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Temporal variation in nucleic acid and protein ratios in four anthozoan-dinoflagellate endosymbioses of the Indo-Pacific: implications for molecular diagnostics

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Abstract

Given the threat of global climate change towards coral reefs and the endosymbiotic anthozoans that construct them, there is an urgent need to better understand the physiological response of these environmentally sensitive organisms. Unfortunately, many aspects of anthozoan biology are poorly understood, such as the cellular and molecular mechanisms underlying the stability of their association with dinoflagellates of the genus *Symbiodinium*. For instance, it has only recently been demonstrated that the molecular composition of these mutualisms is temporally variable; specifically, *Symbiodinium* DNA content has been shown to vary significantly across the light-dark cycle, and such variation could have implications for normalization of macromolecular expression data. For that reason, there was an interest in better understanding both spatio-temporal and interspecific variation in the concentrations of nucleic acids and proteins in reef-building corals and endosymbiotic sea anemones. Upon exploration of both published and unpublished datasets, it was found that total RNA, DNA, and protein quantities and concentrations were variable over time in the majority of the taxa sampled, which included two reef-building corals (*Montipora capitata*

Hawaii, as well as one Taiwanese scleractinian (*Seriatopora hystrix*). Furthermore, RNA/DNA and protein/DNA ratios, which can serve as indicators for total levels of gene and protein expression, respectively, also varied over time and across species, and these differences may be due to spatio-temporal variation in *Symbiodinium* densities driven by heterogeneity in the abiotic environment. Collectively, these data suggest that a high degree of quality control is necessary when employing molecular diagnostics approaches to management of corals and the reefs they build, lest results from biomarker-based endeavors be biased by the potentially unstable biological composition of these globally threatened endosymbioses.

Keywords: anthozoan, coral, endosymbiosis, gene expression, global climate change, molecular biology, *Symbiodinium*

Introduction

Earth's coral reefs are currently threatened by a number of anthropogenic insults, including seawater pollution stemming directly from human population growth (Liu et al., 2009; 2012). However, global climate change (GCC) is largely considered to pose the greatest danger to these fragile ecosystems (Hoegh-Guldberg, 1999); specifically, both elevated temperature and acidity are predicted to have negative consequences for coral reefs and the myriad organisms that inhabit them (Hoegh-Guldberg et al., 2007). The framework-building scleractinians, themselves, which engage in a mutualistic endosymbiosis with dinoflagellates of the genus *Symbiodinium* (Muscatine et al., 1981), are thought to be particularly threatened by GCC (Brown, 1997), as these endosymbioses are environmentally sensitive and prone to disintegrate upon prolonged exposure to environmental changes (Gates et al., 1992; Gates and Edmunds, 1999), a phenomenon known as bleaching (Gates, 1990). Despite these concerns, little is know about fundamental components of the biology of reef-building corals and other anthozoans associated with photosynthetic endosymbionts (van Oppen and Gates, 2006; Mayfield and Gates, 2007). For instance, while it has been known that the association is indeed of mutual benefit to both partners for many years (Muscatine and Cernichiari, 1969), the molecular regulation of this symbiosis has only recently been investigated (Peng et al., 2010; 2011; Chen et al., 2012; Mayfield et al., in review).

Given the threats of GCC towards coral reefs, there is an urgent need to

develop proactive means by which to assess the health of corals and other reef inhabitants prior to more visible, but temporally removed, signs of stress, such as bleaching or disease (Downs et al., 2000). As such, molecular biology proves to serve as a fitting tool to uncover sub-cellular effects of environmental stress on a timescale that is in tighter synchrony with the advent of an environmental insult (Downs et al., 2002). Specifically, relative levels of gene and protein expression represent potentially useful parameters for understanding which cellular processes are most greatly affected by environmental stress, and the underlying macromolecules could additionally function as molecular biomarkers for marine animal health assessment (Downs et al., 2005). Such a proactive management strategy, which depends on both significant research funding for undertaking of the requisite molecular analyses and a government willing to employ adaptive management approaches, could achieve success in relatively affluent and environmentally conscious nations, such as the United States, Japan, and Taiwan, all of which contain vast expanses of coral reefs.

Given the potential for use of routine molecular tools for rapidly and accurately assessing the health of marine organisms, the coral biology field, in particular, has been quick to adopt such genomic and proteomic approaches to developing molecular diagnostic assays for reef coral health assessment (Edge et al., 2005; Mayfield et al., 2011; Putnam et al., accepted). Unfortunately, a plethora of molecular-derived data, particularly from microarray and real-time PCR-based endeavors (e.g., Voolstra et al., 2009; Kenkel et al., 2011; Leggat et al., 2011), have been generated for reef-building corals in an experimental and methodological framework that has neglected to consider the fact that, as reef-building corals and certain sea anemone species are of dual-compartmental nature (Mayfield et al., 2009), traditional macromolecular expression approaches developed in model organisms such as humans may, in fact, need to be modified to account for the mixed nature of the biological material extracted from experimental samples (Mayfield et al., 2010). For instance, a bleached coral will contain significantly less *Symbiodinium*, as well as lower concentrations of macromolecules derived from these dinoflagellates, relative to a healthy coral with high densities of *Symbiodinium*. Therefore, endosymbiosis-specific methodological controls must be taken in order to compare samples of differing biological composition such that reduced levels of *Symbiodinium* gene expression, for instance, are not attributed to bleached corals simply due to the fact that fewer *Symbiodinium* nucleic acids were extracted from those samples relative to

healthy specimens. Unfortunately, the data generated by recent works attempting to uncover the molecular processes underlying pathways such as the establishment (Rodriguez-Lanetty et al., 2006) and breakdown (DeSalvo et al., 2008; Kaniewska et al., 2012) of anthozoan-dinoflagellate endosymbioses must be interpreted with great caution, as the authors neglected to normalize their data to account for spatio-temporal or experimentally-derived variation in the ratio of host: *Symbiodinium* biological material.

In fact, in the few cases when such endosymbiosis-driven biological composition variation was assessed, it was found that *Symbiodinium* DNA content, as deduced by real-time PCR-based genome copy numbers (GCNs), was temporally variable (Mayfield et al., 2010; 2011; Mayfield et al., in review) or varied in response to experimental treatments (Mayfield et al., 2012; Putnam et al., accepted), and the authors took these changes to be indicative of *Symbiodinium* mitosis and, presumably, cell division. Although the anthozoan-dinoflagellate endosymbiosis is stable under ambient environmental conditions (Hoegh-Guldberg and Smith, 1989; Stimson and Kinzie, 1991), there is still a high degree of both *Symbiodinium* and host cell turnover (Baghdasarian and Muscatine, 2000; Fitt, 2000), and the cell cycles of the two predominant eukaryotes of the holobiont may not necessarily progress in a tandem fashion (Wang et al., 2008). As such, Mayfield et al. (2010; 2011) hypothesized that the overall biological composition of the holobiont might vary in response to *Symbiodinium* cell division *in hospite*.

