. GLMs and LMs were run to determine differences in survivorship at t_{28} and t_{72} .

Results

Pre-experimental Acclimatization

Although photochemical efficiency values were lower at the end of the acclimatization period compared to pre-conditioning, there was no significant difference in mean Fv/Fm for juveniles infected with either SS or WT (0.45 ± 0.003 and 0.43 ± 0.003 , respectively) (LM, $F_{1,16} = 0.02$, $p = 0.89$).

At t_0 , juveniles in the WT@27 treatment were larger and experienced significantly greater percentage increase in area compared to SS@27 juveniles (1.71 ± 0.03 versus 1.27 ± 0.02 mm², LM, $F_{1,1,565} = 155.6$, $p < 0.05$). Given this difference, all growth results are presented as percent change in basal area of juveniles between treatments. During the pre-experimental grow-out period, the number of polyps per juvenile was also significantly greater in the WT@27 compared to SS@27 treatment (LM, $F_{1,524} = 20.3$ and $p < 0.001$). Cell density was also significantly greater in WT@27 compared to SS@27 juveniles during acclimatization ($39,754 \pm 5,973$ versus $16,932 \pm 2,892$ cells per mm², LM, $F_{1,22} = 11.9$, $p = 0.002$).

Photophysiology

At 27°C, Fv/Fm was not significantly different between juveniles infected with the SS and WT strains over the course of

the experiment (linear mixed effect model [LME], $p = 0.82$) (Fig. 2A). In contrast, Fv/Fm was significantly higher for juveniles in the SS@31 treatment compared to the WT@31 treatment (LME, $p = 0.04$) (Fig. 2B). This was predominantly driven by differences in Fv/Fm during the second half of the experiment (t_{29} – t_{70}) (LME, $p < 0.001$), as ratios were not significantly different in the first half (t_{-7} – t_{29}) (LME, $p = 0.94$). In contrast, juveniles in the WT@32.5 treatment exhibited significantly higher Fv/Fm compared to juveniles in the SS@32.5 treatment (LME, $p = 0.01$) (Fig. 2C).

Coral Growth

Significant differences in growth were detected between juveniles infected with the WT versus the SS within each of the temperature treatments through time. After 28 days, WT-juveniles had increased their basal area significantly more than SS-juveniles in both the 27°C (LM, $F_{1,431} = 92.5$, $p < 0.001$) and 31°C treatments (LM, $F_{1,427} = 6.1$, $p = 0.01$) (Fig. 3A). Specifically, at 27°C, the change in area from the first timepoint for WT-juveniles was $14.8 \pm 1.5\%$. SS-juveniles over time decreased in area by $-0.67 \pm 0.8\%$. At 31°C, the change in area from the first timepoint for WT-juveniles was $4.6 \pm 1.4\%$. SS-juveniles over time grew $0.8 \pm 0.9\%$. After 28 days at 32.5°C, juveniles infected with SS grew slightly more compared to juveniles infected with WT, although not significantly more (LM, $F_{1,435} = 1.93$, $p = 0.17$).

After 28 days, significant changes in percent area were also detected when the performance of WT- and SS-juveniles were compared between temperature treatments. WT-juveniles suffered greater decreases in growth with increasing temperatures and SS-juveniles showed marginal increases in growth with increasing temperatures. Specifically, WT-juveniles grew significantly more (3.2–7.6 times more) at 27°C compared to WT-juveniles at 31 or 32.5°C (LM, $F_{1,352} = 26.11$ and $p < 0.001$, $F_{1,367} = 52.2$ and $p < 0.001$, respectively). Growth of WT-juveniles did not differ significantly between the 31 versus 32.5°C treatments (LM, $F_{1,369} = 2.42$ and $p = 0.12$). In contrast, after 28 days, SS-juveniles grew significantly more at 32.5°C compared to SS-juveniles at 27 or 31°C (LM, $F_{1,499} = 14.2$ and $p < 0.001$, $F_{1,493} = 6.35$ and $p = 0.01$). No difference in growth was detected between SS-juveniles at 27 and 31°C (LM, $F_{1,506} = 1.3$ and $p < 0.25$).

