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Evidence for a host role in thermotolerance divergence between populations of the mustard hill coral (*Porites astreoides*) from different reef environments

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Abstract

Studying the mechanisms that enable coral populations to inhabit spatially varying thermal environments can help evaluate how they will respond in time to the effects of global climate change and elucidate the evolutionary forces that enable or constrain adaptation. Inshore reefs in the Florida Keys experience higher temperatures than offshore reefs for prolonged periods during the summer. We conducted a common garden experiment with heat stress as our selective agent to test for local thermal adaptation in corals from inshore and offshore reefs. We show that inshore corals are more tolerant of a 6-week temperature stress than offshore corals. Compared with inshore corals, offshore corals in the 31 °C treatment showed significantly elevated bleaching levels concomitant with a tendency towards reduced growth. In addition, dinoflagellate symbionts (*Symbiodinium* sp.) of offshore corals exhibited reduced photosynthetic efficiency. We did not detect differences in the frequencies of major (>5%) haplotypes comprising *Symbiodinium* communities hosted by inshore and offshore corals, nor did we observe frequency shifts ('shuffling') in response to thermal stress. Instead, coral host populations showed significant genetic divergence between inshore and offshore reefs, suggesting that in *Porites astreoides*, the coral host might play a prominent role in holobiont thermotolerance. Our results demonstrate that coral populations inhabiting reefs <10-km apart can exhibit substantial differences in their physiological response to thermal stress, which could impact their population dynamics under climate change.

Keywords: acclimatization, coral, local adaptation, population structure, *Porites astreoides*, thermotolerance

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Introduction

Reef-building corals are cnidarians that exist in obligate symbiosis with photosynthetic dinoflagellates of the genus *Symbiodinium* (Muscatine 1990). Thermal stress results in the functional loss of the endosymbionts in the process known as coral bleaching, which can ultimately result in death if stressful conditions persist (Glynn 1993). Increasingly frequent and severe bleaching episodes in combination with anthropogenic disturbance, eutrophication and ongoing climate change (Harvell *et al.* 1999; Lesser *et al.* 2007) have led to suggestions that these organisms, and the reefs they support, may not persist in the future (Hoegh-Guldberg 1999; Hoegh-Guldberg *et al.* 2007). While there is substantial inter- and intraspecific variation in thermotolerance, most corals appear to exist within one to two

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degrees of their local thermal tolerance limit (Berkelmans & Oliver 1999), rendering them vulnerable to even slight warming. However, corals have persisted through warming episodes in the recent geological past without any noticeable decline (Pandolfi 1996, 1999) and today can be found over broad latitudinal ranges inhabiting a variety of thermal conditions (Hughes *et al.* 2003). This suggests that historically, corals have adapted to both spatial and temporal variation in temperature. Furthermore, evidence is mounting that suggests ongoing adaptation of coral populations to repeated bleaching events, manifested as higher bleaching resistance at sites that experienced frequent or particularly devastating bleaching in the past (Glynn *et al.* 2001; Maynard *et al.* 2008; Thompson & van Woesik 2009; Guest *et al.* 2012). The question remains, however, whether the particular adaptive mechanisms used by corals are efficient enough to keep up with the present rate of climate change, compounded by historically unprecedented stressors such as ocean acidification and declining water quality (Wooldridge 2009; Pandolfi *et al.* 2011).

Like other animals, corals can respond to elevated temperature at the individual colony level (Coles & Jokiel 1978; Brown *et al.* 2002) as well as the population level, resulting in a matching of coral physiology to the local environment (Meesters & Bak 1993; Oliver & Palumbi 2011a). This process can be achieved through physiological plasticity (i.e. acclimatization), changes in population allele frequencies (i.e. adaptation, Kawecki & Ebert 2004) or both. Corals also feature a unique intermediate response mechanism: some species are able to 'shuffle' proportions of resident symbiont genotypes (Berkelmans & van Oppen 2006), which is essentially a plastic change in allele frequencies. Research into coral adaptation and acclimatization capacity has largely focused on variation in coral–*Symbiodinium* associations, as it is a potentially rapid and reversible mechanism by which corals can cope with their thermal environment (Buddemeier & Fautin 1993). However, not all coral species appear to be capable of such temporal flexibility in their symbiont associations (Goulet 2006; Stat *et al.* 2009), although investigation of this phenomenon is still underway (Silverstein *et al.* 2012). Therefore, the coral host must also play a role in shaping thermotolerance variation (Baird *et al.* 2009a). Interactions between host coral species and symbiont types have been shown to alter the ultimate holobiont thermal physiology, providing support for the role of the host (Abrego *et al.* 2008). Host-specific effects have also been implicated in the absence of significant symbiont genetic differences in corals from varying thermal environments that exhibit divergent thermotolerance physiologies (Barshis *et al.* 2010).

While it is clear that both host and symbiont are involved in shaping holobiont thermotolerance limits, it is difficult to evaluate the relative contributions of each partner without evaluating their respective physiologies within the same study system. Furthermore, when investigating population-level variation in response to temperature, genotyping both hosts and symbionts can provide an additional layer of information by suggesting which partner may be driving the adaptive response. We employed this approach to test for local thermal adaptation or acclimatization in the mustard hill coral, *Porites astreoides*, from thermally distinct reef habitats in the Florida Keys. As inshore reefs experience higher temperatures than offshore reefs annually for prolonged periods during the summer, we hypothesized that inshore corals are better adapted to long-term heat stress than offshore corals. We conducted a common garden experiment with heat stress as our selective agent to compare within-genotype responses of *P. astreoides* for growth and bleaching (holobiont fitness proxies) and photosynthesis photochemical efficiency (a *Symbiodinium* fitness proxy). As a host-specific response, we profiled host gene expression, which is described in an accompanying paper (Kenkel *et al.* 2013). Newly developed microsatellite assays were used for coral host genotyping. *Symbiodinium* were genotyped using a novel approach, which involved deep sequencing of the second internal transcribed spacer of the ribosomal RNA gene (ITS2) to detect shifts in symbiont community composition in response to heat stress or between populations.

