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Red fluorescence in coral larvae is associated with a diapause-like state

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Abstract

Effective dispersal across environmental gradients is the key to species resilience to environmental perturbation, including climate change. Coral reefs are among the most sensitive ecosystems to global warming, but factors predicting coral dispersal potential remain unknown. In a reef-building coral *Acropora millepora*, larval fluorescence emerged as a possible indicator of dispersal potential since it correlates with responsiveness to a settlement cue. Here, we show that gene expression in red fluorescent larvae of *A. millepora* is correlated with diapause-like characteristics highly likely to be associated with extended dispersal. We compared gene expression among three larval fluorescent morphs under three coloured light treatments. While colour morphs did not differ in their gene expression responses to light colour, red larvae demonstrated gene expression signatures of cell cycle arrest and decreased transcription accompanied by elevated ribosome production and heightened defenses against oxidative stress. A meta-analysis revealed that this profile was highly similar to the signatures of elevated thermal tolerance in the same coral species, and moreover, functionally resembled diapause states in an insect and a nematode. Our results support a connection between red fluorescence and long-range dispersal, which offers a new perspective on the molecular underpinnings of coral larval dispersal and the biological function of GFP-like fluorescent proteins.

Keywords: coral, diapause, dispersal, fluorescence, gene expression

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Introduction

Corals harbour a stunning diversity of GFP-like fluorescent proteins (FPs) (Alieva *et al.* 2008), which contribute to the vibrant colours of coral reefs (Matz *et al.* 2006). Coral FPs are widely utilized in biomedical science as genetically encoded fluorescent markers (Chudakov *et al.* 2010), however, the biological functions of different spectral types of FPs remain unclear. Coral FPs can constitute up to 7% of total protein in a coral (Oswald *et al.* 2007) and show signatures of diversifying natural selection at residues affecting their spectral properties (Field *et al.* 2006), indicating that FP colour diversity likely plays an important role in coral fitness. Most coral FPs are strongly regulated at the gene expression level in response to stressors despite having a long half life (Leutenegger *et al.* 2007): their expression is generally down-regulated under thermal stress and darkness (Bay *et al.* 2009; DeSalvo *et al.* 2012; Roth & Deheyn 2013) and up-regulated under high light (D'Angelo *et al.* 2008) and UV stress (Aranda *et al.* 2011). There is notably high GFP expression around growing areas of a colony (Salih *et al.* 2000; D'Angelo *et al.* 2012) such as branch tips and after injury (Palmer *et al.* 2008, 2009) leading to the implication that the magnitude of green fluorescence might correlate with coral health (D'Angelo *et al.* 2008, 2012; Roth & Deheyn 2013). It has also been suggested that coral FPs are able to quench free radicals (Bou-Abdallah *et al.* 2006). However, non-fluorescent FP mutants quenched reactive oxygen species even better than fluorescent ones (Palmer *et al.* 2009), making it unlikely that FPs have evolved specifically for this function. There is also evidence that FPs might participate in light-activated electron transport (Bogdanov *et al.* 2009) or energy transfer cascades (Roth

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et al. 2013) as opposed to passive light absorption, and might provide photoprotection through reflectance and dissipation of high-energy solar radiation (Salih *et al.* 2000; Gittens *et al.* 2015). These examples highlight the ambiguity in assigning functions to coral FPs and suggest that FPs play important, and most likely diverse roles in mediating corals' interactions with the environment. To get around the lack of genetic manipulation tools, we take advantage of natural variation in FP expression within a coral species and investigate molecular traits that covary with it.

Acropora millepora, a widespread Indo-Pacific coral, exhibits prominent fluorescence polymorphism in both larval and adult stages (Beltran-Ramirez 2010; Kenkel *et al.* 2011). Larval settlement behaviour appears to be correlated with fluorescent phenotype: greener larvae are more likely to settle in response to natural cues (crustose coralline algae) than redder ones (Kenkel *et al.* 2011). This suggests that the relative proportions of different FPs might reflect complex physiological variation, modulating the ability of the larva to perceive settlement cues and/or appropriately respond to them. Red FP is a particularly promising candidate for a sensory molecule, since it accumulates in epidermal cells of the aboral pole of the larva that is forward-facing during searching behaviour and becomes the initial site of attachment to substratum (Beltran-Ramirez 2010; Kenkel *et al.* 2011).

Here, we evaluated two broadly defined hypothetical functions of coral FPs: (i) stress response and (ii) sensing external stimuli. We compared global gene expression profiles of three distinct larval fluorescent phenotypes (red, green and mega-green) (Fig. 1A) after exposure to different light conditions (darkness, green and red light). The stress response hypothesis predicts an enrichment of stress-related genes among those differentially expressed between colour phenotypes. Of all possible sensory functions, FPs are most likely to participate in the response to light since they are by far the most highly expressed light-absorbing molecules in coral larvae (Meyer *et al.* 2011). If this is the case, we expect to see differences between larval colour morphs in their molecular response to light. The stress and sensory hypotheses are not mutually exclusive, as FPs of different spectral types likely serve different functions (Field *et al.* 2006).

