MOLECULAR ECOLOGY

Molecular Ecology (2011) doi: 10.1111/j.1365-294X.2011.05205.x

Profiling gene expression responses of coral larvae (*Acropora millepora*) to elevated temperature and settlement inducers using a novel RNA-Seq procedure

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Abstract

Elevated temperatures resulting from climate change pose a clear threat to reef-building corals; however, the traits that might influence corals' survival and dispersal during climate change remain poorly understood. Global gene expression profiling is a powerful hypothesis-forming tool that can help elucidate these traits. Here, we applied a novel RNA-Seq protocol to study molecular responses to heat and settlement inducers in aposymbiotic larvae of the reef-building coral *Acropora millepora*. This analysis of a single full-sibling family revealed contrasting responses between short- (4-h) and long-term (5-day) exposures to elevated temperatures. Heat shock proteins were up-regulated only in the short-term treatment, while the long-term treatment induced the down-regulation of ribosomal proteins and up-regulation of genes associated with ion transport and metabolism (Ca^{2+} and CO_3^{2-}). We also profiled responses to settlement cues using a natural cue (crustose coralline algae, CCA) and a synthetic neuropeptide (GLW-amide). Both cues resulted in metamorphosis, accompanied by differential expression of genes with known developmental roles. Some genes were regulated only by the natural cue, which may correspond to the recruitment-associated behaviour and morphology changes that precede metamorphosis under CCA treatment, but are bypassed under GLW-amide treatment. Validation of these expression profiles using qPCR confirmed the quantitative accuracy of our RNA-Seq approach. Importantly, qPCR analysis of different larval families revealed extensive variation in these responses depending on genetic background, including qualitative differences (i.e. up-regulation in one family and down-regulation in another). Future studies of gene expression in corals will have to address this genetic variation, which could have important adaptive consequences for corals during global climate change.

Keywords: Cnidarian, heat shock, recruitment, transcriptomics

Received 21 November 2010; revision received 19 May 2011; accepted 20 June 2011

Introduction

Coral reefs determine the physical and ecological characteristics of entire coastlines, influence global biodiversity patterns (Malay & Paulay 2010; Palumbi 1997; Paulay 1994) and provide essential support for local economies (Moberg & Folke 1999; Wilkinson 1996). Reef-building corals are threatened on a global scale by anthropogenic disturbances including run-off, pollution, ocean acidification and increased sea surface temperatures resulting from elevated levels of atmospheric CO_2 (Brown 1997; Carpenter *et al.* 2008; Hoegh-Guldberg *et al.* 2007; Lesser 2007). The widespread increase in stress-induced coral bleaching and declines in coral populations over the last several decades (Glynn 1993; Harvell *et al.* 1999; Hoegh-Guldberg 1999) have elevated this issue to the forefront of modern coral research. The possible outcomes for coral populations facing these disturbances include adaptation (Day *et al.* 2008), acclimatization (Coles & Brown 2003; Gates & Edmunds 1999), geographical range shifts (Greenstein

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& Pandolfi 2008; Precht & Aronson 2004) and extinction (Carpenter *et al.* 2008), and all of these possibilities are being actively investigated.

Reef-building corals are symbiotic organisms, hosting unicellular dinoflagellate algae of the genus *Symbiodinium* (called 'zooxanthellae') within their gastrodermal (gut) cells. The zooxanthellae gain protection from herbivores, exposure to light and nutrients (nitrogen and inorganic carbon) from the coral, whereas the coral utilizes up to 95% of their photosynthetic product (Muscatine *et al.* 1984). Breakdown of coral–zooxanthellae association under stressful conditions results in the loss of visible pigmentation and is called 'coral bleaching' (Glynn & D'Croz 1990; Goreau & Hayes 1994), which may lead to death of the coral (Glynn 1993). In the past decade, much attention has been devoted to the physiological parameters of various natural strains of zooxanthellae and the mechanisms that may enable corals to exploit this diversity to achieve thermal acclimatization (Baker, 2003; Baker *et al.*, 2004; Berkelmans and van Oppen, 2006; Buddemeier and Fautin, 1993; Rowan, 2004). However, the coral hosts' ability to protect zooxanthellae symbionts or tolerate the adverse effects of their stress may also be important determinants of thermal tolerance in the holobiont (Baird *et al.* 2008; Weis 2010). The existence of coral-specific factors affecting the thermal tolerance of the holobiont is clearly evident from between-species comparisons (Abrego *et al.* 2008; Bhagooli & Hidaka 2003; Fitt *et al.* 2009). Although the nature and magnitude of host effects in these systems is debated (Baird *et al.* 2008; Mieog *et al.* 2009), it is clear that modelling the evolutionary responses of coral populations to climate change will require a detailed understanding of both host and symbiont factors (Day *et al.* 2008).

In the majority of broadcast-spawning corals, early stages are naturally aposymbiotic (i.e. do not host zooxanthellae symbionts) and can be easily maintained in culture, presenting an opportunity to isolate coral host-specific aspects of thermal physiology. Because pelagic larval stages are the dominant dispersal mode for corals, thermal tolerance and settlement responses during these stages are likely to play an important role in the establishment and persistence of coral populations during climate change. The functional consequences of temperature stress have been well studied in aposymbiotic larvae (Baird *et al.* 2006; Randall & Szmant 2009a,b; Yakovleva *et al.* 2009), and we have previously documented the genetic effects on temperature responses in these stages (Meyer *et al.* 2009b). More recently, the gene expression profiles associated with these responses have been measured using microarrays (Polato *et al.* 2010; Portune *et al.* 2010; Rodriguez-Lanetty *et al.* 2009; Voolstra *et al.* 2009). Building on this body of work, our study provides a comparison between short-term stress responses and completion of the entire larval duration at elevated temperatures.

The pelagic duration of invertebrate larvae is often limited by behavioural and life history traits (Shanks 2009), suggesting that variation in these traits could have important implications for coral distributions and the possibility of range shifts in response to climate change. The lecithotrophic development of broadcast-spawning corals occurs rapidly at ambient temperatures: in *Acropora millepora*, a swimming larval stage (planula) is attained 72 h after fertilization (Ball *et al.* 2002) and metamorphic competency at 7–9 day postfertilization (Heyward & Negri 1999). In the absence of a settlement cue, these larvae have been maintained in culture for much longer periods (Graham *et al.* 2008), demonstrating the potential for increased durations and dispersal distances depending on the availability of and response to natural settlement cues. The settlement cues for coral larvae have been extensively studied, including crustose coralline algae (CCA) collected from the reef (Heyward & Negri 1999), individual chemicals isolated from CCA (Kitamura *et al.* 2007) and microbial biofilms (Webster *et al.* 2004). We have previously documented considerable genetic variation (narrow-sense heritability $h^2 = 0.49$) in the responsiveness of *A. millepora* larvae to CCA (Meyer *et al.* 2009b), which might have important consequences for range shifts during climate change. An artificial inducer of metamorphosis is also available: a synthetic neuropeptide analogous to naturally occurring peptides from Hydra (Erwin & Szmant 2010; Iwao *et al.* 2002), which might prove useful for distinguishing between universal and cue-specific components of the settlement response.

The aim of our study was to identify the genes involved in responses to elevated temperatures and settlement cues by coral larvae. Any variation in these expression responses could affect the larval survival and dispersal distance, so these genes are likely targets for selection during global climate change. This study represents an essential first step towards future research on genetic variation in these responses across populations and in response to climate change. Towards that end, we developed a novel procedure for RNA-Seq analysis of gene expression in organisms lacking genomic sequence resources. The term 'RNA-Seq' describes a variety of methods based on the deep sequencing and quantitative analysis of short cDNA reads (Wang *et al.* 2009; Wilhelm & Landry 2009). RNA-Seq offers similar quantitative accuracy as microarrays ('t Hoen *et al.* 2008; Bloom *et al.* 2009; Marioni *et al.* 2008), with a greatly enhanced dynamic range (Wang *et al.* 2009), but is only beginning to see applications outside traditional model systems (Chen *et al.* 2010; Jeukens *et al.* 2010;

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Wolf *et al.* 2010). We describe a tag-based procedure that makes efficient use of sequencing coverage and works with small amounts of input RNA, and an analytical strategy that requires only a 454-derived transcriptome assembly (Meyer *et al.* 2009a) as a reference database. This study is the first global gene expression analysis in corals based exclusively on resources derived from next-generation sequencing technologies (NGS). The RNA-Seq methodology outlined here is widely applicable to other nonmodel organisms and may be expected to broadly facilitate the studies of ecophysiology and adaptation across species and ecosystems.