After 72 days at 31°C, changes in basal area continued to be significantly greater in WT-juveniles than in SS-juveniles ($24.4 \pm 2.7\%$ versus $17.4 \pm 1.7\%$, LM, $F_{1,236} = 5.2$, $p = 0.02$). In contrast, after 72 days at 27°C, growth did not differ significantly between WT- and SS-juveniles (LM, $F_{1,286} = 0.5$ and $p = 0.46$). After 72 days at 32.5°C, all juveniles in this treatment were dead.

Polyp Growth

After 28 and 72 days at 27°C, percent change in the number of polyps was significantly greater in WT-juveniles than in SS-juveniles (GLM, $p = 0.02$ and 0.004) (Fig. 3B). In contrast, percent change in polyp number did not differ significantly

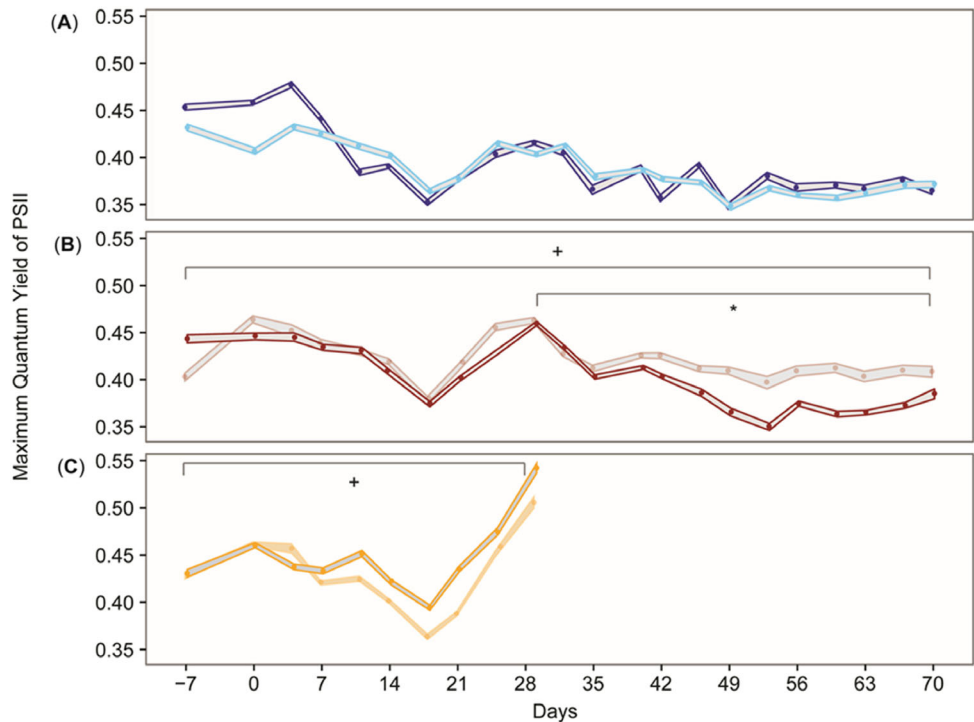


Figure 2. Mean maximum quantum yields (F_v/F_m) \pm standard errors (shading between lines) compared between juveniles infected with mixtures of the wild type (WT) versus selected symbiont (SS) strains of *Cladocopium goreaui* at: (A) 27°C, (B) 31°C, and (C) 32.5°C from pre-acclimatization (t_{-7}) until day 70 (t_{70}). Dark and light colors refer to WT and SS symbionts, respectively. Statistically significant differences between juveniles infected with WT and SS strains and exposed to different temperature treatments over specific periods are represented by a plus sign (significance value p value = 0.05–0.01) or an asterisk (significance value p value < 0.001). Brackets identify comparisons between treatment groups as discrete time points (pre-acclimatization, t_{28} and t_{72}). These were tested separately from those statistical tests examining time as a continuous main effect.

between WT- and SS-juveniles in either the 31 or 32.5°C treatments, at either 28 or 72 days (GLM, $p < 0.90$).