Methods

Experimental procedures

Colonies of *Acropora millepora* were collected from Ningaloo reef, Western Australia, and isolated in bins with filtered seawater (FSW) ~3 days prior to spawning. Colonies were allowed to spawn naturally on March 14, 2012 at approximately 9 PM. Gametes from four individuals were combined and allowed to fertilize for ~4 h. Larvae were reared at a density of 1 larva/mL with slow FSW flow-through and gentle aeration at ambient temperature (28–30 °C). Larval fluorescent phenotypes were visualized using a fluorescent stereomicroscope MZ FL-III (Leica, Bannockburn, IL, USA) as in (Kenkel *et al.* 2011). Four days post fertilization, a subsample of the bulk larval culture was separated by fluorescence phenotypes and exposed to three different LED-light treatments (red light, green light and darkness) for 4 h. The two light treatments were equalized for total photon flux using a USB2000 spectrometer (Ocean Optics) by adjusting the light intensity to equalize the areas under incident light curves. After light exposure, 20 larvae of each of the three phenotypes were preserved in RNAlater (Ambion, Austin, TX, USA) with two replicates larval pools per light treatment per phenotype, 18 samples total.

Parentage assignment

We evaluated relative proportions of larval morphs produced by each parent based on genetic parentage assignment using previously characterized microsatellites (Wang *et al.* 2009). Small branches from each parent and individual larvae sorted by fluorescent phenotype (red, green and mega-green) were preserved in ethanol. Adult and larval DNA was extracted using a phenol-chloroform method as in (Davies *et al.* 2013). Previously characterized microsatellites (Wang *et al.* 2009) were used to genotype the four parents. Two markers that exhibited unique homozygous (locus WGS092) or almost unique heterozygous alleles (only two alleles were shared between two of the parents) (locus EST032) for each of the four parents (Wang *et al.* 2009) were identified (Table S1, Supporting information). Genotypes were assigned to individual larvae using amplification and sequences methods described in (Wang *et al.* 2009). Since only two markers were sufficient to assign parentage, pedigrees of individual larvae were evaluated manually.

Tag-based RNA seq

RNA was extracted for preparation of cDNA libraries using the Ambion RNAqueous-micro kit (Ambion) according to the manufacturers instruction. Isolated RNA was quality checked by gel electrophoresis to confirm the presence of ribosomal RNA bands. 228–500 ng of RNA per sample was used to prepared tag-based RNA-seq libraries (Meyer *et al.* 2011) adapted for Illumina HiSeq. The detailed step-by-step protocol and

detailed walkthrough for the bioinformatics pipeline is available at https://github.com/z0on/tag-based_RNA-seq. Briefly, 50 bp single-end reads were deduplicated based on identity of the degenerate 5′-leading sequence introduced during cDNA synthesis (NNMW followed by 3–5 G bases) and the first 20 bases of the read, after which the 5′ leader and, where observed, the 3′-part containing poly-A stretch and 3′-adaptor were removed. These trimmed reads were quality-filtered using fastx toolkit (Pearson *et al.* 1997), requiring 90% of the bases to be above Phred quality 20 (i.e., <1% error probability). The cleaned reads were mapped to the *A. millepora* transcriptome (Moya *et al.* 2012) using BOWTIE2 (Langmead & Salzberg 2012). The custom perl script samcount.pl was used to count the number of reads mapping to each isogroup (a collection of contigs representing the same gene) while disregarding reads that mapped to more than one isogroup equally well. Lastly, the per-sample counts were tabulated for further analysis in R.

Statistical analysis

All analyses were performed in R3.1.2 (R Core Team 2015). Counts data were analysed using LRT statistics modelling the effect of fluorescent phenotype, light treatment and their interaction using DESEQ2 (Love *et al.* 2014). One sample outlier (green larvae, red light exposed 1), which substantially deviated from the rest in total counts, was identified using the package ARRAYQUALITYMETRICS (Kauffmann *et al.* 2009) and removed from subsequent analysis. For both LRT and Wald tests, the *P*-value cutoff corresponding to the false discovery rate of 10% was determined using simulation-based empirical FDR method (Dixon *et al.* 2015; Wright *et al.* 2015) which achieves power comparable to the independent filtering procedure embedded in the DESEQ2 but does not require discarding low-abundant genes to boost FDR-passing numbers. Principal coordinate analysis using the package ADEGENET (Jombart 2008) and variance stabilized counts data derived from DESEQ2 was performed based on Manhattan distances between samples, which reflects the sum of absolute log-fold-changes across all genes. The effect of larval phenotype was calculated using function ADONIS of the package VEGAN (Dixon 2003).

Pairwise contrasts were performed between the colour morphs using Wald tests (Love *et al.* 2014). For each comparison, samples from one colour morph were compared against the samples without that phenotype [i.e. red larvae vs. larvae that are not red (green and mega-green)], which allowed us to identify genes that were uniquely differentially expressed with respect to each colour phenotype. Two additional comparisons were performed; contrasts between green and mega-green morphs revealed differences in gene expression along the second principal coordinate (Fig. 1B), contrasts between red and mega-green larvae allowed us to eliminate possible parental effects, since both red and mega-green larvae have similar parentage (Fig. 1D).

