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Real-time PCR-based gene expression analysis in the model reef-building coral *Pocillopora damicornis*: insight from a salinity stress study

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Abstract

*Pocillopora damicorni*s has emerged as the model coral for studying global climate change given that it is found in the waters of nearly all nations that invest significantly in coral reef research. To further the development of a molecular blueprint for understanding the sub-cellular response of this coral to changing environmental conditions, three approaches were taken herein. First, a salinity challenge study was conducted with corals from Kaneohe Bay, Hawaii (United States). Specimens were exposed to 24, 28, 32 (control), 36, or 40 partial salinity units (psu) for 1, 2, or 3 hrs, and the expression of three mitogen-activated protein kinase (*mapk*) genes; stress-activated protein kinase 2 (*p38*), extracellular signal-regulated kinase 2 (*erk2*), and big *mapk* 1 (*bmk1*), was measured. *p38* and *erk2* both exhibited large increases in expression in corals exposed for 1 hr to 28 psu, potentially suggesting that the associated proteins are involved in the initial stages of the hypo-osmotic stress response and may be important molecular targets for understanding this coral's response to environmental perturbations. As a second effort to better develop the molecular infrastructure of *P. damicornis* populations in Southern Taiwan

in particular, real-time PCR assays from previously published works were applied to cDNA generated from RNA derived from *P. damicornis* populations of Nanwan Bay in Southern Taiwan. In many cases, assays developed from *P. damicornis* cultures reared in Monaco were found to be unsuitable for use with Taiwanese specimens, and, overall, only ~55% of assays developed by other laboratories could be utilized with confidence with Taiwanese *P. damicornis* specimens. Finally, a next generation sequencing-based transcriptome sequencing study was used as a platform for the identification of genes involved in acclimation to high temperature, and 13 gene expression assays were developed for use with *P. damicornis*. In total, there are now 28 genes (14 in each of the host and *Symbiodinium* compartments) whose expression levels can be measured accurately with real-time PCR in this ecologically important reef builder that exists in many regions of the Indo-Pacific. It is hoped that these assays will not only be employed in Taiwan-based studies, but in molecular biomarker surveys conducted as part of the Living Ocean Foundation's Global Reef Expedition aboard the research vessel *Golden Shadow*.

Keywords: coral, dinoflagellate, endosymbiosis, gene expression, mitogen-activated protein kinase (MAPK), osmoregulation, *Pocillopora damicornis*, real-time PCR, salinity stress

Introduction

Given the threats of global climate change (GCC) towards reef-building corals (Hoegh-Guldberg et al., 2007; IPCC, 2007), there is an urgent need to understand how to identify corals that are approaching their stress tolerance limits (van Oppen & Gates, 2006). Unfortunately, only retroactive approaches are currently available for coral reef health diagnostics; the number of corals bleached, diseased, or overgrown on a reef is merely quantified and reported (e.g., Guest et al., 2012). Such dying corals would have already been stressed for many weeks before manifesting such late-stage signs of health decline. Yet, there are currently few means of detecting sub-lethal stress on a timescale that would allow for managers to mitigate the source of the environmental impact (e.g., water pollution or overfishing) prior to extensive loss of coral.

Molecular biomarkers (Downs et al., 2000) represent a promising means for

proactive health assessment of reef-building corals, as the expression of these molecules (e.g., genes and proteins) responds quickly (minutes to hours) to environmental changes (Downs et al., 2005). Furthermore, such expression differences may be maintained throughout the duration of the stress event (Downs et al., 2002). This suggests that expression levels of such biomarkers could allow for the rapid generation of an index for accurate gauging of coral health on a proactive timescale, provided that their expression is indeed associated with a certain phenotype (Mayfield et al., 2013b) and can, more specifically, predict future decreases in physiological performance.

Pocillopora damicornis (Fig. 1) is amongst the world's most widely dispersed corals (Veron, 2000) and is found off the coasts of nearly all nations that fund coral reef research to a substantial extent: the United States, Mexico, Monaco, Japan, Taiwan, Australia, and Israel. It is also the coral biology field's "lab rat," serving as the model organism in more studies than any other coral (e.g., Jokiel and Coles, 1990; Stimson & Kinzie, 1991; Mayfield et al., 2013a; Putnam et al., 2013). Furthermore, both partial (Traylor-Knowles et al., 2011) and complete (Mayfield et al., unpublished) transcriptomes (population of all expressed mRNAs) have been sequenced for this species, which will greatly facilitate future molecular-based studies. As such, *P. damicornis* has recently been nominated to serve as the model reef-building coral (Mayfield et al., 2013a) for both studies of basic biology (e.g., Cumbo et al., 2013), as well as those aimed at understanding the effects of GCC on coral performance (e.g., Putnam et al., 2013).

To better develop the molecular resources available for *P. damicornis*, and, more specifically, increase the number of prospective mRNA-level biomarkers that could be used for detecting sub-lethal stress in this species, both a traditional PCR/cloning/sequencing endeavor and a next generation sequencing (NGS)-based approach were used with cDNA derived from RNA from *P. damicornis* specimens from Hawaii and Taiwan, respectively. Then, real-time PCR assays were developed for the cloned and differentially expressed genes (DEGs), respectively. For the Hawaii-based assays, the cloned genes were considered to potentially serve as housekeeping genes (HKGs) for assessment of host (but not *Symbiodinium*) gene expression. To determine their efficacy as HKGs, a salinity stress study was conducted; briefly, as changes in osmotic pressure could be hypothesized to influence every cellular process in a reef coral (Mayfield and Gates, 2007), salinity challenge experiments represent an ideal means for determining which genes could serve as HKGs for normalization of host coral gene expression.

Expression of not only the three cloned

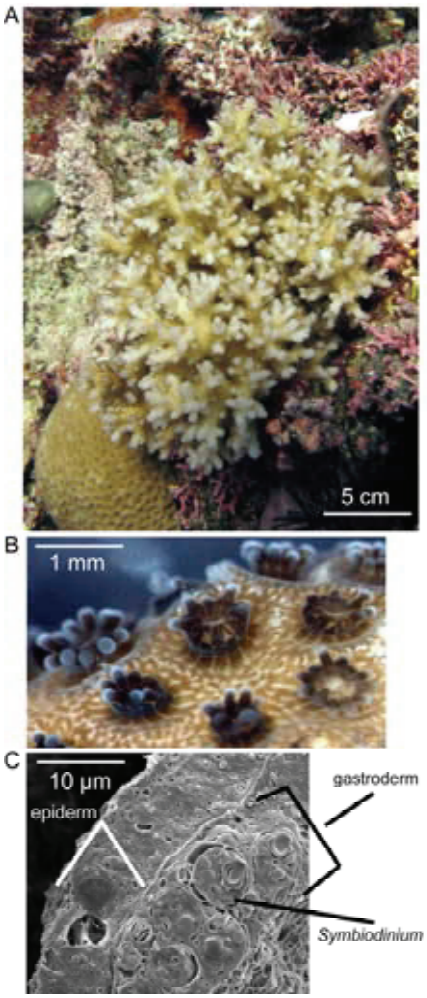

Fig. 1. A healthy *Pocillopora damicornis* colony on a reef in Fiji's Lau Archipelago (A). *P. damicornis* is amongst the world's most widely distributed corals and is found throughout nearly all of the tropical Indo-Pacific. Its biology is driven by an endosymbiotic relationship with dinoflagellates of the genus *Symbiodinium*, which contribute to the typically brown or cream coloration of the holobiont (B) and translocate photosynthetically fixed carbon to their hosts. When seen under a scanning electron microscope (C), the fact that these dinoflagellates contribute significantly to this coral holobiont's biomass, as well as that of all other reef-building scleractinians, is more greatly appreciated. One of the five visible *Symbiodinium* cells has been labeled.

