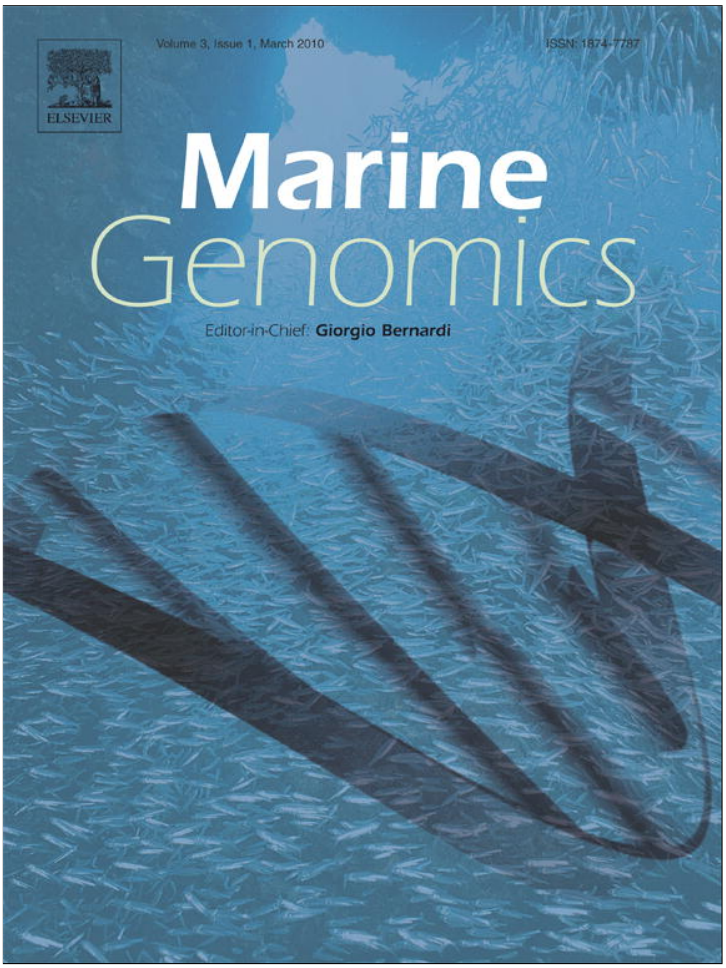

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# Development and heat stress-induced transcriptomic changes during embryogenesis of the scleractinian coral *Acropora palmata*

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ABSTRACT

Projected elevation of seawater temperatures poses a threat to the reproductive success of Caribbean reef-building corals that have planktonic development during the warmest months of the year. This study examined the transcriptomic changes that occurred during embryonic and larval development of the elkhorn coral, *Acropora palmata*, at a non-stressful temperature (28 °C) and further assessed the effects of two elevated temperatures (30 °C and 31.5 °C) on these expression patterns. Using cDNA microarrays, we compared expression levels of 2051 genes from early embryos and larvae at multiple developmental stages (including pre-blastula, blastula, gastrula, and planula stages) at each of the three temperatures. At 12 h post-fertilization in 28 °C treatments, genes involved in cell replication/cell division and transcription were up-regulated in *A. palmata* embryos, followed by a reduction in expression of these genes during later growth stages. From 24.5 to 131 h post-fertilization at 28 °C, *A. palmata* altered its transcriptome by up-regulating genes involved in protein synthesis and metabolism. Temperatures of 30 °C and 31.5 °C caused major changes to the *A. palmata* embryonic transcriptomes, particularly in the samples from 24.5 hpf post-fertilization, characterized by down-regulation of numerous genes involved in cell replication/cell division, metabolism, cytoskeleton, and transcription, while heat shock genes were up-regulated compared to 28 °C treatments. These results suggest that increased temperature may cause a breakdown in proper gene expression during development in *A. palmata* by down-regulation of genes involved in essential cellular processes, which may lead to the abnormal development and reduced survivorship documented in other studies.



## 1. Introduction

Global ocean temperatures have been steadily increasing over the past century (IPCC and Climate Change, 2007), while extreme ocean warming events, which have occurred during several summers over the past 3 decades, are predicted to increase in frequency and severity over the coming decades (Baker et al., 2004; Brown, 1997; Glynn, 1991, 1996; Glynn and Dcroz, 1990; Hoegh-Guldberg, 1999). In the tropics, this has resulted in summer seawater temperatures gradually rising above the maximum temperature tolerance ranges of many species, especially reef-building corals (see above references). Most reef corals broadcast their gametes into the water column, where embryogenesis and larval development take place within the planktonic surface layer (Harrison and Wallace, 1990), where daytime surface seawater temperatures are generally higher than those of the bulk water column due to solar heating. Seawater temperatures elevated by only a degree or more over normal summer temperatures have been shown to affect the physiology of the embryos and larvae of reef corals, causing dramatic effects such as increases in abnormal development, mortality, and reduced settlement (Baird et al., 2006; Edmunds et al., 2001; Negri et al., 2007; Nozawa and Harrison, 2007; Randall and Szmant, 2009a,b; Bassim and Sammarco, 2003; Bassim et al., 2002). However, not all corals are equally sensitive to temperature stress, as developing larvae of *Favites abdita*, *Favites chinensis* and *Mycedium elephantotus* exhibited normal embryogenesis at temperatures up to 5 °C higher than ambient (Negri et al., 2007).

The scleractinian coral *Acropora palmata* was once a dominant Caribbean reef-building species before a major decline in abundance that began in the early 1980s (Aronson and Precht, 2001; Lighty et al., 1982; Shinn, 1976). *A. palmata* broadcast spawns during the late summer,

*Abbreviations:* cDNA, complementary DNA; EST, expressed sequence tag analysis of variance (ANOVA); aRNA, amplified RNA; BLAST, Basic Local Alignment Search Tool; LOWESS, locally weighted scatterplot smoothing; BAGEL, Bayesian Analysis of Gene Expression Levels; TIGR, The Institute for Genomic Research; FOM, Figure of Merit; GO, gene ontology; ECM, extracellular matrix; DEG, differentially expressed gene; ATP, adenosine triphosphate; GTP, guanosine triphosphate; ADP, adenosine diphosphate; GDP, guanosine diphosphate; EBPs, enhancer-binding proteins; FC, fold change.

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when seawater temperatures are at their warmest, and development takes place in the planktonic surface layer (Randall and Szmant, 2009b; Szmant, 1986). *A. palmata* has a similar pattern of embryogenesis to that of its Pacific congener *A. millepora* (previously reported in Hayashibara et al., 1997; Ball et al., 2004; Okubo and Motokawa, 2007), with an irregular, flattened, post-morula stage known as the 'prawn chip' stage preceding the blastula stage, and with gastrulation taking place by invagination. Following completion of gastrulation (30 to 50 h after fertilization (Randall and Szmant, 2009b)), embryos eventually develop into planula larvae that remain in the plankton for a minimum of four days after fertilization before they become competent to settle (Randall and Szmant, 2009b; Szmant and Miller, 2006). Symbiotic *Symbiodinium* are not normally acquired until a week or so after settlement (Szmant personal observations). Given the late summer spawning cycle of *A. palmata* and their slow development to the planula stage, developing embryos of *A. palmata* are likely to be exposed to elevated seawater temperatures for a week or more. Elevated seawater temperatures (above 30 °C), such as those reported in recent years (McWilliams et al., 2005; Strong et al., 2008; Winter et al., 1998), have been shown to cause increased developmental aberrations as well as decreased survivorship and settlement in embryos and larvae in this species (Randall and Szmant, 2009b). Significantly reduced fertilization rates, increased embryonic abnormalities, embryonic mortality, and reduced survivorship also have been observed in the Pacific reef-building coral, *A. millepora*, exposed to temperatures of 31–32 °C (Negri et al., 2007; Rodriguez-Lanetty et al., 2009).

The molecular control of developmental processes in cnidarian embryogenesis is of general interest since cnidarians serve as a model for early metazoan development. Numerous genes homologous to those found in bilaterian animals have been identified in several cnidarians, with functions for some developmental processes such as pattern formation (de Jong et al., 2006; Finnerty et al., 2004; Miller and Miles, 1993). One such gene, *snail*, may play a role in the epithelial-mesenchyme transition during gastrulation in *A. millepora* (Hayward et al., 2004, 2001) and in the sea anemone *Nematostella vectensis* (Fritzenwanker et al., 2004). Recently, Grasso et al. (2008) conducted a large transcriptomic study of gene expression throughout development in *A. millepora* and found an up-regulation of transcripts for cell replication, RNA processing, and intracellular signaling proteins in early prawn chip stages, while extracellular matrix and cell adhesion transcripts were up-regulated in planula larvae and polyp stages.