To elucidate if functional groups of genes were enriched in each comparison between colour morphs, we performed rank-based Gene Ontology enrichment analysis using Mann–Whitney U tests (Kosiol *et al.* 2008; Voolstra *et al.* 2011; Dixon *et al.* 2015), based on uncorrected log-transformed *P*-values (negative if the gene was down-regulated, positive if up-regulated). The scripts and instructions to perform this analysis are available at https://github.com/z0on/GO_MWU. Hierarchically clustered heatmaps were used to visualize significant DEGs between colour morphs and genes associated with specific GO terms using the package PHEATMAP (Kolde 2013).

Correlations with external coral data sets

We compared the current data set to other published gene expression data sets on heat tolerance and response to thermal stress in *A. millepora* adults and larvae. Two of these data sets were from previous experiments on larval response to short-term (4 h) and long-term (5 days) heat stress at 31.5 °C (Meyer *et al.* 2011). The other two data sets include larval thermal tolerance (gene expression observed under benign conditions that predicted the survival rate of the larval cohort under stress), and adult response to heat stress (31.5 °C for 3 days) (Dixon *et al.* 2015).

We used KOG (euKaryotic Orthologous Groups) class assignments (Tatusov *et al.* 2003) to compare the enriched functional categories of genes between data sets (Dixon *et al.* 2015). This analysis was based on same 'signed log *P*-value' measures as GO analysis. A heatmap of KOG delta-ranks (a measure of each KOG class enrichment with up or down-regulated DEGs) in each data set was plotted using the package PHEATMAP (Kolde 2013). The KOG analysis was performed using KOGMWU package in R (Dixon *et al.* 2015). Correlations between KOG categories in each data set comparison were performed using a Pearson's Correlation.

Correlations with external diapause data sets

Two noncoral data sets were chosen for comparison: dauer vs. nondauer *Caenorhabditis elegans* (Sinha *et al.* 2012) and diapause vs. nondiapause midge *Sitodiplosis mosellana* (Diptera) (Gong *et al.* 2013). These data were extracted from the NCBI Gene Expression Omnibus (GEO) database (accession numbers GSE30977 and

GSE48156, respectively). The GEO2R function on the GEO website was run to extract differences between *C. elegans* Dauer vs. Mix-Stage. The *C. elegans* protein database was downloaded from NCBI and KOG annotations were generated using the webMGA server (http://weizhong-lab.ucsd.edu/metagenomic-analysis/server/kog/). Custom perl scripts were used to create a table that associated each gene identifier with a KOG class. The *S. mosellana* data set was re-analysed using DESEQ2 (Love *et al.* 2014) to calculate *P*-values and fold-changes for all genes in the data set using the number of unique reads for DOL (diapause) and NDOL (nondiapause) samples. The *S. mosellana* shotgun transcriptome assembly was downloaded from NCBI, translated based on blastx results against UNIPROT database using a custom perl script CDS_extractor_v2.pl and annotated with KOG classes as described above. For both data sets, 'signed log *P*-values' were calculated for all genes, after which they were subjected to KOG class enrichment analysis together with the coral data sets, as described above. All custom scripts and their documentation are available as part of the transcriptome annotation bundle at https://github.com/z0on/annotatingTranscriptomes.

Results

Fluorescent phenotypes in larvae

Acropora millepora larvae from Western Australia included green fluorescent phenotypes similar to those described in Great Barrier Reef (GBR) populations (Beltran-Ramirez 2010; Kenkel *et al.* 2011). While *A. millepora* larvae from the GBR usually exhibit a continuum of colour phenotypes ranging from purely green to purely red, larvae from Ningaloo segregate strongly between green and red phenotypes with no intermediates between them (Fig. 1A). In addition, *A. millepora* larvae from Ningaloo demonstrated a novel fluorescent phenotype that has never been described on the Great Barrier Reef that we called 'mega-green'. This novel colour morph expresses strong green fluorescence in the epidermis in the oral half of the larva forming a sharp boundary between oral and aboral halves (Fig. 1A). There is variation in expression of known *A. millepora* FPs between colour morphs, however only expression of cyan FP was significantly different among larval colour morphs (p_{LRT} = 0.004) (Fig. 1C) and was highest in green colour morphs.

Parental differences between colour morphs

If there was a strong bias in parentage among colour morphs, gene expression differences between them could be attributable to genetic effects rather than physiological states associated with specific fluorescent phenotypes. Parentage was assigned to a total of 62 individual larvae: 34 green, 18 mega-green and 10 red morphs. Four adults contributed to the larval culture; therefore each individual was assigned two of the four parents (designated A, B, 1 and 2). Colour morphs were not exclusively generated by any particular set of parents, as each parent contributed to each of the three colour morphs, with the exception of parent B, which predominantly begot green morphs and not a single red

