

Genetic variation in responses to a settlement cue and elevated temperature in the reef-building coral *Acropora millepora*

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ABSTRACT: Reef-building corals are threatened by increasing sea surface temperatures resulting from global climate change. Whether corals can adapt to increasing temperatures over the course of generations will depend in part on heritable variation in thermal physiology and dispersal potential, which may serve as the raw material for natural selection. To investigate whether such variation exists in coral populations, and build a framework for identifying the coral-specific genetic factors involved, we performed controlled crosses between 3 genetically distinct colonies of the branching coral *Acropora millepora*. We compared the families of larvae (which in this species naturally lack symbionts) for several physiological traits, and observed between-family differences in nearly every case. Using larvae cultured at standard and elevated temperatures, we measured the developmental decrease in protein content and the expression of candidate heat response genes. We used an *in vivo* assay for mitochondrial enzyme activity to evaluate the metabolic response to temperature changes in individual larvae. We also compared the responsiveness of larvae from different families to a natural settlement cue to gain insights into long-range dispersal potential. Partitioning the components of total phenotypic variance confirmed the existence of additive genetic effects for settlement rates and β -crystallin expression, while variance in mitochondrial Q_{10} and the expression of actin and *Hsp16* were driven by non-additive effects. The phenotypic variance observed among the small number of families analyzed here suggests the existence of considerable heritable variation in natural coral populations, which supports the possibility of effective adaptive responses to climate change.

KEYWORDS: Global climate change · Adaptation · Coral reefs · Thermal tolerance

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INTRODUCTION

The threats posed to reef-building corals by global climate change are now widely recognized. Coral bleaching episodes have occurred with increased frequency in recent decades, corresponding to major declines in coral populations (Wilkinson 1996, Brown 1997, Gardner et al. 2003). These declines are clearly associated with environmental factors including ele-

vated sea surface temperatures (Glynn & D'Croz 1990) and acidification resulting from increased atmospheric CO₂ (Hoegh-Guldberg et al. 2007, De'ath et al. 2009). Current climate models predict increasing ocean temperatures in the coming decades (IPCC 2007), prompting questions about the future of coral populations and coral reef ecosystems. The potential for adaptation, acclimatization, or range shifting by corals in response to global climate change is therefore a major focus of

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contemporary research in coral biology (Coles & Brown 2003, Precht & Aronson 2004, Berkelmans & van Oppen 2006, Lesser 2007).

A key factor determining the thermal sensitivity of corals, and the potential for any adaptation or acclimatization, is their symbiotic association with zooxanthellae (genus *Symbiodinium*). These intracellular symbionts are directly affected by temperatures just 1 to 2°C above ambient summer sea surface temperatures (Jokiel & Coles 1990). Damage to the symbionts is followed by pigment loss, death, or expulsion from the host (Warner et al. 1999). Diverse thermal tolerances are observed among genotypes of the symbiotic zooxanthellae (Robison & Warner 2006), and this variance has a profound impact on the thermal tolerance of corals associated with particular symbiont types (Rowan 2004, Berkelmans & van Oppen 2006). Despite this historical focus on symbionts, it is increasingly clear that thermal responses of corals are affected by both coral- and symbiont-specific factors (Abrego et al. 2008), and data on both partners in the symbiosis will be required for effective modeling and management of coral populations (Baums 2008, Day et al. 2008, Maynard et al. 2008, Baird et al. 2009).

There are several mechanisms through which the coral host might influence thermal responses, including photoprotection, removal of reactive oxygen species, and molecular chaperone activities (Baird et al. 2009). The expression of genes from the coral host has been extensively used as a biomarker system for environmental stress, providing clear evidence that coral traits are involved in stress responses (Brown et al. 2002, Edge et al. 2005). Baseline variation in the expression of these indicator genes has been shown within populations (Edge et al. 2008), some of which is probably genetically determined. Several other lines of evidence also suggest genetic variation in thermal responses. First, variation in bleaching responses has been reported among different coral taxa (Berkelmans & van Oppen 2006), and also within populations of a particular coral species (Edmunds 1994). Reciprocal transplant experiments have identified locally adapted specialist genotypes (D'Croz & Maté 2004). Finally, the development of nuclear genetic markers for corals has revealed more population structure among corals than previously expected (Baums 2008), confirming the genetic plausibility of local adaptation to environmental conditions. Although none of these studies on natural populations have unambiguously demonstrated genetic control of thermal tolerance, together they provide a rationale for experiments aimed at identifying genetic determinants of thermal responses in corals.

One possible scenario for coral populations faced with warming sea surface temperatures is geographic

range shifting, in which corals colonize newly suitable habitats (e.g. at higher latitudes). There is good evidence for range shifting by corals over geological time scales (Greenstein & Pandolfi 2008), and some evidence of range shifting during the current climate change has begun to emerge (Precht & Aronson 2004). Reef-building corals have larval dispersal phases that can range from days to weeks in duration (Wilson & Harrison 1998), underscoring the importance of understanding larval responses to environmental stress during this time period. Differential effects of elevated temperatures on coral embryos and larvae have been found between species and between aposymbiotic and symbiotic larvae (Baird et al. 2006, Negri et al. 2007), but genetic variation in these larval traits within a species remains unexplored. The responsiveness of larvae to settlement cues is one important factor that can affect dispersal potential (Miller & Mundy 2003), in addition to the distribution of these cues in the environment and the time required to reach competence. Settlement cues affecting coral larvae have been extensively studied, including the documentation of natural cues (Heyward & Negri 1999), identification of the active compounds (Kitamura et al. 2007), and characterization of the roles of biofilms in recruitment (Webster et al. 2004). These studies provide a framework for characterizing genetic variation in the responsiveness of coral larvae to settlement cues.

