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Relationships Between Host and Symbiont Cell Cycles in Sea Anemones and Their Symbiotic Dinoflagellates

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Abstract. The processes by which cnidarians and their algal endosymbionts achieve balanced growth and biomass could include coordination of host and symbiont cell cycles. We evaluated this theory with natural populations of sea anemones hosting symbiotic dinoflagellates, focusing on the temperate sea anemone *Anthopleura elegantissima* symbiotic with *Symbiodinium muscatinei* in Washington State, USA, and the tropical anemone *Stichodactyla helianthus* associating with unknown *Symbiodinium* spp. in Belize. By extruding symbiont-containing gastrodermal cells from the relatively large tentacles of these species and using nuclear staining and flow cytometry, we selectively analyzed cell cycle distributions of the symbionts and the host gastrodermal cells that house them. We found no indications of diel synchrony in host and symbiont G2/M phases, and we observed evidence of diel periodicity only in *Symbiodinium* spp. associated with *S. helianthus* but not in the anemone itself. Seasonally, *S. muscatinei* showed considerable G2/M phase variability among samples collected quarterly over an annual period, while the G2/M phase of its host varied much less. Within samples taken at different times of the year, correlations between host and symbiont G2/M phases ranged from very weakly to very strongly positive, with significant correlations in only half of the samples (two of four *A. elegantissima* samples and one of two *S. helianthus* samples). Overall, the G2/M phase relationships across species and sampling periods were positive. Thus, while we found no evidence of close cell cycle coupling, our results suggest a loose, positive relationship between cell cycle processes of the symbiotic partners.

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Introduction

Symbioses with algae that subsidize their hosts' energy demands are widespread among cnidarians living in shallow tropical and temperate habitats throughout the world. Dinoflagellates of the genus *Symbiodinium* are by far the most common cnidarian symbionts and are located within the host's gastrodermal cells, surrounded by a host-derived membrane known as the symbiosome (Wakefield and Kempf, 2001). Within this environment, largely stable populations of endosymbionts are maintained by several processes that remain only partially understood (Davy *et al.,* 2012). These processes generally include restriction of symbiont proliferation and removal of excess symbionts *via* expulsion and degradation (Muscatine and Pool, 1979; Davy *et al.,* 2012). The high proliferation potential of the symbionts, inferred by their higher growth rates in culture than *in hospite* (Wilkerson *et al.,* 1988; Falkowski *et al.,* 1993; Smith and Muscatine, 1999), appears to require considerable regulation of their numbers by the host. It should also be noted, however, that the presence of symbionts may manipulate host cell proliferation, but this has received less attention (Rodriguez-Lanetty *et al.,* 2006; Davy *et al.,* 2012).

An early suggestion of host control over symbiont populations was the observation of remarkably similar areal densities of *Symbiodinium* in several tropical coral species across a range of depths (Drew, 1972). Tropical reef corals are now known to undergo seasonal variations in symbiont areal density that are environmentally linked (Brown *et al.,*

1999; Fitt *et al.*, 2000), but these fluctuations are often closely related to changes in areal host tissue biomass (Thornhill *et al.,* 2011), suggesting a connection between host and symbiont growth. Similarly, in the temperate sea anemone *Anthopleura elegantissima*, host biomass-specific symbiont density changes little despite significant seasonal variation in light and temperature, and host biomass and symbiont mitotic index show parallel seasonal changes that are suggestive of coupled growth (Dimond *et al.,* 2011). Coupled growth of the two symbiotic partners is also supported by observations that the fastest growing apical regions of branching acroporid corals have *Symbiodinium* populations with higher division frequencies than those in older basal regions (Wilkerson *et al.,* 1988; Gladfelter *et al.,* 1989; Jones and Yellowlees, 1997), and similarly, that younger anemone polyps have symbionts with higher division frequencies than those in older polyps (Smith, 1986). One explanation for coupled growth comes from a few studies showing that symbiont proliferation depends on host feeding (McAuley, 1985; Smith and Muscatine, 1999; Fitt, 2000; Fitt and Cook, 2001) and cellular growth (McAuley, 1985; Fitt, 2000). On the other hand, the observation that seasonal increases in symbiont density in Caribbean corals precede increases in host tissue biomass suggests that symbiont energetic contributions may also drive coupling between host and symbiont growth (Fitt *et al.,* 2000). Rather than being contradictory, these two alternative explanations (host feeding *vs.* symbiont photosynthesis) for drivers of positive relationships between host and symbiont growth and biomass may in fact be complementary and simply reflect the mutualistic nature of these symbioses.

As with many fundamental aspects of the cell biology of cnidarian-algal symbioses, progress in understanding the extent of coordination between host and symbiont cellular growth has been slow (Edmunds and Gates, 2003; Weis *et al.,* 2008; Davy *et al.,* 2012). To date, only three studies—two involving the *Hydra-Chlorella* symbiosis (McAuley, 1981, 1985) and the other involving a marine hydroid with *Symbiodinium* (Fitt, 2000)—have directly compared host and symbiont cellular growth, assessing division of both the symbionts and the host cells that house them. Similar studies with anthozoans, the most common and widespread cnidarian hosts of algal endosymbionts, are lacking. To further our understanding of coordination between symbiotic partners in cnidarian-algal symbioses, we tested for positive relationships between host and symbiont cell cycles among wild populations of sea anemones. We focused our study on the temperate sea anemone *Anthopleura elegantissima* in Washington State, USA, and the tropical anemone *Stichodactyla helianthus* in Belize, collecting samples during different seasons for comparison. We extracted symbiont-containing gastrodermal cells from the relatively large tentacles of these sea anemones and used nuclear staining and flow cytometry to measure cell cycle distributions of the symbionts and their host cells.