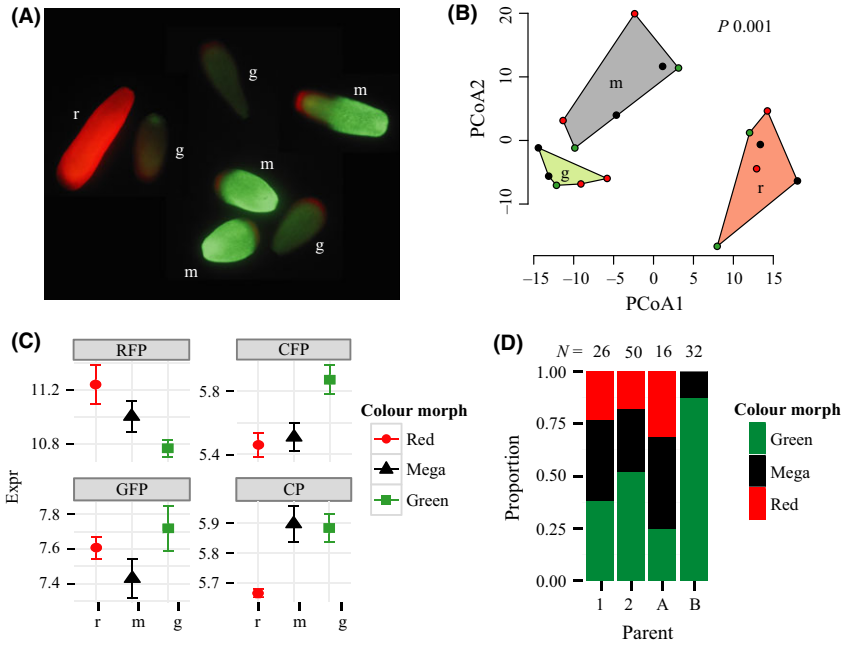

Fig. 1 Fluorescent polymorphism and gene expression in *Acropora millepora* larvae from Ningaloo Reef. (A) Larvae show three categorical phenotypes: red (r), green (g) and a novel colour morph 'mega-green' (m). (B) Principal coordinate analysis based on Manhattan distances. Clusters are grouped by fluorescent phenotype, red, mega-green and green (*p*PERMANOVA = 0.001). Green, black and red points represent light treatments, green light, darkness and red light, respectively. (C) Gene expression ± SE of known fluorescent proteins and nonfluorescent chromoprotein (CP) in colour morphs. (D) Proportions of colour morphs among offspring of specific parental colonies. The numbers above bars indicate sample sizes – the number of larvae attributed to the parent based on microsatellite analysis.

morph among those that were genotyped (Fig. 1D). Therefore, the comparison between green and nongreen morphs might be confounded with the genetic effect of parent B. However, contrasts between red and mega-green morphs should be unaffected by differences in larval parentage (Fig. 1D).

Differential gene expression profiles between colour morphs under light treatments

Tag-based RNA sequencing yielded 63 423 504 total reads, on average 3 523 528 per sample. After trimming, deduplicating and quality filtering, an average of 18.8% of reads were retained, of which approximately 74.83% mapped unambiguously to the *A. millepora* transcriptome (Moya *et al.* 2012), resulting in an average of 366 814 unique transcript counts per sample. Expression of 35 658 genes was analysed using DESEQ2 (Love *et al.* 2014) and a simulation-based empirical FDR method (Wright *et al.* 2015). The empirical FDR approach has comparable power to the independence filtering procedure implemented in DESEQ2 but does not require discarding data to boost statistical significance post-FDR correction. The analysis yielded 1258 differentially expressed genes (DEGs) for larval phenotype and 16 DEGs for the light treatment. No DEGs for phenotype-by-treatment interaction exceeded the simulated false discovery rate (Fig. S1, Supporting information), substantiating the lack of its detectable effect on gene expression. Principal coordinate analysis revealed that each colour morph has a distinctive gene expression profile ($p_{PERMANOVA}$ = 0.001, Fig. 1B).

Analysis of differential gene expression between colour morphs

To characterize gene expression patterns specific for each morph, pairwise contrasts were run using Wald statistics testing each morph against the other two. Simulating the data through *Empirical.FDR* testing (Wright *et al.* 2015) validated the results of the Wald tests and adjusted the 0.1 FDR cut-offs. Between red morphs and both other morphs, 1493 genes were differentially expressed, 816 down-regulated and 677 up-regulated. There were 609 DEGs specific to green morphs compared to red plus mega-green morphs, 356 genes down-regulated and 253 genes up-regulated, and 86 DEGs specific to mega-green morphs compared to red plus green morphs, 39 down-regulated and 47 up-regulated. When comparing mega-green to only green morphs there were 130 differentially expressed genes, 48 genes down-regulated and 82 genes up-regulated. The final comparison between red and mega-green morphs, to avoid potential confounding effect of uneven parentage in the compared larval pools, revealed 502 DEGs, 265 up-regulated and 237 down-regulated in red larvae.