The major aim of the present study was to characterize the extent of heritable variation in traits that might be adaptively important during climate change. Families of aposymbiotic larvae produced from controlled crosses were cultured under controlled conditions, avoiding the confounding effects of different thermal histories and symbiont types. Temperature responses were measured at multiple levels of biological organization (gene expression, enzyme activity, and protein content) and compared between replicate cultures for each family. Responses to a known settlement cue were compared among families, revealing differences that might affect dispersal potential. Our findings suggest substantial heritable variation in these traits, highlighting the need for a more complete understanding of the genetic and phenotypic variance that might be under selection in natural populations of corals facing warming sea surface temperatures.

MATERIALS AND METHODS

Genetic crosses and larval culture. In October 2007, 3 colonies of *Acropora millepora* were collected at Magnetic Island, Queensland, Australia, prior to the natural mass-spawning event characteristic of this and many other reef-building corals (Babcock et al. 1986).

Table 1. Crossing design. Names of 6 larval families produced by crossing sperm and eggs from 3 colonies are given as sire \times dam. Attempted self-crosses (within colonies) showed no fertilization

Egg source	Sperm source		
	Colony A	Colony B	Colony C
Colony A	—	BA	CA
Colony B	AB	—	CB
Colony C	AC	BC	—

All colonies were collected within a small area (Nelly Bay) to ensure that the genetic crosses produced corresponded to realistic possibilities within natural populations. Colonies were isolated in individual bins filled with 1 μ m filtered seawater (FSW) prior to spawning. After spawning, gamete bundles were collected from each colony and gently passed through a 300 μ m nylon mesh to separate sperm from eggs. Samples of sperm were collected from each parent colony and preserved in ethanol for genotyping. Parent colonies were later genotyped at 40 microsatellite loci (Wang et al. 2009), confirming that these colonies were genetically distinct. Based on these data, a similarity index was calculated for each comparison between parent colonies (Kosman & Leonard 2005). Following spawning, parent colonies were returned to the reef.

The eggs isolated from each colony were combined separately with sperm from each of the other 2 colonies to produce 6 different larval families (Table 1). Self-fertilization occurs at low rates in this species (Willis et al. 1997), and control self-cross trials verified that self-fertilization was not detectable in our samples. After fertilization, excess sperm were removed by rinsing with FSW on 300 μ m mesh, and embryos were stocked into 2 l FSW in plastic culture vessels at 2 ml⁻¹. Three independent cultures were established for each larval family, in each of 2 different temperature-controlled rooms, for a total of 36 culture vessels. One room was set at a standard culturing temperature of 28°C, and the other at an elevated temperature of 32°C. Water temperature was recorded at 10 min intervals using data loggers (Onset Computer), confirming that temperature was maintained at within half a degree of target levels (27.7 \pm 0.4°C and 31.4 \pm 0.5°C, respectively). Culture seawater (FSW) was changed 1 d after fertilization to remove unfertilized eggs and cell debris, and at 2 d intervals thereafter. Larvae were sampled from each culture vessel daily and preserved in RNALater (Ambion) for later analysis.

Measurement of protein content. Protein content of individual larvae was measured throughout development (1 to 5 d post-fertilization) using the Bradford assay (Bradford 1976) with the following minor

modifications. Individual larvae were first rinsed in 150 mM NaCl, then transferred individually into a 96-well PCR plate along with 10 μ l of 150 mM NaCl. Larvae were homogenized by adding 28 μ l of 1.33 N NaOH and back-pipetting 10 to 20 times, then incubated at 60°C for 30 min. Samples were neutralized with 10 μ l of 1.67 N HCl and combined with 150 μ l of Bradford Reagent (Sigma) in a 96-well flat-bottom plate. Each plate included duplicate dilutions of a standard curve prepared from bovine serum albumin (BSA), ranging from 2 to 15 μ g per well. Protein content was calculated based on the absorbance at 595 nm using a SpectraMax M2 plate reader (Molecular Devices) and comparison to the standard curve. Multiple individuals (n = 8) were measured from each culture vessel at each developmental time point (1 to 5 d post-fertilization), for 1440 individual measurements in total. The amount of protein lost during development was calculated for each culture vessel as the difference between initial and final contents (1 and 5 d post-fertilization).

***In vivo* measurement of mitochondrial metabolism.**

Mitochondrial metabolism of individual larvae was measured using an *in vivo* test of mitochondrial dehydrogenase activity based on the water-soluble tetrazolium salt WST-1 (Ishiyama et al. 1993). At 5 to 7 d post-fertilization, 25 individual larvae from each of the 32°C culture vessels were assayed individually in a 384-well plate. Larvae were incubated at constant temperature in the WST-1-containing cell proliferation reagent (Clontech) diluted 1:10 in FSW. Absorbance at 450 nm was monitored for 2 h using the SpectraMax M2 well plate reader with the temperature maintained at 28°C. Next, the temperature was increased to 32°C and the absorbance monitored for an additional 2 h. For the 28°C data, a slope was calculated for each individual larva by robust regression of absorbance against time using the MASS library in R statistical software (Venables & Ripley 2002). Next, a slope was calculated for the 32°C data from each larva in the same way. Finally, the increase in slope with increasing temperature was used to calculate a standardized measure of temperature response (Q_{10}) for each larva as: $Q_{10} = (\text{slope}_{32^\circ} / \text{slope}_{28^\circ})^{(10/\Delta T)}$, where ΔT is the change in temperature. Altogether, rates were measured in 450 individual larvae. To satisfy assumptions of linearity, data sets that fit poorly to linear models ($R^2 < 0.5$) were excluded. To minimize the impact of methodological artifacts (e.g. wells containing dead or lysed larvae), the largest and smallest 1% of slopes and Q_{10} values were excluded, along with wells showing negative slopes. The 349 assays that passed these quality filters were used for between-family comparisons of Q_{10} values.

qPCR analysis of gene expression. RNA was extracted from larvae preserved in RNALater using the

RNAqueous-micro kit (Ambion) as follows: Larvae were sampled at 5 d post-fertilization from all culture vessels in each temperature-controlled room (28 and 32°C) for RNA extraction ($n = 36$ extractions). RNA was extracted from samples of 8 to 12 larvae per culture vessel according to the manufacturer's instructions. For each sample, first strand cDNA was prepared from 85 ng of total RNA using SuperScriptII reverse transcriptase (Invitrogen) and a modified oligo-dT primer (5'-CGC AGT CGG TAC TTT TTT TTT TV-3'). An aliquot of first strand cDNA equivalent to 0.2 ng RNA was used for each qPCR reaction.