Materials and Methods

Sample collection

On Fidalgo Island, Washington, USA (48°30.089′N, 122°41.474′W), a population of *Anthopleura elegantissima* (Brandt, 1835) hosting *Symbiodinium muscatinei* (*Symbiodinium* B4, LaJeunesse, 2001) in several shallow mid-intertidal pools was sampled over the course of a year during low tides in August 2011, November 2011, February 2012, and May 2012 ($n = 9–11$ per sample). Samples were obtained in the morning, except in February when they were taken in the early afternoon. On Carrie Bow Cay, Belize (16°48.176′N, 88°4.927′W), *Stichodactyla helianthus* (Ellis, 1768) was sampled from a shallow (1–2-m depth) backreef habitat in December 2011 and June 2012 ($n = 9–10$ per sample). These samples were taken in the late afternoon on both occasions. Although we did not identify the *Symbiodinium* type associated with *S. helianthus*, Finney *et al.* (2010) reported *Symbiodinium* type A4a from *S. helianthus* collected a decade ago at similar depths around Carrie Bow Cay, and the same symbiont type in *S. helianthus* collected in Barbados in 2005. For collections of both sea anemone species, individuals were chosen haphazardly within an area of approximately 2500 m^2, and 4–5 tentacles from each anemone were excised with scissors and placed into individual microcentrifuge tubes.

To evaluate potential diel synchrony in host-symbiont cell cycle patterns in the *A. elegantissima–S. muscatinei* symbiosis and in the *S. helianthus–Symbiodinium* sp. symbiosis, we haphazardly collected tentacle samples from 3–5 individuals at 6-h intervals over an 18-h period between 1800 and 1200 h the following day. For this analysis, *S. helianthus* was sampled in June 2012 and *A. elegantissima* was sampled in October 2012. We then sampled these anemone populations a second time in June 2013 (*S. helianthus*) and August 2013 (*A. elegantissima*) to capture a full 24-h period with a 4-h sampling frequency.

Sample preparation

Tentacle samples were brought back to the laboratory for cell extraction and fixation within 1 h of collection. To extract symbiont-containing gastrodermal cells, tentacles were placed on a microscope slide and gripped with forceps at the tip while a dental pick or the flat edge of another set of forceps was used to squeeze the gastrodermal cells out of the severed end of the tentacle. When sufficient cells (approximated based on the amount of golden-brown symbiont pigmentation evident on the slide) were obtained, a 22-gauge syringe was used to aspirate the cells from the slide and decant them into a clean microcentrifuge tube filled

with 1 ml of 70% ethanol, which served as both a fixative and a solvent to extract pigments from the symbionts. To mechanically dissociate symbionts from host gastrodermal cells and release the host cell nuclei, cell suspensions were repeatedly forced through the syringe. The fixed cell suspensions were kept in 70% ethanol for up to one week before analysis. Upon analysis, cells were pelleted with a microcentrifuge at 2000 $\times$ *g* for 3 min and the ethanol was decanted. The cells were then resuspended in RO water with 0.1% Triton X-100 (to discourage particle clumping and facilitate cell lysis and membrane permeabilization) and again repeatedly forced through a syringe. After 5 min in this suspension, cells were pelleted again and then resuspended for a second time in 70% ethanol for 30 min to remove any remaining symbiont pigments. After a final centrifugation step, the ethanol was decanted and cells were resuspended in 1 ml of a staining solution consisting of 0.01% Triton X-100 in RO water with RNase A (150 μg ml^{-1}) and either propidium iodide or ethidium bromide (10 μg ml^{-1}). These stains produced nearly identical results and the choice of stain was based on availability. Cell and particle clumping was minimized by repeatedly forcing the suspension through a syringe.

We verified that our preparation method yielded stained, intact *Symbiodinium* and sea anemone cell nuclei by viewing a subset of samples under epifluorescence microscopy. Early experimentation with aposymbiotic *Aiptasia* spp. confirmed that sea anemone nuclei were brightly stained particles typically 2–3 μm in diameter present in gastrodermal cell preparations using the methods described above. We reasoned that it was highly unlikely that these particles were prokaryotes because we observed considerably dimmer particles in the suspensions that were more likely to be prokaryotes, and the largest known prokaryote genome is over 16 times smaller (and would emit correspondingly dimmer nuclear staining) than that of the sea anemone *Nematostella vectensis* (Chang *et al.,* 2011; Gregory, 2012).

Passage of cell suspensions through a syringe was essential to avoid excessive particle aggregation, particularly among the host cell nuclei (Fig. 1A, B). We evaluated control samples without mechanical disruption with the syringe and found that aggregation of host cell nuclei was too excessive to permit analysis. We were, however, able to compare *Symbiodinium* with and without syringe use and found no significant differences in cell cycle distributions (paired Student's *t*-test, $n = 4$, $P > 0.05$ for all phases). Extracting only anemone gastrodermal cells, as opposed to homogenizing whole anemone tissues, had the additional benefit of minimizing cellular debris, which was particularly important for flow cytometry. We also found that using RO water as the staining medium instead of seawater or a buffered saline solution produced the best nuclear staining. This is likely due to the sensitivity of nuclear stains to high ionic strength solutions (Marie *et al.,* 1996).

