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SHORT COMMUNICATION

Tenacious D: *Symbiodinium* in clade D remain in reef corals at both high and low temperature extremes despite impairment

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ABSTRACT

Reef corals are sensitive to thermal stress, which induces coral bleaching (the loss of algal symbionts), often leading to coral mortality. However, corals hosting certain symbionts (notably some members of *Symbiodinium* clade D) resist bleaching when exposed to high temperatures. To determine whether these symbionts are also cold tolerant, we exposed corals hosting either *Symbiodinium* C3 or D1a to incremental warming (+1°C week^{-1} to 35°C) and cooling (−1°C week^{-1} to 15°C), and measured photodamage and symbiont loss. During warming to 33°C, C3 corals were photodamaged and lost >99% of symbionts, while D1a corals experienced photodamage but did not bleach. During cooling, D1a corals suffered more photodamage than C3 corals but still did not bleach, while C3 corals lost 94% of symbionts. These results indicate that photodamage does not always lead to bleaching, suggesting alternate mechanisms exist by which symbionts resist bleaching, and helping explain the persistence of D1a symbionts on recently bleached reefs, with implications for the future of these ecosystems.

KEY WORDS: *Symbiodinium*, *Montastraea cavernosa*, Coral bleaching, Symbiosis

INTRODUCTION

Climate change is increasing the frequency and severity of temperature stress on coral reefs (Hoegh-Guldberg, 1999), resulting in coral 'bleaching' – the loss or expulsion of corals' symbiotic algae (*Symbiodinium* spp.; Jokiel and Coles, 1977) – which often leads to widespread mortality, with significant consequences for ecosystem structure and function (Glynn, 1993; Baker et al., 2008). High temperatures have been implicated in most mass-bleaching events (Hoegh-Guldberg, 1999), with 2015–2016 experiencing the most severe global bleaching event on record, attributable to climate warming and El Niño conditions (Eakin et al., 2016). However, cold temperatures, which also may become more frequent and severe under climate change (Kim et al., 2014), can also be detrimental to corals. For example, in 2010, corals in Florida experienced one of the coldest winters and hottest summers on record (Roth et al., 2012), with the cold event causing widespread bleaching that resulted in 11.5% mortality (Lirman et al., 2011).

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Cold stress appears physiologically similar to heat stress in corals, as both cause reduced photosystem II (PSII) quenching, leading to photodamage and symbiont loss (Saxby et al., 2003; Thornhill et al., 2008; Kemp et al., 2011; Roth et al., 2012). Like heat stress, bleaching from cold stress is also light dependent (Saxby et al., 2003), implicating a common mechanism associated with photodamage to PSII (Wicks et al., 2010). Indeed, photodamage is thought to trigger a cascade of events leading to cnidarian bleaching (Weis, 2008, but see Tolleter et al., 2013).

However, *Symbiodinium* are genetically and functionally diverse (Pochon and Gates, 2010), with some symbionts (e.g. some members of clade D including D1a/*S. trenchii* and D1/*S. glynni*) showing greater heat tolerance (Rowan, 2004). Bleaching thresholds are elevated by 1–2°C when hosting these symbionts in the Pacific (Berkelmans and van Oppen, 2006) and the Caribbean (Silverstein et al., 2015), where *S. trenchii* may have been recently introduced (Pettay et al., 2015). However, the tolerance of these symbionts to cold temperatures has not been previously investigated.

Here, we investigated the impact of incremental warming and cooling on the common Caribbean coral *Montastraea cavernosa* Linnaeus 1767 hosting *Symbiodinium* communities dominated by either clade C or clade D (ITS2 type C3 or D1a, each co-occurring with background populations of the other, non-dominant symbiont). Specifically, we tested the hypotheses that: (1) *Symbiodinium* D1a is more tolerant than C3 of both high and low temperature stress, and (2) bleaching severity is correlated with photodamage. This work aims to identify the upper and lower thermal thresholds of these symbioses and explore the mechanistic basis of bleaching at both temperature extremes.