Gene expression approaches have also been used to study the effects of elevated temperatures on both adult and embryonic corals. Desalvo et al. (2008) used DNA microarray experiments to examine the effects of temperature stress on changes within the transcriptomes of adult corals, finding that elevated temperature affected cellular processes such as oxidative stress, $Ca^{2+}$ homeostasis, cytoskeletal organization, cell death, calcification, metabolism, protein synthesis, heat shock protein activity, and transposon activity in the adult coral *Montastraea faveolata*. Voolstra et al. (2009) examined transcriptomic changes in developing *M. faveolata* embryos under temperature stress using the same cDNA microarrays, finding that increased temperatures may depress metabolic processes and cause oxidative stress, apoptosis, and a structural reconfiguration of the cytoskeletal network. cDNA microarrays were also recently used by Rodriguez-Lanetty et al. (2009) to examine transcriptomic changes in 10-day-old *A. millepora* larvae exposed to elevated temperature stress, finding a rapid expression of heat shock protein genes within three hours of hyperthermal exposure and little response from oxidative stress genes.

Reduced reproductive success would be predicted to increase under future global warming scenarios due to the disruption of development in many corals at elevated temperatures, thus becoming an important factor in coral population dynamics and coral reef resilience. However, the mechanisms by which temperature affects coral development remains poorly understood as does much of the normal process of coral embryonic development. This study used cDNA microarrays to follow changes in the transcriptome of *A. palmata* during development from mid-cleavage stages (6 to 8 cleavages) to mature planula larvae, when cultured under a non-stressful summer temperature (28 °C). The 28 °C temperature is slightly lower than the average late summer water temperature for the Caribbean at ~29 °C (McWilliams et al., 2005; Winter et al., 1998). We also examined the effects of two levels of elevated temperature on gene expression: 30 °C, moderate heat stress that can now be expected to regularly occur throughout the Caribbean during the late summer, and 31.5 °C, a more severe heat stress which has been recorded during recent record warm years (Winter et al., 1998). We identified numerous genes that were differentially expressed at defined developmental times, as well as identified patterns of up- and down-regulated genes in response to moderate and elevated heat stress during early development. This study contributes to the growing understanding of gene regulation in cnidarians during embryonic development, and identifies molecular processes that may be affected by elevated temperature in these early stages.

## 2. Materials and methods

### 2.1. Sample collection

*A. palmata* spawn was collected from a dense patch of colonies growing offshore of Tres Palmas beach in Rincon, Puerto Rico, at ca. 9:30 pm August 19, 2008. The gametes were mixed and allowed to fertilize for one hour before excess sperm was washed away. Fertilized spawn was brought back to the Magueyes laboratory of the Department of Marine Science of the University of Puerto Rico, Mayaguez by 1200 am on August 20, 2008. Batches of the spawn containing ~5000 larvae each were placed into 500 ml plastic containers, and two containers each were placed into duplicate IncuFridge® incubators per temperature set at 28 °C, 30 °C, and 31.5 °C (four beakers total per temperature). Subsamples from each Incufridge (200+ embryos or larvae combined from the two beakers within each incubator) were taken at selected time points (12, 24.5, 42.5, 89, and 131 h post-fertilization) over the 6 days of development, which coincided with multiple developmental stages (pre-blastula, blastula, blastula/gastrula transition, gastrula, gastrula/planula transition, and planula stages). Samples for transcriptomic analysis were preserved in 1.5 ml RNAlater (Ambion) in 2 ml cryovial tubes and stored at −20 °C until the samples were returned to the laboratory for microarray analysis. A second set of samples was preserved in 2% glutaraldehyde in 0.05 N sodium cacodylate buffer for later characterization and quantification of developmental stages. The percent of each developmental stage in each sample was determined by counting under a microscope the number of embryos in each developmental stage (12, 24.5, 42.5, 89, and 131 h post-fertilization). Representative photomicrographs of different developmental stages of live embryos and planula larvae taken concurrently with sampling times for this study are displayed in Fig. 1. The criteria for distinguishing the developmental stages were those described by Randall and Szmant (2009b), which were based on those in Okubo and Motokawa (2007). A separate temperature exposure experiment (Randall and Szmant, 2009b), run concurrently with separate batches of *A. palmata* embryos from the same spawn used in this experiment, further examined in detail the patterns of development and percent survivorship during development, as well as abnormalities of development that resulted from exposure to the same elevated temperatures as used in this experiment.

### 2.2. RNA extraction and microarray hybridization

Embryos and larvae were removed from the RNAlater and placed in new 2 ml cryovial tubes for extraction. Nucleic acids were released following bead-beating in 1 ml QIAzol lysis reagent (Qiagen) and 0.3 ml each of 0.1 mm glass and 0.5 mm zirconia/silica beads for each sample, with a Mini-Beater (Biospec Products) for 2 min. Total RNA

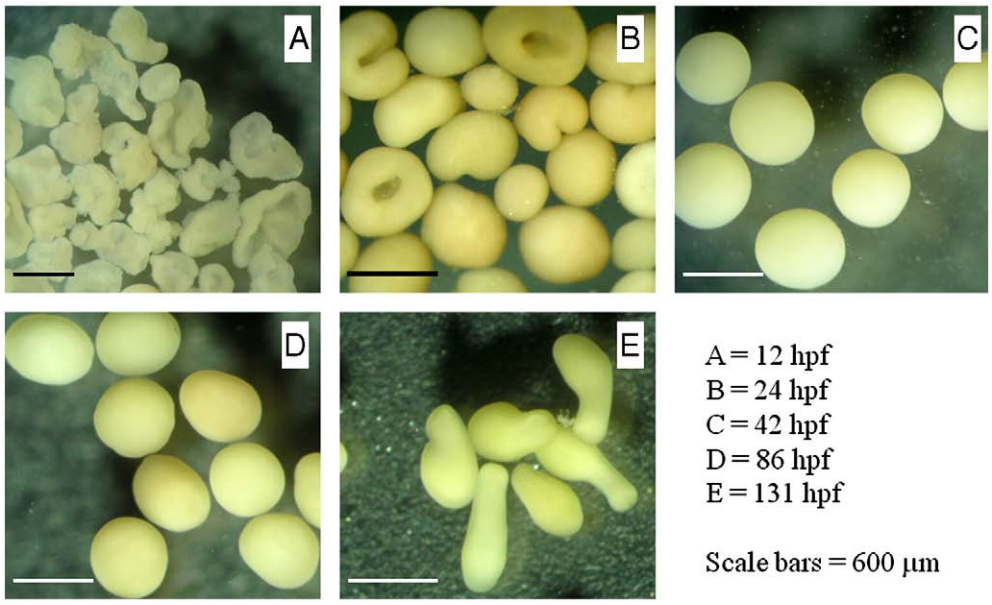



**Fig. 1.** Representative photomicrographs of live embryos and planula larvae of *Acropora palmata* taken concurrently with sampling for microarray analysis during the temperature exposure experiment at (A) 12 hours post-fertilization (hpf) (prawn chip), (B) 24 hpf (blastula to gastrula transition), (C) 42 hpf (early gastrulae), (D) 86 hpf (late gastrulae and early planulae), and (E) 131 hpf (mature planulae).

was isolated with 2 chloroform extractions, isopropanol precipitation, and 2 ethanol (80%) washes. Pellets were re-suspended in 100 µl nuclease-free water and purified with RNeasy Mini columns (Qiagen). RNA quantity and integrity were measured on a NanoDrop ND-1000 spectrophotometer and Agilent 2100 Bioanalyzer, respectively. Total RNA (1 µg) for each sample was amplified using the MessageAmp II aRNA kit (Ambion) according to the manufacturer's instructions. A reference sample was constructed by pooling together total RNA from single replicates from every time point at each temperature, followed by aRNA amplification as described above. Microarray hybridizations were performed for each sample using 3 µg of aRNA according to the methods used in Desalvo et al. (2008). Cy3 and Cy5 dyes were consistently labeled to reference and treatment samples, respectively. Biological duplicate samples for all 5 developmental time points and 3 temperature treatments were hybridized against the same reference sample (30 slides total) so that all samples could be normalized to the reference. 2051 cDNAs from *A. palmata* were spotted in duplicate onto poly-L-lysine coated slides yielding 4102 total features. Complementary DNAs (cDNAs) were chosen from expressed sequence tag (EST) libraries from Schwarz et al. (2008). Annotations of cDNAs were performed using BLASTx (*E*-value range from $8e^{-6}$ to $1e^{-180}$) against the nonredundant (nr) DNA and protein database in GenBank.