Methods

Larval culture, temperature treatments and settlement induction

Full-sibling larval families were produced by cross-fertilizing gametes collected from three different colonies of the branching coral *Acropora millepora*, as previously described (Meyer *et al.* 2009b). The experimental design is depicted in Appendix S2 (Fig. S2.1, Supporting information). Each family was stocked in triplicate culture vessels in two different temperature-controlled rooms, with one set at a standard culturing temperature (27.7 °C) and the other at an elevated temperature (31.4 °C). Five days after fertilization, larvae were sampled from each treatment and preserved in RNAlater (Ambion, Austin, TX, USA) to study the long-term response to elevated temperature. To explore short-term responses, larvae were sampled from the control cultures and divided into four groups. The first group (handling control) was given no additional manipulations. Another group was incubated at the elevated temperature for 4 h. No differences in mortality or developmental stage were apparent in larvae from either short-term or long-term temperature treatments. A third group was incubated for 4 h in the presence of a natural settlement inducer, a mixture of ground CCA (Heyward & Negri 1999). Finally, a fourth group was incubated for 4 h with an artificial inducer of metamorphosis, a GLW-amide peptide EPLPIGLW-amide (Iwao *et al.* 2002). Larvae were monitored by light microscopy to record behavioural and morphological responses to these cues. All samples were preserved in RNAlater and stored at −20 °C.

Four larval families were used in these experiments, with one family (A) sampled for both RNA-Seq and qPCR and the others (B–D) sampled only for qPCR. The long-term culture temperature treatments (27.7 and 31.4 °C) were each conducted in triplicate for all families (n = 24 independent culture vessels). For short-term treatments, larvae from each family (cultured at 27.7 °C) were pooled across culture vessels. Each short-term treatment was applied to a single group of larvae from each family (i.e. short-term treatments were replicated across families but not within families).

Preparation of cDNA for SOLiD sequencing

Duplicate samples were prepared as follows for each culture vessel or treatment. First, total RNA was extracted from ~20–30 larvae from each treatment using the RNAqueous kit (Ambion, Austin, TX, USA) and then treated with DNAse I to remove residual genomic DNA contamination (Roche, Indianapolis, IN, USA). Within each set of preparations, an additional RNA sample was prepared from the control culture (27.7 °C) to evaluate the variation across preparations. Tag libraries for SOLiD sequencing were prepared from each of the resulting seven RNA samples (two from larvae cultured at standard temperature, one from larvae cultured at elevated temperature and one each for handling control, short-term temperature stress, CCA exposure and GLW-amide exposure). Our library preparation procedure was designed to make efficient use of sequencing coverage by producing a single tag from each polyadenylated RNA molecule, corresponding to a random position within a narrow window near the 3′ end of each transcript (Fig. 1). Total RNA was first fragmented by heating and then used to synthesize first-strand cDNA with adapters incorporated at both 5′ and 3′ ends (Matz *et al.* 1999). About 0.5 μg of total RNA was sufficient to amplify a cDNA sample in 17–19 PCR cycles, which ensures adequate representation of all transcripts in the amplified product (Matz 2003). Each cDNA sample was labelled with a specific barcode (Fig. 1) and gel-extracted to purify the size fraction (150–200 bp) appropriate for sequencing on the SOLiD System. The detailed library preparation protocol is available in Appendix S1 (Supporting information).

Sequencing, quality filtering and mapping

Barcoded samples were pooled for sequencing on the SOLiD System at the University of Texas at Austin's Genome Sequencing and Analysis Facility. To take advantage of upgraded chemistry and software for this platform that became available during the course of our analysis, we sequenced each sample three times. The initial libraries prepared from each biological sample (n = 7) were sequenced on the SOLiD System Version 2 with 35-bp read lengths. Next, an additional library was then prepared from each biological sample (a different RNA sample from the same pool of larvae) and sequenced twice using Version 3 chemistry: once with

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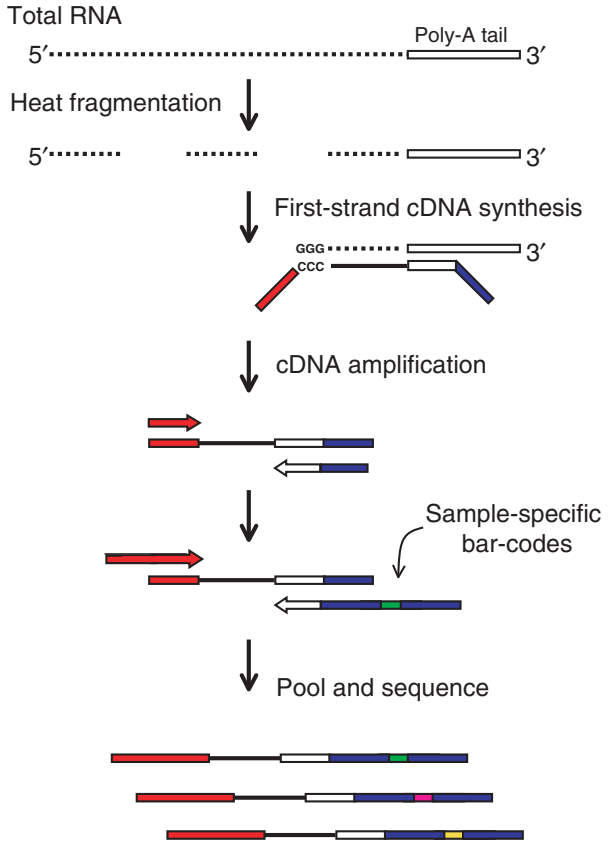

Fig. 1 Overview of the protocol used to prepare 3′ cDNA tag libraries from total RNA. RNA was fragmented at the beginning to eliminate biases resulting from differences in transcript lengths. First-strand cDNA was primed with a modified oligo-dT containing primer to target 3′ ends. Each sample was prepared with a sample-specific oligonucleotide barcode, then quantified and pooled prior to sequencing.

35-bp read lengths and then with 50-bp read lengths. A detailed summary of sequencing yield is provided in Appendix S2 (Supporting information). The raw sequences were first trimmed to remove four nontemplate bases introduced by reverse transcriptase at the 5′ end of each read (Fig. 1). To reduce the computational time required for mapping, we excluded reads containing long homopolymer regions (≥10 bp) and low-quality reads (for the 31-bp reads, we allowed ≤8 positions with quality scores <8; for the 46-bp reads, ≤18 positions with scores <10). The 77 million high-quality (HQ) reads that passed these filters (Appendix S2, Supporting information) were mapped (aligned) against an annotated assembly of cDNA sequences derived from the same larval families(Meyer *et al.* 2009a), using the SHRiMP software package (version 1.2.0) (Rumble *et al.* 2009). The mapping parameters and an overview of mapping efficiency are described in Appendix S2 (Supporting information).

Identification of differentially expressed genes (DEGs)

All calculations and statistical tests were conducted using the R statistical software (R Development Core Team, 2008). For statistical comparisons, we focused on the core set of high-confidence transcripts supported by at least 10 reads per million in one or more samples. Read counts were summed across sequencing runs by sample. Expression differences were evaluated using a test based on the negative binomial distribution, as implemented in the R package DESeq (Anders & Huber 2010). The mean–variance relationship for these comparisons was estimated empirically from duplicate libraries of the long-term control sample. Four comparisons between control and treatment were made: (i) larvae cultured at standard temperature vs. those cultured at elevated temperature; (ii) short-term temperature stress vs. handling control; (iii) CCA exposure vs. handling control; and (iv) GLW-amide exposure vs. handling control. False discovery rate (FDR) was controlled at 5% (Benjamini & Hochberg 1995).

The reference sequences had been previously annotated with gene names and Gene Ontology (GO) biological process terms (Meyer *et al.* 2009a). This made it possible to test for enrichment of a particular term among the DEG, indicating the involvement of that process in the response. The number of genes associated with each process was compared between the DEGs induced in each treatment and the complete reference data set, to evaluate whether any processes were more highly represented than expected by chance (Fisher's exact test, $P \leq 0.05$). Enrichment analysis was carried out for the set of DEG identified in each treatment and for all overlapping categories (e.g. genes up-regulated in both CCA and GLW-amide treatments).