MATERIALS AND METHODS

Coral collection and preparation

Nine colonies of *Montastraea cavernosa* were collected at 20 m depth from Breakers Reef (26°42.18′ N, 80°01.01′W) in Palm Beach County, Florida [where temperatures range seasonally from ∼22 to 30°C (NOAA Buoy LKWF1)], in February 2011, under Florida FWC Special Activity License SAL-11-1182-SRP. Corals were fragmented into replicate ∼2.5 cm diameter cores using a drill press. While all cores initially hosted *Symbiodinium* C3, ∼75% were bleached during a previous study (Silverstein et al., 2015), and mostly recovered with *Symbiodinium* D1a. Corals that were not bleached remained dominated by C3. These treatments produced corals of the same genotype but with different *Symbiodinium* communities, allowing us to test the influence of *Symbiodinium* on responses to heating and cooling, while controlling for host genotype. In total, 119 D1a-dominated corals (mean proportion clade D=97.9%, median=99.9%) and 40 C3-dominated corals (mean proportion clade C=96.4%, median=100%) were placed into the temperature ramping treatments (Table 1), which began after 105 days of recovery from the previous bleaching experiment.

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Table 1. Distribution of C-dominated (*Symbiodinium* C3) and D-dominated (*Symbiodinium* D1a/*S. trenchii*) coral cores in each temperature treatment

(No. of cores)	Cooling	Heating	Sum
C-dominated	19	21	40
D-dominated	53	66	119
Sum	72	87	159

Temperature treatments

Temperature treatments were applied in four replicate 284 liter tanks supplied with sand- and UV-filtered seawater from Bear Cut, Biscayne Bay, Florida. Temperature was controlled ±1°C with heaters and chillers (SeaChill, Jaeger, and ViaAqua), and light was supplied at 190–280 µmol photons m^{-2} s^{-1} (12 h:12 h light:dark). At the end of the previous study (Silverstein et al., 2015), corals were acclimated to either 24 or 29°C, which were used as starting points in the present study. Cores acclimated to 24°C were cooled at a rate of −1°C $week^{-1}$ over 9 weeks, spending the final week at 15°C. Cores acclimated to 29°C (and an additional 11 cores acclimated to 24°C) were warmed at a rate of +1°C $week^{-1}$ over 6 weeks, spending the final week at 35°C.

Symbiont community function

The maximum quantum yield of PSII (F_v/F_m) was measured using an Imaging Pulse Amplitude Modulated Fluorometer (I-PAM, Walz, Effeltrich, Germany) each week, just before the temperature was increased or decreased, and before the lights were turned on. A saturating pulse from an LED array at 460 nm was provided at an intensity of 2800 µmol photons m^{-2} s^{-1} for 800 ms, and the entire 2.5 cm diameter core was selected as the area of interest (AOI) for extraction of F_v/F_m data using Walz ImagingWin software. The measuring light and damping values were each set to 2, and the gain was adjusted to the lowest value between 2 and 8 that yielded minimum fluorescence F_o>130 units for all AOIs.

Symbiont community structure

Small (~1–3 mm^2) tissue samples were taken from each core with a new razor blade to obtain genomic DNA using an organic extraction protocol (Baker and Cunning, 2016). In the warming treatment, samples were collected at the start (29°C), and at the end of weeks 4 (33°C) and 6 (35°C). In the cooling treatment, samples were collected at the start (24°C), and at the end of weeks 4 (20°C), 6 (18°C) and 9 (15°C). The symbiont to host (S/H) cell ratio (Mieog et al., 2009) for *Symbiodinium* clades C and D was measured using quantitative PCR assays for actin loci specific to each *Symbiodinium* clade (Cunning and Baker, 2013) and *M. cavernosa*, following methods described in Silverstein et al. (2015). Cycle threshold (C_T) values were normalized for fluorophore intensity and gene copy number, averaged among technical replicates, and used to calculate S/H ratios with the formula $2^{C_{T,host}-C_{T,symbiont}}$. The total S/H ratio in each sample was calculated as the sum of clade C and D S/H ratios. Based on data from the present study, these assays detect both clades at S/H ratios at or below 0.0001, and relative abundances at or below 0.01%.

Data analysis

Corals were categorized as clade C- or D-dominated (‘C corals’ and ‘D corals’, respectively) based on their dominant symbiont at the beginning of the experiment. When a clade was not detected in a sample, its clade-specific S/H ratios were set to a non-zero value below the minimum detection threshold. Generalized additive mixed models (GAMMs; http://CRAN.R-project.org/package=gamm4) were used to analyze relative F_v/F_m as a smooth function of time with dominant symbiont as a fixed factor and colony and core as random factors. Multivariate normal simulation (n=1000) using fitted GAMM coefficients was used to test for differences between C and D corals in each treatment. S/H ratios were analyzed using linear mixed models (Bates et al., 2015) with time and dominant symbiont as fixed factors and colony and core as random factors. *Post hoc* tests were used to identify differences within each group relative to the initial or previous time point. Data and R scripts to reproduce the analyses and figures presented here are archived at Zenodo (Cunning, 2017), including an R Markdown document providing a detailed description of these analyses, which were conducted in R v3.2.2 (https://www.R-project.org).