For each of the three pairwise comparisons, a rank-based GO analysis was performed (Table 1, Figs S2 and S3, Supporting information) (Dixon *et al.* 2015). In red larvae, the GO term most significantly enriched in up-regulated genes is 'ribonucleoprotein complex', while 'chromatin organization' is most significantly enriched in down-regulated genes (Table 1, Figs S2 and S4, Supporting information). Among DEGs specific to green morphs, the most significant up-regulated categories are 'extracellular matrix structural constituent' and 'col-

Table 1 Top significant gene ontology (GO) terms resulting from functional enrichment analysis of pair-wise comparisons between colour morphs

Colour Morph	Direction	No. Sig genes†	Top GO enrichment (*P*-value*): No. genes with uncorrected *P* val <0.05/all genes within category		
			Biological process	Cellular component	Molecular function
Red	Up	677	Ribosome biogenesis (<0.001): 27/89	Ribonucleoprotein complex (<0.001): 43/209	Structural constituent of ribosome (<0.001): 32/153
Red	Down	816	Chromatin organization (<0.001): 22/151	Microtubule associated complex (<0.001): 12/74	Microtubule motor activity (<0.001): 12/72
Green	Up	353	NA	Collagentrimer (<0.001): 12/27	Extracellular matrix structural constituent (<0.001): 12/27
Green	Down	356	Ribonucleoprotein complex biogensis (<0.001)	Ribonucleoprotein complex (<0.001): 42/209	Oxidoreductase activity (<0.001): 88/872
Mega‡	Up	82	NA	NA	Oxidoreductase activity (0.08): 29/853
Mega‡	Down	48	NA	NA	Extracellular matrix structural constituent (<0.001): 9/27

*MWU test corrected *P*-value.
†nbinomTest, FDR determined through the EMPIRCALFDRpackage (includes unannotated genes).
‡Wald test contrast between green larvae only.

lagen trimer', while 'ribonucleoprotein complex biogenesis' and 'oxidoreductase activity' terms are down-regulated (Table 1, Fig. S3, Supporting information). Significant GO enrichments were found for mega-green larvae only when compared to green, and included up-regulation of 'oxidoreductase activity' and down-regulation of 'extracellular matrix structural constituent' (Table 1).

KOG class enrichment

DEG summary by KOG classes corresponded well with GO enrichment analysis. Red larvae show most pronounced down-regulation among classes 'Chromatin structure and dynamics' and 'Cell cycle control, cell division, chromosome partitioning' while up-regulating 'Energy production and conversion' (both in red-vs.-all