To compare the effects of temperature on gene expression between families, we selected a small heat shock protein gene (*Hsp16*) previously implicated in temperature stress responses in corals (Downs et al. 2000), and 2 additional genes (actin and $\beta\gamma$ -crystallin) based on unpublished experiments performed in our laboratory that showed differential expression of these genes in stressed corals. Analysis of qPCR data using the comparative C_T method, where C_T refers to the cycle at which the fluorescence signal crosses the threshold, requires the use of reference genes for normalizing expression to RNA loading (Livak & Schmittgen 2001); we selected 18S rRNA for that purpose on the basis that rRNA is the major component of total RNA and is therefore a suitable standard for normalizing expression to total RNA. cDNA sequences for these genes were identified by homology searches in annotated sequences from the larval transcriptome (Meyer et al. 2009), and primers were designed for each gene using Primer3 (<http://primer3.sourceforge.net/>). The primers used for qPCR were: actin-F (5'-GCT CCC CCA ACG ATG AAG AT-3'); actin-R (5'-GAC CCT CCA ATC CAT ACA GAG TAT TT-3'); Hsp16-F (5'-TCC TCC TCT GAG CGT TGT TT-3'); Hsp16-R (5'-CTC ACG GGA ATC AAA CGA GT-3'); $\beta\gamma$ -crystallin-F (5'-TTG GAG GAA AGT TTG ACG CT-3'); $\beta\gamma$ -crystallin-R (5'-TCA TTG CCG ATG TTC ATT GT-3'); 18S-F (5'-AAT CCT CAG TGG AGG GAG GT-3'); and 18S-R (5'-CAC CAG ACT TGT CCT CCG AT-3'). qPCR was performed in 15 μ l volumes with SYBR Green Master Mix (Applied Biosystems) and analyzed using the Applied Biosystems 7300 Real Time PCR System. The specificity of each primer pair was confirmed by gel electrophoresis and melting curve analysis, and dilution series were analyzed to confirm equal amplification efficiencies. Each cDNA sample was assayed in triplicate for each gene, and relative expression calculated using the ddC_T method (Livak & Schmittgen 2001).

Evaluating larval response to settlement cue. To quantify the responsiveness of competent larvae to a natural settlement cue, crustose coralline algae (CCA) (Heyward & Negri 1999), samples of 50 to 100 larvae from each culture vessel were transferred into 5 ml of FSW in a 6-well plate. Seven-day-old larvae from cul-

tures grown at 32°C were used for these settlement trials, which were performed at this same temperature. Locally collected samples of CCA were finely ground, then tested for effectiveness on larvae sampled from culture. Preliminary trials with overnight exposure to a strong settlement cue (freshly ground CCA) resulted in 100% settlement, demonstrating that larvae from all families had reached settlement competence. To allow for detection of differences in the settlement response, the cue was weakened by maturation of the ground CCA overnight, followed by several rinses with FSW. A small amount of this diluted cue was distributed to each well, and after 12 h the settled recruits were photographed along with any larvae that remained swimming. Settlement success was calculated as the percent of larvae that had settled and attached to the plate during the experiment.

Statistical analysis. Phenotypic data for each family were compared by ANOVA using R statistical software (R Development Core Team 2008), with age and temperature treated as fixed factors and family as a random factor. Bartlett's test was used to test for heteroscedasticity, and the Kolmogorov-Smirnov test to test for normality. The experimental unit for all comparisons was the average value per culture vessel. Developmental changes in protein content were analyzed using a 3-way ANOVA with the factors age, temperature, and family. For a conservative estimate of the effects of temperature on protein loss, a series of 2-way ANOVAs of initial and final protein content was conducted with the factors age and temperature, and corrected for multiple comparisons using the false discovery rate procedures of Benjamini & Hochberg (1995). Mitochondrial metabolic Q_{10} values were log-transformed to satisfy normality assumptions, and the average Q_{10} compared between families using 1-way ANOVA, followed by pairwise comparisons between families using Tukey's honestly significant difference (HSD) test. Gene expression data (ddC_T values) were analyzed separately for each of the 3 targeted genes using a 2-way ANOVA with the factors temperature and family. The percent of larvae that settled in response to settlement cue was arcsine square root-transformed, as is standard practice for percentage data (Zar 1984), and compared between families using 1-way ANOVA followed by pairwise comparisons using Tukey's HSD.