Methods

Study system

The Florida Keys are a 180-km chain of islands emerging from the southern tip of Florida that separate Florida Bay from the greater Atlantic Ocean (Fig. 1). Florida Bay is shallow (1.2–1.5 m deep on average), and current systems push water in an eastward direction across the Bay, onto nearshore Florida reefs (Smith & Pitts 2001). Temperature variation at inshore reefs is considerable, likely due to the reduced heat storage capacity of shallow nearshore waters in addition to Florida Bay inputs (Chiappone 1996). Temperature variation at offshore reefs is buffered by the along-shore current patterns of Hawk Channel, which disrupt flow from Florida Bay (Smith & Pitts 2001). In addition, offshore reefs also experience the thermal buffering of the Florida Current (Gulf Stream), resulting in less variable annual thermal profiles (Lirman *et al.* 2011). Hourly temperature data from 2006 to 2011 for a pair of inshore and offshore reefs in the lower Keys show that on average, the

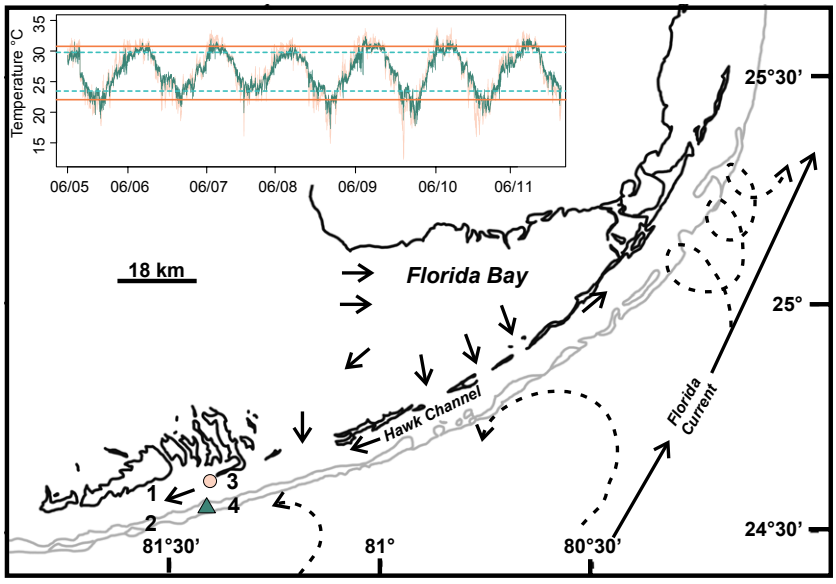

Fig. 1 Current systems influencing reef environments in the Florida Keys (redrawn and modified from Klein & Orlando 1994; Smith & Pitts 2001). Bold arrows indicate predominant direction of current flow. Offshore reef tract shown in grey. Inset shows hourly temperature data for a representative inshore site and offshore site from the lower Keys, marked by the red circle and blue triangle, respectively. Upper and lower solid lines indicate mean June–August and December–February temperatures for the inshore site, while dashed lines indicate means for the offshore site. Numbers correspond to populations sampled for genotyping as shown in Fig. 6.

inshore reef was 1 °C warmer in summer and 1.4 °C cooler in winter than the offshore reef (Fig. 1).

Experimental design

Fifteen colonies of *Porites astreoides* were collected on the same day from a depth of 2–3 m from each of two sites: an inshore patch reef (N 24°35.142, W 81°34.957, site 1, Fig. 1) and an offshore reef (N 24°31.303, W 81°34.605, site 2, Fig. 1) 7.1 km apart near Sugarloaf Key in August 2010 under Florida Keys National Marine Sanctuary (FKNMS) permit 2010-094. Colonies were immediately returned to Mote Marine Laboratory's Tropical Research Laboratory and halved using a hammer and chisel. Fragments were placed in a shaded (70% photosynthetically active radiation reducing) flow-through seawater system (raceway) with an average water temperature of 28.0 ± 0.7 °C and allowed to acclimate for 10 days. After acclimation, one-half of each colony was randomly assigned to a temperature treatment, a tank within that treatment and a specific position within that tank (n = 3 fragments per tank). Temperature treatment consisted of two shaded (70% PAR reducing) raceways, one control and one elevated temperature, each holding ten 40-l aquaria with clear plastic lids. Control temperature treatment was achieved by completely filling the 40-l tanks with seawater, equipping each tank with a 2-W aquarium pump (Hesen) and allowing water to flow-through the raceway as a water bath. The elevated temperature raceway was set up next to the control in exactly the same manner, but each individual tank was also equipped with a 200-W aquarium heater (Marineland) set to maximum heat. Temperatures were 27.2 ± 0.4 °C in the control tanks and 30.9 ± 1.1 °C in the heated tanks (Fig. S1, Supporting information). Treatment continued for 6 weeks (43 days) with tank cleaning and 30–50% water changes performed three times each week to maintain salinity levels at 35 ppt.

Holobiont trait measurements (growth and bleaching)

Immediately prior to turning on the heaters, all fragments were buoyant weighted in duplicate as described in the study by Davies (1989). Following the 6-week treatment, all fragments were cleaned using a small brush to remove any filamentous algal growth and buoyant weighted again in duplicate. Technical replicates of weight measurements for each fragment were averaged. Initial weight measurements were subtracted from final weight measurements and divided by the initial weight measurement to determine the proportion of weight gained over the 6-week treatment for each fragment. Corals were photographed during the acclimation period and again at the end of the 6-week treatment. To quantify colour changes associated with reduced chlorophyll and symbiont densities, Corel PHOTO-PAINT was used to balance exposures across photographs using a common white standard. Mean red channel intensity was then calculated for 10 quadrates of 25 × 25 pixels within each coral fragment as a measure of brightness; higher brightness indicated reduction in algal pigments (i.e. bleaching). This analysis was performed following the study by Winters *et al.* (2009) using the MATLAB macro 'AnalyzeIntensity'.

Symbiodinium trait measurements (photosynthetic efficiency)

The photochemical efficiency of the symbionts' photosystem II was quantified for each experimental fragment

using a pulse-amplitude-modulated fluorometer (PAM) (Diving-PAM, Waltz). Reductions in effective quantum yield (EQY) indicate a temporary down-regulation of photosynthesis under excess heat and/or light, while reduced maximum quantum yield (MQY) values are indicative of sustained damage to the photosystem. Quantum yield measurements of the symbiont photosystem were taken during the last 2 days of the acclimation period, the first 3 days of heat stress treatment and the final 2 days of the heat stress experiment. EQY measurements were taken during the day, between 0800 and 1800 h ($n = 3$ measurements per coral fragment during acclimation, $n = 5$ during treatment days 1–3 and $n = 3$ during treatment days 42–43), while MQY measurements were taken following dark adaptation 1.5 h after sunset ($n = 2$ acclimation, $n = 1$ day 1–3 and $n = 1$ day 42–43).