### 2.3. Microarray data analysis

Microarray slides were scanned according to Desalvo et al. (2008) and spot intensities were identified and separated from background fluorescence using TIGR Spotfinder 2.2.4 software (Saeed et al., 2003). Data were normalized using a printtip-specific LOWESS algorithm and in-slide duplicate spots were averaged using TIGR MIDAS 2.19 software (Saeed et al., 2003). Fluorescence intensities of samples at each time point were normalized to the corresponding reference fluorescence intensity on each hybridization slide. Normalized intensity ratios were then used to compare differences in gene expression across developmental time points and across temperature treatments within time points using BAGEL (Bayesian Analysis of Gene Expression Levels) (Townsend and Hartl, 2002). Conservative gene-by-gene criteria of non-overlapping 95% credible intervals were used to identify significantly differentially expressed genes (DEGs). For comparing gene expression at different temperatures within each developmental time point, genes from samples at 30 °C and 31.5 °C were defined as up- or down-regulated in relation to corresponding genes in 28 °C samples within each respective time point. Gene expression fold changes were calculated by the ratios 28 °C:30 °C and 28 °C:31.5 °C for down-regulated genes when 28 °C values had higher gene expression, and by the ratios 30 °C:28 °C and 31.5 °C:28 °C for up-regulated genes when 30 °C and 31.5 °C had higher gene expression values.

For comparing gene expression across developmental times at 28 °C, significant DEGs between consecutive developmental stages were identified through BAGEL analysis. Each time point was then normalized to the mean fluorescence intensities of each corresponding gene at 12 h post-fertilization. To visualize the temporal expression of DEGs across developmental times, genes were grouped into 7 patterns of gene expression by K-means clustering using a Euclidean distance metric with 50 iterations in TIGR TMEV 4.0 software (Saeed et al., 2003). Optimal numbers of K-means clusters were chosen using Figure of Merit (FOM) tests within the TIGR TMEV 4.0 software.

### 2.4. Functional grouping

Annotated genes spotted on the microarray were sorted into putative function according to Gene Ontology (GO), literature, or protein database searches and assigned to one of the following categories: cell replication/cell division (i.e. genes associated with mitosis or cytokinesis), metabolism (anabolism and catabolism), protein synthesis (translation), ion-binding/ion transport, calcium homeostasis, intracellular signal transduction, transport proteins/binding proteins (for proteins or macromolecules), cytoskeleton proteins, extracellular matrix (ECM)/cell adhesion proteins, oxidative stress, proteases/apoptosis, heat shock/response to cell damage, transcription-related proteins (i.e. transcription factors, histones, transcription enhancers), RNA-binding proteins, RNA processing, and proteins without an annotation (unknown) function (see Supplementary Table 1 [Table S1]). These categories were further grouped into the following major cellular processes: growth and development, cell signaling, structural support, response to stress, and regulation of gene expression (Table S1). The number of unique genes defined within functional groups of cellular processes that were spotted on the microarray ranged from 116 (from the group response to stress) to 294 (growth and development) (Table S1). Genes without

an annotation (unknown function) made up 45.7% of spots on the array with a total of 938 genes (Table S1). All clone ID numbers can be found in the SymBioSys resource database (SymBioSys). Microarray data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE21038.

## 3. Results

### 3.1. Developmental stages for each time point

Microscopic evaluation of each of the samples preserved for developmental analysis showed that at 12 hours post-fertilization (hpf), the majority of embryos were in pre-blastula stages (prawn chip; Randall and Szmant, 2009b), with small percentages (~1–4%) in the blastula stage at all 3 temperatures (Fig. 2). A small percent (~2–3%) of embryos were identified as starting gastrulation (blastula/gastrula transition) in both the 30 °C and 31.5 °C temperature treatments at 12 hpf (Fig. 2). At 24.5 hpf, most embryos had either advanced to the late blastula stage or were in the process of gastrulation, with cultures at 31.5 °C temperatures having a slightly higher percentage of embryos that had completed gastrulation than samples at 28 °C or 30 °C. By 42.5 hpf, the majority of embryos had reached the gastrula phase with no differences in the level of development discernible among temperatures (Fig. 2). By 89 hpf, a large percent of embryos in all 3 temperatures had started the gastrula/planula transition (ranging from 57 ± 0.02% to 74 ± 0.5%), while a large percent of fully formed planulae (ranging from 26 ± 0.5% to 43 ± 0.02%) were also found in these samples (Fig. 2). By 131 hpf, all samples consisted of fully formed planulae at all three temperatures (Fig. 2). Development occurred slightly faster at temperatures of 30 °C and 31.5 °C compared to 28 °C, notably during early developmental stages from 12 to 42.5 hpf (Fig. 2).

### 3.2. Differential gene expression across developmental times

Comparison across successive developmental stages of embryos grown at the control treatment temperatures (28 °C) revealed 771 significant DEGs out of 2051 unigenes represented on the array, with at least one significant difference between any two consecutive developmental stages. Cluster analysis of significant DEGs identified 7 distinct synexpression clusters each with distinct gene expression patterns which map onto different stages of coral development (Fig. 3). Clusters 1, 2, and 3 identified "post-prawn chip stage" genes that were up-regulated during 24.5, 42.5, 89, and 131 hpf compared to those in pre-blastula development at 12 hpf (Fig. 3). Up-regulated genes in post-prawn chip stages with the highest mean fold changes were grouped into Cluster 1, followed by progressively lower fold changes in Clusters 2 and 3 (Fig. 3). Cluster 4 identified gastrula and planula stage genes that were up-regulated during 42.5, 89, and 131 hpf compared to earlier stages of development at 12 and 24.5 hpf (Fig. 3). Clusters 5, 6, and 7 identified "pre-blastula stage" genes that were down-regulated at 24.5, 42.5, 89, and 131 hpf compared to 12 hpf (Fig. 3). Down-regulated genes in post-prawn chip stages with the highest mean fold changes were grouped into Cluster 5, followed by progressively lower fold changes in Clusters 6 and 7 (Fig. 3).

Genes associated with metabolism and protein synthesis were represented in higher numbers (40 and 35 genes, respectively) in post-prawn chip stage clusters (Clusters 1–3) compared to the 17 and 4 genes, respectively, in pre-blastula stage clusters (Clusters 5–7) (Table 1, Supplementary Table 2 [Table S2]). Twenty-two homologs of genes encoding 40S and 60S ribosomal proteins, 5 translation initiation factors, and 4 ATP synthase subunits were among these genes grouped into post-prawn chip stage clusters (Table S2, Clusters 1–3).

High numbers of genes associated with cell replication/cell division (36 genes) and transcription (49 genes) were grouped into pre-blastula stage clusters (Clusters 5–7) in contrast to the 13 genes associated with cell replication/cell division and 18 transcription-related genes grouped into post-prawn chip stage clusters (Clusters 1–3) (Table 1). Within pre-blastula stage clusters, six genes associated with cell replication/cell division include homologs of cyclins and other mitosis-related genes (i.e. Protein cut 8), while 5 genes encode for cytokinesis-related proteins (Table S2, Clusters 5–7). Four homologs of genes encoding transcription initiation and elongation factors, 2 homeobox genes (Homeobox protein Nkx-6.3 and Homeobox protein Nkx-2.8), and 4 histone-related proteins were also grouped into pre-blastula stage clusters (Table S2, Clusters 5–7). Five homologs of transcription–regulation genes and 2 histone-subunit genes were grouped into post-prawn chip stage clusters (Table S2, Clusters 1–3).

Genes associated with the cellular processes involving cell signaling, structural support, response to stress, DNA- and RNA-binding proteins, and RNA processing were similar in total number of DEGs in pre-blastula and post-prawn chip stage clusters (Table 1, Clusters 1–3, 5–7). No clear