Flow cytometry and cell cycle analysis

Samples were analyzed by flow cytometry after at least 30 min in the staining solution. We used a BD FACScalibur flow cytometer equipped with a 488-nm laser to analyze relative cellular DNA content. Regardless of the stain used, DNA fluorescence was acquired using the FL2 (585/42 band pass) detector with linear amplification. By varying the detector voltage, we were able to distinguish symbiont cells and host cell nuclei in a single sample (Fig. 1C–J). In all cases, *Symbiodinium* spp. had stronger FL2 fluorescence than anemone nuclei, so we simply increased FL2 detector voltage to visualize and acquire host nuclei after *Symbiodinium* cells had been acquired in a separate data file. Higher fluorescence of *Symbiodinium* spp. compared to anemone nuclei is consistent with the larger genome sizes of symbiotic dinoflagellates relative to sea anemones (LaJeunesse *et al.,* 2005; Gregory, 2012). Some FL2 overlap of host nuclei and symbiont cells occurred among *S. helianthus* samples, which was addressed by subtracting a well-defined *Symbiodinium* gate on the forward-scatter *versus* side-scatter plot (Fig. 1G–J). All samples were screened for doublets on an FL2-Area *versus* FL2-Width plot. Approximately 10,000 symbiont cells or anemone cell nuclei were acquired for each sample. Cell cycle distributions were visualized as histograms of FL2 pulse area, and the distributions of G1, S, and G2/M cell cycle phases were modeled in Cyflogic 1.2 (CyFlo Ltd., Turku, Finland). The G2/G1 ratio was 2 for host cell nuclei, while for symbiotic dinoflagellates it was about 1.8. This low ratio may be due to the highly condensed nature of dinoflagellate chromosomes and its potential effect on nuclear staining efficacy (Bhaud *et al.,* 2000; Wong and Kwok, 2005).

Statistical analyses

We focused our analyses on the percentages of host and symbiont cells in the G2/M phase, which includes cells that have completed DNA replication and are either preparing for or undergoing division. Temporal differences in G2/M phase percentages were evaluated with ANOVA with time as the factor, following Levene's test of variance homogeneity. Diel G2/M phase data for *A. elegantissima* host cells from October 2012 were inverse-transformed to homogenize variances. Relationships between the percentages of host and symbiont cells in G2/M phase were evaluated with Pearson correlations. All analyses were performed with SPSS Statistics 18, and statistical significance was evaluated at $\alpha = 0.05$.

Results

On the basis of visual assessment alone, host and symbiont G2/M phase in both *Anthopleura elegantissima* and *Stichodactyla helianthus* showed no indication of coordi-