Host genotyping

Eight *P. astreoides* microsatellites were mined from the transcriptome (Kenkel *et al.* 2013) using a custom Perl script (Table S1, Supporting information). Additional individuals from each of the compared populations were collected for genotyping under FKNMS permit 2011-115 for a total of 33 inshore and 40 offshore individuals. In addition, 27 individuals were genotyped from a novel inshore–offshore reef pair near Summerland Key (14 inshore and 13 offshore, sites 3 and 4, Fig. 1) collected under FKNMS permit 2009-078. Tissue samples were preserved in RNALater and extracted using RNAqueous kits, which retained considerable amounts of high-purity DNA along with the RNA. Microsatellite markers were amplified individually following PCR conditions described in the study by Davies *et al.* (2012). Electropherograms were analysed using GeneMarker software 1.70 (Soft Genetics), and alleles were scored manually based on amplicon size. For 12 of the 15 inshore individuals and 14 of the 15 offshore individuals, genotypes were identical between halves of experimental individuals, as expected. The remaining corals, 3 inshore and 1 offshore, exhibited different genotypes between heat and control treatment halves, likely due to cosettlement of individuals at the larval stage, as has been observed in *Acropora millepora* (Puill-Stephan *et al.* 2009). The separate halves of these individuals were coded as novel genotypes in subsequent genetic and statistical analyses. Three pairs of corals in the additional population samples (1 inshore and 2 offshore) exhibited identical genotypes across all loci. We considered these individuals as clones, and one from each pair was removed prior to statistical analyses.

No amplification of expected fragment size was observed for any marker when tested using pure A4 *Symbiodinium* DNA (A4 isolate 368, LaJeunesse 2001). For 41 of the 100 unique *P. astreoides* genotypes, we observed amplification peaks indicating third alleles at one to four loci (of eight); and across all samples, all markers exhibited occasional three-allele states. Such individuals were found in all four subpopulations. These triallelic genotypes remained despite repeated extractions and amplifications and, when observed, occurred in both halves of individuals. Triallelic genotypes were also observed in independent analyses with *P. astreoides* populations from the US Virgin Islands and Bermuda, although at a lower frequency (X. Serrano, personal communication).

Although somatic mutation is known to produce intracolony variation (Maier *et al.* 2012), this process would be unlikely to generate identical novel genotypes in different colony halves. Alternative explanations include physical proximity of planula larvae during recruitment resulting in fine-scale chimerism, the presence of brooded larvae in the genotyped corals or a ploidy >2n. As the karyotype of *P. astreoides* is unknown, we tested for higher order ploidy by modelling the expected multiallele frequency under different ploidy scenarios given observed allelic diversity (Appendix S1, Supporting information). We find no support for polyploidy and conclude that subpopulations include about 55% of diploid and 'noncontaminated' colonies, and 45% of colonies that are probably diploid as well, but are 'contaminated' by extraneous genetic material. Because we cannot rule out the remaining explanations, and because the true diploid genotype of 'contaminated' individuals cannot be determined, we excluded all individuals that exhibited triallelic genotypes at any loci from the allelic analyses, leaving 17 inshore and 20 offshore individuals from Sugarloaf and 11 inshore and 7 offshore individuals from Summerland.

The significance of genetic differentiation between populations was examined using a multilocus *G*-test following (Goudet *et al.* 1996). The pairwise F_{ST}-statistic was calculated following the study by Weir & Cockerham (1984) using FSTAT version 2.9.3.2 (Goudet *et al.* 2002). As a parallel alternative approach, the number of genetically differentiated clusters (K) was estimated using the program STRUCTURE (Falush *et al.* 2003) with an admixture model including sampling site as a prior. Log-likelihood values for each K (1–16) were computed from the multilocus genotypes with a series of 20 independent runs for each K. The most likely K was evaluated using the method of Evanno *et al.* (2005) as implemented in STRUCTURE HARVESTER (Earl & vonHoldt 2011). CLUMPP (Jakobsson & Rosenberg 2007) and DISTRUCT (Rosenberg 2004) were used to visualize results for the most likely K.

Symbiodinium genotyping

Symbionts were genotyped from both halves of experimental individuals using the standard *Symbiodinium* marker, the nuclear internal transcribed spacer region 2 (ITS2). We developed a deep sequencing approach based on 454 (Roche), following the logic of Stat *et al.* (2011), to more accurately quantify the relative proportions of different ITS2 genotypes within each coral fragment (see Appendix S1 for detailed methods, Supporting information).

Internal transcribed spacer region 2 amplicons were only sequenced from samples in which they could be amplified in <35 PCR cycles. In total, sequences were obtained for 49 of the 60 fragments: 12 inshore control, 12 inshore heat, 15 offshore control and 10 offshore heat. Of the 45 192 total reads, 44 393 were left after adaptor trimming, quality filtering and discarding reads shorter than 150 bp. Remaining sequences per sample ranged from 180 to 2886 (mean: 854, median: 687). Pooled reads from all samples were then clustered into 100% identical groups using the program *cd-hit* (Weizhong & Godzik 2006) resulting in 11 395 unique sequence clusters. 48% of all the sequence reads were contained within seven clusters each comprising >200 reads (Fig. S2, Supporting information). These sequences were aligned using the program SeqMan (DNASTAR) to identify SNPs, and the consensus alignment was used as a query to blast against the GenBank (NCBI) nucleotide collection.