Symbiont Cell Density

At 27°C, changes in symbiont cell densities differed significantly between WT- and SS-juveniles at 28 and 72 days. Whereas cell densities increased by $60.6 \pm 13.8\%$ in SS-juveniles, they decreased by $16.5 \pm 19.4\%$ in WT-juveniles after 28 days (LM, $F_{1,30} = 10, p = 0.003$). After 72 days, percent change in cell densities remained positive for SS-juveniles ($+39 \pm 13.2\%$) and negative for WT-juveniles ($-44.2 \pm 11.5\%$; LM, $F_{1,23} = 22.7, p < 0.001$) (Fig. 3C). At 32.5°C, decreases in cell densities were significantly greater in WT- compared to SS-juveniles ($-94 \pm 1.4\%$ versus $-76.3\% \pm 5.3$, GLM, $p = 0.005$). At 31°C, changes in cell densities did not differ significantly between WT- and SS-juveniles, either after 28 days ($-39.8 \pm 7.6\%$ versus $-51.5 \pm 5.1\%$, LM, $F_{1,36} = 1.64, p = 0.21$) or after 72 days ($-56 \pm 10.2\%$ versus $64.8 \pm 5.1\%$, LM, $F_{1,27} = 0.51, p = 0.48$).

Survival

In the 27°C treatment, the probability of survival was significantly greater for WT- compared to SS-juveniles at 28 days ($95 \pm 0.5\%$ versus $89.3 \pm 0.3\%$, LM, $F_{1,4} = 72.25, p = 0.001$). No other comparisons within time points were significantly

different. For example, after 28 days, the probability of survival did not differ significantly between WT- and SS-juveniles at 31°C ($95.7 \pm 0.3\%$ versus $93 \pm 2\%$, LM, $F_{1,4} = 1.4, p = 0.28$) or between WT- and SS-juveniles at 32.5°C ($94 \pm 2\%$ versus $94.7 \pm 1.7\%$, LM, $F_{1,4} = 0.05, p = 0.82$).

After 72 days, the probability of survival did not differ significantly between juveniles infected with WT versus SS symbionts at 27°C ($p_{\text{survival}} = 0.96$), 31°C ($p_{\text{survival}} = 0.08$), or at 32.5°C ($p_{\text{survival}} = 1$) (Fig. 4). Although mean survival of WT-juveniles was greater than that of SS-juveniles after 72 days in the two lower temperature treatments, the probability of survival did not differ significantly between WT- versus SS-juveniles, either at 27°C ($87.3 \pm 8.6\%$ versus $80.3 \pm 4.6\%$, LM, $F_{1,4} = 0.50, p = 0.52$) or at 31°C ($81.7 \pm 7.8\%$ versus $68.7 \pm 8.1\%$, LM, $F_{1,4} = 1.328, p = 0.31$).

Discussion

Results presented here demonstrate that 21 generations of experimental evolution of a mix of selected strains of *Cladocopium goreaui* provided heat tolerance to symbionts but no growth or survival benefits to juvenile hosts when exposed to elevated temperatures. Whereas survival did not differ between juveniles infected with mixtures of SS versus WT in any of the three temperature treatments, growth of juveniles hosting selected strains