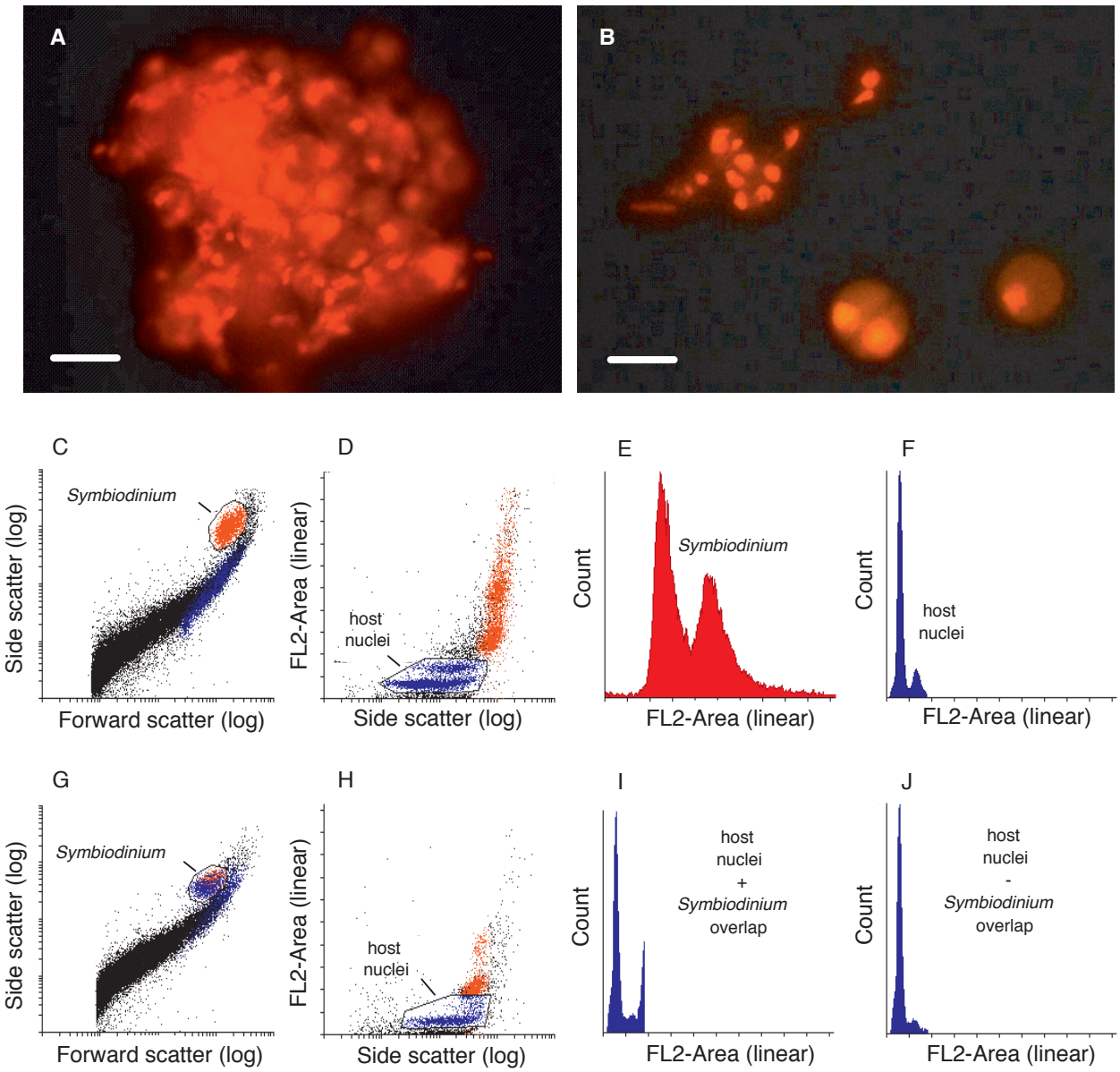

Figure 1. Nuclear staining and flow cytometric analysis methods for sea anemone gastrodermal cell suspensions. Epifluorescence micrographs of PI-stained cell suspensions without (A) and with (B) repeated passage through a 22-gauge needle (scale bars = 10 μm). Needle passage was essential to mechanically dissociate cells and particles, especially host cell nuclei, which had a strong tendency to aggregate (A). Panel (B) shows individual host cell nuclei in the upper left and a dividing and a nondividing *Symbiodinium* cell in the lower right. Cytograms of *Anthopleura elegantissima–Symbiodinium muscatinei* suspensions (C–F) show *Symbiodinium* gating on the forward-scatter *vs.* side-scatter plot (C), host cell nuclei gating on the side-scatter *vs.* FL2 pulse area plot (D), and their respective histograms (E, F). Note lack of FL2 fluorescence overlap between the two cell and nucleus types. This contrasts with the analysis of *Stichodactyla helianthus–Symbiodinium* sp. suspensions (G–J), which had a clearly defined *Symbiodinium* gate (G), but host nuclei gating was more complex on the side-scatter *vs.* FL2 pulse area plot due to overlap with *Symbiodinium* (H, I). This was resolved by subtracting the *Symbiodinium* gate in (G) from the host nuclei gate in (H), with the resulting histogram shown in (J). Note that during actual analyses, by convention, FL2 voltage was always adjusted to center the G1 peak at approximately 200 relative fluorescence units.

nated diel cycling (Fig. 2A–D). *A. elegantissima* host and symbiont G2/M phases (Fig. 2A, B) were largely stable throughout the day during both sampling periods, with no significant temporal variation in October 2012 (host: $F_{3,15} = 1.07$, $P = 0.39$; symbiont: $F_{3,15} = 0.63$, $P = 0.61$) or August 2013 (host: $F_{6,21} = 1.67$, $P = 0.18$; symbiont:

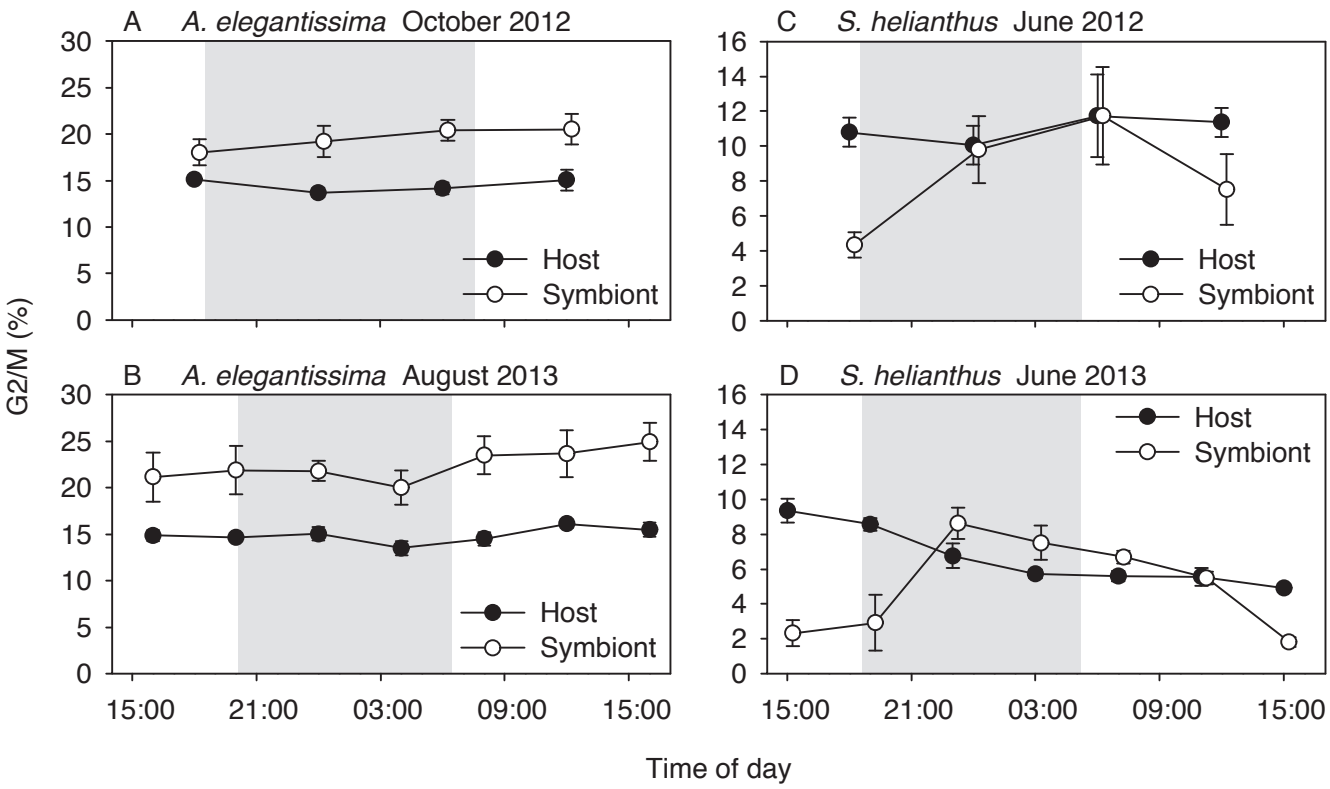

Figure 2. Diel patterns (means ± SE) of host and symbiont cell cycles in (A, B) *Anthopleura elegantissima* symbiotic with *Symbiodinium muscatinei* (n = 4–5 anemones) and (C, D) *Stichodactyla helianthus* symbiotic with unknown *Symbiodinium* sp. (n = 3–4 anemones). *A. elegantissima* was sampled in October 2012 and August 2013 and *S. helianthus* was sampled in June 2012 and June 2013. The nighttime period between sunset and sunrise is shown with a gray background.