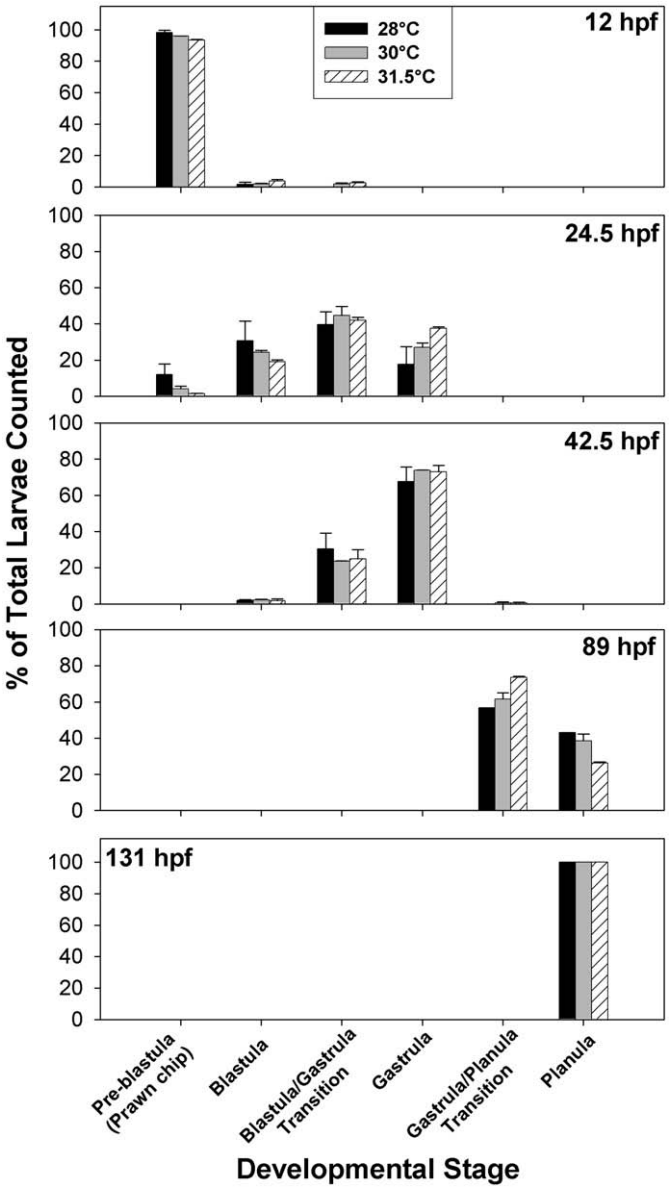
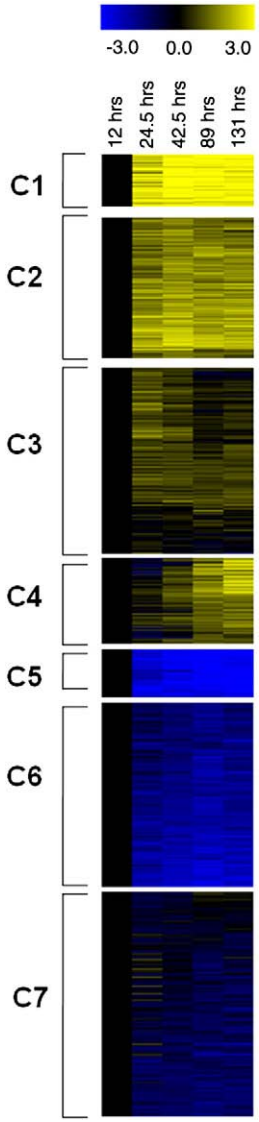



**Fig. 2.** Average percent of total larvae present in samples from each development stage of *Acropora palmata* cultures at the three experimental temperatures (28, 30, 31.5 °C) for the five sample times used in this study: 12, 24.5, 42.5, 89, and 131 hours post-fertilization (hpf). Samples for developmental stage determination were analyzed in duplicate ($N = 2$) for each culture at each time point.




**Mean expression profile (fold changes compared to 12 hrs)**

| Cluster | 24.5 hrs | 42.5 hrs | 89 hrs | 131 hrs | Mean fold change across all times |
|---|---|---|---|---|---|
| C 1 | +2.68 | +3.48 | +3.38 | +3.37 | +3.23 |
| C 2 | +1.51 | +1.83 | +1.63 | +1.63 | +1.65 |
| C 3 | +0.69 | +0.56 | +0.32 | +0.40 | +0.49 |
| C 4 | +0.02 | +0.55 | +1.25 | +1.63 | +0.86 |
| C 5 | -2.62 | -3.24 | -3.71 | -3.61 | -3.30 |
| C 6 | -1.38 | -1.63 | -1.89 | -1.70 | -1.65 |
| C 7 | -0.56 | -0.68 | -0.80 | -0.81 | -0.71 |

**Fig. 3.** Graphical representation of significant differentially expressed genes at 28 °C, with at least one significant difference between all developmental time points. Genes were grouped by K-means clustering into 7 synexpression clusters (C1–C7) according to similar patterns in gene expression over time. Clusters 1–3 consist of genes up-regulated in gene expression during 24.5 to 131 hpf compared to 12 hpf, while Cluster 4 largely consists of genes up-regulated in gene expression in later developmental periods (42.5 to 131 hpf). Clusters 5–7 consist of genes down-regulated in gene expression during 24.5 to 131 hpf compared to 12 hpf. Mean expression profiles for each development time point within respective clusters are expressed in terms of fold changes compared to the first time point (at 12 hpf).

trend in total numbers of DEGs was observed across development for these cellular processes, although several functionally-related genes displayed similar patterns of expression. Among genes of particular interest involved in intracellular signal transduction were several homologs from the Ras superfamily (GTP-binding protein Di-Ras2, Ras-related protein Rap-2a, Ran-specific GTPase-activating protein, Ras-specific guanine nucleotide-releasing factor 1), which grouped into pre-blastula stage clusters (Table S2, Clusters 5–7). Other genes of interest included four heat shock protein genes, which were up-regulated in later developmental stages at 24.5–131 hpf (Table S2, Clusters 1–3). High numbers of DEGs with unknown functional annotations were detected in all clusters (Table 1).

### 3.3. Changes in transcriptome associated with heat stress within developmental time periods

We detected a range of 16–189 DEGs at 30 °C compared to 28 °C within each respective developmental time point (Table 2). The highest number of DEGs (189) at 30 °C was detected after 24.5 hpf, while low total numbers of DEGs (16–21) were expressed in later development (Table 2). At 31.5 °C, DEGs ranged from 17 to 89 within respective time points, with high differential expression occurring between 24.5 and 89 hpf, while the lowest number occurred at 131 hpf (Table 2).

### 3.4. Temperature effect on growth and development genes

Overall, considerable total numbers of significantly down-regulated genes involved in growth and development were observed at 24.5 hpf (19 genes at 30 °C) and 42.5 hpf (10 genes at 31.5 °C), while multiple up-regulated genes (6 and 5 genes for 30 °C and 31.5 °C, respectively) were also observed at 24.5 hpf (Table 3). Among these down-regulated genes are several crucial for cell replication/cell division processes such as regulating mitosis (G2/mitotic-specific cyclin-B, Cyclin-A2, Protein cut8, Tolloid-like protein 2) and several genes associated with cytokinesis (Protein regulator of cytokinesis 1 and Cell division cycle 5-related protein) (Supplementary Table 3 [Table S3]). Multiple homologs of genes for metabolic processes such as glycogenolysis/glycogenesis, glycolysis, and oxidative phosphorylation were down-

**Table 1**
Total differentially expressed genes (DEGs) from each functional group in each K-means cluster (C1–C7) corresponding with Fig. 3, as well as total DEGs from "post-prawn chip stage" clusters (Clusters 1–3) and "pre-blastula stage" clusters (Clusters 5–7).

| Functional groups | Cluster 1 | Cluster 2 | Cluster 3 | Cluster 4 | Cluster 5 | Cluster 6 | Cluster 7 | Clusters 1–3 | Clusters 5–7 |
|---|---|---|---|---|---|---|---|---|---|
| Growth and development | | | | | | | | | |
| Cell replication/cell division | – | 1 | 12 | 3 | 8 | 13 | 15 | 13 | 36 |
| Metabolism | 3 | 21 | 16 | 5 | 1 | 9 | 7 | 40 | 17 |
| Protein synthesis (translation) | 11 | 17 | 7 | 1 | – | 2 | 2 | 35 | 4 |
| Total | 14 | 39 | 35 | 9 | 9 | 24 | 24 | 88 | 57 |
| Cell signaling | | | | | | | | | |
| Ion-binding/ion transport | 1 | 6 | 7 | – | – | 4 | 4 | 14 | 8 |
| Calcium homeostasis | 1 | 5 | 1 | 1 | – | 3 | – | 7 | 3 |
| Intracellular signal transduction | 4 | 3 | 4 | 4 | 1 | 9 | 9 | 11 | 19 |
| Total | 6 | 14 | 12 | 5 | 1 | 16 | 13 | 32 | 30 |
| Structural support | | | | | | | | | |
| Transport/binding proteins | 1 | 13 | 13 | 2 | 1 | 13 | 16 | 27 | 30 |
| Cytoskeleton | 3 | 12 | 5 | 2 | 2 | 5 | 11 | 20 | 18 |
| ECM/cell adhesion | 2 | – | 4 | 1 | – | 8 | 3 | 6 | 11 |
| Total | 6 | 25 | 22 | 5 | 3 | 26 | 30 | 53 | 59 |
| Response to stresses | | | | | | | | | |
| Oxidative stress | 1 | 2 | 2 | – | – | 2 | 1 | 5 | 3 |
| Proteases/apoptosis | 2 | 7 | 10 | 1 | 2 | 9 | 4 | 19 | 15 |
| Heat shock/response to damage | 2 | 3 | 4 | 3 | – | 2 | 2 | 9 | 4 |
| Total | 5 | 12 | 16 | 4 | 2 | 13 | 7 | 33 | 22 |
| Regulation of gene expression | | | | | | | | | |
| Transcription | 1 | 7 | 10 | 4 | 12 | 21 | 16 | 18 | 49 |
| RNA-binding proteins | – | 1 | 2 | 1 | – | 2 | 4 | 3 | 6 |
| RNA processing | – | 1 | 12 | – | – | 2 | 14 | 13 | 16 |
| Total | 1 | 9 | 24 | 5 | 12 | 25 | 34 | 34 | 71 |
| Unknown function | 16 | 33 | 62 | 50 | 18 | 65 | 98 | 111 | 181 |
| Total for all functional groups | 48 | 132 | 171 | 78 | 45 | 169 | 206 | 351 | 420 |

regulated in both 30 °C and 31.5 °C at 24.5 hpf (Table S3). Genes associated with protein synthesis, such as translation initiation and ribosomal proteins, were up- and down-regulated in 30 °C and 31.5 °C at 24.5 hpf, while a homolog for an elongation factor was up-regulated as well (Table 3, Table S3).