HKGs- *sine oculis* (*so*), POU III transcription factor brain 1 (*brn1*), and β-actin (*act1*)- but also three mitogen-activated protein kinase (*mapk*) genes, was measured in coral samples exposed to 24, 28, 32 (control), 36, or 40 psu for 1, 2, or 3 hrs. In contrast to the former three genes, expression of the *mapks*- stress-activated protein kinase 2 (*p38*), extracellular signal-regulated kinase 2 (*erk2*), and big *mapk1* (*bmk1*)- was expected to be dramatically influenced by changes in salinity (Bohm et al., 2002). Although *p38* has been extensively studied and known to be important in regulation of the initial stages of the osmotic stress response (Kultz, 2001), the functions of the other two *mapks* are still unknown, though they are assumed to be involved in the signal transduction response to osmotic stress, as well (Abe et al., 1996). All *mapks* targeted herein were considered to potentially serve as useful biomarkers for early detection of osmotic stress in this coral species.

As an additional effort to better develop the molecular infrastructure for *P. damicornis*, real-time PCR assays developed by other researchers were used with cDNA derived from RNA of *P. damicornis* specimens from Southern Taiwan. It was hypothesized that those designed by scientists from a marine laboratory in Monaco may not function (Vidal et al., 2009; 2013), as the assays were developed by computer software and not actually applied to coral cDNA. However, those developed for both the host and its resident *Symbiodinium* populations in previous studies from locations in Australia and Japan were hypothesized to be functional with samples from Taiwan.

Materials and Methods

Cloning of putative HKGs for gene expression analysis of *P. damicornis*. A HKG-based normalization approach is unsuitable for assessment of *Symbiodinium* gene expression due to the fact that their contribution to the nucleic acid pool of the coral holobiont is temporally variable (Mayfield et al., 2010) and can even vary between samples (Mayfield et al., 2011) due to, for instance, bleaching (Mayfield et al., 2009). However, such a strategy *could* be more confidently applied to the coral host compartment, which, in theory, should contribute a greater fraction of the mRNAs extracted. Herein, three putative HKGs were targeted: *so*, *brn1*, and *act1*. As *so* and *brn1* encode transcription factors involved in light reception system development (Bebenek et al., 2004) and nervous system development (Lee et al., 2003), respectively, their expression was expected to be relatively low and stable across the experimental treatments given that salinity stress was not hypothesized to affect developmental processes. Additionally, only adult corals were used, and so genes encoding proteins involved in development

were hypothesized to be expressed at stable, baseline levels.

On the other hand, *act1* is a highly expressed gene (Mayfield et al., accepted) that has been used as a HKG in numerous other systems given that the components of the cytoskeleton are typically non-responsive to environmental changes (Kreuzer et al., 1999). However, Mayfield et al. (2010) found that another pocilloporid, *Seriatopora hystrix*, demonstrated temporally variable patterns of *act1* expression, possibly due to cytoskeletal re-arrangements that were taking place at night to accommodate *Symbiodinium* cell division. As such, it was hypothesized that only *so* and *brn1* would serve as suitable HKGs for host coral gene expression analysis in *P. damicornis*.

The *P. damicornis* DNAs extracted in Mayfield et al. (2010), which were from coral colonies inhabiting fringing reefs of Coconut Island (within Kaneohe Bay), Oahu, Hawaii, USA (see Mayfield et al., 2010 for coordinates.), were used as templates in PCRs with the degenerate primers described in the associated manuscripts for each gene (Table 1). For *so* and *brn1*, two rounds of PCR were conducted at the Hawaii Institute of Marine Biology (HIMB) with 1 U Immolase™ polymerase (Bioline, Taunton, MA, USA), 3 mM $MgCl_2$, 500 nM each primer, 2.5 mM each dNTP, and 1x ImmunoBuffer (TM) (Bioline) in the following manner: 95°C for 15 min x 1 cycle (necessary to activate the antibody-bound polymerase) followed by 30 cycles of 95°C for 30 s, 50 and 55°C for 45 s (first and second round of PCR, respectively), and 72°C for 2 min. One microliter of PCR product from the first reaction (50 µl) was used as the template in the second reaction, which, otherwise, contained the same components as the first.. For *act1*, only one round of PCR (35 cycles) with the mastermix described above and the degenerate *act1* primers (Table 1) was required to successfully produce the target gene.

For all genes, 5 µl of PCR product were electrophoresed on 2% Tris-acetate-EDTA (TAE) agarose gels, which were stained with ethidium bromide after running at 100 V for 30 min as described in Mayfield et al. (2010). Correctly-sized bands were excised from the gel with a razor blade, purified with a QIAEX® II gel extraction kit, (Qiagen, Dusseldorf, Germany), ligated into TOPO® TA plasmid vectors (Life Technologies, Grand Island, NY, USA), transfected into competent *Escherichia coli* cells, and sequenced in both the 5' and 3' directions as in Mayfield et al. (2010).

Approximately 20 bacterial plaques were screened for each of the three genes, and all containing the correctly sized insert were sequenced. Upon confirming the identity of the consensus sequence of the three genes with NCBI's translating BLAST (tBLASTx) tool, real-time PCR

primers (Table 3) were designed with MacVector (ver. 10, MacVector, Inc., Cary, NC, USA). While trouble-shooting the real-time PCR assays with cDNA, it was found that *so* was not expressed by adult corals. Therefore, it was instead employed as a spike-in control. Briefly, RNA was transcribed *in vitro* from partially digested, recombinant *so* plasmids as described in Mayfield et al. (2009). Then, a poly-A tail was added with poly-A polymerase (Ambion, Austin, TX, USA) as described by the manufacturer in order to generate the *so* RNA spike. Real-time PCR primers were designed (Table 3) and used to amplify a 93-bp amplicon (AGGCGATGGGTATGGGTTGTGCGA GTACCACTCTCGCAATATGTTCCTTG ATTTTTCCTTGAAACAGTAGCTCGT CTCCTCCCCATCCCAAA) as described below. As this sequence, which represents the entirety of the DNA clone, is less than 100 bp, it could not be deposited in the NCBI database.

Salinity stress study. Six *P. damicornis* colonies were collected from 1-2 m depth on snorkel from fringing reefs around HIMB on Coconut Island, Hawaii (see Mayfield et al., 2010 for coordinates and sampling details.) during the afternoon (~15:00) of a Fall day in 2006 and transported to nearby seawater tables, at which point each colony was fragmented into 7-8 nubbins of ~ 2 g each with sterile bone-cutting pliers. The 45 nubbins were randomly mixed in the seawater table, suspended on fishing line as described in Mayfield et al. (2012a), and allowed to acclimate in flow-through seawater aquaria exposed to natural light for three weeks prior to experimentation.

On the day of experimentation, fifteen tanks of approximately 50 l each were randomly assigned to serve as either the 24, 28, 32 (control), 36, or 40 psu treatments (n = 3 tanks per treatment). In the case of the hypo-osmotic treatments (i.e., 24 and 28 psu), filtered seawater (FSW) was diluted with tap water to achieve the desired salinities. For the hyper-osmotic treatments (i.e., 36 and 40 psu), NaCl (Sigma-Aldrich, St. Louis, MO, USA) was mixed with FSW to achieve the desired salinities. A portable refractometer (FG100sa, Sybon, Bethesda, MD, USA) was used to ensure that the salinities were within 5% of their target values. Once the salinities were stable, three nubbins were randomly assigned to each of the fifteen tanks, and one nubbin was sampled after 1, 2, and 3 hrs of treatment exposure. Upon sampling, nubbins were snap-frozen in liquid nitrogen and stored in a -80°C freezer until extraction of their RNA, as described below.

RNA extraction, reverse transcription, and real-time PCR. The 45 samples were removed from the -80°C freezer, and a ~50-mg fragment was removed with sterile bone-cutting pliers. Prior to complete thawing, the fragments were immersed in 500 µl TRIzol (Life Technologies) previously

Table 1. *Pocillopora damicornis* genes targeted in the salinity stress study, as well as those isolated and/or characterized in other studies whose real-time PCR assays were conducted with complementary DNA (cDNA) from Taiwanese *P. damicornis* specimens. The reference corresponds either to the manuscript in which the gene was cloned or sequenced (Vidal-Dupiol et al., 2009; Mayfield et al., 2010; Vidal-Dupiol et al., 2013) or to the manuscript in which degenerate primers for gene cloning were provided (Bebenek et al., 2004). As such, the references do not necessarily correspond to those in which the real-time PCR assay for the respective gene was first published (see Table 3 for primers, real-time PCR mastermix components, and thermocycling conditions.). "gDNA" = genomic DNA. "EST" = expressed sequence tag. "NGS" = next generation sequencing.