$F_{6,21} = 0.45$, $P = 0.84$). *S. helianthus* (Fig. 2C, D) host cell G2/M phase was temporally stable in June 2012 ($F_{3,10} = 0.32$, $P = 0.81$), but not in June 2013 ($F_{6,19} = 13.85$, $P < 0.001$), when it showed a general decline during the 24-h period. In contrast, the symbionts within *S. helianthus* showed G2/M phase diel cycling on both occasions, with distinct peaks at night or just after dawn. There was no significant temporal variation in June 2012 ($F_{3,10} = 2.17$, $P = 0.16$); however, effect size ($\eta^2 = 0.39$) was moderate for this analysis, indicating that 39% of the variance in symbiont G2/M phase was accounted for by time of day. In June 2013, diel variation was significant ($F_{6,19} = 26.47$, $P < 0.001$).

A. elegantissima host and symbiont cell cycle distributions varied among sampling dates (Fig. 3), with both partners showing significant differences in percentages of G2/M phase nuclei between sampling dates in different seasons (host, $F_{3,37} = 13.5$, $P < 0.001$; symbiont, $F_{3,37} = 64.8$, $P < 0.001$). Both partners had the highest percentages of G2/M phase nuclei in August (summer). *S. muscatinei* (Fig. 3B, D) exhibited considerably more variability between sampling dates than did its host (Fig. 3A, C), showing over twice the percentage of G2/M phase cells in August (summer) as in all other months, and very high percentages of S phase cells in May (spring) compared to all other months. Within sampling months, both partners exhibited little individual variation. Correlations of host and symbiont G2/M phase analyzed by month showed significant positive correlations in August (summer; $R = 0.831$, $P = 0.003$) and May (spring; $R = 0.734$, $P = 0.01$), while November (fall; $R = 0.092$, $P = 0.801$) and February (winter; $R = 0.148$, $P = 0.684$) were only weakly positive and non-significant (Fig. 4). When considered as a whole, the seasonal data indicate an overall positive relationship between host and symbiont G2/M phase.

Host and symbiont cell cycle distributions in *S. helianthus* (Fig. 5) also differed significantly between sampling months, with higher percentages of G2/M phase cells and nuclei in June (summer) than in December (winter) (host, $F_{1,17} = 14.7$, $P = 0.001$; symbiont, $F_{1,17} = 8.46$, $P = 0.01$). Correlations of G2/M phase for the two partners were positive and significant in December (winter; $R = 0.731$, $P = 0.016$), but only weakly positive and non-significant in June (summer; $R = 0.028$, $P = 0.943$) (Fig. 6).

Collectively, host-symbiont G2/M phase percentages across species and sampling periods exhibited an overall

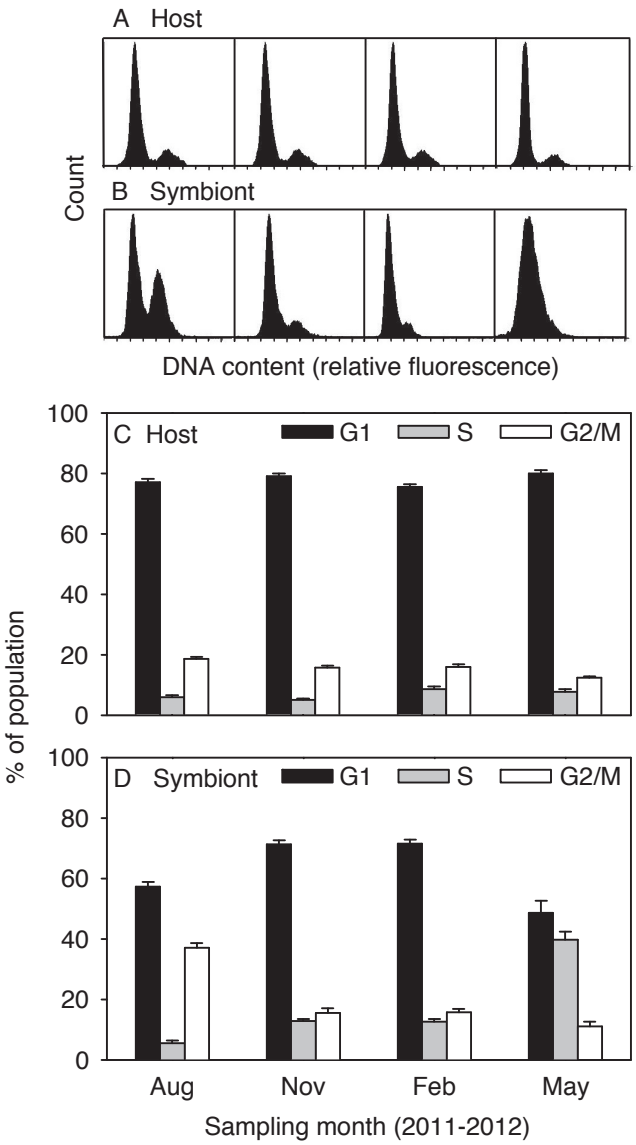

Figure 3. Cell cycle distributions for *Anthopleura elegantissima–Symbiodinium muscatinei* samples taken quarterly over an annual period. Top panels (A, B) show representative histograms from host and symbiont; bottom panels show mean (± SE) percentages of cells in each cell cycle phase (n = 9–11 anemones).

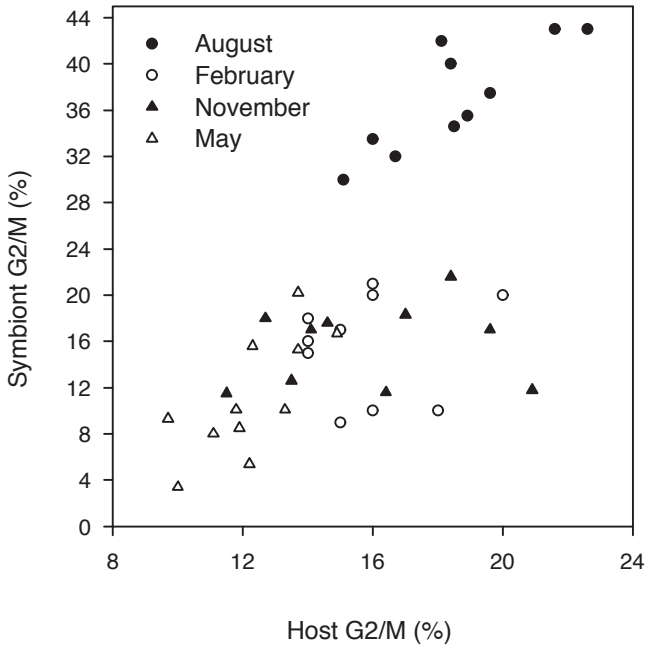

Figure 4. Correlations between host and symbiont G2/M phase percentages for *Anthopleura elegantissima–Symbiodinium muscatinei* samples taken quarterly over an annual period (n = 9–11 anemones). Each point represents an individual host-symbiont pair.