### 3.5. Temperature effect on cell signaling, structural support, and stress response genes

Numerous DEGs (7 down- and 7 up-regulated) involved in intracellular signal transduction were detected at 24.5 hpf in 30 °C or 31.5 °C (Table S3). Of these DEGs, 6 genes that were down-regulated over time in response to elevated temperature were members of the Ras superfamily (Ran-specific GTPase-activating protein, Ras GTPase-activating-like protein IQGAP1, Ras-related protein Rap-2a, Rho GDP-dissociation inhibitor 1, Ras-related protein Rab-35, ADP-ribosylation factor-like protein 13B) (Table S3). Homologous genes associated with ion-binding/ion transport were down- and up-regulated with no clear trend for 30 °C or 31.5 °C (Table 3). Numerous DEGs involved in protein-binding and transport were both up- and down-regulated at 30 °C and 31.5 °C at 24.5 hpf, as well as up-regulated at 89 hpf. Multiple genes involved in cytoskeleton proteins such as actin and microtubule associated proteins were also down-regulated in 30 °C or 31.5 °C at 24.5 hpf (Table S3). Nine homologous genes coding for proteins involved in responses to cell damage were up-regulated in either 30 °C or 31.5 °C during 24.5 hpf (Table S3). These genes included 5 heat shock proteins, 2 heat shock-binding proteins, and an enzyme involved in detecting damage to DNA (Bifunctional polynucleotide phosphatase/kinase) (Table S3). Few genes involved in oxidative stress were differentially expressed at both 30 °C or 31.5 °C over all developmental times (Table 3). Soma ferritin and glutathione peroxidase were both down-regulated during 24.5 hpf at 30 °C and 31.5 °C, respectively (Table S3).

**Table 2**
Total DEGs at 30 °C and 31.5 °C compared to 28 °C at all 5 time points (12, 24.5, 42.5, 89, and 131 h post-fertilization). Total down- and up-regulated DEGs are represented for each temperature treatment.

| Differentially expressed genes for all time points | | | | | | | |
|---|---|---|---|---|---|---|---|
| Temperature treatment | 12 hpf | 25.5 hpf | 43.5 hpf | 89.5 hpf | 132 hpf | Total down-regulated genes | Total up-regulated genes |
| 30 °C | 56 | 189 | 27 | 16 | 21 | 147 | 162 |
| 31.5 °C | 35 | 89 | 67 | 89 | 17 | 168 | 129 |

### 3.6. Temperature effect on genes involved in regulation of gene expression

Multiple down-regulated DEGs coding for proteins involved in transcription were found at all developmental time points in response to temperature stress, with a high number of down-regulated genes detected at 24.5 hpf (12 and 8 genes in 30 °C and 31.5 °C, respectively) compared to other developmental stages (Table 3). Among these genes, there were homologs for genes associated with RNA polymerases (DNA-directed RNA polymerase III subunit RPC2), transcription elongation factors, transcription regulators (CCAAT/enhancer-binding protein epsilon), and 6 histone-subunit genes (Table S3). Three homologous genes associated with transcription were also up-regulated at 24.5 hpf (Table S3). Multiple genes involved in RNA processing were consistently down-regulated at 24.5 and 42.5 hpf (Table 3), including 7 small nuclear ribonucleoproteins and other genes involved in pre-mRNA splicing (Table S3). However, 4 genes involved in pre-mRNA splicing were also up-regulated at 30 °C and 31.5 °C at both 12 and 24.5 hpf (Table S3).

### 3.7. Temperature effect on genes with unknown functions

Large numbers of DEGs detected at each developmental time point in 30 °C (up to 84 genes) and 31.5 °C (up to 36 genes) did not have a known functional annotation (Table 3). The largest amount of DEGs with unknown annotations in both 30 °C and 31.5 °C treatments was detected at 24.5 hpf (105 DEGs), representing 42% of the total number of DEGs at this developmental time (Table S3).

**Table 3**
Total DEGs from each functional grouping at 30 °C and 31.5 °C treatments compared to 28 °C treatments at each time point.

| Functional group | Time 1 (12 h) | | | | Time 2 (24.5 h) | | | | Time 3 (42.5 h) | | | | Time 4 (89 h) | | | | Time 5 (131 h) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Down-regulated | | Up-regulated | | Down-regulated | | Up-regulated | | Down-regulated | | Up-regulated | | Down-regulated | | Up-regulated | | Down-regulated | | Up-regulated | |
| | 30 °C | 31.5 °C | 30 °C | 31.5 °C | 30 °C | 31.5 °C | 30 °C | 31.5 °C | 30 °C | 31.5 °C | 30 °C | 31.5 °C | 30 °C | 31.5 °C | 30 °C | 31.5 °C | 30 °C | 31.5 °C | 30 °C | 31.5 °C |
| Growth and development | | | | | | | | | | | | | | | | | | | | |
| Cell replication/cell division | – | – | 1 | – | 11 | 2 | 3 | 1 | 2 | 4 | 1 | 1 | – | 4 | – | 2 | 1 | 2 | – | – |
| Metabolism | 2 | 1 | 2 | 3 | 5 | 4 | – | 1 | 1 | 3 | – | 2 | – | 2 | 1 | 1 | – | – | – | – |
| Protein synthesis (translation) | 1 | – | – | – | 3 | – | 3 | 3 | – | 3 | – | – | – | – | – | – | 1 | – | – | 1 |
| Total | 3 | 1 | 3 | 3 | 19 | 6 | 6 | 5 | 3 | 10 | 1 | 3 | 0 | 6 | 1 | 3 | 2 | 2 | 0 | 1 |
| Cell signaling | | | | | | | | | | | | | | | | | | | | |
| Ion-binding/ion transport | – | 1 | – | – | 2 | – | 2 | – | – | 2 | – | – | – | 2 | – | 2 | – | – | – | – |
| Calcium homeostasis | – | 1 | 2 | – | 3 | – | – | – | – | – | – | – | – | 1 | – | – | – | 1 | – | – |
| Intracellular signal transduction | 3 | 2 | 3 | 2 | 6 | 4 | 5 | 3 | 2 | 3 | 1 | 1 | – | 3 | 1 | 1 | 1 | – | – | – |
| Total | 3 | 4 | 5 | 2 | 11 | 4 | 7 | 3 | 2 | 5 | 1 | 1 | 0 | 6 | 1 | 3 | 1 | 1 | 0 | 0 |
| Structural support | | | | | | | | | | | | | | | | | | | | |
| Transport/binding proteins | 2 | 2 | 1 | 1 | 6 | 4 | 9 | 5 | – | 1 | – | 1 | 1 | 3 | 1 | 7 | 1 | 1 | 1 | – |
| Cytoskeleton | – | – | – | – | 6 | 2 | 2 | 1 | – | – | 1 | – | – | 3 | – | 2 | 1 | 1 | – | – |
| ECM/cell adhesion | – | – | – | – | 3 | 3 | 2 | 1 | – | – | – | 1 | – | 1 | 1 | 3 | – | – | – | – |
| Total | 2 | 2 | 1 | 1 | 15 | 9 | 13 | 7 | 0 | 1 | 1 | 2 | 1 | 7 | 2 | 12 | 2 | 2 | 1 | 0 |
| Response to stresses | | | | | | | | | | | | | | | | | | | | |
| Oxidative stress | 1 | – | – | – | 1 | 1 | – | 1 | – | 1 | – | – | – | – | – | – | – | – | – | – |
| Proteases/apoptosis | 2 | 2 | 1 | 1 | 4 | 2 | 2 | – | 1 | 2 | – | – | – | 1 | 1 | 2 | 1 | 1 | 2 | 1 |
| Heat shock/response to damage | – | – | – | 1 | – | – | 5 | 6 | – | – | – | – | 1 | 1 | – | 2 | – | – | – | 1 |
| Total | 3 | 2 | 1 | 2 | 5 | 3 | 7 | 7 | 1 | 3 | 0 | 0 | 1 | 2 | 1 | 4 | 1 | 1 | 2 | 2 |
| Regulation of gene expression | | | | | | | | | | | | | | | | | | | | |
| Transcription | 1 | 3 | 1 | 2 | 12 | 8 | 3 | 1 | 1 | 3 | 2 | 2 | – | 4 | 1 | – | 2 | 3 | – | – |
| RNA-binding proteins | – | – | – | 1 | 1 | – | 1 | 1 | – | 2 | – | – | – | 1 | – | 1 | – | – | – | – |
| RNA processing | – | 1 | 1 | 2 | 3 | 7 | 2 | 3 | 4 | 6 | – | – | – | 3 | – | 1 | – | – | 1 | – |
| Total | 1 | 4 | 2 | 5 | 16 | 15 | 6 | 5 | 5 | 11 | 2 | 2 | 0 | 8 | 1 | 2 | 2 | 3 | 1 | 0 |
| Unknown function | 5 | 6 | 27 | 3 | 32 | 16 | 52 | 9 | 4 | 13 | 7 | 16 | 2 | 12 | 6 | 24 | 5 | 3 | 4 | 2 |
| Total genes for all functional groups | 17 | 19 | 39 | 16 | 98 | 53 | 91 | 36 | 15 | 43 | 12 | 24 | 4 | 41 | 12 | 48 | 13 | 12 | 8 | 5 |