Validation of expression profiles using qPCR

We selected a set of 20 DEGs for validation that allowed for 24 comparisons between RNA-Seq and qPCR (some genes were differentially expressed in more than one treatment). Three reference genes were selected for normalization, based on their constant expression in RNA-Seq (coefficient of variation ≤10%): *Eif5*, *Atp6v0c* and *Rpl11*. We compared these against another gene commonly used as a reference in qPCR, 18S rRNA (Meyer *et al.* 2009b; Schmittgen & Livak 2008). The stability of these reference genes across treatments was verified by qPCR analysis of reactions loaded with equal amounts of RNA. Because 18S rRNA was slightly up-regulated in the metamorphosis induction treatments, this gene was excluded as a reference (details of qPCR controls are shown in Appendix S2, Supporting information). Primers for qPCR were

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designed using Primer3 (http://primer3.sourceforge.net/), with amplicon lengths of ~150 bp and $T_M \sim 60$ °C. We tested all primer pairs across a series of cDNA dilutions to verify their per cycle amplification factors, or efficiencies (E), requiring $E = 2.0 \pm 0.1$ and $R^2 \geq 0.95$. Expression data for each sample were normalized by subtracting the average C_T for the three reference genes from the C_T values of all other genes in that sample. This is mathematically equivalent to division by the geometric average of the reference genes' relative expression values, as previously described (Vandesompele *et al.* 2002), after $\log_2$ transformation of the data.

To evaluate the quantitative accuracy of expression profiles produced from RNA-Seq, we first measured the expression of the selected DEGs by qPCR, using the same RNA samples originally used in RNA-Seq. Next, we surveyed biological variation in these responses in a replicated experiment using three independent cultures of larvae produced by crossing different parental colonies, which were cultured and exposed to short-term treatments in the same experiment as the larvae used for RNA-Seq. For this initial survey of variation among families, a single RNA sample was collected from each family and treatment (i.e. one treatment and one control per family). Finally, to evaluate the relative contribution of between-family and within-family sources of variation, we measured the expression of six DEGs in replicated treatments for the long-term stress comparison (i.e. three independent culture vessels for the control and three for the treatment, within each family). All qPCR were performed in duplicate for each gene, treatment and sample.

Results

SOLiD sequencing and mapping to reference transcriptome

A total of 170 million raw reads were produced, 77 million of which passed all quality filters (details in Appendix S2, Supporting information). Using the SOLiD sequencing chemistry version 2.0, 37% of raw reads passed quality filters, and >60% passed similar filters in version 3.0. After processing, an average of 3.7 million HQ reads were produced from each sample in each sequencing run (range: 1.3–8.1 million). To identify the gene from which each read originated, HQ reads were aligned, or 'mapped', against an annotated larval transcriptome (Meyer *et al.* 2009a). On average, 1.5 million reads per sample produced HQ, unambiguous alignments to the reference (range: 0.6–3.2 million). The most current version of the SOLiD sequencing platform in our study (version 3 chemistry, 50-bp read length) yielded 31% gross mapping efficiency (raw reads mapped) and 48% net mapping efficiency (HQ reads mapped). The reads that failed to map uniquely to the reference might result from reads mapping to repetitive sequences, sequencing or assembly artefacts in the reference, sequencing errors and inadequate quality filtering in the SOLiD reads or biological contaminants present in one sample but not the other (larvae were cultured in nonsterile conditions). Whatever the cause of unmapped reads in our coral data, the overall mapping efficiency achieved in our study was comparable to the 39% reported in the publication describing the mapping software we used (Rumble *et al.* 2009), in an analysis that included a fully sequenced reference genome.

Nearly all the cDNA sequences in our reference database (96%) were detected in the RNA-Seq data set. Because the reference is likely to contain sequencing errors and incompletely assembled transcripts, we focused on the high-confidence transcripts supported by at least 10 reads in one or more samples. A large fraction of the reference sequences (derived from assembly of 454 cDNA sequences) were not well supported by RNA-Seq, probably reflecting sequencing or assembly errors in those data. Excluding those poorly supported sequences eliminated many reference sequences (80%, primarily singletons), but very few of the RNA-Seq reads (4% of mapped reads). The remaining 96% of mapped reads correspond to a core set of 20 048 high-confidence reference sequences that were well supported in both platforms. BLASTN comparisons between these core sequences and those represented on previously described *Acropora millepora* microarrays (13k array, Gene Expression Omnibus GPL6774, and 18k arrays, GPL8613 and GPL6941) confirmed that most of the arrayed sequences (77–81%) were detected in our RNA-Seq experiments. In contrast, most of the core sequences detected by RNA-Seq (71–78%) had no matches on the arrays.

About one-third of the 20 048 core cDNAs (31%; n = 6,161) matched known proteins, 7% matched unknown or hypothetical proteins (1348), and the remaining 63% lacked matches (12 539). Based on the redundancy of annotation among the 6161 matching cDNAs (5547 unique gene names), we estimate that the 20 048 core cDNAs correspond to ~18 050 unique genes. Noncoding RNA and RNA derived from organelles contributed negligible amounts to RNA-Seq profiles, with 7% of reads matching nuclear-encoded rRNA genes, 5% matching mitochondrial-encoded transcripts and the remaining 88% presumably derived from nuclear genes. Most of the genes were expressed at low levels (Fig. 2), with a mode at three reads per million and a long upper tail extending to a maximum of

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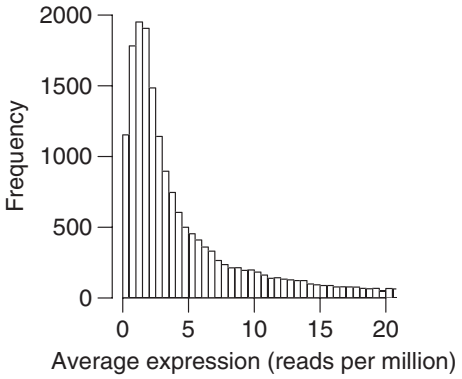

Fig. 2 Distribution of expression levels among the core set of 20 048 reference sequences expressed at ≥10 reads per million in one or more samples. This core set includes 96% of all mapped reads. An additional 1237 reference sequences expressed at ≥100 reads per million extend beyond the margins of this figure. The mode of this distribution is three reads per million.

43 918 reads per million (a single rRNA species comprising ~4% of the total). A few protein-coding genes were highly abundant in our samples (*GolgA6*, a gene associated with the maintenance of the Golgi apparatus at 1.7% of mapped reads; a GFP-like protein, annotated as *FP506* in the larval transcriptome, at 1.5%; and the chaperone protein *Hsp90A1* at 0.8%), suggesting their functional importance in planula-stage coral larvae. Raw counts data for the core genes are available in Table S1 (Supporting information).

Specificity and lack of bias in mapping

Most reads mapped near the 3′ end of a cDNA sequence, as expected, with a dominant peak 60–70 bp upstream of the poly-A tail and additional minor owing to a few over-represented cDNAs including rRNAs and mitochondrial RNA (Fig. 3a). We investigated whether