These seven unique sequences were used as reference haplotypes to which we mapped all reads using the command *runMapping* from the Newbler GS Reference mapper program v2.6 (Roche), with the repeat score threshold (-rst) parameter set to 0. Of the 44 393 trimmed sequences across samples, 39 654 (89%) were successfully mapped to one of the seven reference haplotypes. The most frequent haplotype in our reference was assigned 31% of all mapped reads, whereas the least frequent one was assigned 2% of the reads, five-fold lower than the typical detection limit (10%) in standard *Symbiodinium* genotyping methodologies based on electrophoresis. We chose not to include rarer references because smaller haplotype clusters were increasingly likely to correspond to systematic errors in sequencing rather than unique, low-abundance symbiont haplotypes and also because the physiological impact of such rare genotypes is uncertain. The number of reads assigned to each genotype within sample was divided by the total number of reads uniquely mapped to normalize variation in absolute read number between samples.

Statistical analysis

All analyses were carried out using R 2.13.2 (R Development Core Team 2013). Differences in the physiological response variables' growth, bleaching (brightness in the red channel), EQY and MQY were evaluated with respect to treatment and coral origin using a nested series of linear mixed models implemented in the *lme4* package (Bates 2005). For all models, treatment and origin were modelled as fixed factors, with levels control/heat and inshore/offshore, respectively, as well as their interaction. Colony identity was included as a scalar random factor. Significance of factors was evaluated using likelihood ratio tests (LRT). A random effect of tank was also included if model fit was significantly improved with its addition, according to the LRT. The applicability of model assumptions (linearity, normality, homoscedasticity) to the data was verified using diagnostic plots of residuals formed by fitting a linear model with the fixed factors listed above, using the functions *lm()* and *plot()*. The proportions data (EQY and MQY) were arcsine-square-root-transformed, and independent comparisons were performed at three time points: the final two days of acclimation, the three initial days of treatment and the final two days of treatment. For symbiont sequence data, frequencies of haplotypes within each sample were arcsine-square-root-transformed, and the frequency of each haplotype with respect to colony origin and experimental treatment was evaluated independently as a response variable. Multiple test correction was subsequently applied to LRT *P*-values for symbiont genotype frequency models using the function *p.adjust*, as recommended by Benjamini & Hochberg (1995). Symbiont genotype divergence between origin, treatment and bleaching status was also explored using the entire haplotype frequency data set through a principal components analysis (PCA) using the *labdsv* package (Roberts 2008). For the purpose of this comparison, heat-treated corals were subdivided into two 'bleaching status' categories based on their brightness. The lightest-coloured top 50% of heat-treated coral samples were designated 'bleached', while those in the bottom 50% were designated 'pale'. All the control samples were designated 'normal'.

The divergence between the seven symbiont reference haplotypes was evaluated by constructing haplotype networks using the functions *haplotype* and *haploNet* from the *pegas* package (Paradis 2010). The four top-scoring blast hits from NCBI's GenBank (Pruitt *et al.* 2012) were included in haplotype network reconstruction, in addition to one more distantly related ITS2 outgroup. Haplotypes were manually trimmed to be of identical length, and gaps were coded as a single-base change such that each indel was considered equivalent to a single point mutation. In addition, we conducted pairwise regressions for all of our symbiont haplotype frequency data to look for positive correlations, suggesting that compared ITS2 sequences

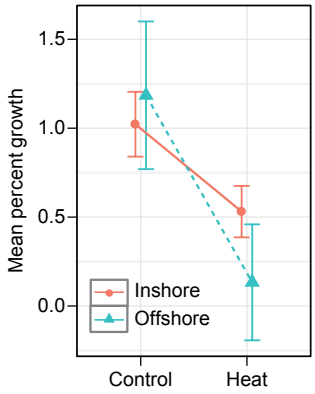

Fig. 2 Mean percent weight gain following 6 weeks of experimental treatment as calculated from a buoyant weighting method (Davies 1989). Red circles: inshore population average (±SEM); blue triangles: offshore population average (±SEM) under control and elevated temperature treatment.

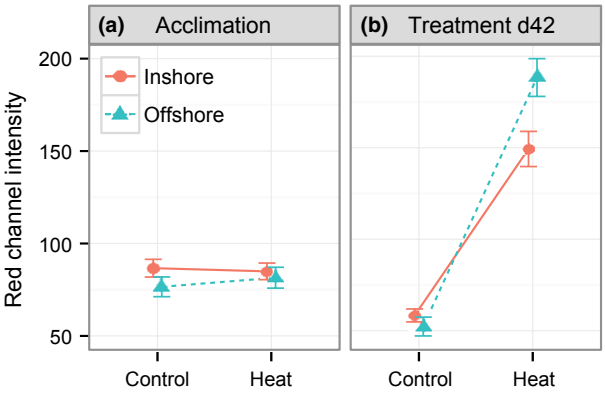

Fig. 3 Bleaching as measured by brightness in the red channel in standardized coral photographs (a) during the acclimation period prior to beginning treatment and (b) at the end of the 6-week experimental period. Red circles: inshore population average (±SEM); blue triangles: offshore population average (±SEM) under control and elevated temperature treatment.

might represent variants within the same genome (Thornhill *et al.* 2007).

Results

Growth and bleaching

Growth was significantly affected by treatment, with heat-stressed corals gaining significantly less weight than controls irrespective of their origin ($P < 0.001$, LRT, Fig. 2). Graphical trends suggest a site of origin by treatment interaction, as offshore corals gained 0.4% less weight on average than inshore corals, although this interaction was not significant ($P = 0.23$, Fig. 2).

Prior to beginning treatment, corals did not differ in their brightness (Fig. 3a). At the end of the 6-week experimental period, however, we observed significant differences in brightness with respect to treatment and the origin by treatment interaction. Heat treatment resulted in a major increase in brightness for both inshore and offshore corals, indicating reduced symbiont and/or chlorophyll densities (i.e. bleaching, $P < 0.001$, LRT, Fig. 3b). In addition, offshore corals exhibited significantly higher brightness under heat treatment than inshore corals, indicating that offshore corals bleached more severely than inshore corals in response to a common thermal stress ($P < 0.01$, LRT, Fig. 3b).