## 4. Discussion

### 4.1. Gene expression patterns in A. palmata embryos and larvae at non-stressful temperature

#### 4.1.1. Growth and developmental genes

As expected, there was an up-regulation and higher expression of genes involved in cell replication and division processes in pre-blastula stages (12 hpf) of *A. palmata* at 28 °C compared to later developmental stages. Several of these genes, such as cyclins and other mitosis-related genes like Protein cut 8 and Protein regulator of cytokinesis 1, play crucial developmental roles that are necessary for rapid cell replication and division during the frequent cleavages integral to early embryogenesis. Protein cut 8 is a heat-inducible regulator protein which recruits the 26S proteasome during anaphase for the destruction of cyclins and cut2 (Tatebe and Yanagida, 2000), and therefore is essential for progression of the cell cycle through anaphase. Protein regulator of cytokinesis 1 is a microtubule bundling protein that is crucial in the formation of the central spindle necessary for accurate cell division (Mollinari et al., 2005). Since rapid cell replication and cell division are required by early developing embryos (O'Farrell et al., 2004), higher relative gene expression for proteins involved in these cellular processes are expected for embryos at early developmental stages (i.e. prawn chip stage). Grasso et al. (2008) found similar changes in the transcriptomes of developing *A. millepora* embryos, in which genes associated with cell replication (such as cyclin genes) were up-regulated during the prawn chip stage. High representation of cell cycle-related transcripts have also been detected in eggs and early embryos in *Drosophila melanogaster* (Arbeitman et al., 2002), *Xenopus laevis* (Baldessari et al., 2005), and in the basal chordate *Ciona intestinalis* (Azumi et al., 2007), due to large deposits of maternal transcripts into fertilized eggs. mRNA transcripts associated with cell replication and division in early *A. palmata* embryos may also derive from stored maternal transcripts, as suggested for *A. millepora* (Grasso et al., 2008), since Schwarz et al. (2008) noted an over-representation of ESTs related to cell division, proliferation, and mitosis in eggs of *A. palmata* compared to planula larvae and adults.

As embryonic development at 28 °C progresses into the blastula, gastrula, and planula stages, the *A. palmata* transcriptome is characterized by an up-regulation of genes involved in protein synthesis and metabolism. In developing *A. millepora* embryos, Grasso et al. (2008) similarly noted a high percentage of up-regulated genes involved in protein synthesis (29.1%) and intermediary synthesis/catabolism enzymes (16%) in planula and polyp stages compared to prawn chip stages. Among particular up-regulated genes of interest in our study include 40S and 60S ribosomal proteins, translation initiation factors, and ATP synthases. 40S and 60S ribosomal proteins have numerous essential functions associated with proper regulation of translation, including the delivery of mRNA to the ribosome, forming the ribosomal entry pore, exerting helicase activity, decoding, translation fidelity, binding and release of tRNAs to the ribosome, and tRNA stability (reviewed in Wilson and Nierhaus, 2005). Eukaryotic translation initiation factors are involved in several processes for protein synthesis, such as the recruitment of the 40S ribosomal subunit to the initiating codon in the mRNA transcript (Neff and Sachs, 1999; reviewed in Merrick and Hershey, 1996), while ATP synthases are well-known enzymes involved in the metabolic catalysis of ATP (Yoshida et al., 2001). Consistent up-regulation of these essential genes in late developmental phases suggests an increasing demand for protein and energy production as embryos develop into motile planulae. High expression of genes for protein synthesis and metabolism have been similarly detected from mid to late embryogenesis on into larval stages in both developing embryos of *Drosophila* (Arbeitman et al., 2002) and *Xenopus* (Baldessari et al., 2005). In contrast, in the ascidian *C. intestinalis*, over-representation of genes involved in protein biosynthesis, respiratory chain proteins, and mitochondrial proteins were highly expressed in eggs and early development with decreased expression in late development (Azumi et al., 2007), possibly suggesting different networks of transcriptional control for these genes during embryogenesis among these groups.

#### 4.1.2. Cell signaling, structural support, and stress response genes

Intracellular signaling is essential for controlling accurate communication of cellular processes during development. In developing *A. millepora* embryos, Grasso et al. (2008) noted a decrease in the percent of genes associated with intracellular signaling in developmental stages after prawn chip stage, suggesting a dependence on these genes during early development in this genus. Schwarz et al. (2008) similarly observed high EST representation of genes associated with signal transduction in *A. palmata* eggs compared to later planula and adult stages. We found that several genes from the Ras superfamily in *A. palmata* may play a particularly important role during pre-blastula prawn chip stages (at 12 hpf), inferred from down-regulation in multiple orthologs of this pathway at later developmental stages at 28 °C. Member genes within the Ras superfamily encode small GTPase enzymes that are involved in many important signal transduction pathways that control cell growth, differentiation, and survival (reviewed in Wennerberg et al., 2005; and Park et al., 2005). Technau et al. (2005) previously detected a high representation of ESTs associated with the Ras-MAPK pathway in both *A. millepora* and the sea anemone *N. vectensis*, and suggested that these homologs played major roles in developmental processes within cnidarians.

Rearrangements in the cytoskeleton, ECM, and cell adhesion proteins during embryonic development are required for cellular processes such as cytokinesis during mitosis, altering morphological structure, cell migration, and the formation of distinct cellular layers. Previous studies with developing *A. millepora* embryos revealed that planula larvae displayed a sharp increase in the percent of up-regulated genes encoding ECM and cell adhesion proteins compared to prawn chip stages, suggesting that higher expression of these genes and subsequent proteins may be required during more differentiated developmental stages (Grasso et al., 2008). Although we detected up-regulation of several genes associated with ECM/cell adhesion proteins in the post-prawn chip stages (from 24.5 to 131 hpf) of *A. palmata*, we also observed a number of genes that were down-regulated after prawn chip stage. These differences may be attributed to different developmental sampling times between the 2 studies, as our samples from 24.5 to 131 hpf consisted of embryos at several developmental stages (including late blastula, gastrula, and early planula larvae), while planula larval samples from Grasso et al. (2008) were taken only at a later time point at 83 hpf, when the majority of larvae had reached the planula stage. Further, since only a few of the genes associated with these cellular functions were represented in our array, this observation remains to be confirmed by future studies.

Heat shock proteins function as molecular chaperones that assist in protein folding, cell signaling, and tumor suppression (Lindquist, 1986). As cells increase their production of newly synthesized proteins, the demand for heat shock proteins as molecular chaperones rises. An increase in relative expression of heat shock genes in post-prawn chip developmental stages (24.5 to 131 hpf) observed in this study may reflect a greater demand for proteins involved in post-translational folding, as increased expression of genes involved in protein synthesis was also observed during this time. This complicates the interpretation of any increase in expression of heat shock genes that may be used as a biomarker for heat stress in other developmental studies, as an up-regulation of heat shock genes may reflect not only stress resulting from increased temperatures, but also an increasing demand for molecular chaperone proteins during times of increased protein production as well.