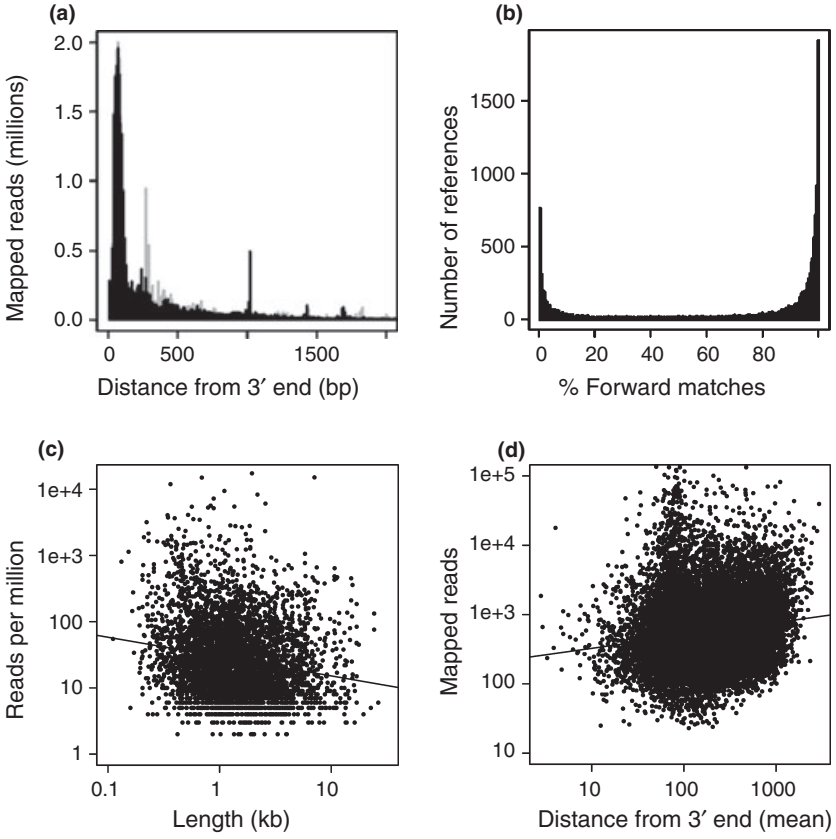

Fig. 3 Mapping specificity and lack of bias in RNA-Seq expression profiles. (a) Distribution of position specificity relative to the 3′ end of each reference sequence. Grey bars: all reference sequences; black bars: mitochondrial and rRNA sequences excluded. Reads that mapped >2 kb from the 3′ end not shown (1.6% of reads). (b) Distribution of strand specificity, calculated as the proportion of reads matching the forward strand of each reference sequence. Strand-specific mappings are indicated by values close to 0 (all reverse matches) or 1 (all forward matches). (c) Expression levels (average reads per million) are not biased by transcript length (estimated from the complete ORF length of the nearest BLAST match). All reference sequences with BLAST matches (n = 6161) are included in this analysis. The relationship is not significant (ANOVA; $P > 0.05$). (d) Bias because of mapping position (distance from the 3′ end of the transcript) is negligible. Although the relationship is significant (ANOVA; $P < 0.001$), this accounts for a small fraction of the total variance ($R^2 = 0.002$). Both regressions (c, d) show trends opposite the expected biases.

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this variation in mapping position affected the sequencing coverage of individual genes. Because the reference database includes a mixture of both sense and antisense sequences without clearly annotated 3′ ends, we estimated distances based on the downstream distance (5′ to 3′) from the mapped position to the end of that reference. Although the correlation between the average mapping start position and average reads count was significant ($P < 0.001$; Fig. 3d), it was quantitatively unimportant, with mapping position accounting for only 0.2% of the total variation in read count ($R^2 = 0.002$). This trend is opposite the expected bias from variation in fragment lengths (over-representation of shorter fragments) and consistent with the possibility that some transcripts produced multiple fragments. Overall, the effects of any variation in mapping position appeared to be minimal.

Our sample preparation protocol is expected to produce strand-specific reads corresponding to the sense strand of the original mRNA (Fig. 1), and in fact, we observed clear strand specificity in mapping (Fig. 3b). This feature was useful for tuning the stringency of quality filtering and mapping: if these parameters are set appropriately, nearly all reads mapping to a reference sequence should match just one strand (Fig. S2.3 in Appendix S2, Supporting information); 97% of reads mapping to the core set of genes matched the 'dominant' strand for each reference, and the average strand specificity across all core genes, calculated individually for each reference, is 90%. Although our data cannot resolve whether the small number of antisense matches represents artifacts or true antisense transcripts, it is clear that these contribute little to the overall expression profile (~3% of reads).

In our method, RNA is fragmented to a uniform size distribution prior to cDNA synthesis, followed by sampling of a single tag from each mRNA molecule (Fig. 1). Therefore, differences in the original length of mRNAs should not bias the read counts across genes. Because gene lengths cannot be accurately estimated from the fragmented cDNA assembly used as a reference (average length = 629 bp), we estimated the transcript length as three times the length of the encoded protein for the subset of reference sequences that matched proteins with known lengths ($n = 6161$). There was no significant relationship between transcript length and expression level in this analysis ($P = 0.09$; $R^2 = 0.0005$; Fig. 3c), and the minor trend towards lower expression in longer mRNAs is opposite the bias that would be expected to result from increased sampling of longer transcripts.

Response to temperature treatments

The long-term temperature treatment induced the differential expression of over six times more genes than the short-term treatment (Figs 4a,b and 5a). There was little overlap between the two DEG lists, and most of

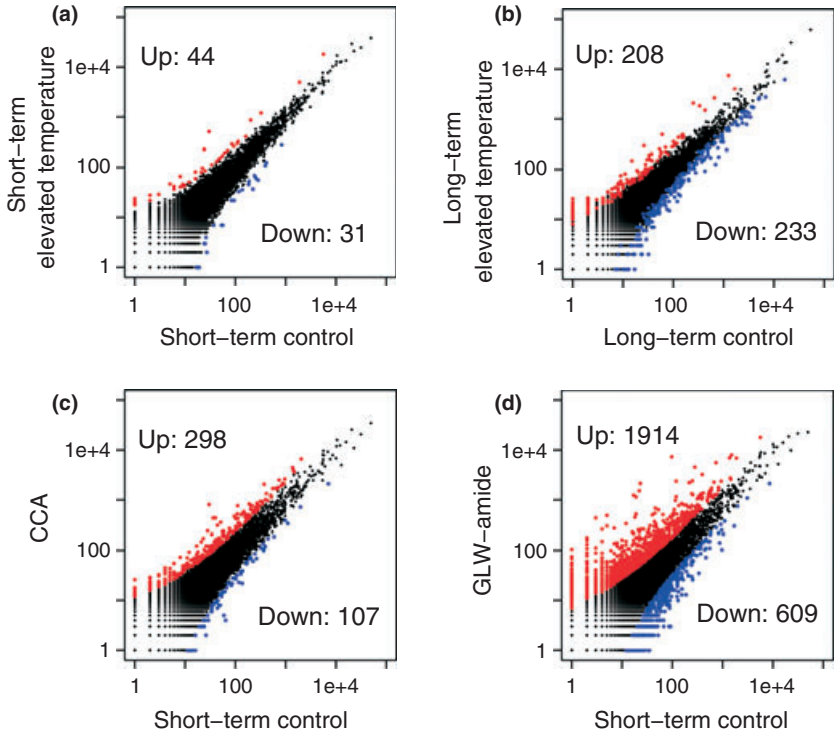

Fig. 4 RNA-Seq identifies differentially expressed genes (DEGs) in the larvae of *Acropora millepora* exposed to elevated temperatures and inducers of settlement and metamorphosis. Each symbol represents the normalized expression level of a single transcript (reads per million) calculated from the sum of all sequencing runs. Significant differences (false discovery rate, FDR = 5%) indicated by coloured symbols: up-regulation in red and down-regulation in blue. Numbers in each panel indicate the number of DEGs induced by that treatment. (a) Short-term exposure to elevated temperature compared with control larvae. (b) Larvae cultured at the elevated temperature compared with those cultured at standard temperature. (d) Exposure to the natural settlement cue, crustose coralline algae (CCA), relative to controls. (e) Exposure to a synthetic metamorphosis inducer, GLW-amide, relative to controls.

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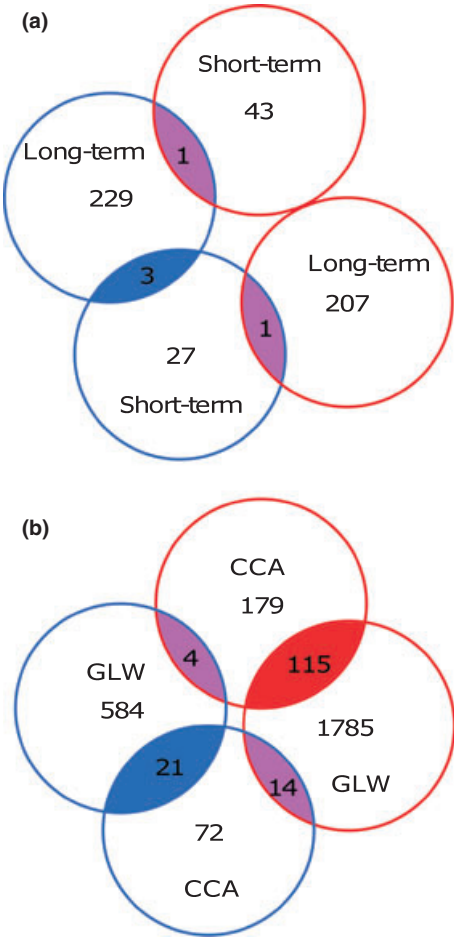

Fig. 5 Overlaps among differentially expressed genes (DEGs) associated with temperature responses and settlement induction. (a) Short-term and long-term exposures to elevated temperatures; (b) exposure to a natural settlement cue (crustose coralline algae, CCA) and a synthetic metamorphosis inducer (GLW-amide). Blue indicates down-regulation, and red, up-regulation. Numbers of DEGs are indicated for each set. Shaded areas of overlap (blue or red) indicate genes showing consistent responses in both treatments; magenta indicates contrasting patterns (up-regulation in one treatment and down-regulation in another).