Full gene name	Abbreviation	Function	NCBI accession	Template/ sequencing	Primer sequence reference	Location	Compatibility w/ Taiwanese *P. damicornis*
Genes targeted in the Hawaii-based salinity stress study							
sine oculis	*so*	transcription factor	This manuscript [a]	gDNA/cloning	Bebenek et al., 2004, cloned herein	Hawaii	Not determined
POU III/brain	*brn1*	transcription factor	FJ858783	gDNA/cloning	Jacobs and Gates, 2003, cloned herein	Hawaii	Not determined
β-actin	*act1*	cytoskeleton	FJ858774	gDNA/cloning	Bebenek et al., 2004, cloned herein	Hawaii	Functional
stress-activated protein kinase 2	*p38*	MAP kinase	FJ858782	gDNA/cloning	Mayfield et al., 2010	Hawaii	Functional
extracellular signal-regulated kinase 2	*erk2*	MAP kinase	FJ858778	gDNA/cloning	Mayfield et al., 2010	Hawaii	Functional
big mitogen-activated protein kinase 1	*bmk1*	MAP kinase	FJ858779	gDNA/cloning	Mayfield et al., 2010	Hawaii	Functional
Real-time PCR assays from other studies applied to cDNA from Taiwanese *P. damicornis* specimens							
galaxin	*galaxin*	calcification	Unpublished	cDNA/NGS	Vidal-Dupiol et al., 2013	Monaco [b]	Non-functional
mannose-binding c-type lectin	*lectin*	cell adhesion	FJ628422	cDNA/EST	Vidal-Dupiol et al., 2009	Monaco [b]	Non-functional
"cyst-rich"	*PDcyst-rich*	cell adhesion	FJ628421	cDNA/EST	Vidal-Dupiol et al., 2009	Monaco [b]	Non-functional
carbonic anhydrase-II	*caII*	metabolism	Unpublished	cDNA/NGS	Vidal-Dupiol et al., 2013	Monaco [b]	Non-functional
UB52	*ub52*	ribosomal assembly	AMU44945	cDNA/ unknown	Takahashi et al., 2008	Australia [c]	Non-functional
heat shock protein 70	*hsp70*	molecular chaperone	AB201749	cDNA/EST	Hashimoto et al., 2004	Japan	Functional

[a] The sequence was too short (93 bp) for NCBI submission and is provided in this manuscript only. [b] Specimens were originally collected in the Red Sea, then cultured in Monaco. [c] The authors inadvertently designed primers from the host (*Acropora millepora*), and not the *Symbiodinium* sequence, as they had reported.

Table 2. Previously published *Symbiodinium* real-time PCR gene expression assays conducted with complementary DNA (cDNA) from *Pocillopora damicornis* populations from Southern Taiwan. For the eight functional assays, the primers, PCR mastermix components, and thermocycling conditions are found in Table 4. "gDNA" = genomic DNA. "EST" = expressed sequence tag. "NGS" = next generation sequencing.

Full gene name	abbreviation	Function	NCBI accession	Template/ sequencing	Reference	Source organism (taxon)	Location	Compatibility w/ Taiwanese *P. damicornis*
heat shock protein 70	*hsp70*	molecular chaperone	EU476880	gDNA cloning	Mayfield et al., 2009 [a]	*Pocillopora damicornis* (reef coral)	Hawaii	functional
heat shock protein 90	*hsp90*	molecular chaperone	GH706802	cDNA EST	Vidal-Dupiol et al., 2009 [a]	*Pocillopora damicornis* (reef coral)	Monaco [b]	non-functional
ascorbate peroxidase	*apx1*	free radical scavenger	HM156698	cDNA EST	Mayfield et al., 2012a [c]	*Seriatopora hystrix* (reef coral)	Taiwan	functional
photosystem I (subunit III)	*psI*	photosynthesis	HM156699	cDNA EST	Mayfield et al, 2012a [c]	*Seriatopora hystrix* (reef coral)	Taiwan	functional
ribulose,1-5,bisphosphate carboxylase/oxygenase	*rbcL*	photosynthesis	AF298221	cDNA unknown	Marendy et al., unpublished [d]	Unknown	Australia	functional
phosphoglycolate phosphatase	*pgpase*	photosynthesis	EU924267	cDNA EST	Crawley et al., 2010 [e]	*Acropora aspera* (reef coral)	Australia	functional
light-harvesting protein	*lhp*	photosynthesis	unpublished	cDNA NGS	Vidal-Dupiol et al., 2013 [a]	*Pocillopora damicornis* (reef coral)	Monaco [b]	non-functional
nitrate transporter 2	*nrt2*	metabolism	HM147134	cDNA EST	Mayfield et al., 2013 [c]	*Seriatopora hystrix* (reef coral)	Australia	functional
carbonic anhydrase	*ca*	metabolism	GH706797	cDNA EST	Vidal-Dupiol et al., 2009 [a]	*Pocillopora damicornis* (reef coral)	Monaco [b]	non-functional
hemoglobin I	*hbI*	unknown	EH035884	cDNA EST	Rosic et al., 2013 [b]	*Acropora aspera* (reef coral)	Australia	functional
hemoglobin II	*hbII*	unknown	EH038142	cDNA EST	Rosic et al., 2013 [b]	*Acropora aspera* (reef coral)	Australia	functional
28s ribosomal RNA	*28s rRNA*	ribosomal assembly	AJ830930	cDNA unknown	Vidal-Dupiol et al., 2009 [a]	*Amphisorus hemprichii* (foraminifer)	Australia	non-functional
18s ribosomal RNA	*18s rRNA*	ribosomal assembly	unpublished	cDNA unknown	Boldt et al., unpublished [e]	*Acropora aspera* (reef coral)	Australia	non-functional

[a] The gene cloning/isolation protocol and real-time PCR assay were described in the same manuscript. [b] Specimens were originally collected in the Red Sea, then cultured in Monaco. [c] The ESTs from which primers were designed were originally published by Mayfield et al. (2011). [d] The real-time PCR assay was developed by Mayfield et al. (2012a). [e] The ESTs from which primer were designed were originally published by Leggat et al. (2007).

inoculated with 10 µl *so* RNA spike. Briefly, the exogenous spike serves as the preferred means to control for reverse transcription (RT) efficiency differences between samples (Bower et al., 2007) and circumvents the need to validate HKGs. That being said, the expression of two putative HKGs was measured across all samples to determine whether they could be used in place of a spike for laboratories without the capacity for cloning and *in vitro* transcription.

Samples were homogenized in the 500 µl spike-inoculated TRIzol in a fume hood until the coral skeletons were pulverized into a fine powder. At that point, the coral tissue-TRIzol slurry was pulse-spun for 1 s to sediment the skeleton, and the supernatant was transferred to a new 1.5 ml microcentrifuge tube containing another 500 µl TRIzol. Then, RNA was isolated according to the manufacturer's recommendations, though as modified by Mayfield et al. (2009).

A DNase treatment was then conducted as described by Mayfield et al. (2011), and the RNA was quantified at 260 nm on a NanoDrop spectrophotometer (Infinigen, City of Industry, CA, USA) and qualified on native 1% TAE agarose gels run at 100 V for 30 min and stained with ethidium bromide as described in Mayfield et al. (2012b).

The 45 DNA-free RNAs were reverse transcribed to cDNA with the High Capacity® cDNA Synthesis Kit (Life Technologies) as recommended by the manufacturer, with 200 ng RNA used in each reaction (20 µl). This cDNA was used in real-time PCR assays (Table 3) designed for quantification of *so* recovery, as well as expression of *act1*, *brn1*, *p38*, *erk2*, and *bmk1*. While the latter three assays were conducted as in Mayfield et al. (2010) with the primers utilized therein, new real-time PCR primers were designed for use with *so*, *act1*, and *brn1* (Table 3).