#### 4.1.3. Genes involved in regulation of gene expression

During development in *A. palmata* embryos at 28 °C, more transcription-related genes were up-regulated during pre-blastula stages compared to later developmental stages, although some important transcription factors and enhancer proteins still displayed higher expression in later stages (24.5 to 131 hpf). Homeobox genes encode DNA-binding homeodomain-containing transcription factors that regulate developmental processes such as pattern formation and cell fate specification in early embryogenesis. A variety of different homeobox genes involved in multiple developmental processes have been detected in many cnidarians, including *Acropora* spp. (Hayward et al., 2001; Finnerty and Martindale, 1997; Gauchat et al., 2000; Putnam et al., 2007, and references therein). The NK homeobox family is one of the major homeobox families that regulates critical steps involved in organogenesis in vertebrates (Kim and Nirenberg, 1989). The *NK6* subfamily controls differentiation in the developing central nervous system and includes the recently identified homeobox gene *Nkx6.3* (Choi et al., 2008). The *A. palmata* homolog of this gene, Homeobox protein Nkx-6.3, was down-regulated in later growth stages at 28 °C, suggesting that this gene plays a role early in development. However, since the formation of potential neural cells in *A. millepora* has been identified only in planula larvae, shortly after the oral/aboral axis was formed following gastrulation (Ball et al., 2002), it remains undetermined whether the *Nkx6.3* homolog detected in our study is involved in neuron formation or controls a different developmental process during early embryogenesis.

Histone proteins are the chief protein components of chromatin and play an important role as transcriptional and cell replication regulators by controlling chromatin condensation and accessibility through binding DNA. Abundant mRNA transcripts encoding histone proteins detected in early embryogenesis (12 hpf) in our study suggests a dependence on these important regulators during early development. Grasso et al. (2008) similarly observed a high representation of histone ESTs in *A. millepora* embryos during prawn chip stages, followed by strong down-regulation in later development. Interestingly, a homolog of a late histone gene (Late histone H2A.2.2) was detected in high expression at 12 hpf (pre-blastula stage) and then subsequently down-regulated in later development. Although abundant transcripts for late histone genes have been detected in unfertilized eggs in sea urchins (Knowles and Childs, 1984), maximal expression of late histone genes are typically found during late blastula/gastrula stages, while early histone genes are typically expressed maximally at early blastula stages (Colin et al., 1988 and references therein). In this study, since abundant mRNA transcripts for the Late histone H2A.2.2 gene were detected at pre-blastula stages, this homolog may be derived from maternal mRNA transcripts during early embryogenesis.

### 4.2. Gene expression patterns in embryos and larvae at elevated temperatures of 30 °C and 31.5 °C

Slightly faster development in 30 °C and 31.5 °C samples compared to 28 °C samples may have partially contributed to the observed DEGs in samples at different temperature treatments for each given sampling time point. Thus, we have 3 possible scenarios; 1) DEGs exclusively due to developmental differences, 2) DEGs exclusively due to temperature differences, and 3) DEGs that may be affected by both development and temperature. Scenarios 1 and 3 may apply when particular DEGs in a given sample time point at 30 °C or 31.5 °C (Table S3) have the same expression patterns (up- or down-regulated) as the same respective genes in 28 °C samples at the following time point (Table S2). Scenario 2 may apply when DEGs in a given time point have opposite expression patterns than the following time point, or if there is no differential expression between developmental stages for those genes in different sample times.

#### 4.2.1. Growth and developmental genes

Temperatures of 30 °C and 31.5 °C may have a pronounced effect on cell replication and cell division processes in developing embryos, as temperature stress caused a down-regulation of several important genes involved in the regulation of mitosis and cytokinesis, particularly during gastrulation (24.5 hpf). Some of these genes include cyclins as well as cell division and embryological patterning proteins such as Protein regulator of cytokinesis 1. Surprisingly, the mitosis-related gene Protein cut8, which has been reported to be up-regulated in response to temperature stress (Tatebe and Yanagida, 2000), was also down-regulated in embryos reared under elevated temperature at 24.5 and 42.5 hpf. Mutations in *cut8* can lead to mis-segregation of chromosomes and abnormal spindle formation which ultimately change the phenotype of mutants (Tatebe and Yanagida, 2000). However, slightly faster development in 30 °C and 31.5 °C samples compared to 28 °C samples at 24.5 hpf may also have contributed to the observed down-regulation of these genes, as Cyclin-A2, G2/mitotic-specific cyclin-B, Protein regulator of cytokinesis 1, and Protein cut 8 were all down-regulated in later developmental stages (Table S2). Down-regulation of these and other important modulators of cell replication and division may cause embryos to progress through cell cycles before all necessary molecular processes are completed, which could ultimately lead to the formation of abnormal embryos. In fact, higher embryo abnormality and increased mortality were observed at the elevated temperatures by Randall and Szmant (2009b) during this developmental period.

Different cellular processes involved in protein synthesis in *A. palmata* seem to be stimulated and repressed at the same time by temperatures of 30 °C and 31.5 °C during gastrulation (24.5 hpf), as several homologs for 40S and 60S ribosomal proteins, translation initiation factors, and elongation factors were up- and down-regulated compared to 28 °C treatments at this time. At least some of these up-regulated protein synthesis genes (Eukaryotic translation initiation factors 1b and factor 2 subunit 2, Elongation factor 1-gamma-A, and 60S ribosomal protein L18a) at elevated temperatures in our study may be attributed to faster developmental differences (Table S2). However, developmental differences do not explain the down-regulated protein synthesis-related genes, nor the numerous genes involved in metabolic processes that were down-regulated during gastrulation (24.5 hpf), which may have disrupted the efficiency of specific metabolic pathways during this critical developmental period. Voolstra et al. (2009) found similar responses in the down-regulation of genes involved in metabolism and also in protein synthesis in embryos of the coral *M. faveolata* in response to heat stress, suggesting that elevated temperatures can adversely affect these cellular processes in several different Anthozoans. Rodriguez-Lanetty et al. (2009) also observed a lack of simple down-regulation of overall protein synthesis genes in *A. millepora* larvae exposed to heat stress, and suggested that the response to thermal stress is a complex adjustment to the metabolic needs of the cell.

#### 4.2.2. Cell signaling, structural support, and stress response genes

Temperatures of 30 °C and 31.5 °C appear to cause a major impact on cell signaling by causing the down-regulation of multiple genes, such as those from the Ras superfamily, involved in essential intracellular signal transduction activities, particularly during 24.5 hpf. Although mild temperature stress has been shown to induce a complex cascade of signaling events associated with the Ras signaling pathway in mammals (reviewed in Park et al., 2005), it appears that elevated temperatures have a negative effect on Ras-related gene expression in *A. palmata* throughout development. Furthermore, developmental differences between temperature treatments do not appear to explain the majority of down- and up-regulated genes associated with intracellular signaling, as most of these genes did not display differential expression between sampling time periods when comparing all samples at 28 °C (Tables S2 and S3). Therefore, potential disruption in normal intracellular signaling

by heat stress in *A. palmata* may lead to improper cell cycle growth, abnormal morphological development, and decreased survivorship.

Heat stress has previously been found to disrupt cytoskeletal arrangements in numerous metazoan embryos with varying effects on different cytoskeletal components (i.e. actin microfilaments, intermediate filaments such as keratin, microtubules) in different organisms (Rivera et al., 2004; references therein). Exposure of adult *M. faveolata* corals to elevated temperatures caused the down-regulation of multiple genes associated with the actin cytoskeleton, suggesting a reorganization or disruption of the actin cytoskeleton due to thermal stress (Desalvo et al., 2008). In our study, *A. palmata* embryos also exhibited down-regulation of several important actin and microtubule associated genes at temperatures of 30 °C and 31.5 °C, particularly during the developmental time when the majority of embryos were undergoing gastrulation (24.5 hpf). Developmental differences do not appear to explain the majority of these down-regulated genes, as most of these genes either did not display differential expression between sampling time periods at 28 °C, or were up-regulated in later developmental time points after prawn chip stages (Tables S2 and S3). Differential expression of these cytoskeleton-related genes may contribute to cytoskeleton rearrangement during this crucial developmental transition which could affect morphological cell structure and proper development.