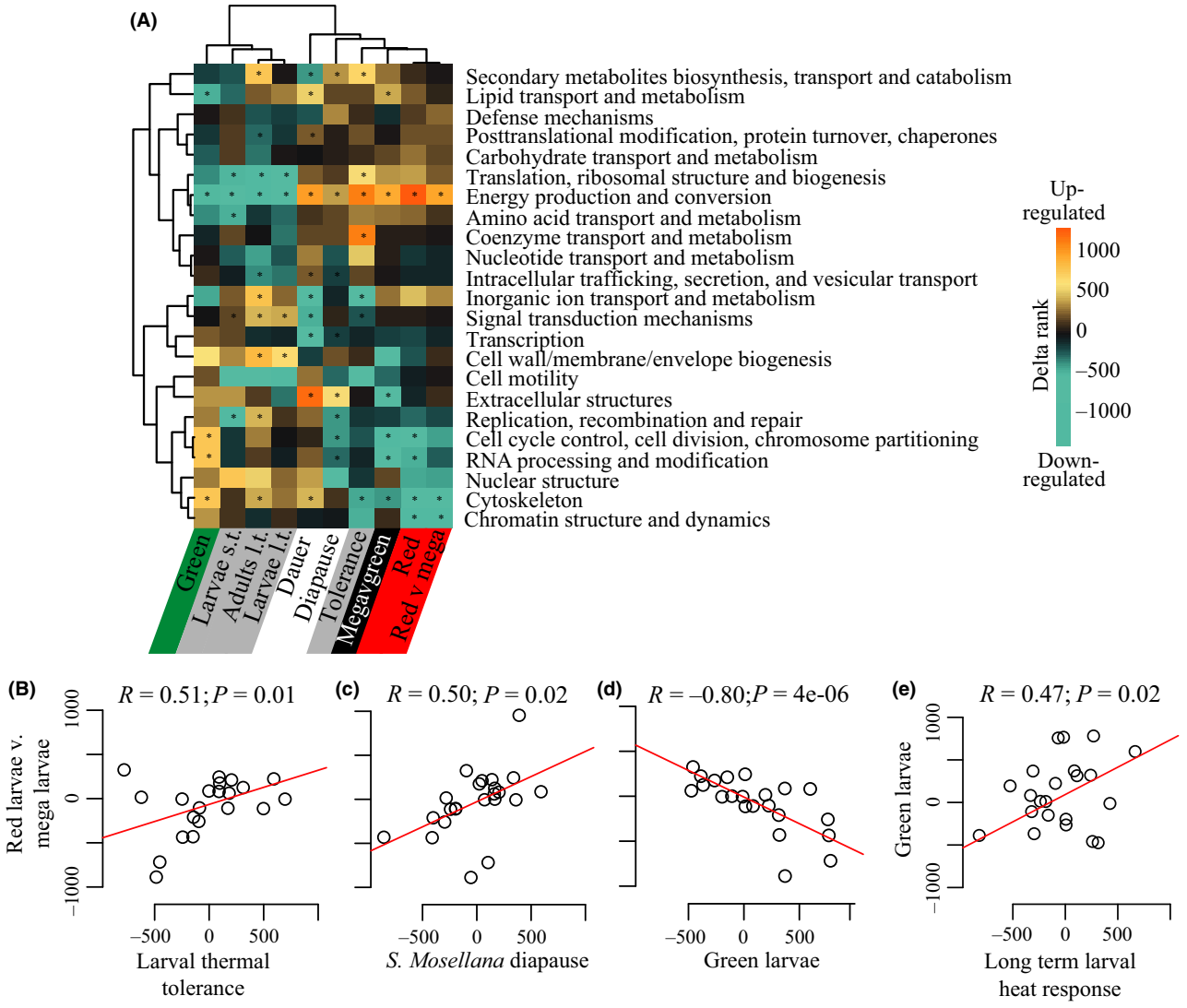

Fig. 2 Gene expression profiles of larval colour morphs correlate to external data sets. (A) Clustered heatmap of KOG class (rows) enriched by differentially regulated genes in colour morphs and in external data sets (columns). Columns labelled 'red', 'green' and 'mega-green' represent this data set. 'Larvae.st' and 'larvae.lt' correspond to larval responses to short-term (4 h) and long-term (5 days) heat stress from (Meyer *et al.* 2011), respectively. 'Tolerance' and 'Adult.lt' correspond to larval heat tolerance and adult 3-day heat stress from (Dixon *et al.* 2015), respectively. 'Dauer' corresponds to the *Caenorhabditis elegans* dauer data set (Sinha *et al.* 2012), and 'diapause' corresponds to diapause in the midge *Sitodiplosis mosellana* (Gong *et al.* 2013). The colour scale is delta-rank: the difference between mean rank of genes belonging to the euKaryotic Orthologous Groups (KOG) class and mean rank of all other genes (positive values indicate enrichment with up-regulated genes). Asterisks denote KOG classes significantly enriched with either up- or down-regulated genes (FDR = 0.1). (B–D) Correlation of KOG delta-ranks between (B) larval heat tolerance and red larvae, (C) between *S. mosellana* diapause and red larvae, (D) between red larvae and green larvae and (E) between green larvae and long-term larval heat response.

and red-vs.-mega-green comparisons). Green larvae have the opposite profile of up- and down-regulated KOG classes, which is expected since this comparison largely corresponds to the same principal coordinate as red vs. all comparison but with an opposite sign (Fig. 2D). In mega-green larvae when compared only to green, 'Energy production and conversion' was the most significantly up-regulated and 'RNA processing and modification' was the most significantly down-regulated KOG term (Fig. 2A).

Meta-analysis including prior data

In comparing the KOG delta ranks with the other coral stress data sets, there is a significant correlation between red larvae and the *A. millepora* larval thermal tolerance data set ($R = 0.51$; P-value = 0.01) (Fig. 2B). Green larvae demonstrated a significant positive correlation with long-term larval heat response ($R = 0.47$; P-value = 0.02) (Fig. 2E).

To assess the possibility that red larvae show gene expression characteristics of diapause quantitatively, we compared gene expression in red larvae to diapause-related gene expression in the dauer stage in the nematode *C. elegans* (Sinha *et al.* 2012) and an insect larvae (midge, order Diptera) *S. mosellana* (Gong *et al.* 2013). Both noncoral external diapause data sets clustered with the red phenotype (defined either as red-vs.-all or red-vs.-mega-green comparison) and *A. millepora* larval thermal tolerance data. In addition, the data set comparing *S. mosellana* diapause with red larval colour morphs shows a significant correlation of KOG class delta-ranks ($R = 0.50$, P-value = 0.02) (Fig. 2C).

Discussion

Phenotypic variation in fluorescence

Acropora millepora larvae from Ningaloo reef exhibit striking fluorescent polymorphism that has never before been observed in the same species on the other side of the continent (the Great Barrier Reef, GBR). The Ningaloo green colour morph resembles the phenotype typically found on the GBR, which happens to be the least fluorescent of the three Ningaloo morphs. Green larvae show expression of GFP around the larval mouth and a high concentration of RFP expressing cells in the epidermal tissue of the aboral pole that thin out in a gradient from aboral pole to about the midpoint of the larva ((Beltran-Ramirez 2010; Kenkel *et al.* 2011), Fig. 1A). The red and mega-green morphs described here are novel and qualitatively different phenotypes. Both of them are very bright, the red morph featuring abundant red fluorescence throughout the epidermis and the mega-green morph exhibiting abundant green fluorescence in a sharply delimited domain of the epidermis in the oral half of the larva (Fig. 1A). Interestingly, the expression of GFP in mega-green morphs was the lowest of the three morphs (Fig. 1C). This discrepancy between presumed GFP protein abundance from the photos and transcript levels suggests the mega-green phenotype may be a result of earlier transcriptional activity of the gene. This is plausible given the long half-life reported for some coral fluorescent proteins [20 days, (Leutenegger *et al.* 2007)]. Another possible explanation is that high expression in a subset of ectodermal cells might be masked by lower expression of the same gene in the rest of the larva.