Photochemical yield of Symbiodinium photosynthesis

The results for both effective (EQY) and maximum quantum yield (MQY) recapitulate bleaching patterns. Prior to beginning the experimental treatment, EQY and MQY were not significantly different between population and treatment groups (Fig. 4a, d). After the first three days of experimental treatment, both EQY and MQY showed significant reductions under heat treatment by 7% and 11%, respectively ($P < 0.001$, LRT, Fig. 4b, e). A significant treatment effect was also observed at the end of the experiment. Individuals under heat treatment showed a 47% reduction in EQY and a 46% reduction in MQY in comparison with their paired controls ($P < 0.001$, LRT, Fig. 4c, f). Final MQY measurements also differed between inshore and offshore corals, with offshore corals exhibiting slightly reduced MQY values overall in comparison with inshore corals ($P = 0.045$, LRT, Fig. 4f). MQY measurements >0.6 can be used as an indicator of a healthy photosystem (Chalker 1983). Mean MQY values were above 0.6 for the duration of the experiment, except for heat-treated corals at the final time point, where inshore and offshore MQY values were 0.42 and 0.29, respectively. Final EQY measurements revealed a significant origin by treatment interaction ($P < 0.01$, LRT). Inshore and offshore controls did not differ significantly from each other, whereas under heat treatment, offshore origin individuals showed a 37% reduction in EQY, compared with inshore origin individuals (Fig. 4c).

To explore the correlation between symbiont photosynthesis and growth, we plotted mean percent weight gain values against the average of both EQY and MQY measurements taken during the final days of the experiment. A subtle but significant positive relationship was found, where quantum yield measurements explain approximately 12% of the variation in weight gain across individuals (adjusted $r^2 = 0.124$, $P = 0.004$, Fig. 5).

Genotyping (host)

Multilocus genotyping revealed a substantial frequency of triallelic genotypes across all loci. As polyploidy was

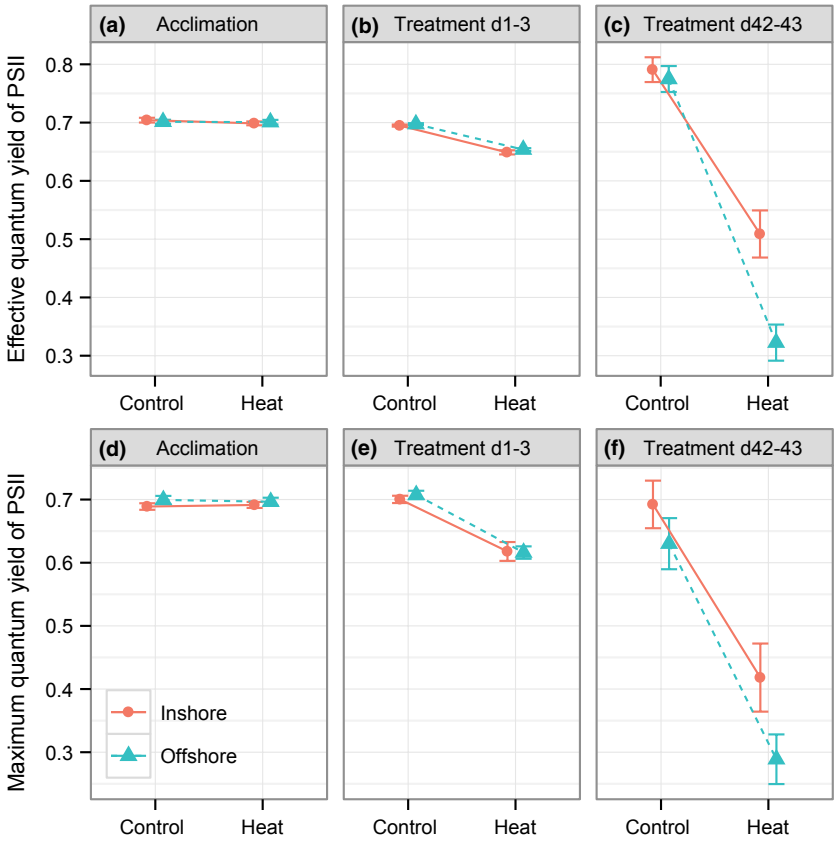

Fig. 4 Photochemical yield of *in hospite Symbiodinium*. (a–c) Effective quantum yield and (d–f) maximum quantum yield for inshore (red circles) and offshore (blue triangles) coral fragments under control and heat treatments (±SEM) represent three time periods: prior to beginning the temperature treatment (a, d), during the initial 3 days of elevated temperature treatment (b, e) and during the final 2 days of the 6-week temperature treatment (c, f).

deemed unlikely (see Host Genotyping Methods), we only included individuals showing diallelic genotypes across all loci for the F_{ST} and STRUCTURE analyses. The frequencies of multilocus genotypes differed significantly between inshore and offshore origin corals for the entire data set ($n = 46$ inshore, $n = 51$ offshore) as well as for the diallelic-only data set ($n = 28$ inshore, $n = 26$ offshore, $P < 0.001$, multilocus *G*-test). Allele frequencies, as calculated for the diallelic data set, also differed significantly between inshore and offshore origin individuals (Summerland Key inshore/offshore $F_{ST} = 0.0402$, $P < 0.05$; Sugarloaf inshore/offshore $F_{ST} = 0.0575$, $P < 0.05$) as well as between the two offshore reef sites ($F_{ST} = 0.0487$, $P < 0.05$). Allele frequencies between the two inshore sites showed no significant differentiation ($F_{ST} = 0.0134$, $P = 0.15$). Plots of delta-*K* (Evanno *et al.* 2005) from STRUCTURE indicate that the most likely number of genetic clusters is 4. Visualization of STRUCTURE results reveals that inshore individuals show a higher probability of assignment to clusters not observed at offshore sites (Fig. 6).

Genotyping (symbiont)

All genotyped *Porites astreoides* hosted *Symbiodinium*-type A4/A4a, as has been reported previously for individuals of this species in the Florida Keys (Thornhill *et al.* 2006). Haplotype network reconstruction clusters the seven reference haplotypes with A4 and A4a sequences from GenBank (NCBI), while the more distantly related A1 (*S. microadriaticum*) is a clear outgroup. GenBank sequence A4.3 aligned against the reference sequence 1 with 100% identity (Fig. 7a).