To determine whether the previously documented temporal differences in *Symbiodinium* DNA content and density impact the nucleic acid and protein concentrations of endosymbiotic anthozoans, both previously published (Mayfield et al., 2009; Mayfield et al., 2010) and unpublished datasets were re-explored, and it was found that, indeed, RNA, DNA, and protein content and concentrations were highly variable over time in both endosymbiotic sea anemones and reef-building corals. These macromolecular concentration differences could have significant implications for the use of genomic and proteomic tools in molecular diagnostics of these organisms. Specifically, both temporal and interspecific differences in nucleic acid and protein concentrations suggest that biological composition controls must become an integral component of all expression-based analyses conducted with such dual-compartmental organisms.

Materials and Methods

***Aiptasia pulchella* infection experiment**. A 6-week infection of experimentally bleached *A. pulchella* (Fig. 1) clones from the laboratory of Professor Robert Kinzie of the Hawaii Institute of Marine Biology (HIMB, Coconut Island, Oahu, Hawaii,

USA, 21°26.2′N, 157°47.6′W) was conducted as described by Mayfield et al. (2009), and RNA, DNA, and protein were extracted from three anemones from each of five clones sampled after 0, 3, and 6 weeks of infection with clade B *Symbiodinium*. Briefly, anemones were immersed in 500 µl TRIzol™ (Invitrogen) and homogenized in 1.5-ml microcentrifuge tubes with a micropestle attached to an electronic rotator until the tissues had completely disintegrated. Then, RNA, DNA, and protein were extracted and quantified from each of the 45 anemones as described by Mayfield et al. (2009). Briefly, while the RNA extraction protocol differed only slightly from that recommended by the manufacturer and involved precipitating with both a "high salt" solution (250 µl) and isopropanol (250 µl) at room temperature (RT) for 10 min, the DNA extraction and purification protocols differed dramatically, as the use of the DNA extraction protocol recommended by Invitrogen did not allow for the recovery of high molecular weight DNA. Specifically, a back extraction buffer (BEB, 500 µl) was incubated with the TRIzol-tissue slurry (~600 µl) at RT for 20 min. The samples were then spun at 12,000 *xg* for 10 min at 4°C, and the aqueous phase was precipitated with 0.1 volume (vol, ~60 µl) sodium acetate (3 M, pH 5.2), 1 vol isopropanol (~600 µl) and 1.5 µl Pellet Paint® NF-co-precipitant (EMD Biosciences) at RT for 10 min. After precipitation, both RNAs and DNAs were spun at 12,000 *xg* for 10 min at 4°C, and the supernatants were decanted. Then, both RNA and DNA pellets were washed once with 75% ethanol, spun at 7,500 *xg* for 10 min at 4°C, and dried inverted on the benchtop for 20-30 min prior to dissolving in DEPC-treated water and 0.1x TE buffer for the RNAs and DNAs, respectively.

RNA and DNA quantity was assessed on a NanoDrop Spectrophotometer (Thermo-Scientific), and their quality was assessed on 3-(N-morpholino) propanesulfonic acid (MOPS, 1x)-formaldehyde (18%)-agarose (1%) and tris-borate-EDTA (TBE) agarose (1%) gels, respectively. Both gels, which also contained ethidium bromide (EtBr), were electrophoresed at 100 v for 40 min and visualized under ultraviolet (UV) fluorescence. Proteins were dissolved in Laemmli sample buffer and quantified with the RC:DC kit (Bio-Rad) as recommended by the manufacturer. Protein quality was determined by electrophoresis of 30 µg protein on SYPRO® Ruby (Invitrogen)-stained 4-10% tris glycine sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels for 20 and 120 min through the stacking and separating gels, respectively, as detailed in Mayfield et al. (2011). RNA concentration ([RNA], ng μl^{-1}), total RNA (µg), DNA concentration ([DNA], ng μl^{-1}), total DNA (µg), protein concentration ([protein], ng μl^{-1}), total protein (µg), and RNA/DNA and

protein/DNA ratios (unit-less) were calculated for each sample, and the variation in total RNA, total DNA, total protein, and the two ratios was assessed over time with one-way ANOVAs conducted with JMP® (version 5) with anemone clone nested within sampling time. After determining that there was no significant effect of clone ($p > 0.05$), the clone factor was dropped from the model, and non-nested one-way ANOVAs were then used. An α-level of 0.05 was used in all analyses.

***Montipora capitata* recovery experiment**. To understand the degree of stress induced by the "nubbin" generation process, whereby small portions (typically branches from branching corals or "cores" from plating corals) are broken from coral colonies to serve as biological replicates in later experiments (*sensu* Mayfield et al., 2012), a study was conducted in May 2007 in which randomly selected colonies of the common, Hawaiian scleractinian, *M. capitata*, from Coconut Island, Hawaii (Fig. 1) were fragmented *in situ*, and the recovery of the respective nubbins generated from each colony was tracked over a one-month period (Hirst, M.B., A.B. Mayfield & R.D. Gates, unpublished data). Prior to experimentation, various fragmentation strategies were employed, and it was found that generation of cores from *M. capitata* colonies characterized by plating morphologies with a pneumatic drill *sensu* Barshis et al. (2010) did not allow for the generation of biological replicates that readily recovered from the fragmentation process. On the other hand, the creation of branch-like nubbins from branching *M. capitata* colonies with bone-cutting pliers did allow for the generation of samples characterized by a high degree of survival (> 90%). As such, eleven branching nubbins were created from each of 10 branching colonies (Fig. 1), resulting in a total of 110 replicates. One replicate from each colony was sampled after 0 (the source colony sampled prior to nubbin generation), 0.25, 1.25, 3.25, 7.25, 14, and 21 days (n = 10 biological replicates day^{-1}), and four replicates were taken from each colony after 28 days (n = 40 biological replicates day^{-1}). A 50 mg fragment was removed from each nubbin with bone-cutting pliers, immediately snap-frozen in liquid nitrogen, and the samples were stored in a -80°C freezer until the day of extraction. As with the sea anemones described above, the TRIzol method was used to extract RNA and DNA, including the use of the modified, high salt precipitation step recommended by the manufacturer for use with samples containing high concentrations of polysaccharides. RNA and DNA quantity and quality were assessed as above. One-way ANOVAs were conducted with JMP® in order to test for the effect of sampling time on total RNA (µg), total DNA (µg), and the RNA/DNA ratio (unit-less).