We subsequently applied a diallel model to these data to evaluate the relative contributions of additive genetic variance and other sources to the total phenotypic variance. For traits measured at a single culture temperature, we explored genetic effects using the standard random effects diallel model:

$$Y_{ijk} = \mu + g_i + g_j + s_{ij} + r_{ij} + e_{ijk}$$

where μ is the grand mean, g_i and g_j are the general combining abilities for the i th and j th parents, respectively, s_{ij} is the specific combining ability between the i th and j th parent, r_{ij} corresponds to reciprocal cross effects, and e_{ijk} is the residual (Griffing 1956, Falconer & Mackay 1996, Lynch & Walsh 1998). Under this framework, differences in general combining abilities (GCA) are attributable to additive genetic variance ($V_a = 4 \times \text{GCA}$), specific combining abilities (SCA) are attributable to non-additive genetic variance ($V_d = 4 \times \text{SCA}$), and reciprocal cross effects are attributable to parental environments or sex-linkage. For traits measured at different culture temperatures, we explored genotype \times environment interactions using an expanded diallel model. Here, we included a fixed factor associated with our temperature manipulation and interactions with genetic terms specified as:

$$Y_{ijkm} = \mu + t_i + g_j + g_k + s_{jk} + t_i^* g_{ij} + t_i^* g_{ik} + t_i^* s_{jk} + r_{jk} + e_{ijkm}$$

where μ is the grand mean, t_i is the temperature treatment, g_j and g_k are the general combining abilities for the j th and k th parents, respectively, s_{jk} is the specific combining ability between the j th and k th parent, $t_i^* g_{ij}$ and $t_i^* g_{ik}$ indicate treatment by general combining ability interactions, $t_i^* s_{jk}$ indicates the treatment by specific combining ability interaction, r_{jk} corresponds to reciprocal cross effects, and e_{ijkm} is the residual; m refers to the unit of replication (culture vessels). In each case, models were fit using restricted maximum likelihood with Proc Mixed in SAS (Littell et al. 1996). Under both frameworks, the significance of components of variance was evaluated with loglikelihood ratio tests. Initial models included all terms, but we also explored subsequent reduced models depending on lack of support for more complex effects (e.g. reciprocal effects or treatment interactions). Because no strong evidence for reciprocal effects was found, the results presented are for models with this term removed. Finally, we estimated narrow-sense heritability of traits with significant GCA as V_a/V_p , where $V_a = 4 \times \text{GCA}$ and $V_p = 2 \times \text{GCA} + \text{SCA} + \text{residual}$ (Falconer & Mackay 1996).

RESULTS

Genetic diversity among parent colonies

Genotyping using microsatellite markers confirmed the genetic diversity of the 3 colonies selected for these experiments. Ninety-five percent of markers ($n = 38$) were polymorphic among these colonies. Allelic diversity among colonies was high, with 136 unique alleles detected for these 40 markers (3 unique alleles per marker, on average). Of the unique alleles detected,

most (69%) were found in only one colony, with 19% found in 2 colonies and only 12% shared among all colonies. Similarity indices (the average percentage of shared alleles per locus) were calculated for each pairwise comparison, revealing substantial genetic differences between parental colonies: 0.41 for A – B, 0.28 for A – C, and 0.34 for B – C. This provides a genetic context for our subsequent comparisons of within- and between-family phenotypic variance.

Effects of family, temperature, and age on protein contents

At the earliest developmental stage studied (1 d post-fertilization), no differences were detected in protein content of larvae from the 6 different families ($F_{5,30} = 0.74$, $p = 0.6$), with an overall mean (\pm SE) protein content of $7.7 \pm 0.3 \mu\text{g embryo}^{-1}$ (Fig. 1a). Two general trends in these data are apparent from simple analyses of the single factors of age and temperature. Firstly, the average protein content across families declined significantly during development ($F_{4,20} = 9.0$, $p = 3 \times 10^{-4}$), for an overall average decrease of 31% by 5 d post-fertilization (Fig. 1b). Secondly, comparison of the average protein loss between culture temperatures revealed a marginally significant difference, with higher protein loss in larvae grown at 28°C (39%) than in larvae grown at 32°C (13%) ($F_{1,5} = 6.7$, $p = 0.049$; Fig. 1c). A complete analysis of the effects of family, temperature, and age on protein content revealed a significant family \times temperature \times age interaction effect ($F_{20,118} = 1.9$, $p = 0.02$), indicating between-family differences in the effects of temperature on developmental changes in protein content (Table 2). Two families lost significantly more protein at the elevated temperature than at the standard culture temperature (family AB: $F_{1,8} = 11$, adjusted $p =$

Table 2. Three-way ANOVA table describing the effects of the fixed factors of culture temperature and larval age, and the random factor of family, on the average protein content of individual larvae from each culture vessel. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Source	df	SS	MS	F	p
Age	4	173.4	43.3	7.6	6.8×10^{-4} ***
Temperature	1	6.1	6.1	1.9	0.23
Family	5	74.4	14.9	5.7	9.2×10^{-5} ***
Age \times Temperature	4	40.5	10.1	2.1	0.12
Age \times Family	20	114.2	5.7	2.2	4.9×10^{-3} **
Temperature \times Family	5	16.5	3.3	1.3	0.28
Age \times Temperature \times Family	20	96.8	4.8	1.9	0.02*
Residuals	118	306.6	2.6		