the overlapping DEGs were not annotated. The only exceptions were a heat shock transcription factor (*Hsf1*) and an antiproliferative signalling gene (*BTG2*), which were down-regulated in both short- and long-term treatments. This highlights the strong dependence of expression responses on treatment duration. A total of 24 DEGs from the short-term treatment and 136 DEGs from the long-term treatment matched known genes. GO enrichment analysis revealed that DEGs from the short-term treatment included significantly more genes associated with response to stress and protein folding than would be expected by chance (Table 1). Examples

Table 1 Biological processes implicated in larval responses by Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs)

Gene set/Biological process	Genes (*n*)	Adjusted *P*-value
DEG in short-term temperature treatment		
GO:0006457: protein folding	3	8.5E-3
GO:0006950: response to stress	3	8.8E-4
DEG in long-term temperature treatment		
GO:0006412: translation	31	2.2E-16
DEG in CCA treatment		
GO:0006979: response to oxidative stress	5	9.0E-4
Up-regulated in CCA and GLW treatments		
GO:0006355: regulation of transcription, DNA dependent	5	0.047
GO:0006979: response to oxidative stress	4	1.9E-4
GO:0007018: microtubule-based movement	3	0.047

CCA, crustose coralline algae; FDR, false discovery rate. *P*-values obtained from Fisher's exact test comparing the proportion of DEG associated with each process relative to the proportion of such genes in the transcriptome (FDR controlled at 5%). Gene sets not shown here lacked any significant enrichment.

from this category include heat shock proteins *HspA5* and *Hsp90B1*, both of which were up-regulated in the short-term treatment. DEGs from the long-term treatment included significantly more genes associated with translation than expected by chance (Table 1): 31 different ribosomal protein genes were down-regulated in that treatment.

The contrast between short-term and long-term responses is clearly evident in heat shock proteins (Hsp), a well-studied component of the coral temperature stress response. Of 16 Hsp or Hsp-associated genes detected in the temperature treatments, three were up-regulated in short-term stress and down-regulated or unchanged in long-term stress. These include some of the most highly expressed Hsp genes in our data set: *Hsp90AA1*, *Hsp90B1* and *HspA5* (up-regulated 3.2-, 3.7- and 3.4-fold, respectively, in short-term treatment and −1.1-fold, 1.4-fold and 1.2-fold, respectively, in the long-term treatment). Conversely, a set of genes associated with the transport and metabolism of calcium and other ions was specifically associated with the long-term response. The calcium channel *Cacna1s* was up-regulated 5-fold in the long-term treatment but remained essentially constant in the short-term (1.2-fold). A mitochondrial adenine transporter (*Slc25a44*) was down-regulated in the long-term treatment (−3.2-fold), but not in the short-term treatment (1.2-fold). The carbonic anhydrase genes *CA2* and *CA3* were down-regulated in the long-term treatment (−3.3-fold and −2.9-fold, respectively), but not in the short-term treatment (−1.6-fold

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and −1.1-fold, respectively). Finally, 31 different ribosomal protein genes were down-regulated in the long-term treatment (−2.1-fold down-regulation on average) but not in the short-term treatment (average fold difference = −1.03).

Response to inducers of settlement and metamorphosis

Both the natural (CCA) and synthetic (GLW-amide) inducers elicited visible larval responses. The natural cue resulted in the larvae becoming elongated, actively exploring the substrate ('substrate searching' behaviour), followed by attachment and finally metamorphosis. In contrast, larvae exposed to GLW-amide assumed rounded shapes in the water column and proceeded to metamorphosis immediately, with most larvae failing to attach to the substrate. Although both treatments induced metamorphosis, GLW-amide appears to bypass the premetamorphic morphological and behavioural changes associated with the normal recruitment process in response to the natural settlement cue.

The CCA treatment induced differential expression of 405 transcripts, 147 of which matched known genes (Fig. 4c). GO enrichment analysis of these DEGs revealed more genes associated with oxidative stress response than expected by chance (Table 1). These included lactoperoxidase (up-regulated 4.4-fold), catalase (3.2-fold) and three genes annotated as peroxidasin (3.9-fold, 5.5-fold and 11-fold).

GLW-amide produced the most dramatic changes of any treatment in our study (Fig. 4d). This treatment induced differential expression of 2523 genes (714 of which were annotated), including some large changes in expression levels (ranging from 35-fold down-regulation to 146-fold up-regulation). Although GO enrichment tests did not identify any significantly over-represented processes among these genes, the DEG list included numerous homeobox proteins with clear relevance for metamorphosis (*Arx*, *Rx3*, *Hoxa3a*, *Hox3*, *Hoxd4*, *Dmbx1b*, *Nkx2.2a*, *Otx1*, *Otx2b* and *Six3*). Nearly all of these were up-regulated in the GLW-amide treatment (range: 2.8 to 24-fold), with the exception of *Hoxa3a* that was down-regulated 16.8-fold. Additional transcription factors were up-regulated in this treatment, including *Ahctf1*, *Foxj3*, *Tfap2a*, *Dmrta2*, *Hltf*, *Smad4*, *Jun*, *Sox9*, *Foxq1*, *Etv6*, *Tfe3* and *Dmtf1* (range of expression changes: 2.1- to 21.7-fold up-regulation). A small number of transcription factors were down-regulated, including *Notch1* (down-regulated −9.5-fold), *Elf4* (−7.1-fold) and *Tcf7l2* (−16.8-fold). The large number of genes in these categories, the magnitude of their expression changes, and their known roles in developmental processes all support their functional involvement in the metamorphic response to the GLW-amide treatment.

In contrast to the lack of overlap between temperature treatments, there was considerable overlap in DEG between the two metamorphosis inducer treatments; 39% of the genes up-regulated in the CCA treatment were also up-regulated in the GLW-amide treatment, and 20% of those down-regulated by CCA were also down-regulated by GLW-amide (Fig. 5b, Table S2, Supporting information). The lists of DEGs up-regulated in both treatments, putatively related to metamorphosis, were significantly enriched for the regulation of transcription, response to oxidative stress and microtubule-based movement (Table 1). Additional details of the genes responding to both treatments are available in Appendix S3 (Supporting information).

The genes affected by CCA but not by GLW-amide are especially interesting, as they might be responsible for the morphological and behavioural changes in the larvae associated with the natural recruitment process. Of the 405 DEGs identified in the CCA treatment, 140 showed contrasting patterns in the GLW-amide treatment: 108 were up-regulated in the CCA treatment, while remaining constant (≤1.25-fold) or down-regulated in the GLW-amide treatment, and 32 genes were down-regulated in the CCA treatment but constant or up-regulated in GLW-amide. Examples from this category include *Sqstm1*, a signalling gene (2.5-fold in CCA and −1.1-fold in GLW); *Dirc2*, a membrane protein with uncharacterized activity (3.8-fold in CCA and −4.2-fold in GLW); and *Trpm2*, a calcium channel (2.8-fold in CCA and 1.1-fold in GLW).

Validation of gene expression profiles using qPCR

To evaluate the accuracy of expression profiles obtained from RNA-Seq, we first measured a selected set of DEGs genes by qPCR, using the same samples originally used for RNA-Seq. This analysis revealed a close correlation between the expression changes (fold difference) measured by each method (regression $P < 0.001$; Pearson's correlation coefficient $r = 0.86$; Fig. 6). This was based on within-gene comparisons (i.e. treatment vs. control within each gene). Examining the relationship between absolute RNA-Seq expression levels and raw dC_T values across genes revealed a weaker but still highly significant relationship ($P < 0.001$; $r = 0.73$). Overall, the close agreement between qPCR and RNA-Seq provides strong evidence for the quantitative accuracy of our implementation of the RNA-Seq methodology.