Response to light does not differ among fluorescent morphs

The effect of light treatments on gene expression was subtle. Two annotated genes were significantly down-regulated under green light compared to red light and darkness; an ATP-dependent RNA helicase and a sodium/potassium/chloride transporter (SLC 12) (Fig. S5, Supporting information). SLC12 transporters function in maintaining epithelial ion homeostasis and secretory processes and are found in all cell types (Russell 2000). Reduction in expression of this homeostasis regulator suggests that exposure to green light may affect ion balances in coral larvae. Cryptochromes, which absorb blue light and are known to be regulated during diel cycles (Levy *et al.* 2011) and synchronous coral spawning events (Levy *et al.* 2007) are up-regulated under green light. Since we did not detect an effect of fluorescent phenotype on gene expression under different light treatments, it's likely that FPs do not modulate an early molecular response to light and are therefore unlikely to be directly involved in light perception.

Mega-green larvae show signatures of stress susceptibility

Although mega-green morphs are very phenotypically distinct, their gene expression profile is quite similar to green morphs (Fig. 1B). The trends in KOG enrichment in the comparison between green and mega green larvae (up-regulation of 'Energy production and conversion' and down-regulation of 'Cell cycle control, cell division, chromosome partitioning') made this comparison cluster with red-specific expression (Fig. 2A), ostensibly reflecting the alignment along the first principal coordinate (Fig. 1B). Mega-green larvae up regulate HSP90 and Caspase, genes that are known to be up regulated during acute thermal stress in corals (DeSalvo

et al. 2008; Tchernov *et al.* 2011), as well as Transketolase, which is up-regulated in adult corals of *Acropora hyacinthis* that exhibit symptoms of white syndrome-like disease (Wright *et al.* 2015) (Fig. S6, Supporting information). Therefore, it is possible that mega-green morphs are experiencing stress, however this would need to be experimentally tested in the future. Since high abundance of GFP in mega-greens is observed in the absence of the high expression of the GFP transcript, accumulation of GFP in oral ectoderm might be a factor making the larvae more susceptible to these threats rather than being a direct response to them. Larvae and adults express different versions of GFP (Beltran-Ramirez 2010) and observations in adult corals contradict this conjecture: adult green fluorescence has been associated with elevated stress tolerance (Salih *et al.* 2000) and better overall health as indicated by the correlation with coral growth (Roth *et al.* 2013). This suggests that GFP may play different functional roles when expressed in different tissues and life-stages.

Red larvae may be geared for long-range dispersal

A previous study of *A. millepora* in the GBR found that higher red fluorescence in the larvae is associated with lower responsiveness to a settlement cue and therefore might be a marker of long-range dispersal potential (Kenkel *et al.* 2011). Here, we evaluated whether the molecular differences associated with red fluorescence in larval colour morphs from Western Australia could be interpreted in favour or against this hypothesis. It should be noted that response to settlement cues was not tested in these larval colour morphs from Western Australia and in theory could be different from what was found before in *A. millepora* larvae from the GBR. We found that both comparisons (red compared to both green morphs and red compared to only mega-green morphs) yielded very similar results, aligning with the first principal coordinate of the gene expression diversity in our experiment (Fig. 1B).

The major difference between the gene expression signatures of green and red colour morphs is expression of genes associated with cell growth, cell division and stress tolerance. Red larvae down-regulate cell division related genes, including several histones types and subunits as well as histone-modifying enzymes, reflected in the enrichment of the GO category 'chromosome organization' (Table 1, Figs S4 and S5, Supporting information). Expression of histones is typically regulated by the cell-cycle, where histone proteins must be balanced with DNA-replication to ensure accurate chromosome replication (Rattray & Müller 2012). Transcription also appears to be down-regulated in red larvae, which is manifested by down-regulation of several subunits and regulators of RNA polymerase II along with components of the mediator complex, which is a necessary component of the pol II transcriptional machinery (Conaway *et al.* 2005). We also detected a significant down-regulation of protein kinase regulators (Fig. S4, Supporting information). Although serine/threonine protein kinases can potentially be involved in all signal transduction pathways, we see kinases specific to the regulation of the cell cycle including two cyclin-dependent kinases (Fig. S4, Supporting information).

Despite these indications of suppressed cell division in red larvae, they also show an up-regulation of ribosomal subunits (Table 1), which is a strong indication of cell growth (Rudra & Warner 2004). Therefore, cells in red larvae seem to arrest their division but maintain healthy physiological state. Notably, a similar pattern is also observed among gene expression signatures associated with elevated larval thermal tolerance (Dixon *et al.* 2015), which also include significant up-regulation of the KOG category 'Translation, ribosomal structure and biogenesis' and down-regulation of 'Chromatin structure and dynamics'. Enrichments of KOG classes with up- or down-regulated genes were significantly correlated among red larvae and heat tolerant larvae, indicating that red fluorescent larvae likely exhibit elevated stress tolerance.

Perhaps the most striking gene expression signature in the red larvae is up-regulation of the whole diversity of transcripts involved in deactivation of reactive oxygen species, including Cu/Zn superoxide dismutase, ferritin, catalase, glutathione peroxidase and thioredoxin (Fig. S4, Supporting information). In adult corals, these proteins provide protection from oxidative stress due to algal photosynthesis (Griffin *et al.* 2006; Lesser 2006; Portune *et al.* 2010; Seneca *et al.* 2010) and are up-regulated under heat stress and during bleaching (Császár *et al.* 2009). The *A. millepora* larvae in this experiment are aposymbiotic and were maintained in the same environmental conditions. Therefore, the up-regulation of antioxidant proteins specifically in red larvae is not likely a response to stress but could be a preemptive mechanism to increase baseline stress tolerance (Barshis *et al.* 2013). Elevated antioxidant protection could be beneficial for long-term survival in sunlit surface waters, where reactive oxygen species are very abundant (Blough & Zepp 1995), which aligns well with the hypothesis of red fluorescence being a marker for long-range dispersal.