***Pocillopora damicornis* field experiment**. Three specimens of the common, ubiquitously-distributed (Veron, 2000), Indo-Pacific reef coral, *P. damicornis* (Fig. 1) were collected on reef flats surrounding Coconut Island, Hawaii as described in Mayfield et al. (2010). Briefly, to document diel variation in expression of a variety of mitogen-activated protein kinase (*mapk*) genes, the authors selected corals (n = 3 sampling time^{-1}) at 12:30, 18:45, 19:30, 23:00, 5:45, and 6:30 on triplicate days in May 2008, and RNA and DNA were extracted from each of the 54 samples as described therein. RNA and DNA quantity were assessed as above, and native 1% TBE-agarose gels containing EtBr were used for assessment of RNA and DNA quality after electrophoresis at 100 v for 30 min. The same molecular parameters as for *M. capitata* were analyzed over time with one-way ANOVAs with sampling day nested within sampling time. After determining that there was no effect of sampling day ($p > 0.05$), the data were pooled across triplicate days and standard, non-nested one-way ANOVAs were then used instead.

***Seriatopora hystrix* mesocosm experiment**. Fifteen colonies of the common (Dai, 1991), ubiquitously-distributed (Veron, 2000), Indo-Pacific reef coral, *S. hystrix* (Fig. 1), were reared in suspension on fishing lines 10 cm below the surface in each of two 50-kl mesocosms located at Taiwan's National Museum of Marine Biology and Aquarium (NMMBA) as described in Mayfield et al. (2010). Briefly, in order to document diel variation in expression of a variety of cytoskeleton genes, the authors selected corals (n = 3 colonies sampling time^{-1} mesocosm^{-1}) at 12:00, 18:00, 21:00, 5:00, and 8:00 on each of three days in July, 2008, and RNA and DNA were extracted from each of the 90 samples as described therein at NMMBA. The pseudo-replication employed was justified by the large size of the mesocosm systems, which had an approximately 10^6-fold greater volume than the experimental colonies. On each sampling day, no colony was sampled more than once to avoid physiological artifacts derived from stress induced by the sampling process, in which bone-cutting pliers were used to remove small (~50 mg) branches from entire *S. hystrix* colonies (Mayfield et al., 2010). RNA and DNA were quantified as above, and quality was assessed on native 1% TBE-agarose gels, which were electrophoresed at 100 v for 45 min and post-stained in an EtBr bath for 20 min prior to visualization under UV fluorescence.

The same molecular parameters as for *M. capitata* were analyzed over time with one-way ANOVAs with mesocosm nested within sampling time for each of the three sampling days with JMP®. Data were pooled across the two mesocosms after determining absence of mesocosm

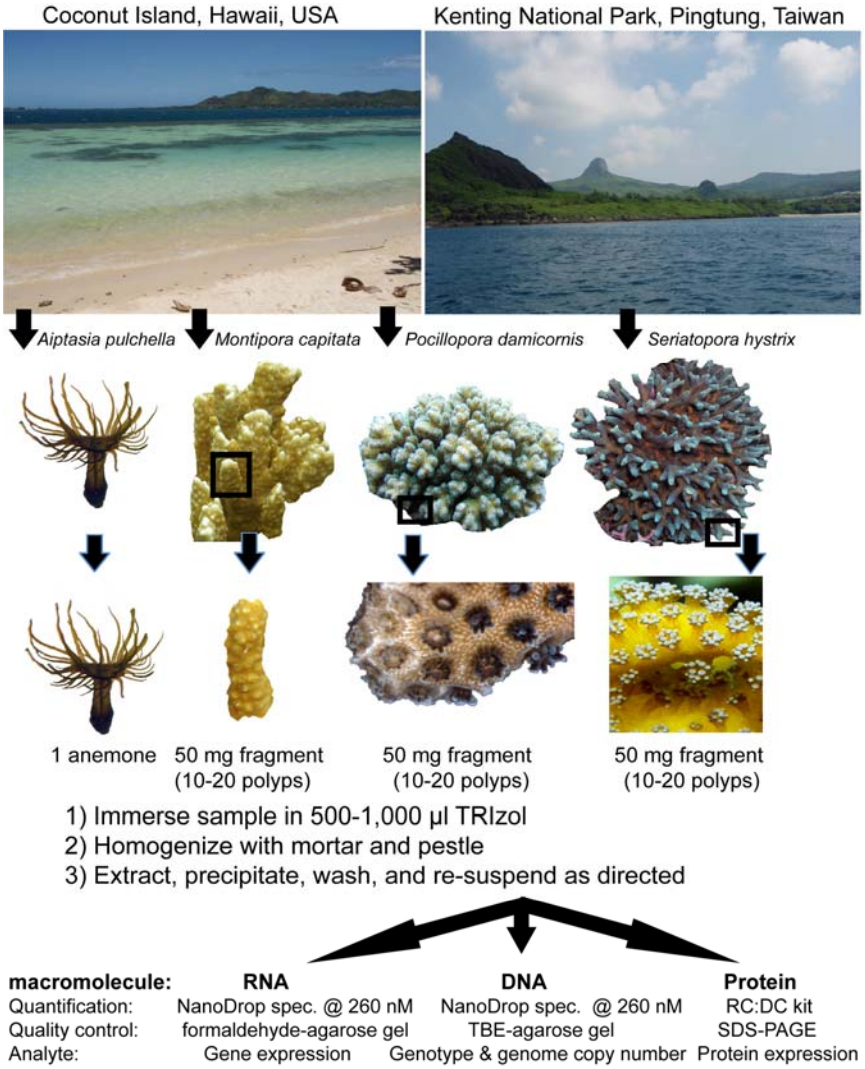

Fig. 1. Schematic of experimental design. Clones of the anemone *Aiptasia pulchella* originally collected from reefs abutting Coconut Island, Hawaii, USA and kept under total darkness for over 20 years by Prof. R. Kinzie were experimentally re-infected at the Hawaii Institute of Marine Biology, and RNA, DNA, and protein were extracted from each specimen. Two species of coral, *Montipora capitata* and *Pocillopora damicornis*, were sampled from reefs surrounding Coconut Island, as described in the text, and RNA and DNA were extracted from replicate samples for each species. Similarly, specimens of *Seriatopora hystrix* originally from Nanwan Bay in Kenting National Park, Taiwan and housed within Taiwan's National Museum of Marine Biology and Aquarium were sampled as described in the text, and their RNAs and DNAs were also extracted. Boxed insets portray the approximate size of the anemone and coral samples used in the RNA/DNA/protein extractions, though images are not presented on the same scale. The analytes listed were assayed in previously published works and are not mentioned herein.

effects ($p > 0.05$), and one-way ANOVAs were then conducted with sampling day nested within sampling time. As there was found to be no effect of sampling day on any molecular parameters ($p > 0.05$), the sampling day term was dropped from the model, and data were pooled across the three sampling days, resulting in 18 biological replicates for each sampling time. Then, non-nested one-way ANOVAs were used to determine the effects of sampling time on total RNA (µg), total DNA (µg), and the RNA/DNA ratio (unit-less).