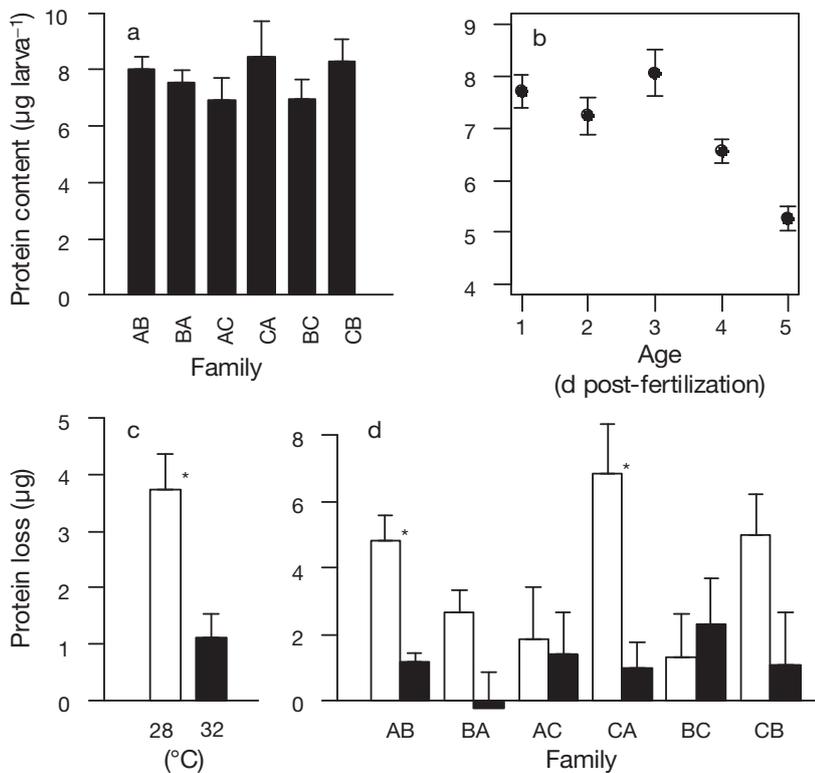


Fig. 1. *Acropora millepora*. Protein content of individual embryos and larvae from different families and culture temperatures. Data shown for each family are the means \pm SE across culture vessels. (a) Average protein content of the 6 families at 1 d post-fertilization was not significantly different. (b) Developmental decrease in protein content, averaged across families and culture conditions. (c) Developmental decrease in protein content between 1 and 5 d post-fertilization, expressed as a percent of the Day 1 value, averaged across families. (d) Average developmental protein loss for each of the 6 larval families at each culture temperature. For (c) and (d), white bars represent larvae grown at 28°C and black bars larvae grown at 32°C; *: significant differences between culture temperatures

0.03; family CA: $F_{1,8} = 17$, adjusted $p = 0.02$). In contrast, other families (e.g. BC) lost less protein overall during development, and culture temperature did not significantly affect their protein loss (Fig. 1d). A separate analysis of protein loss using the expanded diallel model revealed a significant effect of temperature ($F_{1,3} = 7.8$, $p = 0.038$), and a SCA \times temperature interaction effect that would be considered significant at a relaxed threshold of $\alpha = 0.1$, but not at the $\alpha = 0.05$ level $\chi^2 = 2.8$, $p = 0.09$).

Family-specific differences in mitochondrial temperature responses

The response of mitochondrial dehydrogenase activity to increased temperatures differed between families, based on a comparison of average Q_{10} values

($F_{5,12} = 6.5$, $p = 3.9 \times 10^{-4}$). Pairwise comparisons revealed several significant differences between families, including between half-siblings CA and CB (adjusted $p = 0.023$). These 2 families span the range from a 'typical' Q_{10} value of 3.8 ± 0.5 for family CB (similar to the median Q_{10} value across all families and culture vessels of 3.1), to an unexpectedly high value of 16.0 ± 0.7 for family CA (Fig. 2a). To illustrate the primary data upon which these Q_{10} values were based, examples of raw data for individual larvae from families CA and CB are shown in Fig. 2b, each of which closely matches the average values for that family. A separate analysis of mitochondrial Q_{10} values using the diallel model revealed a significant SCA effect $\chi^2 = 12.8$, $p = 3.5 \times 10^{-4}$), demonstrating a non-additive component of phenotypic variance in this trait.

Effects of family and temperature on gene expression

Expression of the 3 genes measured in the present study differed between families and culture temperatures (Fig. 3). Analysis of actin expression by 2-way ANOVA revealed significant differences between families (family main effect: $F_{5,24} = 3.4$, $p = 0.02$), and in family-specific responses to temperature (family \times temperature interaction effect: $F_{5,24} = 3.4$, $p = 0.019$). The large variance in these measurements obscured any pairwise differences between temperatures and families, after adjusting p-values to correct for multiple tests (e.g. for the comparison of actin expression between temperature treatments in family BC, $p = 0.043$; adjusted $p = 0.17$). Nevertheless, differential responses between families were clear for certain comparisons; e.g. actin was down-regulated by 62% at 32°C in family AB, and up-regulated by 282% at 32°C in family BC (Fig. 3a). The other 2 genes assayed showed similar patterns (Fig. 3b,c). Expression of *Hsp16* differed significantly between families ($F_{5,24} = 5.9$, $p = 0.001$), as did expression of $\beta\gamma$ -crystallin ($F_{5,24} = 4.3$, $p = 0.006$). Analysis of these gene expression data using the expanded diallel model revealed different patterns for the 3 genes. Expression of $\beta\gamma$ -crystallin was significantly affected by

GCA ($\chi^2 = 5.2$, $p = 0.023$) and by temperature ($F_{1,29} = 5.48$, $p = 0.026$), suggesting that variance in $\beta\gamma$ -crystallin expression includes both environmental and additive genetic components. Actin expression showed a significant SCA \times temperature effect ($\chi^2 = 5$, $p = 0.025$), indicating that the effects of temperature on actin expression differed between specific parental combinations (i.e. families). Expression of *Hsp16* showed significant effects of SCA ($\chi^2 = 16.2$, $p = 5.7 \times 10^{-5}$) and temperature ($F_{1,29} = 4.87$, $p = 0.036$). Overall, these data indicate that gene expression in coral larvae is affected by both genetic and environmental factors, and the relative contributions of these factors differ between genes.