Gene expression in red larvae is similar to diapausing organisms

Molecular characteristics of diapause, which have been intensively studied in organisms such as *Caenorhabditis elegans* that produce a long-lived dauer stage, include

the reduction of cell division and ion transport coupled with an increase of molecular chaperones and ROS scavenging enzymes, including superoxide dismutase (MacRae 2010). Gene expression observed in red larvae appears to mirror some of these characteristics, such as possible cell cycle arrest and elevated tolerance to thermal and oxidative stress. Indeed, at the level of KOG class enrichment, red larval gene expression clustered with diapause data sets and exhibited statistically significant similarity to a midge diapause state (Fig. 2A). In the midge and the worm, diapause is a discrete physiological state triggered by an environmental factor such as population density and low resources in *C. elegans* (Golden & Riddle 1984), and decreased temperature and soil moisture in *Sitodiplosis mosellana* (Gong *et al.* 2013). In coral larvae, a discreet diapause-like state has never been described, but the striking fluorescent phenotype of red larvae (Fig. 1A) as well their clear separation from the rest in terms of gene expression profile (Fig. 1B) suggest this may be a possibility. In this experiment, there was no obvious environmental trigger that could have induced this state in some, but not all of the larvae in the bulk culture. Although this study finds red fluorescent larvae were produced in similar proportions (20–30%) by three of the four parents (Fig. 1D), there remains a possibility of maternal effects on larval fluorescence and physiology. It's possible that larval phenotype, both fluorescence and potential diapause-like physiological characteristics, are determined by the health state or environmental history of the dam, especially since maternal effects on larval traits have been demonstrated previously (Dixon *et al.* 2015). In-depth quantitative genetic studies are required to disentangle the contribution of sire, dam and environment in determining larval fluorescent phenotype.

It would be beneficial if broadcast-spawning corals were able to modulate the dispersal potential of their offspring, promoting faster settlement when conditions are good and gearing the larvae up for long-range dispersal when conditions are stressful. Notably, the existence of such a mechanism would lead to intensified long-range genotype exchange under global warming, thus promoting rapid adaptation via 'genetic rescue' (Dixon *et al.* 2015). It's possible that environmental stress or history can trigger a diapause-like state that promotes long-distance dispersal and 'escape' from this regional stress/environment. Thus far, the possibility of such mechanism has not been investigated in any marine invertebrate, as larval dispersal potential continues to be regarded as a fixed species-specific property that cannot be switched or modulated (Cowen & Sponaugle 2009). This study raises multiple hypotheses about the significance of fluorescent variation in *A. millepora* larvae, which need to be directly tested. It is critical to test thermal tolerance between colour morphs in larvae from Western Australia as well as whether or not mega-green larvae are experiencing stress. Both of these questions could be addressed using assays as in (Dixon *et al.* 2015). One possible explanation of the observed larval fluorescent polymorphism could be difference in developmental timing between colour morphs; however, we believe that this explanation is unlikely. If the small differences in developmental timing among our larvae were responsible for the striking colour polymorphism, we would have seen rapid colour transitions in the whole culture through days 4–6, which was not the case. Kenkel *et al.* 2011 found differences in settlement between green and red larvae, however whether this results holds true for the novel colour morphs from Western Australia is yet to be seen. In the future, to fully substantiate our assertion that bright red *A. millepora* assume a diapause-like state it will be necessary to directly test their responsiveness to settlement cue and stress tolerance as well as characterize the environmental cues that trigger induction and exit out of diapause and the molecular changes necessary to do so.

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M.V.M. designed the study and performed experiments in the field. G.V.A. completed laboratory work. M.E.S. analysed the gene expression data and performed the meta-analysis. M.E.S. and M.V.M. both contributed to writing the manuscript.

Data accessibility

Raw data files and R scripts have been deposited into Dryad and are available at doi:10.5061/dryad.m3685. Raw sequence reads can be found on NCBI-SRA, Accession: SRP062326.

Supporting information

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

Fig. S1 Results of EMPERICALFDR.DESEQ2 testing for phenotype by treatment interaction.

Fig. S2 Gene ontology categories enriched by genes differentially expressed between red morphs and both green morphs.

Fig. S3 Genes and gene ontology (GO) analysis of genes differentially expressed between green morphs and all other morphs.

Fig. S4 Gene names corresponding to significant GO terms (FDR = 0.1) in pairwise comparisons between colour morphs with unadjusted *P*-values <0.1.

Fig. S5 Heatmaps of annotated genes differentially expressed after 4-h exposure to light treatments.

Fig. S6 Heatmaps of annotated genes differentially expressed between mega-green morphs and other morphs.

Table S1 Summary of two microsatellite *Acropora millepora* SSR markers used in parentage analysis (Modified from Wang *et al.* 2009) and corresponding parental genotypes.