Ten microliters (1x) of Power™ SYBR® Green mastermix (Life Technologies) were used with two microliters of undiluted cDNA and the primer concentrations found in Table 3 in a total volume twenty microliters for each reaction. An Applied Biosystems 7500 real-time PCR machine (Life Technologies) was used for all assays, and all reactions (conducted in triplicate for each sample) were initiated with an incubation at 95°C for 10 min followed by the number of cycles stated in Table 3, each of which being conducted at 95°C for 15 s followed by the respective annealing temperature for 60 s. A melt curve was conducted after all analyses to ensure that the primers were specific to the gene target of interest, and two serial dilutions of a randomly chosen cDNA sample were run simultaneously to ensure that the PCR efficiency of the assays was ~100% (*sensu* Bower et al., 2007). In certain cases (Table 3), bovine serum albumin

(BSA) was added to improve amplification efficiency.

After calculating the quantity of *so* reverse transcribed in each of the 45 RT reactions, as inferred from the threshold cycle (C_t) values, expression of the remaining five target genes was normalized to recovery of this exogenous *so* RNA spike as in Mayfield et al. (2009). It should be noted that no DNA-based biological composition normalization (*sensu* Mayfield et al., 2009; 2010; in press) was conducted, as it was hypothesized that the relative proportion of host RNA would be similar between samples. In other words, due to the short-term nature of the experiment, no bleaching (loss of endosymbiotic *Symbiodinium*) was anticipated, and so the host/*Symbiodinium* biological composition ratio was assumed to be similar across samples and not bias interpretation of the gene expression data. Had *Symbiodinium* genes been targeted, as well, such an endosymbiosis-tailored gene expression normalization strategy would need to be conducted to produce accurate data (Mayfield et al., 2011; 2012a; 2013a).

Statistical analyses. All data analysis was conducted with JMP® (ver. 5, SAS Institute Inc., Cary, NC, USA). Normality of datasets and homogeneity of variance were tested with Shapiro-Wilk W and Levene's tests, respectively. When datasets did not meet these two criteria, $\log_e$, square root, or rank transformations were conducted prior to performing the repeated measures ANOVAs. In short, as time and salinity were not independent, a standard 2-way ANOVA could not be employed. Instead, the repeated measures MANOVA model was utilized in JMP, though univariate tests were instead used when certain data points were missing; such was the case for the corals sampled after 3 hrs of exposure to 24 psu, whose cDNA did not readily amplify with the *bmk1* primer set. Tukey's honestly significant difference (HSD) tests were used to determine individual means differences when an overall effect was found in the model, and in all cases, a result was considered to be statistically significant when $p < 0.05$. All error terms displayed below and in the figures correspond to standard error of the mean.

Use of published real-time PCR assays with cDNA from Taiwanese *P. damicornis* specimens. Real-time PCR assays designed for *P. damicornis*-focused research in Hawaii, Australia, Japan, and elsewhere (Tables 1-2) were applied to cDNA generated from RNA isolated from *P. damicornis* specimens collected from upwelling reefs of Houbihu, Nanwan Bay, Southern Taiwan (see Mayfield et al., 2012a for coordinates.). Specifically, the same RNA/cDNA samples of Mayfield et al. (in press) were utilized with both published and unpublished real-time PCR assays developed either specifically for *P. damicornis* (Table 1), or, alternatively, for

Symbiodinium sp. found to associate with this widely distributed reef builder (Table 2).

In most cases, the PCR reaction mixtures (i.e., mastermixes) and thermocycling conditions were not published in the respective manuscripts in which the real-time PCR assays were first presented, and so a variety of primer concentrations (typically from 50-500 nM), annealing temperatures (typically from 58-62°C), and cycle numbers (typically from 30-40) were utilized with each set. All primers were purchased from EuroFins MWG Operon (Huntsville, AL, USA), and real-time PCRs were conducted on a StepOnePlus™ real-time PCR machine (Life Technologies) located at the University of Louisiana, Lafayette in the laboratory of Dr. Joseph Neigel. EZ-TIME™ SYBR® Green I mastermix with ROX® passive reference dye (Yeastern Biotech., Ltd., Taipei, Taiwan;1x [10 µl]) was used in triplicate 20 µl reactions with a gradient of primer concentrations and annealing temperatures, as these two PCR parameters appear to have the most significant impact on the efficacy of the assays in our experience.

Undiluted cDNA, as well as 10- and 100-fold diluted cDNA (2 µl for all reactions) was used with all primer sets, and an assay was considered to be functional when the PCR efficiency was between 90 and 110% and only a single peak was observed in the melting curve, which was conducted after all reactions. The latter indicates that the primers were specific to the amplicon of interest. It should be noted that the specific PCR mastermix and thermocycling information provided in Tables 3-4 is only present for assays that worked with cDNA derived from *P. damicornis* from Taiwan, with the exception of *so* and *brn1*, whose respective assays were not applied to cDNA from these samples but were instead used in the Hawaii-based salinity challenge study. In total, 10 and 13 previously published host (Table 1) and *Symbiodinium* (Table 2)-targeted real-time PCR assays, respectively, were applied to the Taiwanese samples.

Next generation sequencing. As a final step to increase the number of mRNA-level biomarkers for health assessment of *P. damicornis*, a NGS-based approach was taken whereby cDNAs from *P. damicornis* specimens that had been exposed to either a control temperature (26.5°C, n = 3 at 2 and 36 weeks) or an elevated one (29.7°C, n = 3 at 2 and 36 weeks)(Mayfield et al., in press) were sequenced. Each RNA (n = 12) was converted to a modified cDNA library with the Illumina Tru-Seq™ kit (ver. 2, San Diego, CA, USA), which was then sequenced on an Illumina Genome Analyzer IIxe at HIMB in Hawaii. Prior to library construction, the RNAs were re-purified with the GeneMark® Plant Total RNA Miniprep purification kit (Hopegen Biotechnology, Taipei, Taiwan)

Table 3. Real-time PCR assays for gene expression analysis of the host compartment of *Pocillopora damicornis*. "bp" = base pairs.

PROCESS Primer name	Full gene name Notes/results from NGS temperature study	amplicon size (bp)	Sequence ('5-3')	[primer] (nM)	Annealing temp. (°C)	Cycles	Reference
STRESS RESPONSE							
PD-*hsp70*-F1	heat shock protein 70	62 [a]	ATCCAGGCAGCGGTCTTGT	300	60	35	Mayfield e al., 2013a
PD-*hsp70*-R1			TCGAGCAGCAGGATATCACTGA				
METABOLISM/TRANSPORT							
PD-*ca*-F1	carbonic anhydrase	122	AGGATGATGAGGAGGATGAGG	250	60	30	This manuscript [b]
PD-*ca*-R1	up-regulated in high temperature samples		ATAGCAGGGAGGGGTGGTAA				
PD-*iontrans*-F1	ion transporter	81 [c]	CTGGCTTCTGGTGGTCTTTT	500	59.5	40	This manuscript [b]
PD-*iontrans*-R1	up-regulated in high temperature samples		ATAACTTGGCTGGGACGGA				
PD-*sftase*-F2	sulfotransferase	149	CCAGTCACACCAACTTTACTTG	500	60	35	This manuscript [b]
PD-*sftase*-R2	down-regulated in high temperature samples		CTCACCATCTCCAACTCTTTC				
TRANSCRIPTION FACTOR							
PD-*so*-F1	*sine oculis*	93	AGGCGATGGGTATGGGTT	500	59	30	This manuscript
PD-*so*-R1	used as an exogenous spike herein		TTTGGGATGGGGAGGAGA				
PD-*brn1*-F1	POU III transcription factor brain 1	109	AACCACCATTTGCCGTTTCGA	500	59	35	This manuscript
PD-*brn1*-R1	targeted in the salinity stress study as a HKG		AGCCCCGTTGTTGTTATCCG				
CYTOSKELETON							
PD-*act1*-F1	β-actin	101	TTGGTTACGATGCCGTGTTC	500	59	35	This manuscript
PD-*act1*-R1	targeted in the salinity stress study as a HKG		GTTGGTATGGGTCAGAAAGATT				
SIGNAL TRANSDUCTION							
PD-*p38*-F1	stress-activated protein kinase 2	100	CCCACAGAGAATGAGATGACT	500	59	40	Mayfield et al., 2010
PD-*p38*-R1	targeted in the salinity stress study		ATTTCAGGAGCCCTGTACCAT				
PD-*bmk1*-F1	big MAPK 1	101	CCGTGGTCTATCTTCCTCT	500	59	40	Mayfield et al., 2010
PD-*bmk1*-R1	targeted in the salinity stress study		CACTTAGCGACAGCATCAGT				
PD-*erk2*-F6	extracellular signal-regulated kinase 2	96 [c]	GGAACCCAGTGTGGTCGT	500	59	60	Mayfield et al., 2010
PD-*erk2*-R6	targeted in the salinity stress study		CCTCAAATTTGCGATTTCGGT				
CELL ADHESION							
PD-*selectin*-F1	selectin	180	TTCCTGTTCTGGTCTCGCTTT	500	60	35	This manuscript [b]
PD-*selectin*-R1	up-regulated in high temperature samples		CTTGGAGGTTGCGTTCTTTC				
PD-*lectin*-F4	lectin	147	AGCCTGGACCCTTGTCAT	300	61	40	This manuscript [b]
PD-*lectin*-R4	up-regulated in high temperature samples		CTTGTGGTTAATTGGTGCGTC				

Table 3. (cont.) Real-time PCR assays for gene expression analysis of the host compartment of *Pocillopora damicornis*. "bp" = base pairs.