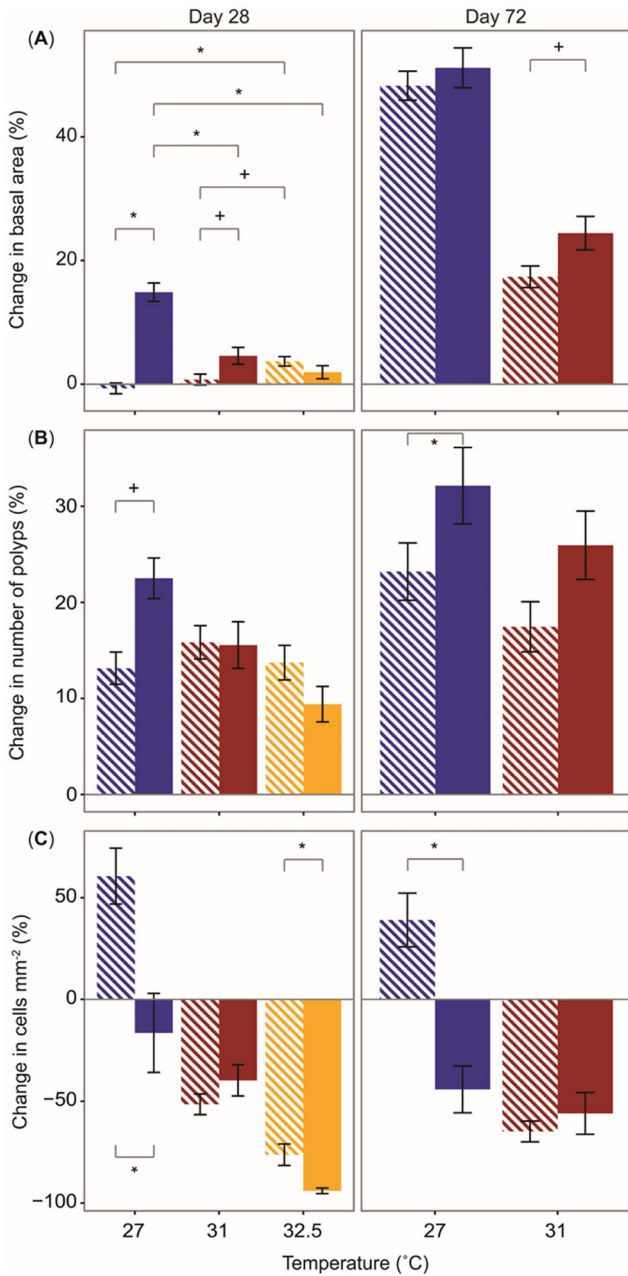


Figure 3. Mean percentage change (\pm SE) in parameters measured on juvenile *Acropora tenuis* infected with mixtures of the wild type (WT) versus selected symbiont (SS) strains of *Cladocopium goreaui*: (A) basal area, (B) number of polyps, and (C) symbiont cells in juveniles at 27, 31, and 32.5°C. Solid and dashed colors refer to juveniles infected with WT or SS, respectively. Statistical significances between temperature treatments are represented by a plus sign (significance value p value = 0.05–0.01) or asterisks (significance value p value < 0.001). Brackets identify comparisons between treatment groups.

was significantly lower than growth of juveniles hosting wild type strains. Even after 73 generations of conditioning (Chakravarti et al. 2017), experimental selection of *C. goreaui* provided no net benefit to the host. However, it is noteworthy that, although not statistically different after 73 generations,

the mean percent change in area of SS-juveniles was positive at 31°C, in contrast to the mean negative growth of WT-juveniles (Chakravarti et al. 2017). More recently, after approximately 130 generations of experimental evolution, three of 10 monoclonal strains tested therein provided bleaching resistance to coral larvae (Buerger et al. 2020). It is important to note that these three strains were not tested individually in studies using fewer than 130 generations (e.g. at either 21 or 73 generations), so it is possible that those three specific strains acquired heat tolerance sooner than 130 generations. Whereas 21 generations of experimental evolution was detrimental to the algal-coral symbiosis (this study), by 73 generations the association had improved (Chakravarti et al. 2017). By 130 generations, experimental selection resulted in at least one strain that, when *in hospite*, produced positive net benefits in bleaching tolerance to their coral hosts at elevated temperatures (Buerger et al. 2020). Therefore, 21–73 generations may not be long enough to provide significant physiological benefits for the coral-algal symbiosis. Our study also uniquely shows that the early overall lack of host benefit is a consistent response even over more prolonged and extreme stress (32.5°C, 72 days). Variability in acquired tolerance amongst strains (Buerger et al. 2020) and through time highlights the need to maintain multiple strains over many generations to develop experimentally selected Symbiodiniaceae that positively impact holobiont phenotypes. Although the results in Chakravarti et al. (2017) suggest only a preliminary trend in the acquisition of host fitness benefits, more recent work by Buerger et al. (2020) more definitively shows that bleaching responses can be reduced in coral larva with the provisioning of selected symbiont strains. Important to note is the length of time for symbiont culture to achieve this (i.e. years), and also that the effect sizes and long-term benefits and consequences of this symbiosis are unclear. In combination, these studies demonstrate that host benefits may be possible using experimental evolution of selected strains of *C. goreaui* and potentially with others. However, time scales for relevant evolution may be lengthy because of the potential stochasticity of mutations relevant to enhancing the thermal tolerance of corals in symbiosis.

Recommendations for Practitioners of Restoration Interventions

Time Scales for Relevant Experimental Evolution of Symbionts.