Cross-experimental comparisons and statistical analyses. To determine the effect of species on the RNA/DNA ratio (unit-less), global mean RNA/DNA ratios were calculated across all samples within each of the four taxa, and a one-way ANOVA was conducted with JMP on rank-transformed data. The average coral RNA/DNA ratio was then compared against the average anemone RNA/DNA ratio with a Kruskal-Wallis test. For all statistical analyses, the Shapiro-Wilk W and Levene's tests were used to determine whether datasets were normally distributed and of equal variance, respectively (Quinn and Keough, 2002). When either or both conditions were not met, transformations were conducted prior to statistical tests. In the cases in which transformation still did not lead to suitable datasets for ANOVA, non-parametric tests were used to test for temporal variation.

Results

***A. pulchella* infection experiment.** High quality and quantity RNA, DNA, and protein were extracted from each of 45 anemones collected after 0, 3, and 6 weeks ($n = 15$) of infection with clade B *Symbiodinium* (Mayfield et al., 2009). Total RNA (Fig. 2A), but not total DNA (Fig. 2B), was shown to vary significantly across sampling times (Table 1). Specifically, total RNA was ~1.5-fold higher in samples sacrificed prior to infection with *Symbiodinium* relative to the latter sampling times (Fig. 2A). Furthermore, total protein (Fig. 2C) co-extracted from the same samples from which RNA and DNA were isolated also varied significantly over time (Table 1), and anemones sampled after three weeks of infection with *Symbiodinium* were shown to have ~1.5-fold higher total protein content than those sacrificed prior to infection and after six weeks of infection (Fig. 2C). Both the RNA/DNA (Fig. 2D) and protein/DNA (Fig. 2D) ratios varied significantly over time (Table 1). The former was approximately 50% higher in uninfected anemones (Fig. 2D), whereas the protein/DNA ratio was approximately 1.5-fold higher in anemones infected with *Symbiodinium* for three weeks relative to uninfected controls (Fig. 2D).

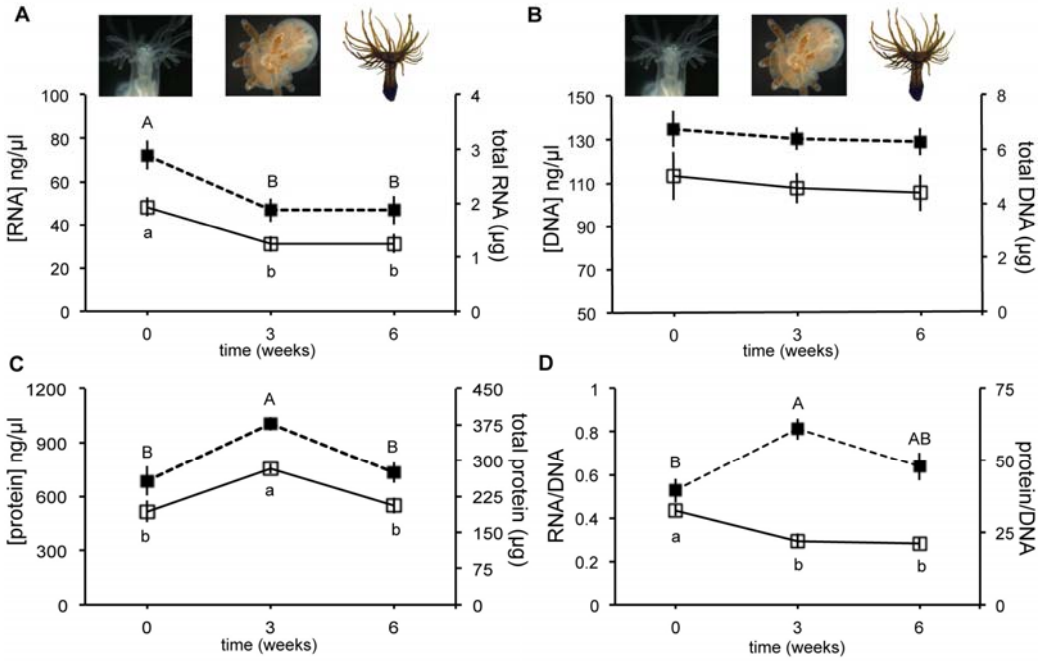

Fig. 2. *Aiptasia pulchella* infection experiment. Three specimens from each of five anemone clones were sampled prior to infection with *Symbiodinium*, as well as after three and six weeks post-infection. Representative images are shown above the corresponding sampling times. The filled (black) icons connected by dotted lines in panels A-C represent the total RNA, DNA, and protein data, respectively, and correspond to the right y-axis. The hollow icons connected by solid lines in panels A-C represent [RNA], [DNA], and [protein] data, respectively, and correspond to the left y-axis. In panel D, the hollow icons and left y-axis correspond to the RNA/DNA ratio data, whereas the filled icons and right y-axis correspond to the protein/DNA ratio data. In all panels, error bars represent standard error of the mean, and in panels A, C, and D, the capitalized letters correspond to Tukey's post-hoc honestly significant difference (HSD) groups ($\alpha < 0.05$) for data of the right y-axis, and lowercase letters correspond to HSD groups for data of the left y-axis.

Table 1. One-way ANOVA table testing temporal differences in biological composition parameters assessed across four anthozoans**.** Parameters marked with "*" and "**" were rank and log-transformed, respectively, in order to meet the assumptions for utilizing ANOVA. Statistically significant differences (α = 0.05) are highlighted in bold font.