COLOR DETERMINATION/LIGHT ABSORPTION							
PD-*gfp-cp*-F1	green fluorescent protein-like chromoprotein up-regulated in high temperature samples	199	AGGCAAACAAACGGGGACATT	500	59	35	This manuscript [b]
PD-*gfp-cp*-R1			GGCACTCCCTCCATCTTCA				
DNA REPLICATION							
PD-*smc-csp*-F2	structural maintenance of chromosome-chromosome segregation protein down-regulated in high temperature samples	174	AGGGAGGAGAGGGATACTTTT	400	60	35	This manuscript [b]
PD-*smc-csp*-R2			ACGCGGTAGTGCAAGAATGAA				

[a] This assay is also routinely used with *P. damicornis* DNA in order to calculated the host coral genome copy proportion (*sensu* Mayfield et al., 2011). [b] The NCBI accession number will be published at a later date. [c] The expression of this gene is typically very low ($C_t > 30$).

by first diluting to 100 µl with DEPC-treated water and mixing with 350 µl of Lysis Buffer-A. Then, 350 µl ethanol were added, and the entire 700 µl volume was vortexed and added to silica-based spin columns. The manufacturer's recommendations were then followed, including an on-column DNase treatment. DNA, salt, and ethanol-free RNAs were eluted into 30 µl of DEPC-treated water after incubating the columns in a 60°C oven for 5 min to evaporate all residual ethanol.

Quality control for these samples was conducted on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) with the RNA 6000 Nano chip (Agilent Technologies), and only samples with RNA integrity numbers (RINs) greater than 8 were used in cDNA library preparations. Although the RIN is likely not of great utility to coral biologists, as high amounts of *Symbiodinium* rRNAs might be interpreted by the instrument to represent degraded host coral rRNAs, it nevertheless provides a high resolution image of RNAs that is important for quality control.

The 12 RNA samples were diluted to 40 ng μl^{-1} in 50 µl of DEPC-treated water, and the entire quantity (2 µg) was used in library preparations as instructed by the manufacturer. Each double-stranded cDNA (dscDNA) sample was ligated to a unique barcode such that all 12 samples could be sequenced on the same flow cell lane. Barcodes 2, 4-7, 12-16, and 18-19 were utilized. The final, indexed dscDNA products were analyzed on an Agilent 2100 Bioanalyzer with the 7500 DNA chip according to the manufacturer's recommendations. Then, real-time PCR was conducted as suggested by Illumina to estimate the number of sequenceable clusters in each sample by comparison to a standard curve of previously sequenced,

serially diluted dscDNA standards (i.e., of known cluster number) from *P. damicornis* larvae (Hollie Putnam, unpublished data). Clusters were generated as recommended by Illumina, and 100 sequencing cycles were conducted on each end to generate paired end reads.

A detailed description of the extensive bioinformatics required to analyze the approximately 2.5 x 10^6 DNA sequences that were generated can be found at http://ips.iis.sinica.edu.tw/coral/. In short, approximately 330,000 contiguous sequences were assembled, and ~175,000 of these passed the rigorous quality control pipeline and were thus analyzed. A one-way, repeated measures ANOVA was used to determine the effects of sampling time (2 vs. 36 weeks), treatment (26.5 vs. 29.7°C), and their interaction on gene expression (n = 3 at each combination of time and treatment). Eight genes that demonstrated statistically higher expression at the high temperature (either at only one sampling time or both) and five that were expressed at higher levels in the controls (either at only one sampling time or both) were targeted for real-time PCR primer design, and the resulting primers can be found in Tables 3 (host genes, n = 7) and 4 (*Symbiodinium* genes, n = 6). Assays were considered to be functional when they passed the same quality control criteria described above.

Results and Discussion

Salinity study

so *recovery and HKG expression*. There was a significant effect of salinity, as well as an interaction effect of salinity and time (Table 5), on recovery of the *so* RNA spike (Fig. 2A). Specifically, within the 15 samples collected after one hour of exposure to the five different salinities, there was approximately 10-fold less *so* reverse transcribed in corals of the 28 psu treatment versus the experimental controls incubated at 32 psu (Fig. 2A). This suggests that the efficiency of the RT reaction differed dramatically between these two treatments. There were no other significant differences within either of the other two sampling times. These data highlight the importance of using an exogenous RNA spike; had such an approach not been used, low expression of the target genes may have been misreported at this treatment and time, not due to physiological differences, but simply due to the fact that less of the target gene mRNA was reverse transcribed to a cDNA that could be amplified in the real-time PCR.

There were significant effects of salinity, time, and the salinity x time interaction on *act1* expression (Table 5 and Fig. 2B), suggesting that it would not be a suitable HKG for gene expression analysis in *P. damicornis*, at least not for salinity challenge studies. After one hour of treatment exposure, there were 4- and 5-fold decreases in expression of this gene

in samples of the 24 and 36 psu treatments, respectively, relative to the experimental controls. Decreases in *act1* expression relative to control values were also evident in the 28 and 40 psu treatments (Fig. 2B), though these differences were not statistically significant due to high degrees of variation. In order to achieve a statistical power of 0.8 ($p < 0.05$), a sample size of six, rather than three, would be required.

After 2 hrs of treatment exposure, there was a significant (~50%) increase in *act1* expression in samples of the 28 psu treatment relative to controls. On the other hand, there were ~3- and 4-fold reductions in expression of this gene in samples of the 26 and 40 psu treatments, respectively, relative to controls at this sampling time. After 3 hrs of treatment exposure, there were 4-fold increases in *act1* expression in samples of the 28 and 36 psu treatments relative to the controls, which showed reduced expression levels at this sampling time. Finally, despite a 48-fold increase in *act1* expression in corals exposed to 24 psu relative to controls at the final sampling time, this difference was not statistically significant due to a high degree of variation. As with the 1-hr samples, a doubling of sample size from three to six would be required to achieve the statistical power necessary to determine a significant difference between these treatments at this sampling time. Therefore, it may be fruitful in future studies of this gene in reef corals to utilize a larger sample size in order to determine whether such differences in expression of this gene across salinities are statistically significant.

This variability in *act1* expression in response to different salinities may speak to the dual role of the cytoskeleton under periods of osmotic stress; on the one hand, swelling due to hypo-osmotic stress might cause a temporary up-regulation of cytoskeleton components as the cells would be expanding at such times (Lang et al., 1998). On the other hand, hyper-osmotic stress would cause cell shrinking, which would then necessitate an increase in expression of cytoskeletal elements to restore cell volume (i.e., regulatory volume increase; Mayfield et al., 2010). In other words, *act1* expression could be hypothesized to either increase or decrease in response to either type of osmotic stress, though the exact molecular mechanisms underlying such expression changes in this coral are currently unclear. Regardless of the explanation, the dramatic differences in *act1* expression between control corals and those exposed to hypo- or hyper-osmotic conditions demonstrate that this gene would not be an ideal HKG for salinity stress studies of *P. damicornis*. Its high degree of temporal variation in another pocilloporid, *S. hystrix*, which may have been due to light-induced changes in *Symbiodinium* cell division

Table 4. Real-time PCR assays for gene expression analysis of the *Symbiodinium* compartment of *Pocillopora damicornis*. "bp" = base pairs.