To optimally operationalize the experimental evolution of cultured algal symbionts for the purpose of enhancing coral heat tolerance, the temporal period of selection of symbionts is a prime consideration. Our results, combined with those of two subsequent studies (Chakravarti et al. 2017; Buerger et al. 2020), highlight the need for long-term conditioning of symbionts over many generations/years and repeated testing to assess host thermal tolerance to determine if selection has yielded improved host benefits. Physiological costs of selection should also be considered. Increased heat tolerance, for example, may come at a cost. Those costs could be associated with reductions in nutrient sharing (Baker et al. 2018) or reduced tolerance to particular light conditions. Given the extreme functional diversity of Symbiodiniaceae across many traits related

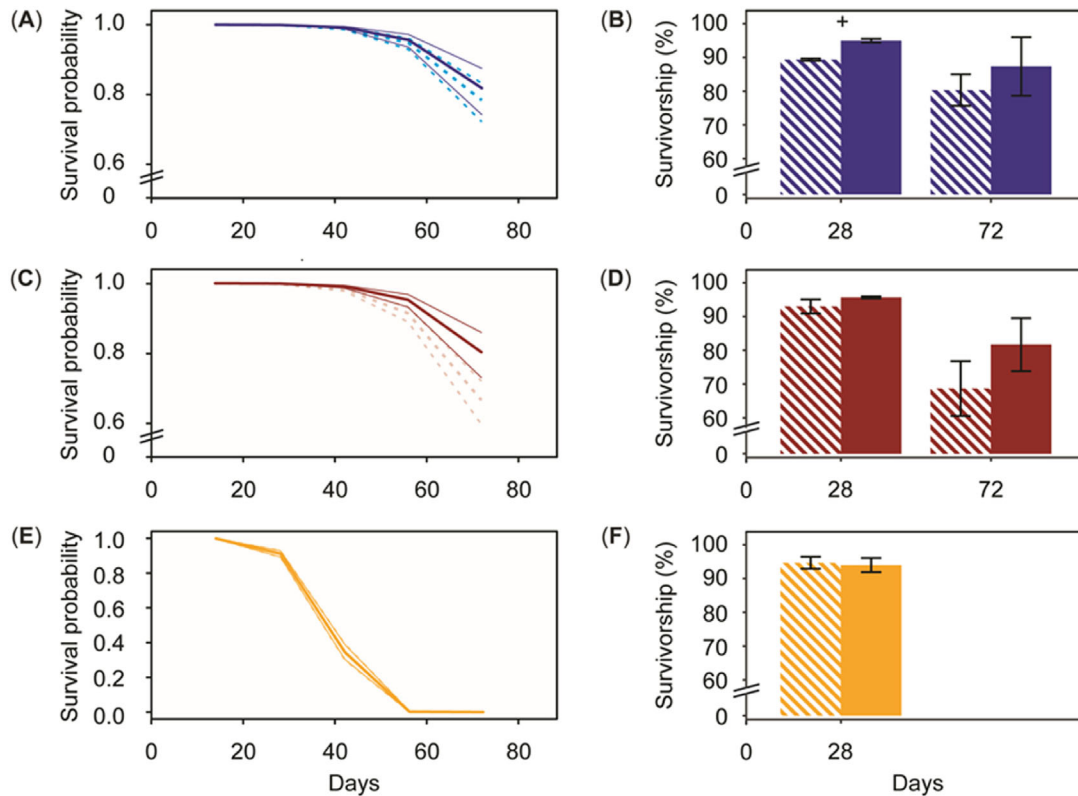


Figure 4. Mean survival probability (\pm CI) over time (left column) along with mean percentage change in juveniles infected with mixtures of the wild type (WT) versus selected symbiont (SS) strains of *Cladocopium goreaui*. Juvenile survivorship (\pm SE) at specific time points (right column) at the following temperature treatments: (A–B) 27°C, (C–D) 31°C, (E–F) 32.5°C. Solid and dashed colors refer to WT and SS symbionts, respectively. Statistical significance is represented by a plus sign (p value = 0.05–0.01).

to light, thermal tolerances, and growth (Swain et al. 2017; LaJeunesse et al. 2018), a longer-term understanding of both costs and benefits of experimental evolution of symbionts is critical.

Future work is urgently needed to identify the minimum number of generations of symbiont selection required to achieve relevant ecological outcomes. Such studies are especially relevant as new Symbiodiniaceae taxa are used for experimental evolution, particularly slower growing, heat-tolerant taxa, including *Durusdinium trenchii* and *D. glynnii*.