The majority of reads were assigned to haplotypes 1, 2, 4 and 7, together comprising 89% of the mapped reads. None of these haplotypes showed enrichment with respect to origin, treatment or the interaction. Differences in symbiont genetic composition between populations were limited to two minor-frequency haplotypes. Haplotypes 3 ($P < 0.05$, LRT) and 6 ($P = 0.05$, LRT) together accounting for 7% of the mapped reads were significantly more represented in inshore individuals (Fig. 7a). A PCA suggested that the increase in representation of haplotypes 3 and 6 might result from a reduction in haplotype 1 (Fig. 7b, c), because haplotypes 3 and 6 are the strongest positive loadings on PC1, while haplotype 1 is the strongest negative loading, although this decrease was not statistically significant in the individual tests. Apart from this trend, there was no apparent clustering of corals for either the origin/treatment interaction (Fig. 7b) or

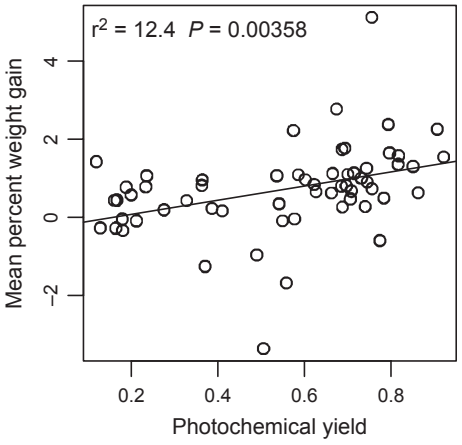

Fig. 5 Percent weight gain as a function of symbiont photosynthetic function (mean Chlorophyll *a* fluorescence) at the end of the 6-week experimental treatment.

bleaching status (Fig. 7c) with respect to total haplotype representation.

The proportion of reads mapped to haplotype 3 showed a significant positive relationship with those mapped to haplotype 6 (adjusted $r^2 = 0.41$, $P < 0.001$). ITS2 is multicopy and can vary intragenomically (Thornhill *et al.* 2007); therefore, a positive correlation between ITS2 haplotype frequencies could indicate that they represent variants co-occurring within the same genome. To distinguish co-occurrence from enrichment of both haplotypes in inshore individuals, we ran separate regression of haplotype 3 on haplotype 6 within each population. Both showed significant positive relationships, although the correlation was stronger in inshore corals (adjusted $r^2 = 0.37$, $P = 0.001$; offshore: adjusted $r^2 = 0.26$, $P = 0.005$, Fig. S3, Supporting information). Relationships between the major haplotypes all show either negative or neutral relationships (Fig. S4, Supporting information) indicating that they probably represent different symbiont genomes.

Discussion

Elevated thermotolerance of inshore Porites astreoides

Significant differences in bleaching and symbiont photophysiology accompanied by a trend towards differential growth in inshore and offshore corals under common heat stress indicate elevated thermotolerance in inshore corals (Figs 2 and 3). This could be due to local thermal adaptation but may also be the result of long-term acclimatization, the effects of which did not dissipate over the relatively short acclimation period. Coral growth and reproduction are significantly influenced by symbiont type and condition (Szmant & Gassman 1989; Little *et al.* 2004; Jones & Berkelmans 2011). It has been estimated that up to 95% of host energy requirements are met by symbiont-derived photosynthetically fixed carbon (Muscatine 1990), which contributes to skeletal deposition (Vago *et al.* 1997). Bleaching has been shown to reduce reproduction (Szmant & Gassman 1989) and growth, which can persist for months following the event (Jones & Berkelmans 2010). The effects of heat observed at the holobiont level (growth and bleaching) might therefore be driven by the decrease in symbiont photosynthetic function (Fig. 4). This decrease, in turn, can be attributed to higher stress susceptibility of offshore symbionts, failure of offshore hosts to mitigate the effects of heat stress on their symbionts or a combination of the two. Below we discuss our results in the light of these alternatives.

Reproductive strategy and local adaptation: general considerations

In broadcast-spawning coral species, the role of symbionts in shaping holobiont thermotolerance physiology has been more thoroughly explored. Some species are known to associate with more thermotolerant symbiont types in warmer environments (van Oppen *et al.* 2001; Oliver & Palumbi 2011b), and others are able to shuffle proportions of symbionts *in hospite* to favour more thermotolerant types in stressful conditions (Berkelmans & van Oppen 2006; Jones *et al.* 2008).

Given the fact that the majority of broadcast-spawning coral species do not receive their symbionts from parent colonies (van Oppen 2004; Baird *et al.* 2009b), and assuming wider connectivity ranges for coral larvae than for symbionts (Rodriguez-Lanetty *et al.* 2001; Howells *et al.* 2009; Kirk *et al.* 2009; Barshis *et al.* 2010; Baums *et al.* 2010; LaJeunesse *et al.* 2010), the driving role of symbionts in local thermal adaptation of these species is not surprising. Establishing a relationship with locally adapted symbionts should maximize holobiont fitness irrespective of the coral's own genetic background, which would allow the coral to maintain high genetic connectivity across environmental gradients while reducing the detrimental effect of gene flow on local adaptation.

Such a strategy does not seem feasible for most brooding species like *Porites astreoides*, which transmit their symbionts vertically from parent to larvae. Vertical transmission may instead potentiate long-term co-evolution of hosts and symbionts, which has been proposed previously for *P. astreoides* and other vertical transmitters (Diekmann *et al.* 2003; Thornhill *et al.* 2006). We further propose that such a co-evolutionary scenario would result in adaptation of both symbiotic partners to the local environment as well as to each other, due to the reduced dispersal capacity typical of brooding

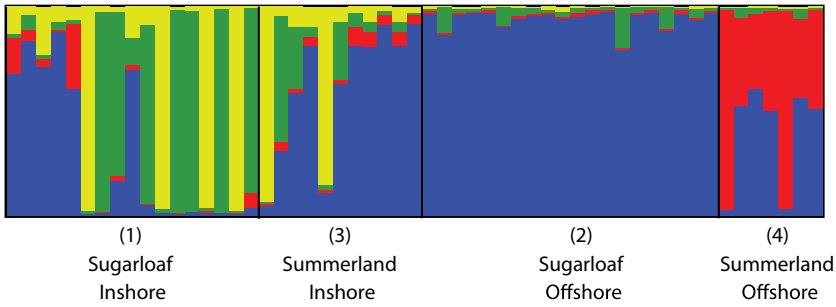

Fig. 6 Genotypic composition of inshore and offshore *Porites astreoides* populations in the lower Florida Keys based on analysis of eight microsatellite loci using STRUCTURE (Pritchard *et al.* 2000). Colours correspond to distinct genotypic clusters, and the proportion of colours within columns indicates the probability of an individual coral's assignment to that cluster. Numbers associated with sites correspond to sampling locations indicated in Fig. 1.