Source of variation	df	MS	F	p	Figure
		Aiptasia pulchella			
total RNA (µg anemone^{-1})	2	3.89	4.77	**0.0150**	Fig. 2A (right y-axis)
Error	36	0.816			
total DNA (µg anemone^{-1})	2	0.739	0.211	0.811	Fig. 2B (right y-axis)
Error	36	3.51			
total protein (µg anemone^{-1})	2	21700	9.102	**0.000**	Fig. 2C (right y-axis)
Error	36	23900			
RNA/DNA	2	0.0820	7.36	**0.00200**	Fig. 2D (left y-axis)
Error	36	0.0110			
protein/DNA	2	20.99	5.92	**0.00600**	Fig. 2D (right y-axis)
Error	36	3.55			
		Montipora capitata			
total RNA (µg fragment^{-1})*	7	46.2	3.059	**0.006**	Fig. 3A (right y-axis)
Error	115	15.1			
total DNA (µg fragment^{-1})**	7	0.545	3.760	**0.00100**	Fig. 3D (right y-axis)
Error	116	0.145			
RNA/DNA**	7	0.585	2.006	0.0600	Fig. 3G
Error	115	0.292			
		Pocillopora damicornis			
total RNA (µg fragment^{-1})**	5	0.543	2.407	**0.0460**	Fig. 3B (right y-axis)
Error	63	0.225			
total DNA (µg fragment^{-1})**	5	0.227	0.961	0.448	Fig. 3E (right y-axis)
Error	64	0.237			
RNA/DNA**	5	0.984	2.51	**0.0390**	Fig. 3H
Error	63	0.392			
		Seriatopora hystrix			
total RNA (µg fragment^{-1})	4	11.9	0.849	0.5080	Fig. 3C (right y-axis)
Error	25	14.08			
total DNA (µg fragment^{-1})**	4	0.177	1.21	0.333	Fig. 3F (right y-axis)
Error	25	0.147			
RNA/DNA**	4	0.607	2.54	0.0650	Fig. 3I
Error	25	0.239			

***M. capitata* recovery experiment**. Total RNA (Fig. 3A) varied significantly over time in *M. capitata* nubbins sampled over a four-week recovery experiment *in situ* (Table 1). However, the only significant pairwise difference was between biopsies taken from nubbins left to recover for 0.25 days and those nubbins sampled after 21 days of recovery from the fragmentation process (Fig. 3A); specimens of the former sampling time possessed approximately 1.5-fold higher RNA levels than those of the latter. Similarly, total DNA extracted from the same biopsies from which RNAs were extracted (Fig. 3D) also varied over time (Table 1). As with total RNA, the highest DNA content was measured in nubbins sampled six hours after fragmentation from their colony of origin. Significantly diminished holobiont DNA content was measured after 3.25, 21, and 28 days of recovery (Fig. 3D). Finally, despite a notable peak in the RNA/DNA ratio in nubbins sampled after 3.25 days of recovery from fragmentation (Fig. 3G), due to high biological variability, there was not a significant temporal difference (Table 1).

***P. damicornis* field experiment.** Total RNA (Fig. 3B) varied significantly over time in replicate *P. damicornis* colonies sampled *in situ* from a small, Hawaiian (Fig. 1) patch reef (Table 1). However, there were no pairwise differences among the six sampling times (Table 1). On the other hand, total DNA (Fig. 3E) did not vary significantly over time in these same samples (Table 1). Finally, the RNA/DNA ratio (Fig. 3H) was significantly affected by sampling time (Table 1), though there were no pairwise differences among individual times, despite a notable ~2-fold peak observed in colonies sampled at 18:45 (i.e., the sampling time just prior to sunset).

***S. hystrix* mesocosm experiment.** Unlike observations made in the anemone infection experiment and the two coral field experiments, there were no significant effects of sampling time on total RNA (Fig. 3C), total DNA (Fig. 3F), or the RNA/DNA ratio (Fig. 3I) in replicate *S. hystrix* specimens collected at five sampling times across two mesocosms within Taiwan's NMMBA over three days in July 2008 (Table 1).

Interspecific variation in the RNA/DNA ratio. The RNA/DNA ratio (Fig. 4) differed significantly between the four anthozoans of this study (see statistics within figure caption.). Tukey's HSD tests suggested that all pairwise differences were significant, and, most notably, the RNA/DNA ratio of *M. capitata* was over 5-fold higher than that of *A. pulchella*. In fact, the average coral RNA/DNA ratio of 1.32 was approximately 4-fold higher than that of the sea anemone *A. pulchella*, and this difference was statistically significant (Kruskal-Wallis test, $Z = 9.477$, $p < 0.0001$).