positive relationship (Fig. 7). Compared to *A. elegantissima–S. muscatinei, S. helianthus* and its symbionts had G2/M phase percentages that were considerably lower. While many host-symbiont G2/M phase averages were located near the line of equality, *A. elegantissima–S. muscatinei* samples in August were a notable exception, with disproportionately high G2/M phase proportions in the symbiont compared to those of its host.

Discussion

We found only partial evidence for close, positive relationships between host and symbiont cell cycles in *Anthopleura elegantissima* and *Stichodactyla helianthus*. We did not observe coupled diel cycling between host and symbiont cell cycles in either sea anemone species, and we found significant positive correlations between partner cell cycles in only half of the samples taken in different seasons (two of four *A. elegantissima* and one of two *S. helianthus* samples). However, overall patterns across seasons and species suggested a generally positive relationship between host and symbiont G2/M phases. While these data are limited in their temporal scope and do not address potential differences in cell cycle duration, they suggest that cnidarian-algal cell cycles are loosely coordinated, with the degree of coordination perhaps influenced by variable environmental factors such as nutrients and food availability. Although nutrient supplementation can increase symbiont growth and population density, particularly in low-nutrient tropical waters (Muscatine *et al.,* 1989, 1998), a nutrient-independent constraint on symbiont cell cycle progression beyond the G1 phase within the host has been found by Smith and Muscatine (1999). Those authors determined that the G1 phase of *Symbiodinium* sp. is dramatically lengthened *in hospite* compared to in culture, and that host feeding shortens the G1 phase and promotes cell cycle progression much more strongly than does N and P supplementation. Host feeding therefore appears to be required for sustained symbiont proliferation and retention (McAuley, 1985; Smith and Muscatine, 1999; Fitt and Cook, 2001), supporting the

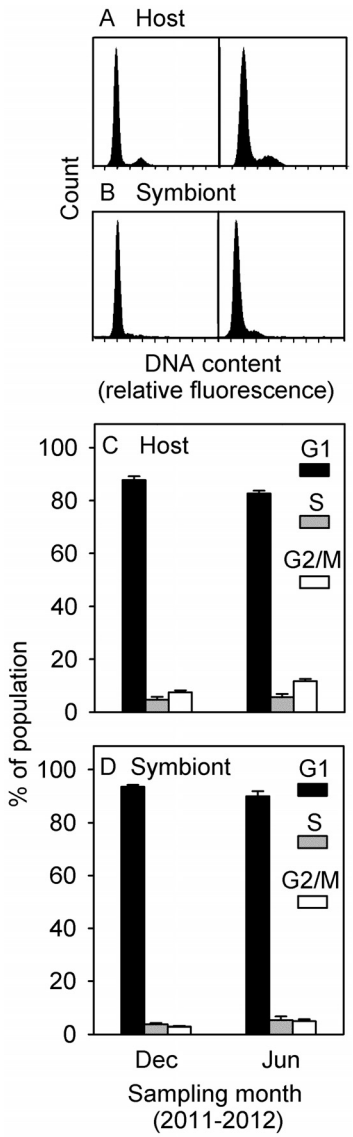

Figure 5. Cell cycle distributions for *Stichodactyla helianthus–Symbiodinium* sp. samples taken during summer and winter. Top panels (A, B) show representative histograms from host and symbiont; bottom panels show mean (± SE) percentages of cells in each cell cycle phase (n = 9–10 anemones).

hypothesis that symbionts are primarily space-limited and that the environment of the host cell sets the upper limit on *in hospite* symbiont population density (Jones and Yellowlees, 1997).

Given the strong evidence that the nutritional status and growth of the host and symbiont are linked, how can we explain the inconsistent strength of host–symbiont G2/M phase correlations? Temporal lags between the proliferation of the two symbiotic partners are a likely explanation. Even where some evidence of synchrony between host and symbiont cell cycles has been found, a temporal disconnect has been observed (McAuley, 1981, 1985; Fitt, 2000). Fitt

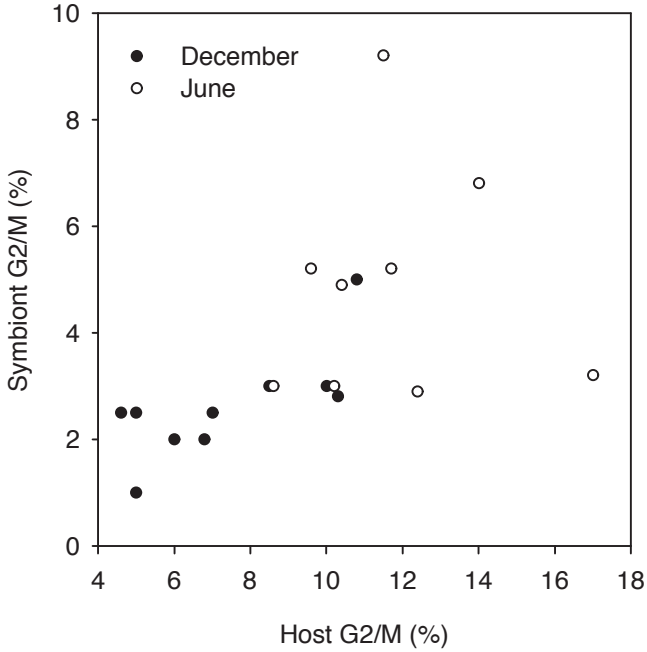

Figure 6. Correlations between host and symbiont G2/M phase percentages for *Stichodactyla helianthus–Symbiodinium* sp. samples taken during summer and winter (n = 9–10 anemones). Each point represents an individual host-symbiont pair.