The classic response in metazoans to elevated temperature stress is an up-regulation of heat shock proteins that is well characterized in a multitude of organisms. Up-regulation of heat shock proteins in response to elevated temperature has been observed in heat stress studies by different corals (*M. faveolata*, *Acropora grandis*, and *Goniopora djiboutiensis*) and sea anemones (*Aiptasia pallida*) (Desalvo et al., 2008; Black et al., 1995; Fang et al., 1997; Sharp et al., 1997). Recently, Rodriguez-Lanetty et al. (2009) observed a rapid expression of heat shock protein genes within three hours of hyperthermal stress in 10-day-old *A. millepora* larvae, indicating that at least in larval stages, up-regulation of heat shock genes can occur quickly after heat stress exposure in this species. Although up-regulation of heat shock proteins was observed in our study at several developmental stages in *A. palmata* at 30 °C and 31.5 °C, a particularly high number of up-regulated genes for this study were detected during 24.5 hpf, suggesting that *A. palmata* may be particularly vulnerable to heat stress during the gastrulation process. However, developmental differences at 24.5 hpf may have contributed to the observed up-regulation of some of these genes (Table S2).

Oxidative stress has been identified as a well-known response in corals to elevated temperature (Hoegh-Guldberg, 1999; Lesser, 1997; Lesser, 2006). Previous transcriptomic studies with both embryonic and adult *M. faveolata* coral have revealed numerous up-regulation of genes associated with oxidative stress when exposed to elevated temperature (Desalvo et al., 2008; Voolstra et al., 2009). In this study, we identified only a few DEGs involved in oxidative stress throughout development, although the small representation of genes associated with oxidative stress on our arrays (0.4% of total genes = 9 genes) may explain this low number of observed DEGs. This discrepancy may also be due to the absence of symbiotic *Symbiodinium* in the developing embryos/larvae, given that the algal symbionts are a primary source of oxygen radicals in adult corals exposed to temperature stress (Lesser, 1996). Yakovleva et al. (2009) demonstrated that developing *Acropora intermedia* larvae experienced higher activity of antioxidant defenses, higher oxidative cellular damage, and higher mortality in developing larvae with *Symbiodinium* compared to those without. Recent cDNA microarray studies by Rodriguez-Lanetty et al. (2009) found little response in oxidative stress genes from 10-day-old aposymbiotic *A. millepora* larvae exposed to three hours of heat stress, although several oxidative stress genes were non-significantly up-regulated in 31 °C treatments compared to 24 °C control treatments after 10 h. Although our array contained homologous genes encoding the classic antioxidant enzymes Cu–Zn superoxide dismutase and catalase, neither of these genes were differentially expressed in response to heat stress, suggesting that developing *A. palmata* embryos in the absence of symbiotic *Symbiodinium* may not be subjected to the same levels of oxidative stress that are commonly observed in adult corals or larvae with *Symbiodinium* at similar elevated temperatures.

#### 4.2.3. Genes involved in regulation of gene expression

Temperature stresses of 30 °C and 31.5 °C may depress transcription-related processes throughout *A. palmata* development as multiple genes associated with transcription were down-regulated at several developmental time points, particularly during 24.5 hpf. Down-regulation of transcription elongation factor homologs, transcription regulators, and proteins associated with RNA polymerases may decrease the overall transcription of many downstream cellular products that are regulated by these proteins. For example, since CCAAT-enhancer-binding proteins (C/EBPs) are transcription factors that regulate multiple cellular processes such as cellular differentiation, inflammation, cellular proliferation, and metabolic control (reviewed in Ramji and Foka, 2002), the down-regulation of this gene observed during 24.5 hpf may disrupt numerous developmental processes under the control of this regulator. Mutations in subunit RPC2, a major subunit of RNA polymerase III, have been found to disrupt digestive organ development in zebrafish (Yee et al., 2007). Down-regulation of the *A. palmata* homolog to DNA-directed RNA polymerase III subunit RPC2, as well as other important transcription-regulating genes could have similar consequences in disrupting developmental processes in numerous systems in *A. palmata*. Although developmental differences in 31.5 °C may have contributed to the observed down-regulation of RNA polymerase III subunit RPC2 (Table S2), elevated temperatures alone appear to cause the down-regulation of the transcription regulator CCAAT/enhancer-binding protein epsilon, as this gene was heavily up-regulated in post-prawn chip stages compared to 12 hpf.

Temperature stress appears to have a negative effect on histone-related gene expression, as substantial down-regulation of multiple histone-related homologs were observed, although developmental differences may have also contributed to this observed down-regulation since the majority of these genes were down-regulated in post-prawn chip stages (Table S2). Down-regulation of histone variants in response to elevated temperature stress has been previously documented in *M. faveolata* corals in both adults and developing embryos (Desalvo et al., 2008; Voolstra et al., 2009). Depletions in the typical concentrations of these essential transcription regulators may cause a severe reduction in accurate transcription rates for multiple genes, with potentially severe downstream consequences for proper cell growth and development. However, since transcriptional regulation through histone proteins is directed by numerous post-translational modifications that enhances or inhibits transcription rates (reviewed in Fischle et al., 2003), the effect of the down-regulation of many of these histone-related genes on transcription rates is unknown.

#### 4.2.4. Unknown differentially expressed genes

Almost half (46%) of the genes represented on our microarrays could not be annotated by BLAST searches or assigned a known function, resulting in a large number of unclassifiable proteins. However, a large number of genes were up-regulated at12 hpf and subsequently down-regulated during 24.5 to 131 hpf in samples at 28 °C (Table 1, Clusters 5–7), indicating that many of these unknown genes may be necessary for early developmental processes. Those genes are of particular interest as they might be coral-specific genes that are important for proper development, and future studies will be directed to elucidate the nature and function of some of these transcripts. In temperatures of 30 °C and 31.5 °C, a large majority of the unclassified genes were also significantly differentially expressed during 24.5 hpf compared to 28 °C treatments, suggesting that many of these genes may also be particularly affected

during this developmental time similar to other genes with known functions.

### 4.3. Transcriptome patterns of total differentially expressed genes at 30 °C and 31.5 °C

Similar patterns in the numbers of up- and down-regulated genes in 30 °C and 31.5 °C treatments at each sampling time were observed for most functional categories, including metabolism, transport/binding proteins, ECM/cell adhesion, protease/apoptosis, heat shock/response to cell damage, transcription, RNA-binding, and RNA processing (Table 3). The number of DEGs in 30 °C treatments, however, was slightly higher for several functional categories at 24.5 hpf, while higher DEGs in 31.5 °C treatments were observed in later time points at 42.5 and 89 hpf, suggesting that moderate (30 °C) and more severe (31.5 °C) heat stresses may elicit different responses within the transcriptome at various stages of development.

The total numbers of DEGs in *A. palmata* embryos at 30 °C and 31.5 °C decreased during later development compared to earlier development (Table 2), suggesting that either the transcriptomes may become acclimated to higher temperatures over time, or that a subset of the embryos genetically more tolerant of elevated temperature gradually dominated the cultures. However, DEGs still remained in high abundances during 42.5 and 89 hpf at temperatures of 31.5 °C but not at 30 °C, suggesting that transcriptome acclimation may be delayed at slightly higher temperatures. Similar results were observed in developing embryos of the scleractinian coral, *M. faveolata*, as transcriptomes acclimatized between 12 and 48 hpf at a temperature stress of 29 °C (compared to the 27.5 °C control temperature treatments), but did not acclimate at 31.5 °C (Voolstra et al., 2009). Randall and Szmant (2009b) observed increased survival of *A. palmata* embryos after they passed gastrulation and became planulae in both 30 °C and 31.5 °C temperature treatments, further supporting the acclimation of this species to elevated temperatures. However, it is not known whether these patterns are due to genetically determined differences in temperature tolerance among embryos, or whether chance survival to a certain stage can induce or allow changes in transcription that convey temperature tolerance.

### 4.4. Conclusion

Elevated seawater temperatures pose a potentially serious threat to the recovery of *A. palmata* by disrupting embryogenesis and causing abnormal development and increased mortality, which can result in reductions in larval supply and thus reduce recruitment potential. Our detailed examination of the transcriptome in developing *A. palmata* embryos at temperatures of 30 °C and 31.5 °C has revealed differential expression patterns of numerous genes involved in cellular developmental process such as cell replication/division, metabolism, intracellular signal transduction, transport proteins, cytoskeleton proteins, heat shock proteins, transcription, and RNA processing. Some of these DEGs appear to be particularly affected during the critical developmental transition from blastula to gastrula stages, coinciding with the increased frequency of abnormal *A. palmata* embryos and increased mortality observed during gastrulation in a companion study (Randall and Szmant, 2009b). However, we also find evidence of transcriptome acclimation over time in *A. palmata* embryos in response to elevated temperatures that ultimately may allow this species to withstand adverse physiological impacts caused by projected elevations of sea surface temperatures. Although our microarray includes only a small subset of the total *A. palmata* genome, this study still identifies many individual genes and general pathways that may be critically affected by temperature stress in developing embryos, thus giving a starting point for future research efforts and functional studies to further examine the complex interactions of these gene networks as they are subjected to elevated temperatures.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.margen.2010.03.002.

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