Identify the Strain. Comparisons of our results presented here with two subsequent studies (Chakravarti et al. 2017; Buerger et al. 2020) demonstrate that knowing strain identity is critical for assessing the effects of host inoculation. Our study was based on a mix of 10 SS strains, thereby precluding our ability to know which selected strains were present in juveniles for comparisons with later studies. Given that Buerger et al. (2020) found high variability in the capacity to confer thermal tolerance on coral larvae across multiple monoclonal strains of *C. goreaui*, it is likely that lack of differential bleaching or survival between WT- and SS-juveniles in both our study and Chakravarti et al. (2017) was because juveniles had not been infected or dominated by any of the three tolerant SS strains identified by

Buerger et al. (2020). This variability is in line with information showing that Symbiodiniaceae exhibit a high level of natural physiological plasticity across a range of temperatures (Swain et al. 2017). Within *C. goreaui* for example, growth and physiological assessments demonstrate significant variability across strains, even when different strains were isolated from the same host species (*Acropora tenuis*) and collected from the same reef (Magnetic Island) (Beltran et al. in press). Therefore, it could be expected that multiple strains within the selected cultures would exhibit high physiological plasticity in response to selective pressures generated by ratchet temperature treatments. The use of monoclonal strains may also be beneficial to maintain co-evolved host-symbiont mutualisms, which may reduce any physiological costs associated with switching from one symbiont genus to another that could reduce host benefits.

Recommendation: Given the complexity introduced by variability amongst strains identified as belonging to a single species of Symbiodiniaceae, results of experimental evolution studies are best interpreted when monoculture strains are used. The huge variability found within Symbiodiniaceae taxa highlights the urgent need for further culturing of many more strains, which should be characterized in conjunction with high-quality whole genome sequencing (Voolstra et al. 2015). Taxa from *Durusdinium* and *Fugacium* that have demonstrated high potential to further enhance heat tolerance in corals (Gierz et al. 2017;

Chakravarti & van Oppen 2018) are recommended as targets for experimental evolution.

More Comprehensive Assessments of Thermal Limits of Symbiodiniaceae Are Needed. Short-term experimental evolution resulted in rapid but conserved shifts in the thermal optima of *C. goreaui*. This shift in the thermal optimum of SS after 21 cell generations represents a 3-fold decrease in the time needed for evolution compared to previous estimates (Chakravarti et al. 2017), and a 2–3-fold faster response compared to Symbiodiniaceae from other genera (Chakravarti & van Oppen 2018). Both WT- and SS-infected juveniles had similar Fv/Fm at 27°C, indicating that photosystem II of the two symbiont strains were similarly efficient at capturing light energy (Genty et al. 1989). The higher Fv/Fm of selected symbionts compared to WT *in hospite* at 31°C suggests that not only is enhanced heat tolerance of selected *C. goreaui* possible, but it can be acquired rapidly by symbionts after only 21 generations of experimental evolution and remain stable during symbiosis with coral juveniles (although the maintenance of superior photosynthetic performance *in hospite* did not translate into increased host survival).

In our study, SS-juveniles demonstrated higher Fv/Fm at 31–32.5°C compared to SS-juveniles at 27°C. This suggests that SS shifted their temperature tolerance range after 21 generations of experimental evolution. In contrast, after 73 generations of exposure to elevated temperatures, the SS-juveniles widened their temperature tolerance, as shown in a lack of differential photosynthetic performance from 27 to 31°C (Chakravarti et al. 2017). After 130 generations, the optimal temperature for the three top-performing monoclonal selected strains also exhibited a widening of photosynthetic performance *in hospite*, although responses were variable (Buerger et al. 2020). Taken together, these combined results suggest that heat selection influences the shape of symbiont thermal performance curves, either by shifting tolerance away from 27°C to higher temperatures or by widening tolerance across a greater temperature range. The need for a greater understanding of how thermal performance curves change is recognized for the coral host (e.g. shifting, narrowing, or widening (Baums et al. 2019; Jurriaans & Hoogenboom 2019; Silbiger et al. 2019; Parkinson et al. 2020)) but is relatively unknown for the algal symbionts (Howells et al. 2012). Although our experimental design was not identical across these three studies (this study; Chakravarti et al. 2017; Buerger et al. 2020) combined, results highlight that short-term conditioning may be more likely to cause directional shifts in temperature tolerance compared to an overall widening of tolerance. Importantly, the shapes of thermal performance curves have important implications for the prediction of how phenotypic traits respond to selection and therefore rates of adaptation (Angilletta 2009). These rates will have important implications for quantifying the effectiveness of genetic intervention methods utilizing assisted evolution.