(2000), for example, found evidence for phased division in both host and symbiont cells of the hydroid *Myrionema ambionense*, but whereas host cell division peaked in the middle of the night, symbiont cell division peaked in the

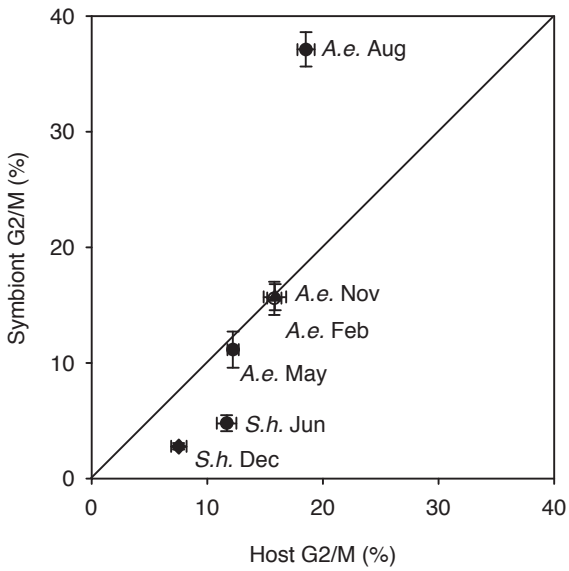

Figure 7. Overall relationship between host and symbiont G2/M phase percentages for all species and time points examined, showing means (± SE) for each sample (n = 7–11 anemones). *A.e.* = *Anthopleura elegantissima, S.h.* = *Stichodactyla helianthus.*

early morning. McAuley (1985) documented close coupling of host and symbiont division in the *Hydra-Chlorella* symbiosis when the host was well fed, but temporal lags occurred if feeding followed prolonged starvation. Both studies (McAuley, 1985; Fitt, 2000) found that host mitosis peaked 12–24 h after feeding, with symbiont mitosis occurring an additional 12–24 h later. These studies suggest that greater frequency of host feeding should be associated with reduced lags between host and symbiont growth. Within this context, therefore, it is noteworthy that the strongest host–symbiont G2/M phase correlations we found in *A. elegantissima* occurred in May and August, times of the year when *A. elegantissima* in Washington State is typically growing and food is plentiful (Sebens, 1982, 1983).

Phased cell division over a diel period, typically with a peak in the early morning, is a prominent feature of many algal cells, including *Symbiodinium* spp. (Fitt and Trench, 1983; Smith and Muscatine, 1999; Wang *et al.,* 2008). These patterns are primarily light-driven in cultured *Symbiodinium* spp. (Fitt and Trench, 1983; Wang *et al.,* 2008), but they may be driven by both light and host feeding for symbionts *in hospite* (Fitt, 2000). Host cells of the reef coral *Acropora cervicornis* and the hydroid *Myrionema ambionense* have also been reported to have phased cell division patterns, both with peaks in the middle of the night, perhaps as a result of feeding cycles (Gladfelter, 1983; Fitt, 2000). The only instance where we observed any indication of a phased cell cycle was in *Symbiodinium* sp. associated with *S. helianthus* (Fig. 2), but the anemone host cells showed no evidence of phasing. *Symbiodinium* spp. living *in hospite* sometimes exhibit dampened diel patterns or a complete absence of phasing (Wilkerson *et al.,* 1983, 1988; Smith and Muscatine, 1999). This was the case for *S. muscatinei*, which is corroborated by previous reports of a stable diel mitotic index in this symbiont (Wilkerson *et al.,* 1983; Verde and McCloskey, 1996). The nutrient-replete growing conditions typical of temperate waters (Jensen and Muller-Parker, 1994; Davy *et al.,* 2006) could be responsible for this stability. For example, Hoegh-Guldberg (1994) reported a dampening of symbiont mitotic diel periodicity when colonies of the coral *Pocillopora damicornis* were incubated in elevated ammonium concentrations. On the other hand, cultured *Symbiodinium* spp. also grow under nutrient-replete conditions yet still show diel periodicity (Smith and Muscatine, 1999; Wang *et al.,* 2008). We hypothesize that diel stability in *S. muscatinei* could also be a result of (1) preferential expulsion of symbionts in the latter stages of the cell cycle that could dampen diel periodicity (McCloskey *et al.,* 1996; Baghdasarian and Muscatine 2000), or (2) the longer duration of the cell cycle in *S. muscatinei* compared to symbionts in warmer tropical environments (Wilkerson *et al.,* 1983, 1988; McCloskey *et al.,* 1996; Verde and McCloskey, 2007). These hypotheses are discussed further below.