PROCESS Primer name	Full gene name Notes/results of NGS temp. study	amplicon size (bp)	Sequence ('5-3')	[primer] (nM)	Annealing temp. (°C)	Cycles	Reference
STRESS RESPONSE							
Sym-*hsp70*-F1	heat shock protein 70	86 [a,b]	CTGTCCATGGGCCTGGAGACT	500	62.5	35	Mayfield et al., 2009
Sym-*hsp70*-R1			GTGAACGTCTGTGCCTTCTTGGTT				
Sym-*apx1*-F1	ascorbate peroxidase	107	GCCAAGTTCAAGGAGCATGTA	500	61	40	Mayfield et al., 2012a
Sym-*apx1*-R1			AGCTGACCACATCCCAACT				
PHOTOSYNTHESIS							
Sym-*psI*-F1	photosystem I (subunit III)	136	GTGGAGTTGACATTGACTTGGA	500	59	35	Mayfield et al., 2012a
Sym-*psI*-R1			TGCTGCTTGGTGGTCTTGTA				
Sym-*rbcL*-F3	ribulose,1-5,bisphosphate	126	CAGTGAACGTGGAGGACATGT	200	60	35	Mayfield et al., 2012a
Sym-*rbcL*-R4	carboxylase/oxygenase		AGTAGCACGCCTCACCGAAA				
Sym-*pgpase*-F1	phosphoglycolate phosphatase	100 [a]	TGACAAA CAATTCCACCAAGAG	250	60	35	Crawley et al., 2010
Sym-*pgpase*-R1			GCTGCAAAGGATGATGAGAAG				
METABOLISM/TRANSPORT							
Sym-*nrt2*-F1	nitrate transporter 2	97	CCACCCATTTCAGGACCTAT	200	62	40	Mayfield et al., 2013a
Sym-*nrt2*-R1			CCAGGGACCTAGCAAACAA				
Sym-*vdic*-F1	voltage-dependent ion channel	108	CTTTCCGCGAGCTTCACA	500	60	40	This manuscript [c]
Sym-*vdic*-R1	up-regulated at high temperature		TCAACATAGCCCAGACGGT				
Sym-*kchannel*-F1	potassium channel	78	GTTTCCAATTCTGCGCTCCTTT	400	59.5	35	This manuscript [c]
Sym-*kchannel*-R1	up-regulated at high temperature		ACACATCCAACTATCCACCAC				
Sym-*zifl1l*-F1	zinc-induced facilitator-like 1-like	69	GGAAGAAGGCCTGTGATGATAA	500	60	35	This manuscript [c]
Sym-*zifl1l*-R1	down-regulated at high temperature		AGACAGACCAAAGCAAACCATAC				
Sym-*tktlase*-F1	transketolase	152	GCCCAGCATTTTCATCTTCAC	500	60	33	This manuscript [c]
Sym-*tktlase*-R1	down-regulated at high temperature		TCCACATCTCCACACATTCG				
Sym-*Cachannel*-F1	calcium channel	148	GCCTTCTTCCGTCATACACAT	500	60	35	This manuscript [c]
Sym-*Cachannel*-R1	down-regulated at high temperature		ACTTCCTCCTCCTGTTCCT				
TRANSCRIPTION							
Sym-*helicase*-F1	RNA helicase	190	AGGAGGCGCAGAAGATGAA	500	60	35	This manuscript [c]
Sym-*helicase*-R1	up-regulated at high temperature		GCAGAGTGGAAGGTGAACT				
UNKNOWN FUNCTION							
Sym-*hb1*-F1	hemoglobin-like 1	63	CCGACGAGCCKTTGGAT	500	60	35	Rosic et al., 2013
Sym-*hb1*-R1			CCGCCACCTTCTTGAAAGTG				
Sym-*hb2*-F1	hemoglobin-like 2	64	TTGGTGCCCATGTTGCAA	450	60	35	Rosic et al., 2013
Sym-*hb2*-R1			AGTATTCTGGCTTCAGGCCATATC				

[a] 1/2x bovine serum albumin (BSA) is required to achieve 100% PCR efficiency. [b] This assay is also routinely used with *P. damicornis* DNA in order to calculated the *Symbiodinium* genome copy proportion (*sensu* Mayfield et al., 2011). [c] The NCBI accession number will be published at a later date.

Table 5. One-way, repeated measures ANOVA of gene expression data from the Hawaii-based salinity study. Statistically significant differences are highlighted in bold font.

Parameter	**Exact F**	**p**	**Fig.**
Source of variation			
***so* recovery** [a]			Fig. 2A
Treatment	24.2	**< 0.0001**	
Time	1.55	0.264	
Treatment x Time	7.88	**< 0.0001**	
***act1* expression** [b]			Fig. 2B
Treatment	13.1	**< 0.001**	
Time	19.6	**< 0.001**	
Treatment x Time	18.2	**< 0.0001**	
***brn1* expression** [c]			Fig. 2C
Treatment	4.10	0.353	
Time [d]	7.011	0.125	
Treatment x Time [d]	1.39	0.483	
***p38* expression** [b]			Fig. 3A
Treatment	1.63	0.241	
Time	2.86	0.1090	
Treatment x Time	3.80	**< 0.01**	
***erk2* expression** [b]			Fig. 3B
Treatment	10.71	**< 0.01**	
Time	6.30	**0.0200**	
Treatment x Time	7.37	**< 0.001**	
***bmk1* expression** [b]			Fig. 3C
Treatment	0.763	0.567	
Time	19.5	0.158	
Treatment x Time	14.0	0.0678	

[a] root-transformed data. [b] rank-transformed data.
[c] $\log_e$-transformed data. [d] univariate test was used due to lack of data sphericity (Mauchley's test, $p < 0.05$).

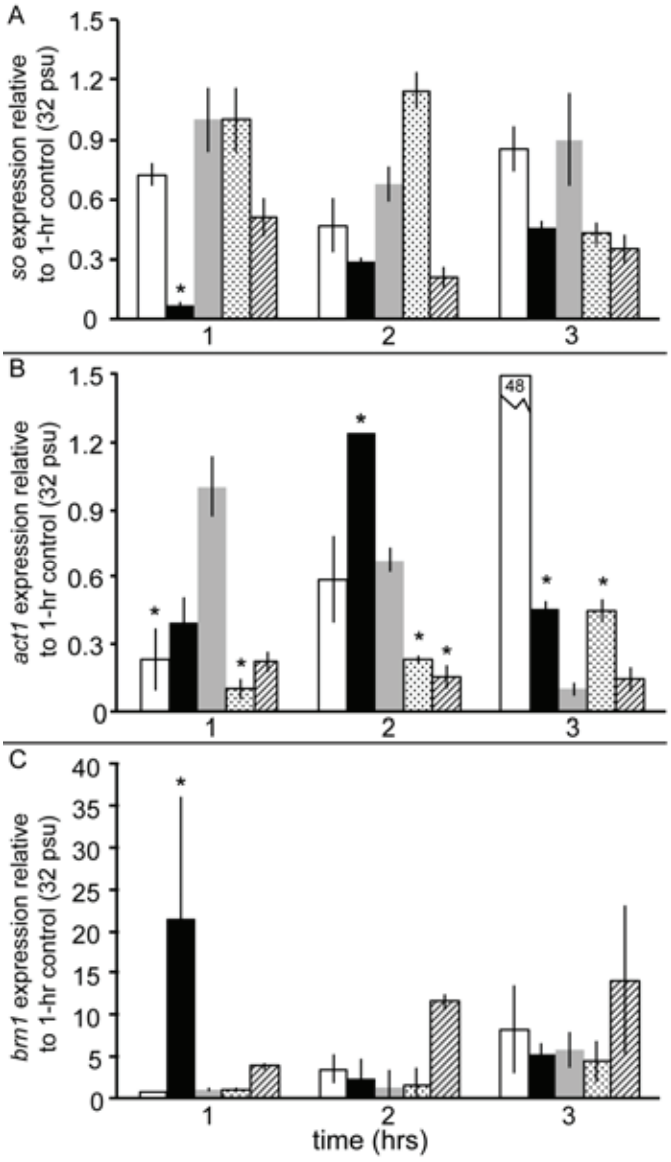

Fig. 2. Forty-five specimens of the reef-building coral *Pocillopora damicornis* were exposed to 24 (white columns), 28 (black columns), 32 (control; gray columns), 36 (speckled columns), or 40 (hatched columns) partial salinity units (psu; n = 3 tanks per treatment), and one was sampled from each of the fifteen tanks after 1, 2, and 3 hrs of exposure. RNA was isolated as described in the text, inoculated with an exogenous *sine oculis* (*so*) RNA spike, converted to cDNA, and used in real-time PCR assays designed for *so* (A), as well as two putative housekeeping genes, β-actin (*act1*; B) and POU III transcription factor brain 1 (*brn1*; C). Errors bars represent standard error of the mean, and astrices denote a significant difference (Tukey's HSD, $p < 0.05$) between the experimental group and the control within each of the three sampling times.