Given the essential nature of knowledge concerning thermal tolerance limits for Symbiodiniaceae, we recommend that comprehensive assessments of thermal performance curves under a

range of temperature conditions are needed to quantify how selection influences the thermal limits (rates and breadth) of selected Symbiodiniaceae.

Looking Forward: Changes in Cell Densities May Indicate Potential Trade-off Between Symbiosis and Temperature Tolerance.

Cell densities in juveniles decreased in all treatments, except for those infected with SS at 27°C. It is unclear if changes in cell density represent bleaching per se or a sign of aquarium stress. It is interesting to consider why juveniles with SS would show an increase in cell densities at 27°C, potentially representing a difference in energy allocation between SS and WT. The increased density of selected cells may indicate an upregulation in cell proliferation by the host to maximize autotrophic nutrition if the selected strain reduces its translocation of nutrients to the host. Alternatively, an increased number of symbiont cells may not maximize host benefits as high densities of commensal symbionts may not equal the high densities of more self-ish symbionts in terms of net benefits to the host coral. A reduction in nutrient translocation at elevated temperatures by symbionts has been postulated in other Symbiodiniaceae-coral symbioses (Cunning & Baker 2014; Wooldridge 2017) but not demonstrated with experimentally evolved symbionts. However, studies examining nutrient translocation in wild-type symbionts show changes in carbon and nitrogen can coincide with bleaching events, suggesting changes in translocation and the nutrient dynamics may be part of host-symbiont rebalancing upon exposure to heat stress (Baker et al. 2018; Cui et al. 2019). Our results also show that, concurrently, densities of the wild type strains at 27°C were comparably lower, potentially as more photosynthate was transferred and therefore fewer symbionts were required to meet host demands. The aforementioned shift in thermal performance curves in SS after 21 generations may also indicate that SS are providing less photosynthates at 27°C, causing the host to upregulate the number of cells needed to maintain homeostasis. Cell number is an important feature of the coral-symbiont association (Cunning & Baker 2014; Baker et al. 2018; Cui et al. 2019) and a redistribution of symbiont abundances may be one mechanism available to the host to modulate nutrient dynamics in its tissues.

In our study, juvenile growth also varied significantly by symbiont strain and temperature treatment. This may result from differences in photosynthate translocation to the host, given that a large proportion of the hosts' metabolic demands are fulfilled by coral symbionts (Baker et al. 2018), especially for carbon (Muscatine & Porter 1977). For example, differences in growth of *A. millepora* juveniles when infected with *C. goreaui* or *Durudinium* (Little et al. 2004) have been attributed to variability in total carbon incorporation associated with the two symbiont taxa (Yellowlees et al. 2008; Cantin et al. 2009). Potential differences in carbon allocation by WT and SS strains when *in hospite* might similarly lead to differences in growth (Cooper et al. 2011). Our results suggest these strains maintained the same potential for nutrient production (i.e. Fv/Fm were maintained) but that at 27 and 32.5°C, the selected strain may translocate less photosynthate to juveniles (i.e. no increased growth

or survival). The lack of improvement in growth and survival at 32.5°C in SS-juveniles but maintenance of photosynthetic efficiencies suggests that heat conditioning may have induced SS to share less photosynthate, which has been documented in other systems including insects (Bronstein 1994; Herre et al. 1999; Wooldridge 2017). Other factors such as light can also influence the ability for symbionts to survive in response to temperature stress, as seen in corals sourced from inshore locations (Jones et al. 2016). Targeted studies to quantify potential differences in the nutritional biology between wild type and selected strains of Symbiodiniaceae and their hosts as well as fully factorial experimental designs incorporating other environmental factors such as light and nutrients is needed to confirm this. Although we demonstrate that very short-term experimental evolution of cultured *C. goreaui* in response to elevated temperatures results in rapidly acquired heat tolerance by the symbiont, we highlight that continued work is needed to comprehensively assess the effectiveness of this intervention, and stress that such work must occur in conjunction with strict and rapid action on climate change.

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Supporting Information

The following information may be found in the online version of this article:

Supplement S1. Supplementary methods