RESULTS AND DISCUSSION

All corals showed declining F_v/F_m in response to cooling (Fig. 1A), reaching ~40% of initial values after 9 weeks. At all temperatures below 24°C, F_v/F_m declined more in D corals than in C corals. No corals lost symbionts during cooling to 18°C, but by 15°C, C corals lost 94.0% of symbionts (Fig. 1A). Meanwhile, D corals still showed no change in total symbiont abundance, but lost 80.2% of their background C symbionts (Fig. 2A). No mortality was observed during cooling.

During warming, F_v/F_m declined steadily in C corals but remained higher in D corals up to and including 32°C (Fig. 1B). However, at 33°C, F_v/F_m was equally reduced in all corals, and C-dominated corals had lost 98.8% of their symbionts; those few remaining were mostly clade D (Fig. 2B). Meanwhile, total symbiont abundance in D-dominated corals remained unchanged (Fig. 1B), despite the loss of 96.0% of their background clade C cells (Fig. 2B). At 35°C, F_v/F_m in both groups declined to ~10–20% of initial values, and C corals had lost 99.7% of their symbionts (Fig. 1B). D-dominated corals also experienced significant symbiont loss (95.3%) at 35°C (Fig. 2B), but mean total S/H ratios still remained more than two orders of magnitude higher than in C corals. Ten cores died by the end of the heating treatment.

While photodamage (i.e. reduction in F_v/F_m) in response to cooling was more severe in *Symbiodinium* D1a compared with C3, the impact of warming (up to 32°C) was more severe in C3. F_v/F_m declines when PSII accumulates damage as a result of excitation energy exceeding the quenching capacity of photochemistry and photo-protective pathways (Roth, 2014). Quenching capacity may be reduced (and photodamage increased) as a result of temperature stress impacting various aspects of photosynthesis, including thylakoid membrane stability (Tchernov et al., 2004; Thornhill et al., 2008), D1 protein synthesis and repair (Warner et al., 1999), or Calvin cycle enzyme activity (Jones et al., 1998; but see Oakley et al., 2014). Consequently, variable effects of temperature on photodamage in different *Symbiodinium* may arise from differences in any of these traits.

Despite accumulating less photodamage during cooling, C corals ultimately bleached while D corals did not (Fig. 1A), suggesting that symbiont loss may be decoupled from photochemical impairment. D corals also did not bleach when heated to 33°C, despite experiencing declines in F_v/F_m equal to those of C corals, which bleached severely (Fig. 1B). Together, these results suggest that D symbionts can resist eviction even when impaired. This may be due to either reduced production of reactive oxygen species (ROS) (McGinty et al., 2012), which signal host cellular bleaching cascades (Lesser, 1997; Weis, 2008), or more effective antioxidant networks that detoxify ROS (Krueger et al., 2014). Alternatively, like some parasitic microbes, clade D symbionts may evade and/or

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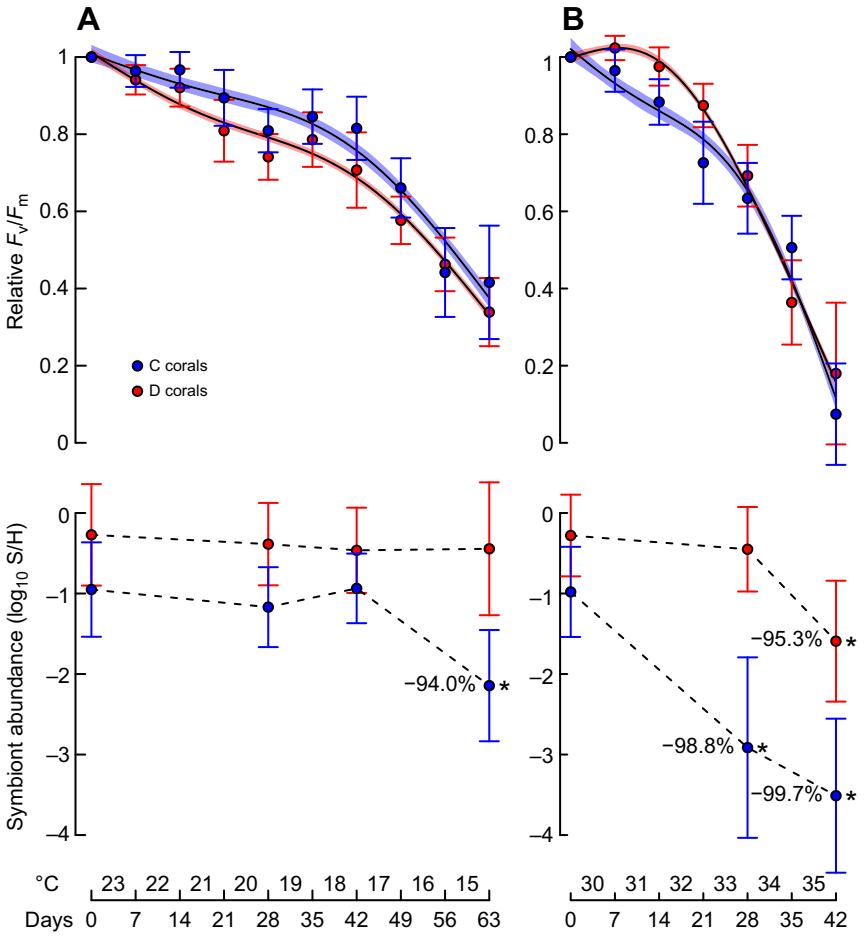