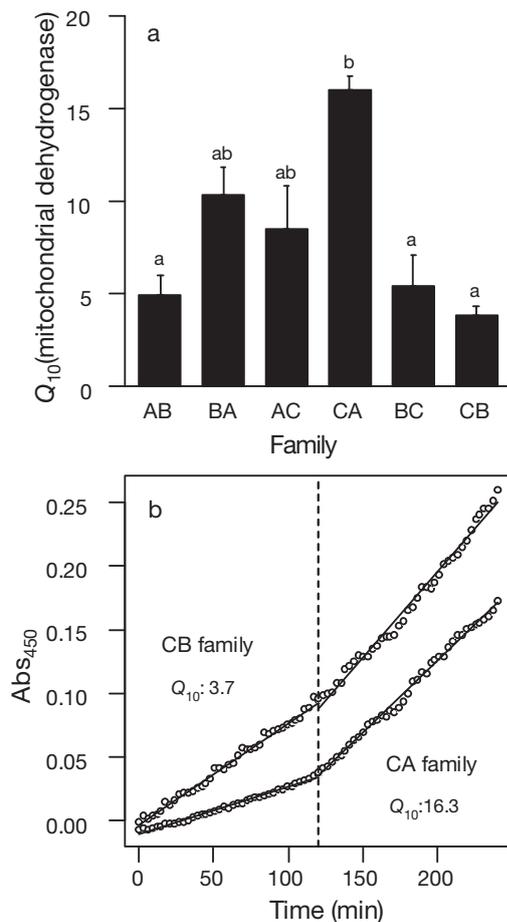


Fig. 2. *Acropora millepora*. Effects of elevated temperature on mitochondrial enzyme activity in individual larvae. (a) Q_{10} values (+SE) calculated for each of the 6 families, averaged across culture vessels. Lowercase letters indicate significant differences between families. (b) Example data illustrating the extremes shown for families CA and CB. The individual larval assay that most closely matched the average for that family was chosen for this example. Abs_{450} indicates absorbance at 450 nm. Vertical dashed line indicates the time at which temperature was increased from 28 to 32°C

Family-specific differences in responsiveness to settlement cue

Larvae from all families were presented with a known settlement cue, CCA, and the proportion of larvae that settled in response to this cue was compared between families. This experiment showed a significant difference in settlement rates between families ($F_{5,12} = 8.5$, $p = 1.2 \times 10^{-3}$; Fig. 4). Pairwise comparisons between families revealed significantly lower settlement success in families AB and BA (~30%) than the ~80% observed in the other 4 families (Tukey's HSD, adjusted $p < 0.05$). Analysis of settlement rates using the diallel model revealed a significant effect of GCA ($\chi^2 = 8.6$, $p = 0.0034$), suggesting the existence of additive genetic variance for this trait.

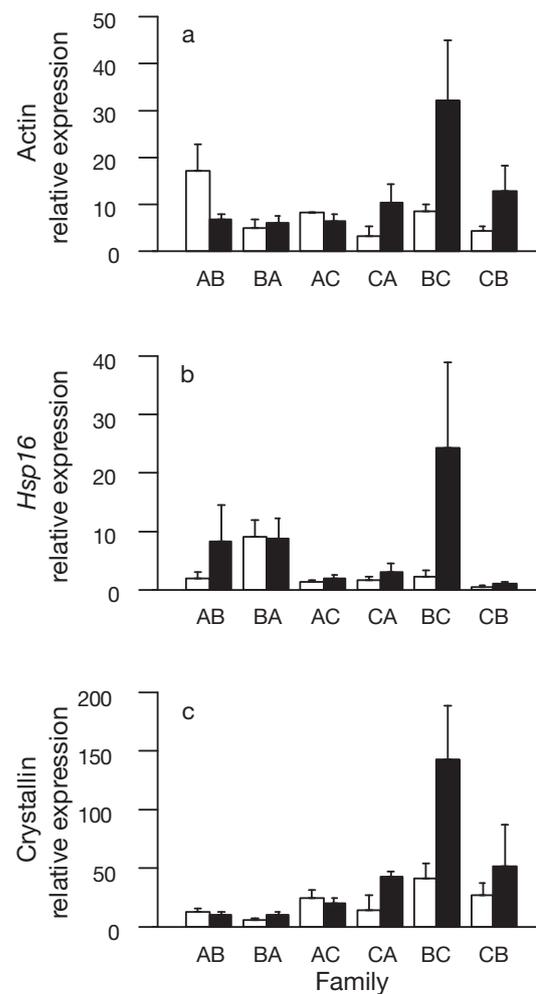


Fig. 3. *Acropora millepora*. Relative expression of candidate thermal stress genes in 5 d old larvae. Data shown represent the means \pm SE across culture vessels. White bars represent larvae grown at 28°C and black bars larvae grown at 32°C. Relative expression data are shown for (a) actin, (b) the small heat shock protein *Hsp16*, and (c) $\beta\gamma$ -crystallin

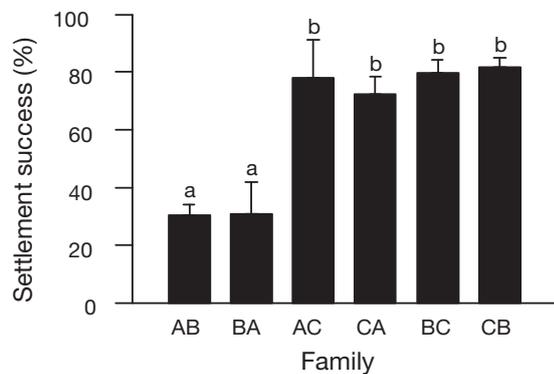


Fig. 4. *Acropora millepora*. Response to a natural settlement cue (crustose coralline algae; CCA) compared among families of 7 d old larvae grown at 32°C. Settlement success (calculated as the proportion of larvae that had settled after 12 h exposure to the settlement cue) is given as the mean \pm SE percent for each family. Lowercase letters indicate significant differences between families

DISCUSSION

Although previous research on variance in temperature stress responses of adult coral colonies has focused largely on the contributions of the zooxanthellae symbionts (Buddemeier & Fautin 1993, Berkelmans & van Oppen 2006), it is now widely recognized that both host and symbiont factors will have to be characterized for effective modeling of adaptation in response to climate change (Day et al. 2008) and conservation efforts (Baums 2008). Our findings demonstrate variation in thermal responses associated with genetic differences between corals, building on previous evidence from natural populations that had suggested a role for host factors in determining coral thermal tolerance (Brown et al. 2002, Baird et al. 2006, Abrego et al. 2008). The present study exploited several advantages of larval stages (relative to the more widely studied adult colonies) to identify differences in temperature phenotypes. Culturing larvae under controlled conditions allows for comparisons between individuals for which the complete thermal histories are known, minimizing the confounding effects of differences in thermal histories. The simultaneous culture of thousands of larvae allows for phenotypic analysis of large numbers of individuals, at a scale that would be difficult with adult corals. Finally, because larvae of *Acropora millepora* lack zooxanthellae symbionts, the observed differences among families are attributable to the coral rather than different symbiont types.