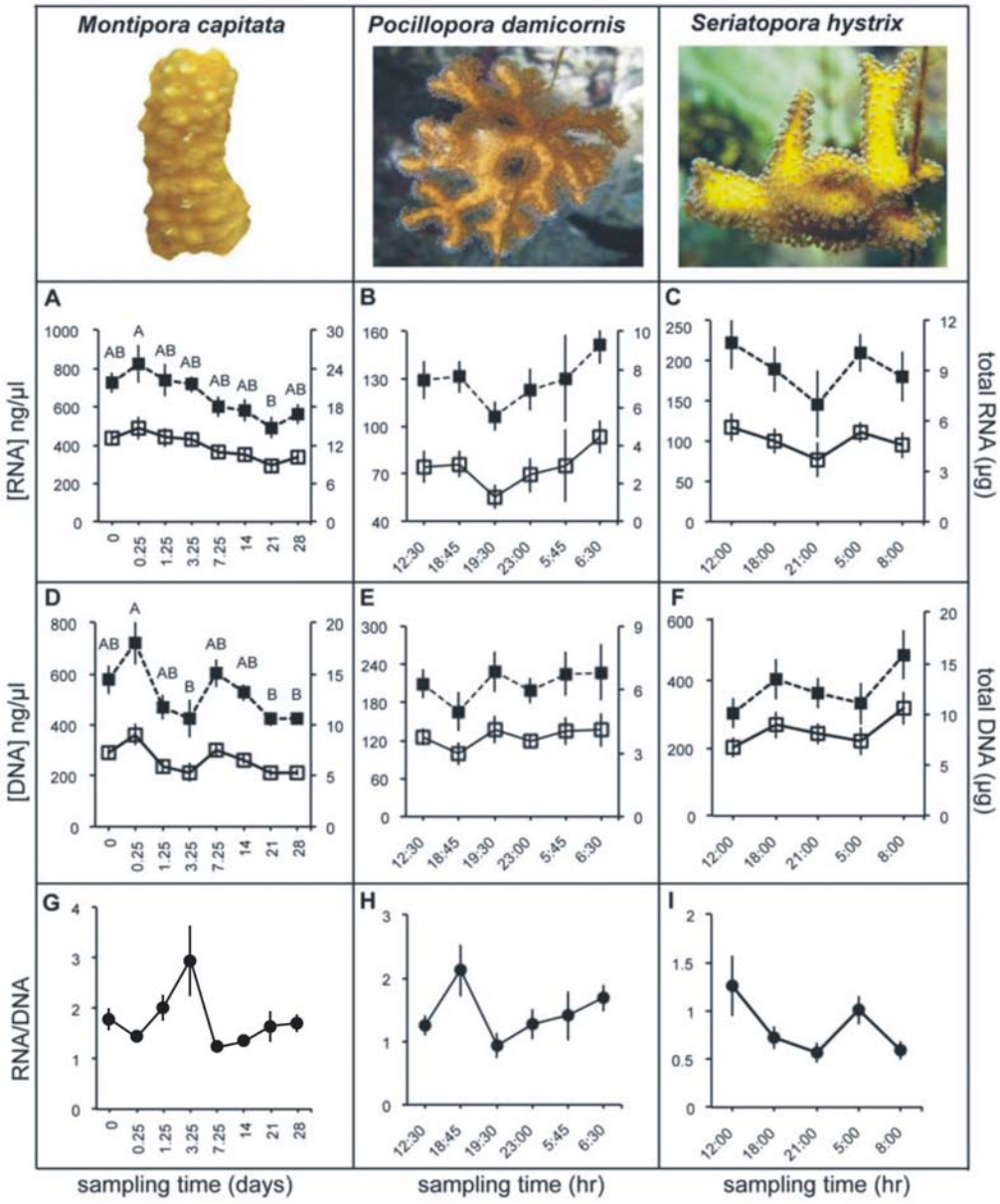

Fig. 3. Reef-building coral recovery, field, and mesocosm experiments. Specimens of *Montipora capitata* (panels A, D, and G) and *Pocillopora damicornis* (panels B, E, and H) from Coconut Island, Hawaii and *Seriatopora hystrix* from mesocosms within Taiwan's National Museum of Marine Biology and Aquarium (panels C, F, and I) were sacrificed at various times, and their RNAs (panels A-C) and DNAs (panels D-F) were extracted and quantified as described in the text. In panels A-F, the filled icons correspond to the total RNA and DNA (right y-axes), and the hollow icons correspond to the [RNA] and [DNA] (left y-axes). RNA/DNA ratios (panels G-I) were also calculated. In all panels, error bars represent standard error of the mean, and capitalized letters correspond to Tukey's HSD groups ($\alpha < 0.05$).

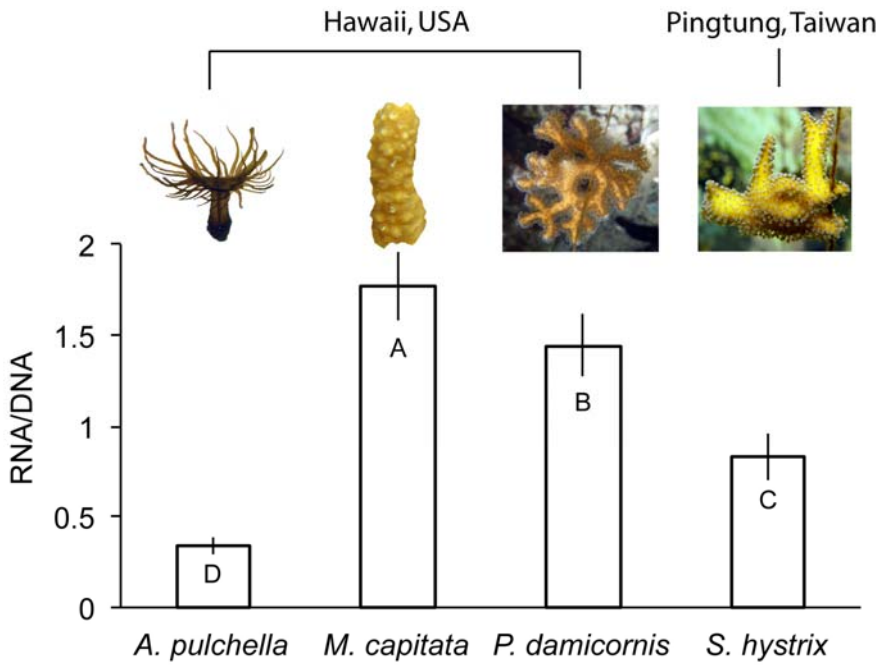

Fig. 4. RNA/DNA ratios of four endosymbiotic anthozoans of the Indo-Pacific. Data from Figs. 2 and 3 were pooled over time and across replicates to calculate global mean RNA/DNA ratios for each species. There was a statistically significant effect of species (one-way ANOVA on ranks, df = 3, F = 199, $p < 0.00001$), and the capitalized letters within the columns correspond to Tukey's HSD groups ($\alpha < 0.05$). Error bars represent standard deviation.

Discussion

Given that *Symbiodinium* actively photosynthesize, grow, and divide within coral and anemone gastrodermal cells, it was hypothesized that the biological composition of these holobionts could vary not only upon exposure to environmental changes (i.e., bleaching; Douglas, 2003), but also across diel cycles in response to dinoflagellate or host cell division (Wang et al., 2008). For instance, while *Symbiodinium* mitosis would lead to a doubling of *Symbiodinium* DNA, the host cell may also divide at such times in order to diminish the cellular spatial constraints driven by housing two or more dinoflagellates within a single cell (Mayfield and Gates, 2007). In fact, most coral and sea anemone gastrodermal cells possess only one *Symbiodinium* (Muscatine et al., 1998), and even in these symbiosomes, the host's cytoplasmic allotment is small (Huang et al., 2008). In short, *Symbiodinium* cell division *in hospite* could also drive

increases in host DNA content, and such would lead to a dramatic increase in the total DNA quantity of the holobiont at such times. As such, this discussion will attempt to integrate the molecular composition data analyzed herein with previously published findings on *Symbiodinium* [DNA] derived from real-time PCR data (Mayfield et al., 2009; 2010) to determine whether *Symbiodinium* cell division indeed influences the overall biological composition and biomass ratios of anthozoan-dinoflagellate holobionts.

Given the massive genomes of dinoflagellates relative to those of animals (Stat et al., 2006), endosymbiotic anthozoans would be expected to have significantly higher concentrations of DNA relative to apo- or asymbiotic con-specifics. Such was, however, not documented herein. Specifically, anemones infected with *Symbiodinium* did *not* possess higher levels of total DNA relative to uninfected controls. In fact, total DNA of aposymbiotic anemones was nearly identical to those of anemones that had been infected with *Symbiodinium* for three or six weeks. On the other hand, the RNA content of the anemone holobionts significantly decreased after infection with *Symbiodinium*, suggesting that *Symbiodinium* may be less transcriptionally active than the hosts in which they reside. In support of this notion, the RNA/DNA ratio of the anemone holobiont also decreased after infection with *Symbiodinium*. Collectively, these data suggest that, while the DNA content of aposymbiotic and endosymbiotic anemones is similar, endosymbiotic anemones display lower levels of total gene expression than uninfected anemones. Whether this decrease in gene expression is more greatly attributed to the host or the endosymbionts remains to be proven, though Mayfield et al. (2011) suggested that *Symbiodinium* may contribute only 10% of the RNA transcripts in the coral *S. hystrix*, and so it is likely that the host anemone, in this case, is a larger contributor to the variation in the RNA content over time.