Next, we surveyed the variation in expression responses across genetic backgrounds, using three additional larval families from the same experiment used

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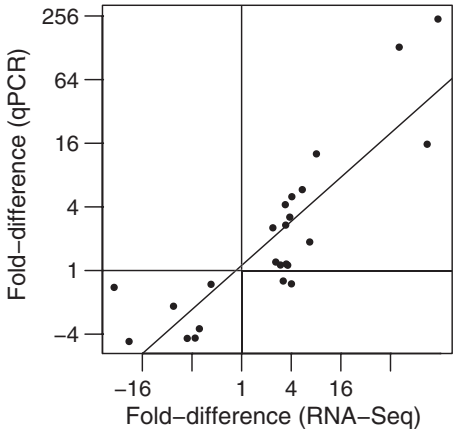

Fig. 6 Quantitative comparison of expression profiles obtained from RNA-Seq and qPCR analysis of the same samples. A subset of differentially expressed gene (DEG) identified by RNA-Seq was measured by qPCR in the appropriate treatment and control samples. Each point represents the expression in the treatment relative to the corresponding control, as measured by RNA-Seq and qPCR. The average fold difference across sequencing runs is presented for RNA-Seq, and the average of 2–3 technical replicates, for qPCR. The regression indicates a close correlation between methods (linear regression of $\log_2$ fold differences: $P < 0.001$; $r = 0.86$).

for RNA-Seq. This analysis revealed extensive variation in responses across families (Fig. 7). Of all comparisons between treatment and control in these qPCR data (24 comparisons between control and treatment for three families = 72 comparisons), most showed expression changes in the same direction observed in RNA-Seq analysis of a single family (76%), although the magnitude of these responses varied between families. The remaining 24% of comparisons revealed gene expression changes in the opposite direction of those originally observed in RNA-Seq analysis of a single family (open symbols in Fig. 7).

To evaluate the extent to which these between-family differences reflect genetics rather than random variation between culture replicates, we performed qPCR on samples from a replicated elevated long-term temperature experiment (three replicate culture vessels for each of four families in each of two culture temperatures, including the family originally used for RNA-Seq). This analysis revealed extensive variation among families in gene expression responses to elevated temperatures (Fig. 8). Larvae from family A consistently showed expression differences in the same direction observed in RNA-Seq analysis of the same family, while the other families differed substantially in the direction and magnitude of this response. For three of the six genes tested (*Fp486*, *Tnfsf10* and *Hsf1*), significant family-specific differences in expression were observed (ANOVA of $\log_2$ fold-changes; $P < 0.05$). For those genes, the family-specific effects accounted for 61–66% of the observed variance, supporting the notion of considerable genetic variation in larval responses to long-term temperature stress.

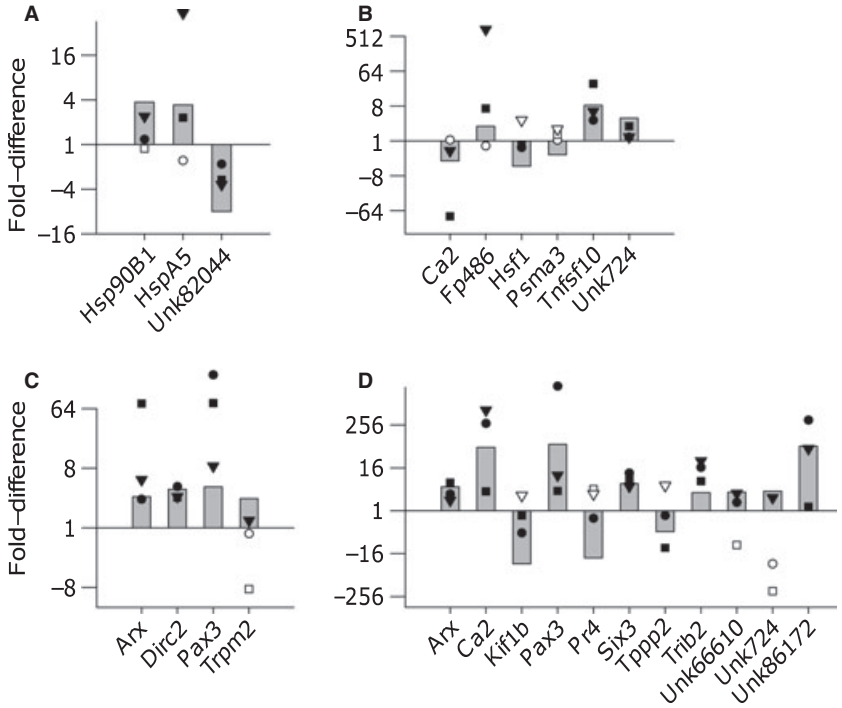

Fig. 7 Surveying gene expression responses in different genetic backgrounds. Grey bars indicate fold differences obtained from RNA-Seq analysis of a single family. Symbols overlaid on those bars represent qPCR data for three other families, with symbol shapes varying by family. Gene expression responses in the same direction as observed in RNA-Seq are indicated by filled symbols, and gene expression responses in the opposite direction are indicated by open symbols. (a) Short-term elevated temperature, (b) long-term elevated temperature, (c) the settlement inducer crustose coralline algae (CCA), (d) the synthetic metamorphosis inducer GLW-amide.

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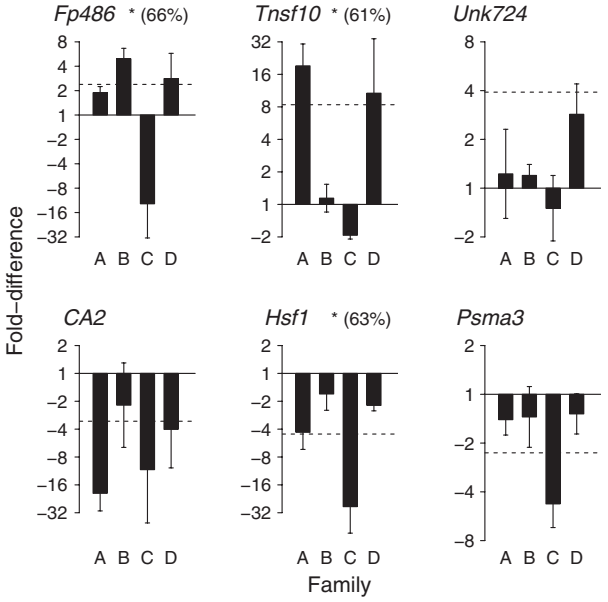

Fig. 8 Family-specific differences in gene expression responses to elevated temperature. Candidate genes from RNA-Seq profiles measured in replicated comparisons using qPCR. Each bar represents the average fold difference (±standard error) calculated from $n = 3$ control sample and $n = 3$ treatment samples in each of four families. Family A is the same full-sibling family of larvae used for RNA-Seq, and families B–D were cultured as part of the same experiment. Horizontal dashed lines indicate fold differences expected from RNA-Seq. Asterisks indicate significant differences in expression among families (ANOVA of $\log_2$ fold differences; $P < 0.05$). The contribution of between-family variance is shown in parentheses as a percentage of total variance.

Discussion

Effectiveness of RNA-Seq analysis in corals

The tag-based RNA-Seq approach outlined here proved successful for expression profiling in corals. The quantitative accuracy of these expression profiles is supported by the close correlation between expression changes measured by RNA-Seq (fold difference between treatment and control) and the expression changes measured in the same samples using qPCR ($r = 0.86$; Fig. 6). The strength of this correlation is comparable to those observed in previous RNA-Seq studies: e.g. $r = 0.35$ ('t Hoen *et al.* 2008), $r = 0.82$ (Bloom *et al.* 2009) and $r = 0.92$ (Tang *et al.* 2009). Our analysis quantified 20 048 reference sequences that probably correspond to ~18 050 genes (based on the low redundancy among annotated sequences). Although the complete gene complement of *Acropora millepora* is not known, this number is reassuringly similar to the ~18 000 genes found in a fully sequenced Cnidarian, *Nematostella vectensis* (Putnam *et al.* 2007). This is substantially higher representation (three times more unique sequences) than microarrays recently used for expression profiling in the same species (Bay *et al.* 2009; Grasso *et al.* 2008, 2011; Rodriguez-Lanetty *et al.* 2009). Although the lack of a complete genome sequence might be viewed as an obstacle to such analyses, the representation and quantitative accuracy of our RNA-Seq analysis confirms that mapping short RNA-Seq reads against an assembly of cDNA sequences derived from 454 reads (Meyer *et al.* 2009a) is an efficient strategy for genomewide expression profiling in nonmodel species.

The sample preparation procedure described in this study targets a narrow window near the 3′ end of polyadenylated transcripts, allowing for efficient use of sequencing coverage. Like the 'digital gene expression' (DGE) approach ('t Hoen *et al.* 2008), our method is tag based. The sequencing of a single tag per mRNA presumably accounts for the lack of length bias in our data (Fig. 3c), because this bias stems from oversampling longer genes in approaches that cover the entire mRNA (Oshlack & Wakefield 2009). In fact, our data show a slight trend towards higher expression of short transcripts, in agreement with the negative relationship between protein length and expression level previously reported (Munoz *et al.* 2004). Like two recently described methods (Cloonan *et al.* 2008; Lister *et al.* 2008), our procedure produces strand-specific reads corresponding to the sense strand of each mRNA. This feature proved useful for tuning the mapping parameters, on the basis that spurious matches should occur on both strands but true matches only on the sense strand (Appendix S2, Supporting information). Like several recently described methods (Cloonan *et al.* 2008; Lister *et al.* 2008; Mortazavi *et al.* 2008), our procedure begins with fragmented RNA to minimize length bias. Many of the previously described RNA-Seq procedures require large amounts of input RNA (4–100 μg) (Cloonan *et al.* 2008; Lister *et al.* 2008; Mortazavi *et al.* 2008; Nagalakshmi *et al.* 2008), with one notable exception (Tang *et al.* 2009). In contrast, our method is based on small amounts of input RNA (0.5 μg total RNA) and does not require poly-A selection or ribosomal RNA depletion.