In addition to the experimental advantages of studying larvae, there are important biological questions specific to pelagic larval stages in corals. Gametes and early developmental stages of broadcast spawning corals are positively buoyant, possibly reflecting an

adaptation to improve fertilization success by concentrating gamete bundles at the surface (Babcock et al. 1986). An obvious consequence of this buoyancy is that these stages are subjected to higher seawater temperatures and higher irradiance at the water surface than those experienced by adult colonies deeper in the water column. Like adult colonies, earlier stages are critically sensitive to temperature; elevated temperatures reduce fertilization success, larval motility, and survival during the development of *Acropora millepora* and other coral species (Edmunds et al. 2001, Bassim & Sammarco 2003, Negri et al. 2007). One factor that contributes to variance in thermal tolerance among larvae is the presence or absence of zooxanthellae symbionts, with symbiotic larvae showing lower thermal tolerance than aposymbiotic larvae (Edmunds et al. 2001, Baird et al. 2006, Yakovleva et al. 2009). Although the larvae used in the present study were aposymbiotic, the elevated temperature we used (32°C) is known to result in thermal stress for both the larval stages and adult colonies of *A. millepora* (Baird & Marshall 2002, Negri et al. 2007). In contrast with these expectations, larvae in the present study fared well at elevated temperatures, with no evidence of impaired growth or development. In addition, larvae that were grown at 32°C survived well (60 to 100% survival; data not shown) during subsequent exposure to even higher temperatures (34°C for 48 h). The contrast between our findings and previous studies on larvae of this species might result in part from differential effects of temperature on fertilization versus growth, and in part from differences (whether environmentally induced or genetically determined) among the corals used in this and other studies.

Larvae survived and developed normally at both culture temperatures, but the temperature difference produced measurable changes in physiology, biochemical contents, and gene expression. Despite the limited statistical power associated with analysis of the small number of parental genotypes used in the present study, the differences that we observed between families (gene expression, mitochondrial enzyme activities, and developmental changes in protein content) were large enough to reach statistical significance. The phenotypic differences we observed did not always lead to clear biological interpretations. For example, larvae grown at elevated temperatures lost less protein during development than those grown at the standard culturing temperature. This unexpected observation leads to the following speculation: protein balance is determined by the relative rates of synthesis and degradation and, in the absence of exogenous nutrients, cells use free amino acids derived from degraded proteins for new protein synthesis (Mizushima & Klionsky 2007). Both synthesis and degradation rates would be ex-

pected to increase at higher temperatures, but if the effects of temperature on synthesis were greater than the effects on degradation (i.e. a higher Q_{10} value for synthesis), this might result in lower protein loss at higher temperatures, as we observed. Regardless of the mechanistic explanation for this pattern, there is no clear basis for associating a particular developmental change in protein content with greater or lesser thermal tolerance, so the observed differences do not predict the fitness of a particular family. Temperature also had a substantial effect on mitochondrial dehydrogenase activity, corresponding to a Q_{10} value of 3.1 (the median value across all families), and this effect differed significantly between families. The effects of temperature on actin expression differed significantly between families, and the expression of 2 other candidate heat stress genes showed similar patterns that did not reach significance. Overall, temperature responses differed between families, and in some cases showed concordant patterns across traits, as clearly demonstrated by the half-sibling families BC and CA. The effects of culture temperature on developmental protein loss were larger for family CA than for family BC (Fig. 1d). Similarly, larvae from family CA showed a higher Q_{10} for mitochondrial dehydrogenase activity (Fig. 2a). Temperature had a larger effect on mitochondrial dehydrogenase activity and developmental protein loss for family CA than for family BC. In contrast, the 3 candidate stress response genes were expressed at higher levels overall in family BC, and showed a greater tendency toward up-regulation at elevated temperatures in that family (Fig. 3). It is tempting to speculate that the increased expression of stress response genes in family BC might account for the reduced effects of temperature on other traits in that family, but both the mechanisms that underlie this variation and any fitness consequences of that variation fall outside the scope of the present study. Nevertheless, the differential temperature responses reported in the present study provide convincing evidence of a genetic component to variation in temperature phenotypes.

In general, the thermal tolerance of larval stages has important implications for the dispersal potential as sea surface temperatures increase, and some of the phenotypic differences we observed suggest possible dispersal consequences. In many non-feeding (lecithotrophic) larval forms, endogenous protein content serves as a substrate for energy metabolism during development (Jaekle & Manahan 1989, Vavra & Manahan 1999). In this context, the developmental decreases in protein content observed in the present study can be interpreted as catabolism of protein reserves for energy, and the observed differences in protein loss suggest differential effects of temperature on protein catabolism between families. One important

caveat for this interpretation is that coral larvae contain slightly more lipid than protein (Richmond 1987), and this lipid also serves as a metabolic substrate during development (Harii et al. 2007), so a full energy budget would require data on both protein and lipids. Coral larvae might utilize dissolved nutrients present in seawater, similar to other marine invertebrate larvae (Manahan 1990), which might also affect the observed differences in protein content. Metabolic rates are profoundly affected by changes in temperature, and the potential for this to affect dispersal potential in marine larvae is widely appreciated (O'Connor et al. 2007). We measured mitochondrial dehydrogenase activity because this represents an essential component of aerobic metabolism. Our observation of different temperature responses (Q_{10}) among larval families (Fig. 2) suggests that temperature affects the metabolic rates of larvae differently during dispersal, depending on their genetic backgrounds. Interactions between the energetic content and metabolic rates of larvae might also be important, although no significant relationship between these traits was observed within the limited sample of genotypes in the present study.