Unlike the RNA data, total protein increased after three weeks of infection with *Symbiodinium*, though reduced to the same levels as those of aposymbiotic controls after six weeks. This may suggest that, while the *Symbiodinium* were not transcriptionally active to a high degree, they did produce more proteins in the earlier stages of infection. The host anemones likely also translated higher levels of proteins during this period given the significant modification of the gastrodermal cell architecture that is required to accommodate *Symbiodinium* (Mayfield and Gates, 2007), which occupy nearly the entire volume of the host cell. After approximately one month of infection, the endosymbiosis is characterized by peak densities of *Symbiodinium* (Smith and Muscatine, 1999), and so it is likely that, at that time, protein expression returned to baseline

levels for each partner. Thus, the 3-week sampling time might reflect a period in which the endosymbiosis has not yet stabilized with respect to the biomass ratio of host: *Symbiodinium*, while at the 6-week sampling time, such a equilibrium point might indeed have been reached. It should be noted, that while total protein expression levels were similar between uninfected anemones and those infected with *Symbiodinium* for six weeks, expression of specific proteins, particularly those involved in osmoregulation (Mayfield and Gates, 2007) and cell adhesion (Chen et al., 2003; Rodriguez-Lanetty et al., 2006) would likely differ between symbiotic states. Similarly, expression of a variety of *Symbiodinium* genes and proteins has been hypothesized to vary as a function of lifestyle. For instance, while ribulose, 1-5 bisphosphate carboxylase/oxygenase (Rubisco) gene (*rbcL*) expression was expressed at elevated levels during the day in cultured *Symbiodinium*, its expression instead peaked at night in *Symbiodinium* within anemone gastrodermal cells (Mayfield et al., in review).

In contrast to the *A. pulchella* infection experiment, all other data analyzed herein were derived from corals, and no other samples were taken from aposymbiotic specimens. In the case of the Hawaiian reef coral *M. capitata*, biological composition was tracked across a one-month recovery period in nubbins artificially fragmented from adult colonies. It was hypothesized that such forceful removal from the source colony could cause stress in the coral nubbins, which could influence their nucleic acid and protein concentrations. In fact, both total RNA and total DNA varied significantly over time, and, specifically, appeared to decrease over the one-month sampling period. Although real-time PCR-derived *Symbiodinium* genome copy numbers, a proxy for *Symbiodinium* DNA content, were not measured in these samples, it is possible that transient bleaching from the stress induced by tissue damage at the time of fragmentation could have led to the significantly reduced holobiont DNA content measured after 3.25, 21, and 28 days. The anthozoan hosts may also have lost DNA over the course of the recovery period, especially as tissue may have continued to slough off of the nubbins at the site of fracture.

These interpretations would suggest that even nubbins sampled after 28 days had not recovered from fragmentation, as their total levels of DNA were significantly lower than controls. On the other hand, although the total RNA content data may also suggest that both *Symbiodinium* and host cells were lost over the course of the sampling period, total RNA levels measured at the last sampling time were similar to those of the source colonies. This may indicate that the host and *Symbiodinium* had indeed recovered, though targeted gene and protein analyses with real-time

PCR and western blotting, respectively, would be needed to conclusively determine if, for instance, expression of stress-sensitive molecules such as heat shock proteins (HSPs) had returned to baseline levels by the end of the experiment. Given the complexity in the biological composition response across a one-month recovery period of experimentally fragmented corals, it is recommended that those seeking to use coral nubbins as biological replicates in future experiments both allow for sufficient time for recovery, which is currently rarely achieved or proven (e.g., DeSalvo et al., 2008; Leggat et al., 2011), *and* track the biological composition of the samples across the acclimation period in an attempt to document recovery and control for biological composition differences due to tissue damage caused during the nubbin fragmentation process.

Unlike the *A. pulchella* study, in which the biological composition of the anemone holobiont was deliberately modified through infection with *Symbiodinium*, and the *M. capitata* experiment, in which stress was induced by the fragmentation process, in the *P. damicornis* experiment, only the natural variation in biological composition was of interest. In other words, healthy colonies were directly sampled *in situ*, and no experimentation was conducted (Mayfield et al., 2010). In this case, it was found that the RNA content and the RNA/DNA ratio were significantly variable over time, though there was no apparent diel trend in the former. On the other hand, though the notable, ~2-fold peak in the RNA/DNA ratio was not statistically significant in corals sampled at 18:45, such pre-sunset periods have previously been proposed to reflect times at which coral gastrodermal cells undergo substantial molecular and biochemical changes to prepare for the shift from diurnal autotrophy to nocturnal heterotrophy (Levy et al., 2006). As such, future work should attempt to uncover the modulation of gastrodermal cell behavior at times just before and after the onset of photosynthesis to uncover, for instance, how significant cellular activities, such as osmoregulation, are affected by photosynthesis and the light cycle.

Both total RNA and the RNA/DNA ratio varied significantly over time in *P. damicornis*, whereas total DNA did not. Interestingly, Mayfield et al. (2010) documented a 2-fold nocturnal increase in *Symbiodinium* DNA content in these sample samples, suggesting that, although the total DNA concentration of the holobiont was similar over time, the relative proportion of the total DNA of *Symbiodinium* origin was higher at 23:00. This suggests that some host cells were shed from the coral tissues at night. In fact, Baghdasarian and Muscatine (2000) suggested that corals and sea anemones may preferentially release gastrodermal cells with dividing *Symbiodinium* as a

means of control of their *in hospite* dinoflagellate densities. Thus, while elevated *Symbiodinium* DNA content was measured at night, the total DNA concentration of the holobiont may not have changed in tandem due to a release of some gastrodermal cells from the tissue layer. Alternatively, epidermal, gastrodermal, or *Symbiodinium* cells could also have been released at night due simply to senescence, and the doubling of *Symbiodinium* DNA content in non-senesced cells could have masked molecularly-based detection of this phenomenon. Similar conjectures could be made to explain the *S. hystrix* results; while neither total RNA, total DNA, nor the RNA/DNA ratio changed significantly over diel cycles in samples from duplicate mesocosms of Taiwan's NMMBA, a 2-fold nocturnal increase in *Symbiodinium* DNA content was measured in these same samples (Mayfield et al., 2010). Given that *S. hystrix* and *P. damicornis* are both pocilloporids, it is possible that they share a similar mechanism of controlling *in hospite Symbiodinium* densities. Future work should attempt to investigate the molecular mechanisms underlying the dynamic turnover of *Symbiodinium* cells within the anthozoan gastrodermal tissue layer.