Temperature stress responses depend on duration

We found substantially different responses between short-term and long-term exposures of the same larvae to the same temperatures, with little overlap in the DEG sets from those treatments (Fig. 5). Most of the studies of gene expression responses to thermal stress in coral larvae have focused on shorter-duration treatments (3–48 h) (Polato *et al.* 2010; Rodriguez-Lanetty

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et al. 2009; Voolstra *et al.* 2009), although one recent study (Portune *et al.* 2010) has extended this to 5 day as in our experiments, to investigate the expression profiles associated with completion of the entire larval lifespan at elevated temperatures. Comparing the findings across treatment durations (including our RNA-Seq analysis and the previous array studies) suggests two major themes. First, heat shock proteins (Hsp) tend to be up-regulated in short-term treatments, but down-regulated or constant in longer-duration treatments. Our findings confirm this general pattern, identifying three Hsp genes up-regulated in short-term temperature stress, but not in long-term (*HspA5*, *Hsp90AA1* and *Hsp90B1*). Second, previous studies have reported the up-regulation of oxidative stress response genes in long-duration treatments, but not in shorter-duration treatments. Our findings are consistent with this trend: two oxidative stress response genes were differentially expressed in our long-term treatment (peroxiredoxin-5 and glutaredoxin), but none in our short-term treatment. One of the most conspicuous signals in our long-term treatment was the widespread down-regulation of ribosomal protein genes, in agreement with previous studies of larval stress responses (Polato *et al.* 2010; Voolstra *et al.* 2009). Considering the central role of ribosomes in protein synthesis, this suggests a mechanism by which elevated temperature might reduce growth rates. Our findings also suggest that long-term exposure to elevated temperatures alters the metabolism and transport of ions including Ca^{2+} and CO_3^{2-}, suggesting a possible explanation for the effects of elevated temperatures on *in situ* calcification rates (Cantin *et al.* 2010). Although the functional roles of these processes in larvae that lack skeletons remain unclear, it is tempting to speculate that these metabolic changes would affect the deposition of the carbonate skeleton upon metamorphosis.

An important caveat for this comparison is that culture temperatures obviously affect developmental rates, and expression profiles change dramatically during coral development (Grasso *et al.* 2008). It is therefore likely that some of the genes induced in long-term but not short-term exposure resulted from temperature-induced changes in developmental rates. Although no visible signs of developmental differences were apparent in these cultures, consistent with previous studies of these stages in another coral species (Portune *et al.* 2010), the lack of morphological landmarks during these stages means that this possibility cannot be excluded.

Transcriptional responses to settlement inducers

Larvae in natural populations encounter diverse and heterogeneous cues that induce settlement and metamorphosis, and their responsiveness to these cues can be expected to affect the distribution of coral species as well as the overall reef resilience. The identity and characteristics of those cues have been extensively studied (Erwin & Szmant 2010; Heyward & Negri 1999; Iwao *et al.* 2002; Kitamura *et al.* 2007; Ritson-Williams *et al.* 2010; Webster *et al.* 2004), but the mechanisms that determine larval responses to these cues remain poorly understood. For our RNA-Seq experiments, we focused on two widely studied inducers: the natural cue CCA and a synthetic peptide (GLW-amide).

RNA-Seq revealed substantial overlap between the genes induced by these cues (Fig. 5), including genes with known developmental roles. For example, several of the transcription factors up-regulated in both treatments (*Pax3*, *Six3* and *Arx*) are associated with pattern formation during Cnidarian development (Galliot 2000; Matus *et al.* 2008; Miller *et al.* 2000; Ryan *et al.* 2006). GO enrichment analysis revealed that the set of DEG up-regulated in both treatments was significantly enriched for transcription factors (Table 1); examples include the homeobox protein *Nkx2.2a* and the segmentation protein *Cnc*. Considering that both treatments result in metamorphosis, the differential expression of these regulatory genes is not surprising. However, many of these were not found in a microarray analysis of these same treatments (CCA and GLW-amide) published, while the present study was in review (Grasso *et al.* 2011). Our RNA-Seq analysis identified many of the same gene expression changes as in this recent microarray study (e.g. the up-regulation of tetraspanin, carbonic anhydrase and the homeobox gene *Otx* in response to GLW-amide treatment). Two aboral genes associated with searching and settlement behaviour in the array study were also up-regulated in our RNA-Seq profiles (*SH3PXD2A* and *Dmrta2*). However, RNA-Seq identified substantially more DEGs than microarrays. For example, no expression changes were detected by arrays after 4 h of exposure to CCA fragments, while RNA-Seq identified >400 DEGs in that treatment. Similarly, array analysis of the GLW-amide treatment uncovered ~400 DEGs, while RNA-Seq identified >2500. These results do not support the notion that larvae are 'primed' for settlement, being able to undergo recruitment processes without transcriptional changes (Grasso *et al.* 2011). This disagreement may be attributed in part to differences between platforms (RNA-Seq vs. microarray) and in part to the inevitable biological differences among samples of CCA and families of coral larvae.

Our analysis also identified many genes that responded specifically to one cue but not the other. As in previous studies, (Erwin & Szmant 2010; Grasso *et al.*

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2011; Iwao *et al.* 2002; Meyer *et al.* 2009a), larvae in our experiments showed markedly different responses to the two cues, supporting the notion that GLW-amide invokes a 'shortcut' through the developmental programme leading to metamorphosis, bypassing the behavioural and morphological changes normally associated with recruitment. In the context of these different responses, the genes associated with CCA but not with GLW-amide are especially interesting, as these might play a role in CCA-specific aspects of the response (e.g. elongation, searching behaviour and attachment to the substrate). These included receptors for GABA (*Gabbr1*), natriuretic peptides (*Npr1*), ADP-ribose (*Trpm2*) and serotonin (*5htr1*). Although sequence comparisons are not sufficient for identifying these receptors' ligands in corals, the localization of these proteins in the cell membrane and their known sensory and signalling functions in other systems suggest that they play similar roles during larval exploration of substrates prior to attachment. The CCA-specific DEGs also included genes implicated in morphogenesis, potentially regulating pre-metamorphic changes in larval morphology: Kremen1 (a transmembrane protein associated with central nervous system development), dentin sialophosphoprotein (tooth and craniofacial development) and apextrin (anteroposterior axis formation). Another noteworthy gene from the CCA-specific list was C-type lectin, six homologs of which were previously found to be up-regulated in larval and juvenile stages in the microarray study (Grasso *et al.* 2008). Lectins are a diverse family of transmembrane proteins with carbohydrate binding activities that are widely implicated in allorecognition, including the establishment of symbiosis (Wood-Charlson *et al.* 2006). These genes could be involved in the detection and acquisition of suitable algal symbionts from the environment during recruitment, suggesting a connection between responses to settlement cues and the initiation of symbiosis.

Comparing expression responses across stages, species and platforms

The effects of elevated temperatures and settlement cues on gene expression have been previously studied using microarrays in a variety of coral species and developmental stages. Comparing our RNA-Seq results with those studies reveals substantial differences across studies (i.e. different genes were differentially expressed). Some of this difference is probably attributable to the greater representation of RNA-Seq relative to microarrays. Species-specific differences probably also play a role (e.g. *Montastrea faveolata* in family Faviidae compared with *A. millepora* in family Acroporidae), along with environmental differences between habitats (e.g. *Acropora palmata* in the Caribbean compared with *A. millepora* in the southern Pacific). Differences in developmental stage present one of the clearest contrasts. For example, one analysis of adult *M. faveolata* colonies (Desalvo *et al.* 2008) identified 74 DEGs associated with stress responses, only five of which were differentially expressed in our RNA-Seq analysis of larvae. A similar analysis of *A. palmata* colonies identified 43 DEGs (DeSalvo *et al.* 2010), of which only three were differentially expressed in our study. Even comparisons among larval studies, a wide range of developmental stages have been profiled: 1–2 day old (Polato *et al.* 2010; Voolstra *et al.* 2009), 10 day old (Rodriguez-Lanetty *et al.* 2009) or 12 h to 5 day old depending on treatments (Portune *et al.* 2010). Because all our experiments used 5-day-old larvae, stage-specific differences in expression profiles (Grasso *et al.* 2008) are likely to confound comparisons across these larval studies.