Fig. 1. Photochemical impairment and symbiont loss. The impact of incremental cooling (A) and heating (B) on relative maximum quantum yield of photosystem II (F_v/F_m; upper panels) and total symbiont abundance (lower panels). F_v/F_m (mean±s.d.) is plotted at each time point along with fitted GAMMs (solid lines) and 84% confidence intervals (shaded regions) for C corals (blue; n=19 in cooling, n=21 in heating) and D corals (red; n=53 in cooling, n=66 in heating). Non-overlapping 84% confidence intervals indicate a significant difference (P<0.05) between C and D corals. For symbiont abundance, log-transformed total symbiont/host (S/H) ratios (mean±s.d.) are plotted at each time point. Significant differences within each group relative to initial values (P<0.05) are indicated by an asterisk, along with the percent reduction in total S/H ratio.

suppress downstream host immune responses (Detournay et al., 2012) that would normally result in symbiont expulsion. Indeed, other features of D1a/*S. trenchii* in the Caribbean, such as its occurrence in many corals at low levels (Silverstein et al., 2012) with temporary opportunistic increases in abundance (LaJeunesse et al., 2009), also reflect more parasitic traits (Lesser et al., 2013). The ability to remain at high densities under stress despite reduced F_v/F_m was also observed in certain clade A *Symbiodinium* (Kemp et al., 2011), another group (along with clade D) within which specific members have been identified as potentially less mutualistic (Stat et al., 2008; Lesser et al., 2013).

While further work is necessary to understand just how some members of clade D are able to remain in reef corals during stress, this finding challenges the notion that photodamage invariably leads to cnidarian bleaching, and suggests an important role for a mechanism sequentially intermediate (or alternative) to the onset of