and/or host osmotic pressure (Mayfield et al., 2010), also seems to suggest that *act1* is a poor HKG candidate for molecularly-based studies of Taiwanese pocilloporids.

brn1 (Fig. 2C) appears to be a better candidate as a HKG for salinity challenge studies with *P. damicornis*, as it exhibited no salinity, time, or salinity x time interaction effects (Table 5). That being said, there was a statistically significant, 20-fold increase in expression of this transcription factor gene in samples exposed to 28 psu for one hour relative to experimental controls at this same sampling time. In fact, the use of HKGs for gene expression analyses should only ever be attempted by laboratories that either lack the funding or capacity to produce or purchase exogenous RNA spikes; only spikes are added to RNAs in a known quantity that does not vary between samples. Therefore, the exact efficiency of the RT reaction can be calculated, unlike for the HKG, in which it is not possible to determine whether purported stability of expression can be attributed more to lack of RT efficiency differences or to actual stability in expression *in vivo*. For instance, a putative HKG might show very similar expression levels between samples A and B. However, it could be that less of this gene was expressed in sample A, though the RT reaction was more efficient. On the other hand, sample B may have expressed more of this gene, though the RT reaction was less efficient. As such, the HKG in this case would not actually help to control for RT efficiency differences between samples, as the exogenous spike would (Bower et al., 2007).

mapk *expression*. Of the three *mapk*s targeted, *p38* (Fig. 3A) and *erk2* (Fig. 3B) demonstrated significant expression differences in response to the interaction of time and salinity (Table 5). Furthermore, *erk2* expression was also responsive to both salinity treatment alone and time alone (Table 5). For both of these genes, it is evident that the most pronounced changes occurred in samples exposed to 28 psu for one hour (Fig. 3). Specifically, there were 102- and 66-fold increases in expression of *p38* and *erk2*, respectively, relative to experimental controls (32 psu) at the 1-hr sampling time, and both of these differences were statistically significant. In fact, no other changes, as determined by Tukey's HSD tests, were detected within the latter two sampling times for either gene, suggesting that the transcription level response of these two *mapks* is rapid, yet ephemeral and not sustained. This is what could be expected given that MAPK enzymes regulate the initial stages of the osmotic stress response and are, specifically, involved in linking the initial sensing of the osmotic stress to the protein-level response pathways necessary to restore homeostasis (Cowan and Storey, 2003).

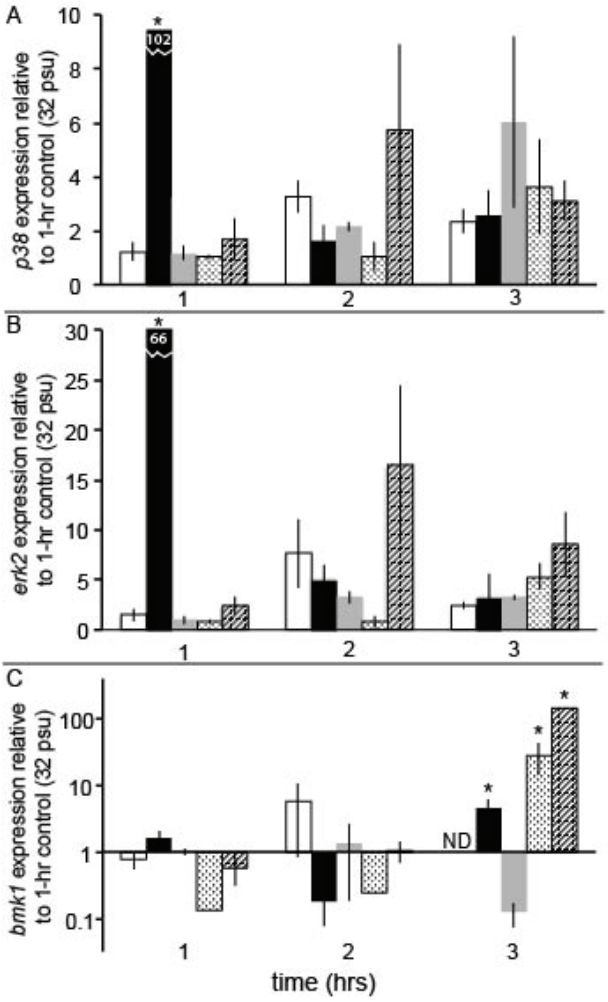

Fig. 3. Forty-five specimens of the reef-building coral *Pocillopora damicornis* were exposed to 24 (white columns), 28 (black columns), 32 (control; gray columns), 36 (speckled columns), or 40 (hatched columns) partial salinity units (psu; n = 3 tanks per treatment), and one was sampled from each of the 15 tanks after 1, 2, and 3 hrs of exposure. RNA was isolated as described in the text, inoculated with an exogenous *sine oculis* (*so*) RNA spike, converted to cDNA, and used in real-time PCR assays designed for three mitogen-activated protein kinase (*mapk*) genes: stress-activated protein kinase 2 (*p38*; A), extracellular signal-regulated kinase 2 (*erk*; B), and big *mapk* 1 (*bmk1*; C). Errors bars represent standard error of the mean, and astrices denote a significant difference (Tukey's HSD, $p < 0.05$) between an experimental group and the control within each of the three sampling times. In A and B, certain columns were "broken" in order to emphasize that the associated values are beyond the scale of the figure. In C, a $\log_{10}$ scale was used due to extensive differences between certain experimental groups and the control group in the final sampling time. The "ND" in C stands for "not determined" and reflects the fact that the RNA isolated from corals exposed to 24 psu for 3 hr did not readily amplify in the real-time PCR, possibly due to poor RNA quality.

The fact that neither gene demonstrated an mRNA-level response to hyper-osmotic stress (i.e., 36 and 40 psu), on the other hand, is puzzling, as *p38* is involved in regulation of the high osmolality glycerol (HOG) pathway, in which glycerol is accumulated in cells exposed to high external osmolality in order to prevent loss of water (Mao et al., 2004). It cannot currently be stated, though, whether expression and/or activity of the respective p38 proteins would demonstrate a similar absence of effect at high salinities; indeed, such a protein-based study represents a fruitful avenue for future research. Ultimately, although an understanding of the behavior of the respective proteins would help to develop a better picture of this coral's cell-level response to osmotic stress, these *mapk* mRNAs would *not* serve as useful molecular biomarkers for coral health assessment given how quickly their expression returned to baseline levels.

In contrast to what was documented for *p38* and *erk2*, *bmk1* expression (Fig. 3C) did not differ in response to salinity treatment, time, or their interaction (Table 5). That said, several post-hoc differences were detected by Tukey's HSD tests, all of which were documented at the final sampling time (3 hrs); samples of the 28, 36, and 40 psu treatments all were characterized by significantly higher expression levels than experimental controls at this sampling time and were 37-, 233-, and 1,160-fold up-regulated, respectively. The fact that such dramatic increases in expression were not detected in the model attests to the significant variability within the dataset. It appears, then, that, unlike *p38* and *erk2*, which appear to be involved in the initial stages of the osmotic stress response, *bmk1* is involved in the latter stages. Indeed, Pi et al. (2004) found that *bmk1* acts to prevent apoptosis of cells exposed to osmotic stress in mammals, a "rescue" process that would likely occur only in cells that had been exposed to a stress-inducing salinity for several hours. If the role of the BMK1 protein in corals is involved with prevention of cell death in stressed cells, it could, unlike the other two MAPKs, serve as a useful biomarker for detecting sub-lethal stress in this coral species.