Despite these differences, some aspects of the gene expression response observed in our RNA-Seq analysis matched those observed in other species or stages. For example, the ubiquitin conjugating enzyme E2 was down-regulated in RNA-Seq analysis of larval temperature stress (−1.6-fold in short-term treatment and −5-fold in the long-term treatment), in agreement with studies of adult coral colonies in *M. faveolata* (Desalvo *et al.* 2008), but contrasting with findings from *A. palmata* (DeSalvo *et al.* 2010). A coral-specific group of genes called Scleractinian cysteine-rich peptides (SCRiPs) are associated with thermal stress responses in adult *M. faveolata* (Desalvo *et al.* 2008) and show differential expression during the development of *A. millepora* (Sunagawa *et al.* 2009). Our study found two SCRiP genes down-regulated in response to both short- and long-term elevated temperatures (range: −3.6 to −5.5-fold), confirming the association of these genes with thermal stress responses throughout the life history and across diverse coral taxa.

The up-regulation of heat shock proteins (Hsp) in response to elevated temperatures is an obvious and broadly conserved aspect of temperature stress responses. Hsps are induced in response to elevated temperatures in adult colonies of *A. palmata* and *M. faveolata* (DeSalvo *et al.* 2010; Desalvo *et al.* 2008) and in larvae of *A. millepora*, as measured by microarray (Rodriguez-Lanetty *et al.* 2009) and as measured by RNA-Seq (present study). Similarly, oxidative stress responses are a major component of both larval and adult responses. Oxidative stress responses have been widely studied in the context of thermal stress and coral bleaching (Downs *et al.*, 2000; Lesser, 1997), as the reactive oxygen species (ROS) produced by symbionts and mitochondria during exposure to elevated

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temperature are thought to play a central role in bleaching (Weis 2008). Our findings confirm that oxidative stress responses are induced in heat-stressed larvae lacking symbionts, in agreement with previous studies of aposymbiotic larvae (Polato *et al.* 2010; Voolstra *et al.* 2009). Calcium homoeostasis is implicated in both larval and adult responses: a variety of calcium binding domains and channels were down-regulated in adult colonies (DeSalvo *et al.* 2010; Desalvo *et al.* 2008), while calcium channels were up-regulated in larvae. Overall, these comparisons suggest that similar processes are involved during larval and adult responses to elevated temperatures. Because the larvae lack symbionts, these represent host-specific components of the coral response to thermal stress, identifying candidate genes and processes for future studies of variation in stress responses.

One of the most ubiquitous findings in studies of coral gene expression is the differential expression of fluorescent proteins (FPs) in response to environmental perturbations. These reports include down-regulation of FPs in response to heat stress or transplantation (Bay *et al.* 2009; Desalvo *et al.* 2008; Rodriguez-Lanetty *et al.* 2009; Smith-Keune & Dove 2008) and up-regulation in response to elevated light or infections (D'Angelo *et al.* 2008; Palmer *et al.* 2009; Seneca *et al.* 2010). These findings suggest important (although poorly understood) functional roles of FPs in corals. RNA-Seq revealed that FPs are among the most highly expressed genes in larvae (range: 1685–15 692 rpm across FP genes; Appendix S3, Supporting information). Comparing these sequences with the complete coding regions of *A. millepora* FP genes (Alieva *et al.* 2008) tentatively identified three FP genes in larvae: a red version (complete gene accession AY646073; larval reference sequence ID 15490); a green or cyan version (AY646070 or AY646067; reference ID 15491); and a nonfluorescent chromoprotein (AY646075; reference ID 5057). These three genes show conspicuously different expression patterns. The putatively green/cyan version (annotated as *Fp486* in the larval transcriptome) was significantly up-regulated in the long-term treatment (2.4-fold), while remaining nearly constant in the short-term treatment (1.5-fold). The nonfluorescent chromoprotein was down-regulated −2.1-fold in the long-term treatment and also showed a trend towards down-regulation in the short-term treatment (−1.7-fold; not significant). The putatively red version remained constant in both treatments (−1.2-fold and −1.1-fold, respectively). These findings are generally consistent with previous suggestions that FPs play a role in corals' response to environmental stress, and emphasize that FP function is not necessarily directly linked to symbiosis, as previously suggested (Salih *et al.* 2000), because our larvae were aposymbiotic. Our results highlight additional sources of variation in FP expression: (i) different responses for the multiple, co-expressed FPs; and (ii) a strong dependence on the duration of exposure to elevated temperatures. Future studies aiming to characterize the functional role of FPs in coral stress responses will benefit from considering these factors.

Genetic variation in gene expression responses

In the present study, we used RNA-Seq to obtain detailed profiles of gene expression in a single family of coral larvae, and qPCR analysis of the same samples confirmed the quantitative accuracy of these profiles. However, qPCR analysis of additional larval families revealed extensive variation in these responses for both elevated temperature and settlement inducers (Fig. 7). These findings align well with the variation in gene expression observed in adult colonies of *A. millepora* (Bay *et al.* 2009; Seneca *et al.* 2010). Expression profiles obtained from a single individual (or full-sibling family, as in the present study) clearly cannot be generalized to the species as a whole. However, we found reasonably reproducible expression levels within the same genetic background; variance within families was less than among families, with family-specific differences accounting for >60% of total variance in some genes (Fig. 8). This strongly suggests genetic variation in expression levels, contradicting a previous suggestion of low heritability for coral gene expression (Császár *et al.* 2010). One common strategy for dealing with this variation in larval research is to analyse bulk cultures derived from multiple parents (Polato *et al.* 2010; Rodriguez-Lanetty *et al.* 2009; Voolstra *et al.* 2009). While analysis of a single larval family is clearly inadequate for describing the population response, the bulk culture approach obscures much of the potentially important biological variation. Taking *Fp486* as an example (Fig. 8), neither the estimate obtained from RNA-Seq analysis of family A (2.4-fold up-regulation) nor the average response across families, as in a bulk culture composed of all four families (1.8-fold up-regulation, based on qPCR), is a useful description of the response in family C (−12.5-fold down-regulated). This variation among families may reflect diversity in the corresponding traits and therefore signify the potential for corals to adapt to the changing environment. Understanding the biological consequences of this variation will require replication of treatments across multiple genotypes or crosses and comparison with phenotypic characteristics of those genotypes (e.g. survival or recruitment). The RNA-Seq method described in this study will facilitate such experiments by enabling high-throughput, genome-wide measurements of gene expression in corals.

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Conclusions

Our study demonstrates the effectiveness of RNA-Seq as a tool for expression profiling in corals. The overall strategy of mapping short reads produced by RNA-Seq against a cDNA assembly derived from 454 reads provides a rapid and cost-effective route to gene expression profiling that should be widely applicable in other organisms lacking sequence resources. Our analysis of coral responses to elevated temperatures highlighted important differences in responses to short-term and long-term exposures. Responses to settlement inducers included some genes induced by both synthetic and natural cues (ostensibly related to metamorphosis) and other genes responding only to the natural cue. qPCR analysis of multiple larval families revealed extensive family-specific variation. Although this variation precludes generalizing these results to the coral species as a whole, it may signify heritable variation in the corresponding traits, which could serve as raw material for natural selection during climate change.

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E.M. is interested in genes and mechanisms underlying fitness-related traits in marine invertebrates. G.A. is working on development of new methods for the application of next-generation sequencing in marine organisms. M.V.M is interested in genetics of adaptation in reef-building corals.

Data accessibility

All sequence data for this study were archived at NCBI's Short Read Archive (SRA) under Accession no SRA029780.1. Gene expression data (number of reads mapped to each reference sequence) are provided as online supplemental material with this article.

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Supporting information

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

Appendix S1 Detailed protocol for preparation of cDNA samples for RNA-Seq analysis using the SOLiD System.

Appendix S2 Additional experimental details.

Appendix S3 Additional details of the expression profiles obtained from RNA-Seq.

Table S1 Raw expression data from RNA-Seq analysis of larvae of *Acropora millepora*.

Table S2 Differentially expressed genes identified by RNA-Seq analysis of *A. millepora* larvae.

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