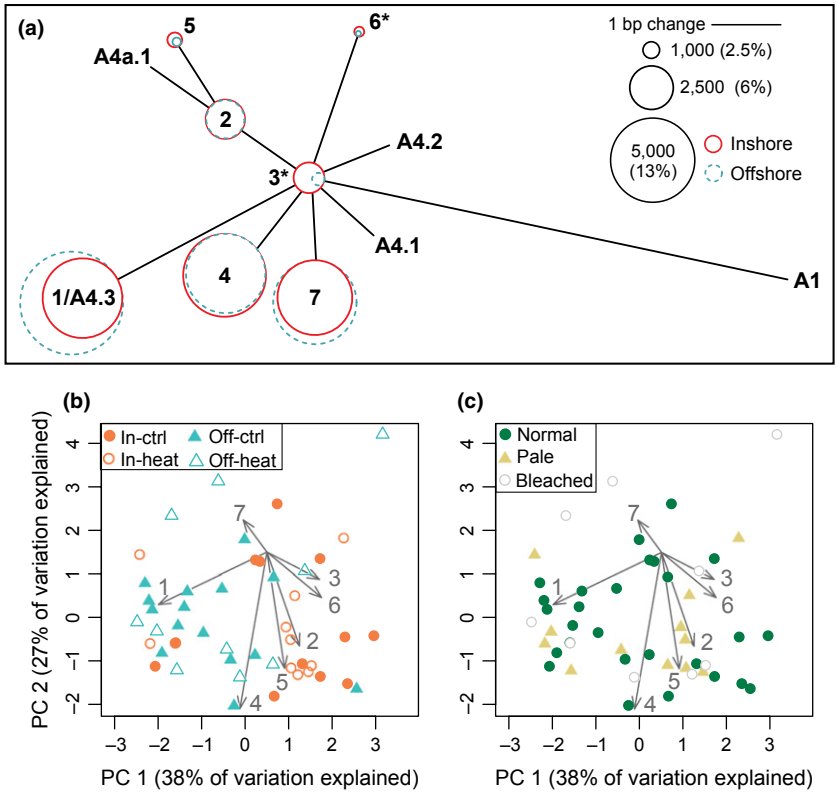

Fig. 7 *Symbiodinium* diversity at the internal transcribed spacer region 2 (ITS2) locus. (a) Haplotype network built using an infinite site model for the seven major haplotypes (circles 1–7). Four additional clade A4/A4a sequences and one A1 outlier were obtained from GenBank to serve as references. Haplotype 1 and reference sequence A4.3 are identical. The linear distance between haplotypes is proportional to the number of point mutations or indels, and the circle size corresponds to the proportion of mapped reads recorded from inshore corals (solid red circles) and offshore corals (dashed blue circles). Asterisks mark significant differences ($P < 0.05$) between locations. (b, c) Principal components analysis of *Symbiodinium* communities as defined by relative proportions of ITS2 types. Both plots contain the same points and loading vectors, but are colour-coded according to different factors. (b) Differences with respect to origin (inshore: red circles, offshore: blue triangles) and treatment (control: solid symbols, heat: open symbols). (c) Differences with respect to bleaching phenotype: normal (i.e. control, green circles), pale (tan triangles) and bleached (white circles).

species (Ayre & Hughes 2000; Underwood *et al.* 2007) that keeps successive holobiont generations within the same environmental conditions. One prediction following from this scenario is a limited flexibility in host–symbiont association; another, that host genotype should play a more prominent role in determining spatial variation in thermotolerance physiology than in broadcast-spawning coral species.

Genetic divergence between inshore and offshore coral hosts

We found that *P. astreoides* inhabiting reefs separated by only 7–8 km exhibit significant population genetic subdivision, consistent with previous studies of other brooding corals (Fig. 6, Ayre & Hughes 2000; Maier *et al.* 2005; Underwood *et al.* 2007; Goodbody-Gringley *et al.* 2010), which increases the potential for adaptive divergence in the coral host in response to local selection. The reduced gene flow observed in this study has three explanations that are not mutually exclusive: *P. astreoides* larvae have difficulty traversing the current systems of Hawk Channel that separate inshore and offshore reefs; larvae have an innate propensity for rapid recruitment; and/or there is selection against immigrants from different reef habitats. Empirically distinguishing between these explanations is nontrivial (Hedgecock 1986). Assuming that variation at our microsatellite loci is neutral or nearly neutral, natural selection should not have precluded the exchange of alleles across habitats if even a small proportion of immigrants survived to reproductive age. Limited dispersal appears more likely, as brooded larvae are competent to settle within 4 h of release (Stake & Sammarco 2003; Goodbody-Gringley 2010) and tend to recruit locally, in some cases within 100 m of the parent colony (Underwood *et al.* 2007).

Similarity of Symbiodinium communities

The constancy of the *P. astreoides–Symbiodinium* clade A4/A4a association in the Florida Keys suggests consistent vertical transmission in this system (Thornhill *et al.* 2006). However, *P. astreoides* is known to host different symbiont types elsewhere throughout its range (LaJeunesse 2002). Moreover, clade A4/A4a is by no means specific to *P. astreoides* and has been found in association with a wide variety of cnidarians (LaJeunesse 2001, 2002) as well as in a free-living state (Porto *et al.* 2008). This raises the possibility that *P. astreoides* might be able to associate with local subtypes of A4 found in inshore or offshore environments. However, our results from deep sequencing of the *Symbiodinium* ITS2 suggest otherwise (Fig. 7). The relative proportions of major A4-related haplotypes, together accounting for 89% of all mapped sequences, remained constant across locations. These major haplotypes probably represent different symbiont genomes, as they are predominantly negatively correlated in frequency. Both inshore and offshore corals hosted the same symbiont haplotypes at similar frequencies, indicating the lack of horizontal acquisition of novel strains. We also did not detect a change in the symbionts' proportions ('shuffling') in response to heat treatment despite pronounced paling and bleaching of the holobionts. This suggests that all haplotypes are equally susceptible to heat stress and none appears to be more heat tolerant, at least in terms of a more dominant association with the host.