Interestingly, although total RNA and RNA/DNA ratios were temporally variable in the anemone *A. pulchella* and the corals *M. capitata* and *P. damicornis*, these parameters did not vary significantly over time in *S. hystrix*. This could be due to the fact that the latter samples were incubated within mesocosms with relatively stable and shaded diel light regimes. Furthermore, the *A. pulchella* and *M. capitata* samples were experimentally modified, whereas *P. damicornis* samples were taken directly off the reef. As such, only *S. hystrix* samples were both un-manipulated and sampled from an environment with stable seawater quality, and these two factors could have led to the similar levels in total gene expression documented over time. That being said, expression of three cytoskeleton genes were found to vary significantly over time in these same samples (Mayfield et al., 2010), and the authors suggested that such was due either to the elevated nocturnal *Symbiodinium* densities described above or a reduction in osmotic pressure stemming from nocturnal photosynthetic quiescence. In short, only light varied significantly over time in the *S. hystrix* experiment (data not shown), and the more stable environment in which the samples lived may have led to the lack in statistically significant variation in total RNA measured over diel cycles.

The stability of the mesocosm environments in which the *S. hystrix* samples were reared may also have influenced the overall RNA/DNA ratio averaged across all 90 samples, which was significantly lower than that of the

other two corals. In other words, the two Hawaiian corals living on reef flats characterized by diel changes in seawater quality may have had a greater need to express higher levels of certain suites of genes and proteins at particular times, such as those required for the organismal stress response (e.g., *hsps*/HSPs]). High constitutive expression of *hsp70* mRNAs was indeed found in *S. hystrix* specimens originating from Nanwan Bay, Taiwan (Mayfield et al., 2011).

The notion that less stable environments may demand higher levels of gene expression, as evidenced by the RNA/DNA ratio, could also help explain the significantly reduced ratio averaged across all 45 anemone samples. The anemones sampled in Mayfield et al. (2009), from which the data assessed herein were taken, lived in flasks with filtered seawater for several years prior to experimentation. As such, there was little variation in the salinity, temperature, or even light, as they were kept in the dark to remain aposymbiotic. While samples used in the experiment were kept in the light, all other aspects of their abiotic environment were tightly controlled, and such stability may have allowed for the minimal arsenal of housekeeping genes to be expressed at any given sampling time. As such, the average RNA/DNA ratio of the anemones, which was approximately 4-fold lower than those of the three corals, could be attributed to the simplicity and stability of their culture environments, which, furthermore, did not even allow them to come into contact with other life forms, as was possible in the coral studies.

Although a plethora of hypotheses can be made as to why the changes in nucleic acid and protein concentrations over time documented herein occurred in each of the four sampled taxa, it is difficult to conclusively understand the molecular mechanisms underlying such variation in biological composition without a better knowledge of the expression of the myriad macromolecules necessary to enact such changes in, for instance, rates of *Symbiodinium* cell division. Therefore, it is hoped that future work will attempt to determine the sub-cellular regulation of the biological composition of the anthozoan-dinoflagellate holobiont. What *can* be stated with confidence is that such changes in global molecular concentrations have significant implications for molecular diagnostics of reef-building corals and other endosymbiotic marine creatures. Specifically, it has been argued for a number of years that the biological ratio of host: endosymbionts must be approximated and used as a normalizing agent for gene and protein expression data (e.g., Mayfield et al., 2012), whereas it is further advocated herein that not only *Symbiodinium* DNA content, but also holobiont [RNA], [DNA], and [protein] be measured in samples to be used for health assessment. These

metrics may, indeed, be useful not only for normalization of macromolecular expression data, but could be interesting parameters on their own. For instance, a decrease in the RNA/DNA or protein/DNA ratio could reflect metabolic suppression, whereas the opposite trend could indicate a stress response. As importantly, the temporal variation in RNA, DNA, and protein content in even healthy corals and sea anemones demonstrated herein suggests that the molecular quality control steps undertaken herein and in previous studies (Mayfield et al. 2009; 2010; 2011) with respect to understanding the contribution of each member to the total RNA, DNA, and protein pools, respectively, are essential even in analyses of samples that appear healthy to the naked eye and have perceivably similar ratios of anthozoan host to dinoflagellate endosymbiont. In conclusion, only by comprehensively understanding the temporal variation in both the ratio of host: *Symbiodinium* and the nucleic acid and protein concentrations of the anthozoan-dinoflagellate holobiont can macromolecular expression data be normalized within a framework that allows for the generation of accurate data that can be used to calculate an index of health for, for instance, a reef-building coral exposed to GCC scenarios.

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印度太平洋四珊瑚綱-雙鞭毛藻共生體核苷酸及蛋白質比例隨時間改變對分子檢測法的意義

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摘　要

由於全球氣候變遷的影響，主要組成珊瑚礁的珊瑚蟲及共生藻的共生關係正在快速的改變。因此，瞭解對環境改變極度敏感的珊瑚蟲及共生藻生理上如何應對快速氣候變遷爲非常急迫的議題。但是，目前對於珊瑚蟲的瞭解依然十分貧乏。尤其是如何與共生藻建立並維持其共生關係依然缺乏深入的瞭解。舉例來說，近來始有研究指出共生關係中的分子成份會隨著時間改變；詳細來說，共生藻的 DNA 數量會隨著亮暗週期而改變，而此現象會影響基因表現的測量。由於上述理由，有需要同時瞭解分子和蛋白質成份在珊瑚、海葵共生體中隨時間、空間以及物種間的改變。在分析已發表和未發表的數據後，發現總 RNA、DNA、蛋白質數量在夏威夷採集的疣表孔珊瑚 （*Montipora capitata*) 、細枝鹿角珊瑚（*Pocillopora damicornis*）、美麗海葵（*Aiptasia puchella*）和臺灣海域中的尖支列孔珊瑚（*Seriatopora hystrix*）中會隨著時間變動。更進一步，可作爲基因表現指標的 RNA/DNA 比例和蛋白質表現指標的蛋白質/DNA 比例亦隨著時間而變動。此現象可能由於共生藻密度隨時間改變而造成。綜合上述資料指出在利用分子技術進行珊瑚礁的檢測時，對於數據的品質控管十分的重要；以防止在利用分子生物指標時造成偏差。

關鍵詞：珊瑚綱，珊瑚，細胞內共生，基因表現，全球氣候變遷，分子生物，共生藻（*Symbiodinium*）。