In contrast to its largely stable diel cell cycle, *S. muscatinei* cell cycle characteristics varied considerably among samples taken quarterly over an annual period. This high degree of variation also contrasted with the relatively small changes in the cell cycle patterns of its host over the same period. G2/M phase ranges (the difference between the highest and lowest measured values) were about 25% and 5% for *S. muscatinei* and *A. elegantissima*, respectively, with G2/M phase percentages of *S. muscatinei* particularly high in August. While the large differences between host and symbiont cell cycle patterns suggests a lack of coordination over longer time scales and the potential for symbiont growth to greatly exceed host growth, other evidence suggests that *A. elegantissima* and *S. muscatinei* biomass and growth are well balanced throughout the year. Symbiont densities normalized to host protein biomass in *A. elegantissima* are largely constant seasonally (Bergschneider and Muller-Parker, 2008; Dimond *et al.,* 2011), and symbiont mitotic index is highest during summer and autumn when host biomass is also highest (Dimond *et al.,* 2011). We suggest two potential explanations for the apparent incongruence between the widely different cell cycle patterns of *A. elegantissima* and *S. muscatinei* and their predominantly stable symbiosis. First, expulsion of symbionts from cnidarian hosts is a common post-mitotic regulatory mechanism that removes excess symbionts, and in *A. elegantissima* it appears to play a prominent role in regulating symbiont densities (McCloskey *et al.,* 1996). Second, the cell cycle data presented here reflect only cell cycle distributions and do not provide any information on cell cycle durations, and thus provide no information on cell proliferation rates. A higher percentage of *S. muscatinei* cells than host cells in G2/M phase is therefore not necessarily an indication of higher proliferation rates of *S. muscatinei* cells. This underscores the need for data on cell cycle durations in cnidarian-algal symbioses; the limited available data pertain only to algal symbionts and have been obtained indirectly through mathematical approximations (*e.g.,* Wilkerson *et al.,* 1983; McCloskey *et al.,* 1996; Smith and Muscatine, 1999).

Lack of information on cell proliferation rates notwithstanding, positive relationships between host and symbiont G2/M phase percentages would indicate host–symbiont growth coordination; again, the fact that we found only partial evidence for this in either *A. elegantissima* or *S. helianthus* suggests incomplete synchrony between partner cell cycles. This loose coordination likely necessitates an important "clean up" role for post-mitotic symbiont regulatory processes such as expulsion and degradation, which in some cases can remove substantial numbers of symbionts on a daily basis (Davy *et al.,* 2012). Indeed, the very existence of these processes is suggestive of imperfect coordination of host and symbiont cellular growth. Moreover, evidence that symbionts can be selectively targeted for

removal in the latter stages of the cell cycle (Baghdasarian and Muscatine, 2000) suggests that expulsion may also function as an effectively pre-mitotic regulatory process. McCloskey *et al.* (1996) documented a disproportionately high mitotic index of expelled *S. muscatinei* in *A. elegantissima*, but provisionally ascribed this phenomenon to post-expulsion release from host control rather than to preferential expulsion of dividing symbionts. Recent experiments in our laboratory with *A. elegantissima* provide more support for a role for preferential expulsion (J.D., Z.R., B.B., unpubl. data).

Temperate and tropical sea anemones live in markedly different environments in terms of light, temperature, nutrients, and food availability—the former two factors being generally higher in the tropics and the latter two being generally higher in temperate regions (Muller-Parker and Davy, 2001). Thus, whereas cellular growth of tropical anemones and their symbionts is probably limited by nutrients and food, temperate hosts and symbionts are more likely to be limited by light and temperature. The higher G2/M phase percentages of host and symbiont cells of *A. elegantissima* compared to those of *S. helianthus* could suggest that *A. elegantissima* and its symbionts experience higher growth than the tropical species due to food and nutrient sufficiency (Jensen and Muller-Parker, 1994; Davy *et al.,* 2006). The G2/M phase of nearly 40% among *S. muscatinei* in August is similar to that observed by Smith and Muscatine (1999) for exponentially growing cultures of *Symbiodinium* sp. Indeed, summer conditions in the Salish Sea are typically ideal for blooms of both micro- and macroalgae as a result of long days, plentiful sunshine and nutrients, and relatively warm temperatures (Nelson *et al.,* 2003; Dimond *et al.,* 2011). Host growth and biomass are also at their peak during this period (Sebens, 1982,1983; Dimond *et al.,* 2011), probably due in part to abundant food. As discussed earlier, this would be expected to further enhance symbiont growth.

Alternatively, or perhaps as a compounding factor, higher G2/M phase percentages of *A. elegantissima* and its symbionts could reflect prolonged cell cycle durations as a result of slowed cellular metabolism due to lower temperatures (Francis and Barlow, 1988). The duration of cytokinesis, for example, is estimated to be over twice as long in *S. muscatinei* associated with *A. elegantissima* as in *Symbiodinium* sp. associated with the tropical jellyfish *Mastigias* sp. (Wilkerson *et al.,* 1983; McCloskey *et al.,* 1996). Estimates of population doubling times for symbiotic dinoflagellates are typically based on mitotic indices and estimated durations of cytokinesis. These doubling time estimates are very sensitive to changes in both parameters and vary widely; calculated doubling times for *Symbiodinium* spp. in tropical hosts range from 4 to 74 days (Wilkerson *et al.,* 1988), and 20 to 320 days for *S. muscatinei* living in *A. elegantissima* (McCloskey *et al.,* 1996; Verde and McCloskey, 2007; Bergschneider and Muller-Parker, 2008). Improved data on the duration of cytokinesis, including its potential dependence on temperature and other factors, would be useful considering the widespread use of just a few estimates of the duration of cytokinesis to calculate symbiont growth rates from mitotic indices.

While we cannot conclude that cnidarian-algal symbioses are likely to achieve balanced growth through close cell cycle coordination, we have found further support for the hypothesis that host and symbiont cellular growth are positively related. It remains clear that the host cell environment imposes limits on symbiont growth, with these limits most closely related to host growth and nutrition (Smith and Muscatine, 1999; Fitt, 2000; Fitt and Cook, 2001). Beyond this, the basis of symbiont growth regulation is still largely unresolved, although some hypotheses include loss of algal photosynthate to support host growth, nutrient limitation, pH oscillations within the host cell, and manipulation of the light environment by host pigments (reviewed by Davy *et al.,* 2012). Even less is known about how the host may be affected by the presence of symbionts, although recent studies suggest that alteration of the sphingosine rheostat in symbiotic hosts may promote host cell survival and tolerance of symbionts (Rodriguez-Lanetty *et al.,* 2006; Detournay and Weis, 2011). Elucidation of the processes that regulate host–symbiont biomass remains essential to our understanding of the fundamental cell biology of cnidarian-algal symbioses.

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