Host and symbiont roles in holobiont thermotolerance

If the predominant symbiont types are not changing in this system, where do population-level differences in holobiont fitness and symbiont photophysiology come from? First, they might be attributable to the effect of minor *Symbiodinium* ITS2 haplotypes (3 and 6) that are significantly elevated in inshore corals. However, such low-frequency *Symbiodinium* strains, together accounting for only about 7% of mapped reads, have never been demonstrated to play an important role in modulating holobiont fitness, although this possibility cannot be formally excluded. Additionally, these haplotypes do not increase in frequency as a result of heat treatment as would be expected if they conferred elevated thermotolerance to their host (Berkelmans & van Oppen 2006).

The internal transcribed spacer region 2 is a single, moderately variable marker; therefore, it is hardly surprising that differences in *Symbiodinium* thermotolerance can arise in different environments in isolates of identical ITS type (Howells *et al.* 2011). However, given that we observe multiple ITS2 haplotypes within inshore and offshore corals, it is more challenging to explain how holobiont thermotolerance varies without an accompanying change in the relative proportions of these hosted types. If ITS2 variants represent alleles segregating within a sexually reproducing panmictic population of symbionts, habitat-driven selection could act on fitness-associated loci elsewhere in the *Symbiodinium* genome without affecting the frequencies of unlinked loci such as ITS2. However, existing evidence suggesting that *Symbiodinium* reproduction *in hospite* is predominantly asexual (Correa & Baker 2009; van Oppen *et al.* 2011), arguing against this explanation. Moreover, the free-living stage in the symbionts' life cycle is likely bypassed due to the vertical mode of symbiont transmission in *P. astreoides*, so this asexual phase may last for several coral generations.

Conversely, ITS2 types could represent different nonrecombining *Symbiodinium* lineages (LaJeunesse 2001, 2005; Litaker *et al.* 2007), but see Correa & Baker (2009). In this case, their relative frequencies *in hospite* might depend more strongly on other lineage-specific traits rather than on their contributions to holobiont thermotolerance. The evolution of higher thermotolerance could then happen within individual lineages without accompanying changes in the overall composition of

the community. However, thermotolerance appears to be a key determinant of a strain's success *in hospite*, as evidenced by symbiont community modulation in response to temperature stress ('symbiont shuffling') at least in some coral species (Berkelmans & van Oppen 2006)).

Alternatively, population-level differences in holobiont performance may be attributable to divergence in host populations. Two lines of evidence support this notion: first, the coral host does show significant genetic divergence between inshore and offshore reefs; and second, host gene expression patterns suggest that inshore and offshore corals manage their energetic metabolism differently in response to thermal stress (Kenkel *et al.* 2013). It must be acknowledged that the use of higher-resolution genetic markers might uncover additional variation within individual *Symbiodinium* ITS2 types (LaJeunesse & Thornhill 2011), some of it with respect to location. Even so, the lack of change in ITS2 frequency profiles between corals from different locations or during heat exposure remains a strong indication that none of these putative within-ITS2-type variants is more heat-tolerant than other *Symbiodinium* types and subtypes hosted by *P. astreoides*.

Adaptation vs. acclimatization

Further work is needed to determine the mechanism underlying observed differences in holobiont thermotolerance between inshore and offshore populations. Divergent responses may be due to physiological plasticity or they could be the result of genetically based adaptation resulting from generations of selection under different thermal regimes, aided by restricted migration between reef environments. The former implies that both inshore and offshore populations have the potential to achieve the full range of observed thermotolerances if given enough time to acclimatize; the latter, that offshore populations might be unable to adapt to increased temperature stress without genetic input from more thermotolerant inshore populations (Sanford & Kelly 2011). Distinguishing between these scenarios is critical to understanding current population dynamics and predicting the response of populations to a rapidly changing climate.

Conclusions

Our results indicate that inshore and offshore *Porites astreoides* populations in the Florida Keys are either adapted or have acclimatized to local thermal conditions, with inshore corals exhibiting higher thermotolerance than offshore corals. In contrast to the typical scenario observed in broadcast-spawning coral species, this physiological divergence does not seem to be the result of differences in hosted *Symbiodinium* ITS types. Genetic divergence between coral host populations in combination with location-specific modifications to host energy metabolism (Kenkel *et al.* 2013) argue in favour of a prominent role of the host in shaping holobiont thermotolerance responses in this system. Further work will aim to clarify the details of host–symbiont interactions governing these population-level differences.

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C.D.K. and M.V.M. conceived and designed the long-term stress experiment. C.D.K., G.G.G. and E.B. conducted the experiment. C.D.K. analysed the data. S.W.D. developed microsatellite assays. D.C. completed ploidy modelling. All authors contributed to writing the manuscript.

Data accessibility

Raw phenotypic trait data (including photographs), symbiont genotyping data (both raw .fna files and mapped read data), host genotyping data (for STRUCTURE input and ploidy modelling) and R scripts: DRYAD doi: 10.5061/dryad.s706 g.

Microsatellite sequences: GenBank Accession numbers listed in Table S1.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Detailed methods descriptions for ploidy modeling and preparation of ITS2 samples for 454 sequencing.

Fig. S1 (a) Mean temperature (±one standard deviation) in each treatment tank for the duration of the 6-week experiment. (b) Continuous temperature data (measurements taken every 8 min) for the one control and the one heat tank equipped with HOBO (Onset) data loggers for the duration of the 6-week experiment.

Fig. S2 (a) Histogram of high-frequency 100% identity sequence clusters indicating size of the cluster, for example the '>25' bar indicates the number of clusters that were assigned at least 25 individual sequence reads. (b) The proportion of total read data represented in the high-frequency clusters.

Fig. S3 Correlations of frequencies of haplotypes 3 and 6 for inshore and offshore populations.

Fig. S4 Pairwise correlations between frequencies of dominant haplotypes (1, 2, 4 and 7) among individual colonies across all populations and experimental treatments.

Fig. S5 Mean parameter $Q \pm 95\%$ profile likelihood confidence intervals for individual subpopulations and the entire dataset.

Table S1 Summary of eight polymorphic *Porites astreoides* SSR markers assessed across 21 individuals from the offshore site of Sugarloaf Key, Florida.