For marine larvae in general, dispersal potential is correlated with the amount of time spent in the plankton (the pelagic larval duration, PLD) (Shanks et al. 2003). Within the constraints of energetic considerations like those outlined above, which might impose upper limits for PLD, the responsiveness of coral larvae to natural settlement cues is likely to play an important role in determining PLD (Miller & Mundy 2003). Our findings show clear differences in responsiveness to settlement cues between families (Fig. 4), suggesting that substantial variation in this trait exists among natural cohorts of coral larvae. An important caveat for this interpretation is that exogenous factors also affect the dispersal of marine larvae, including currents, predation, and the availability of suitable habitat (Cowen & Sponaugle 2009). For some coral populations, genetic data and dispersal models have shown that exogenous factors like these limit larval dispersal (Baums et al. 2006), which might reduce the importance of responsiveness to settlement cues for those populations. Nevertheless, the magnitude of the phenotypic differences observed in the present study suggests that genetically determined variation in this trait could be an important determinant of dispersal potential in some populations, with potential implications for range shifts during global climate change.

The expected response of a trait to selection depends on the strength of selection and the narrow-sense heritability of that trait, defined as the proportion of phenotypic variance due to additive genetic effects (Lynch & Walsh 1998). Obviously the strength of selection on thermal tolerance is expected to be high, based on the

well-documented declines in coral populations associated with increased sea surface temperature (Glynn & D’Croz 1990, Gardner et al. 2003, Hoegh-Guldberg et al. 2007) and current predictions for continued increases (IPCC 2007). Our findings show variance in responses to elevated temperature and settlement cues; this corresponds to total phenotypic variance, which includes both additive and non-additive effects. Partitioning of this variance with the diallel model (Lynch & Walsh 1998) revealed significant additive genetic effects for 2 traits (responsiveness to settlement cue and expression of $\beta\gamma$ -crystallin), implying the potential for an adaptive response to selection on those traits. Further supporting this conclusion, heritability (h^2) estimates for these traits were high: $h^2 = 0.49$ for settlement propensity, and $h^2 = 0.38$ for $\beta\gamma$ -crystallin expression. In contrast, we observed significant non-additive effects for other traits (mitochondrial Q_{10} and the expression of actin and *Hsp16*), which might result from dominance or more complex genetic interactions. The contrasting patterns observed for different traits highlight the importance of identifying both the traits that selection might act on and the genetic basis of phenotypic variance in those traits. Overall, our findings support the conclusion that additive genetic variance exists within coral populations for several traits that might reasonably be expected to have fitness consequences during global climate change. Although our data set is small for estimating variance components or making inferences about population-level heritability, the diallel analysis provides an informative initial screening of genetic variance in corals. Future efforts should include expanded crossing designs to more robustly estimate patterns of genetic variance in natural populations, and comparisons between populations to evaluate the effects of natural selection on this variance in different environments. Direct measurements of thermal tolerance would also provide a valuable addition in future studies.

The phenotypic and genetic variance in our experimental material represents a small sample from a single population, and should be considered in the context of the total genetic variation within and between coral populations. We have previously demonstrated high levels of polymorphism among colonies of *Acropora millepora* within a reef, averaging 8 to 9 alleles per locus (Wang et al. 2009), in agreement with other studies of genetic diversity in this species that used microsatellite markers (Van Oppen et al. 2006). Analysis of allozymes in an extensive set of samples for this species has also revealed high levels of genetic diversity both within and between reefs (Smith-Keune & van Oppen 2006), and studies in other coral species have also uncovered substantial genetic diversity within populations (Ayre & Hughes 2000, Underwood 2009,

Wang et al. 2009). The general agreement between these studies with regard to the high genetic diversity within coral populations suggests the possibility that the phenotypic variance we observed might similarly be widespread within natural populations. Further investigations of this subject should include parents from different populations to compare the within- and between-reef components of phenotypic variance.

Genetic and phenotypic diversity are not the only considerations; the potential for corals to adapt will also depend critically on life-history traits and population dynamics. The importance of effective population size (N_e) for adaptation has been extensively studied (Charlesworth 2009), and the response to selection by natural populations is expected to vary in proportion to N_e . For broadcast spawning marine invertebrates in general, N_e is frequently orders of magnitude lower than the census size (Palumbi & Wilson 1990, Hedgecock 1994). Clonal reproduction by reef-building corals can further depress N_e in some populations (Lasker & Coffroth 1999), potentially constraining the rate of adaptation. Another possible constraint is the generation time, which would obviously affect the absolute rate of any adaptive response to selection. Generation times in acroporid corals are generally 3 to 5 yr (Wallace 1999). Current climate models predict that thermal stress bleaching episodes could occur at an annual frequency in 30 to 50 yr (Donner et al. 2005), suggesting that adaptation to warming ocean temperatures would have to take place within ~10 generations for coral populations to persist.

Ultimately, identifying the genetic determinants of thermal tolerance and dispersal potential will require genetic approaches like quantitative trait loci mapping or association studies. To make such studies possible, our laboratory is developing genomic resources for *Acropora millepora* that include genetic markers (Wang et al. 2009), sequencing and annotation of the larval transcriptome (Meyer et al. 2009), and a genetic map. In combination with other sequencing efforts underway for corals (Schwarz et al. 2008), as well as their algal symbionts *Symbiodinium* (Leggat et al. 2007, Voolstra et al. 2008), these resources should enable the identification of the loci that control expression of temperature response genes in corals. In the long term, this will allow for population-level studies of allele frequency and adaptation, leading to a more complete understanding of the fates of coral populations during climate change.

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