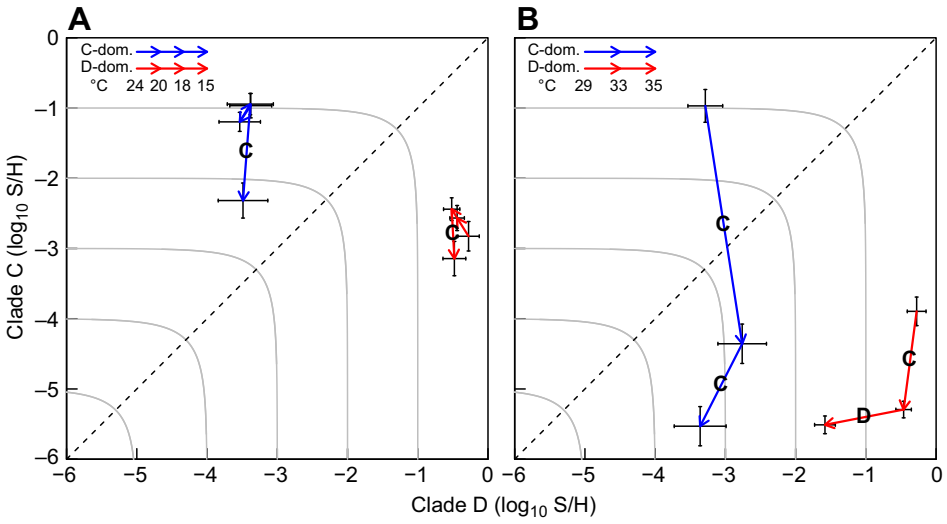

Fig. 2. Dynamics of both dominant and subdominant symbiont loss under temperature stress. For both cooling (A) and heating (B) treatments, fitted values of mean abundance of clade C and D symbionts in both C-dominated (blue) and D-dominated (red) corals are plotted (±s.e.m.) with arrows connecting consecutive sampling time points, as indicated in the key. A 'C' or 'D' at the midpoint of an arrow indicates a significant reduction in that clade during the time interval [e.g. during cooling (A), both C- and D-dominated corals lost C symbionts during the final interval, etc.]. The dashed diagonal line represents equal abundances of clades C and D, and gray contours represent equal sum total abundance of C and D (i.e. constant total S/H) at order of magnitude intervals.

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photodamage and loss of symbiont cells. Indeed, recent work has called into question the ubiquity of symbiont photo-oxidative stress as the trigger of bleaching, suggesting that host regulation of cellular redox status may be equally important (Krueger et al., 2015; Hawkins et al., 2015). Our findings add further nuance to this decoupling of bleaching from photodamage by showing an interactive effect of symbiont identity, with *Symbiodinium* D1a being more resistant to bleaching despite greater photodamage.

The high resistance of D1a symbionts to expulsion relative to C3 is also exemplified by the community changes that occurred within individual corals: while not losing any clade D cells, D-dominated corals lost 80% of their sub-dominant clade C cells at 15°C, and 96% at 33°C (Fig. 2). These observations suggest the fate of symbionts depends on their genetic identity and is mediated at the level of individual cells, consistent with previous work showing selective expulsion of particular symbiont types (Yamashita et al., 2011).

While all corals lost most of their symbionts at 35°C, associated with a 90% reduction in F_v/F_m, D corals still had two orders of magnitude more symbionts than C corals remaining in their tissues. Furthermore, despite a 60% reduction in F_v/F_m at 15°C, clade D symbionts still remained at high abundance. It is unclear whether retention of dysfunctional D symbionts confers a cost or benefit to the host at thermal extremes. Do these symbionts continue to damage their coral hosts by generating ROS, or do they benefit their hosts (or offset ROS damage) by continuing to produce photosynthate? Future investigations should measure oxidative stress and carbon translocation to better address the functional consequences of these 'tenacious' symbionts in clade D.

The lack of mortality (or even bleaching) in D corals at 15°C suggests that *M. cavernosa* may survive at even lower temperatures, particularly when hosting clade D. Indeed, these data indicate that the cold-bleaching threshold for D corals is at least 1–2°C lower than C corals, and that the heat-bleaching threshold is at least 1–2°C higher. Even though our relatively slow warming and cooling rates (1°C week^{-1}) may have allowed time for acclimation (or alternatively, caused greater cumulative stress than a shorter, acute exposure), the difference in bleaching threshold we found between C and D corals is remarkably similar to previous work showing a 1–1.5°C increase in heat tolerance associated with clade D during acute stress (Berkelmans and van Oppen, 2006).

In conclusion, in *M. cavernosa*, association with *Symbiodinium* D1a (*S. trenchii*) extends bleaching thresholds by at least 1–2°C at both hot and cold extremes. However, these symbionts remain in symbiosis despite accumulating significant photodamage, suggesting that the unusually high bleaching resistance of certain *Symbiodinium* may not be due to their ability to resist photodamage, but rather their tenacity as intracellular symbionts (i.e. their ability to resist expulsion). If different mechanisms are involved, it is possible that other thermally tolerant symbionts, such as C15 (LaJeunesse et al., 2003) or C3/*S. thermophilum* (Hume et al., 2015), may be resistant to bleaching for reasons other than those identified for the D1a symbionts investigated here. These results underscore a clear need to better understand the physiological costs and benefits of hosting thermotolerant symbionts, and their contributions to the coral host when under stress, as well as under normal conditions. The increasing abundance of symbionts in clade D on reefs following mass bleaching events (Baker et al., 2004), and their tendency to remain for extended periods, suggests these investigations will have relevance to understanding real-world transitions that are occurring on today's reefs.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
R.N.S. and A.C.B. designed the study; R.N.S. and R.C. performed experiments and analyzed data; R.N.S., R.C. and A.C.B. wrote the paper.

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Data availability
All data and analysis scripts are available at Zenodo (doi:10.5281/zenodo.260218).

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