Application of both previously published and new, NGS-derived real-time PCR assays to cDNAs from Taiwanese *P. damicornis* specimens

Of the 12 host genes for which real-time PCR assays had been published (Table 1), 10 were applied to cDNA from *P. damicornis* specimens from Southern Taiwan. Of these 10, only 5 were functional (efficiency between 90-110% and a single melting curve peak). Specifically, four of the assays developed for work with this coral in Hawaii (*p38*, *erk2*, *bmk1*, and *act1*) produced single peaks in the melting curves conducted at

the completion of the reactions. However, the expression of the *mapk* genes was quite low (C_t > 30 in many cases), meaning that it may still be preferable to clone these genes with DNA or cDNA from Taiwanese samples, then re-design the respective real-time PCR primer sets.

Of the five non-functional assays, four were developed from *P. damicornis* populations originating in the Red Sea, suggesting that genetic differences may have accounted for failure of primers designed from corals of one population to bind to the homologous gene of those of another population. However, in the case of the Vidal-Dupiol et al. (2009; 2013) assays, primers were developed by a computer, and the associated real-time PCR assays do not appear to have been utilized with coral RNA. Therefore, it is possible that these real-time PCR assays are not functional with any *P. damicornis* specimens. It is urged herein that researchers do not publish primers whose functionality they have not verified. If such primers are nevertheless published, it is recommended that the authors explicitly note that they have not actually been tested.

From the NGS-based transcriptome characterization of Taiwanese *P. damicornis* specimens exposed to either 26.5 or 29.7°C for 2 or 36 weeks, seven real-time PCR assays were developed for the coral host compartment (Table 3), all of which produced melting curves with a single peak and amplified host cDNAs in under 32 cycles at a high efficiency (98-102%). There are now 14 genes whose levels of expression can be measured accurately in the host compartment of *P. damicornis* holobionts from Southern Taiwan (Table 3). Interestingly, both carbonic anhydrase (*ca*) and green fluorescent protein-like chromoprotein (*gfp-cp*) were found to be up-regulated in specimens that had been exposed to 29.7°C for 36 weeks (Mayfield et al., unpublished data) and so could serve as biomarkers for the assessment of coral health. On the other hand, host *hp70*, which encodes a molecular chaperone involved in refolding of denatured proteins, was shown to lack temperature sensitivity in this coral at both the larval (Putnam et al., 2013) and adult (Mayfield et al., 2013a; in press) life history stages.

Of the 13 real-time PCR assays developed for *Symbiodinium* in previously published studies (Table 2), which, unlike for the coral host, is not an exhaustive list, 8 successfully amplified cDNA from *P. damicornis* specimens from Southern Taiwan. As with the host coral assays, the majority of the non-functional assays were developed from *P. damicornis* populations originating in the Red Sea and cultured in Monaco. Again, these primers were designed by computer software and were likely not applied to actual coral samples, which may explain their lack of utility for the Taiwanese samples used herein. The non-specificity of the rRNA

primers (28 and 18 s) is not surprising given the diversity of rRNA sequence types both within and across *Symbiodinium* populations (Stat et al., 2011). In fact, intragenomic variation in *its2* and other rDNA and rRNA genes continues to thwart efforts by the coral biology field to accurately assess the genetic diversity of *Symbiodinium* (Pochon et al., 2012).

From the NGS-based approach, six real-time PCR assays were successfully developed for the *Symbiodinium* populations of *P. damicornis* holobionts from Southern Taiwan (Table 4). Briefly, all six produced single melting curve peaks at the completion of the PCRs, indicating that they were specific for the target amplicon. Furthermore, C_t values were generally below 32, and PCR efficiencies were always between 95-105%, suggesting that the assays can be used confidently. In combination with the 8 functional assays from previously published works (Table 2), there are now 14 real-time PCR-based assays that can be used to accurately measure gene expression in the dominant *Symbiodinium* populations residing with *P. damicornis* colonies of Southern Taiwan.

Alongside the 14 host-targeted genes (Table 3), there are now 28 genes whose expression can be assessed in this dominant reef-builder of many regions of the Indo-Pacific. There are also six DNA-based assays that have been developed for this coral (Mayfield et al., 2013a; in press); two, the *Symbiodinium* and host genome copy proportions (GCPs) are used for gene expression normalization, whereas the other four were created for clade-level genotyping of *Symbiodinium* (clades A-D, *sensu* Correa et al., 2009). Future works should seek to multiplex several of these assays within a single reaction using Taqman® probe (Life Technologies)-based technology to minimize the number of reactions conducted. For instance, both the *Symbiodinium* and host GCPs could be multiplexed within a single reaction, and genotyping assays could be conducted in a single tube.

Currently, researchers aboard the Khaled bin Sultan Living Oceans Foundation's (www.livingoceansfoundation.org) research vessel, the *Golden Shadow*, are traversing the globe as part of the "Global Reef Expedition". As the cruise will spend all of 2014 in the Indo-Pacific, scientists will be collecting *P. damicornis* samples across a gradient of anthropogenic impact, from relatively pristine reefs (e.g., Cook Islands) to those abutting major population centers (e.g., certain islands in Indonesia). There is an urgent need, then, to continue to develop real-time PCR-based assays for expression analysis of potential biomarkers that could be employed with samples collected during these research missions such that the health of this widespread species could be monitored on a proactive timescale. For instance, were

high levels of stress-indicative biomarkers measured in samples of a certain reef, scientists could contact the local managers to see if proactive measures could be taken to prevent, for instance, a mass coral bleaching episode driven by deterioration of seawater quality (e.g., from land-based pollution). Future work will, then, be focused on developing a "stress test" for this model reef-building coral, using not only the 28 gene expression assays discussed herein, but also others emerging from NGS-based transcriptome studies.

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即時聚合酶鏈鎖反應分析細枝鹿角珊瑚之基因表現：鹽度壓力研究

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摘 要

細枝鹿角珊瑚的廣泛分布全球，使其逐漸成爲研究全球氣候變遷時所選擇的珊瑚代表物種。爲了更進一步發展可有效確實瞭解珊瑚細胞內對環境改變之反應的分子生物技術，本研究從三個角度深度探討珊瑚對壓力的細胞分子反應。 一，鹽度壓力實驗。樣本暴露在五種不同的濃度下 （24，28，32(控制組)，36 或 40 psu）經過不同時間長度（1，2，或 3 小時）後測量下述基因的基因表現：三個 mitogen-activated protein kinase (MAPK) genes; stress-activated protein kinase 2 (*p38*), extracellular signal-regulated kinase 2 (*erk2*), and big MAPK1 (*bmk1*)。本研究發現在 1 小時 28 psu 的處理下 *p38* 和 *erk2* 大量的增加其基因表現。此結果可能代表這兩個蛋白質和珊瑚細胞初期如何應對低鹽度壓力環境有關並可能爲未來研究珊瑚如何對環境變動反應之重要分子目標。 二，本研究建立臺灣南部海域細枝鹿角珊瑚的分子藍圖已進一步發展可適用的分子分析試驗。目前各國研究團隊所發展的分子分析試驗中，僅有大約 55%可用於台灣的細枝鹿角珊瑚。舉例來說，由摩納哥細枝鹿角珊瑚所發展的即時聚合酶鏈鎖反應試驗（real-time PCR assay）並不適用於臺灣的細枝鹿角珊瑚。三，使用基因轉錄體序列研究辨識與適應高溫環境有關的基因。目前總共有 28 組基因（14 組珊瑚基因以及 14 組共生藻基因）已可利用即時聚合酶鏈鎖反應準確地測量其基因表現並廣泛運用於印度太平洋區域。未來將利用上述基因分子指標研究臺灣海域珊瑚，並更進一步用於參與蘇爾坦海洋基金會的全球珊瑚考察團進行廣泛的分子指標調查。

關鍵詞：珊瑚, 雙鞭毛藻, 胞內共生, 基因表現, mitogen-activated protein kinase (MAPK), 滲透壓調節, 細枝鹿角珊瑚, 即時聚合酶鏈鎖反應, 鹽度壓力