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NOTE

Symbiosis regulation in a facultatively symbiotic temperate coral: zooxanthellae division and expulsion

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Abstract Zooxanthellae mitotic index (MI) and expulsion rates were measured in the facultatively symbiotic scleractinian *Astrangia poculata* during winter and summer off the southern New England coast, USA. While MI was significantly higher in summer than in winter, mean expulsion rates were comparable between seasons. Corals therefore appear to allow increases in symbiont density when symbiosis is advantageous during the warm season, followed by a net reduction during the cold season when zooxanthellae may draw resources from the coral. Given previous reports that photosynthesis in *A. poculata* symbionts does not occur below approximately 6°C, considerable zooxanthellae division at 3°C and in darkness suggests that zooxanthellae are heterotrophic at low seasonal temperatures. Finally, examination of expulsion as a function of zooxanthellae density revealed that corals with very low zooxanthellae densities export a significantly greater proportion of their symbionts, apparently allowing them to persist in a stable azooxanthellate state.

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Introduction

Many anthozoans and some other invertebrates are well known for their endosymbiotic associations with zooxanthellae (*Symbiodinium* sp. dinoflagellates). Living within gastrodermal cells, zooxanthellae utilize host wastes and translocate photosynthetic products to the animal, in some cases fulfilling nearly all of the host's energy demands (Muscatine 1990). Host cells have a flexible, but finite capacity for zooxanthellae, and must therefore either grow additional cells to accommodate dividing symbionts or regulate their numbers (Muscatine et al. 1998). Pre-mitotic mechanisms of zooxanthellae regulation which curb zooxanthellae growth include density-dependent negative feedback via nutrient or space limitation (Jones and Yellowlees 1997), host-induced release of photosynthate from the symbionts (Gates et al. 1995), and manipulation of symbiont cell cycle by the host (Smith and Muscatine 1999). Removal of existing zooxanthellae, or post-mitotic regulation, includes degradation or digestion of symbionts (Titlyanov et al. 1996) and expulsion of intact symbionts (Hoegh-Guldberg et al. 1987).

Zooxanthellae expulsion has been widely documented as an active regulatory mechanism in corals and anemones. Although expulsion may not be equally important among different host species (Hoegh-Guldberg et al. 1987; Baghdasarian and Muscatine 2000), expulsion rates are responsive to environmental changes (McCloskey et al. 1996) and may be modified to facilitate recovery of symbiont populations following coral bleaching (Jones and Yellowlees 1997). Furthermore, some hosts appear to

preferentially target dividing cells for removal (Baghdasarian and Muscatine 2000).

In contrast to the obligate associations between most tropical scleractinians and zooxanthellae, many temperate corals maintain facultative symbioses that result in substantial natural variability in their symbiont density. Such a continuum of zooxanthellae density can be found in populations of *Astrangia poculata* (=*Astrangia danae*; Peters et al. 1988) throughout its broad distribution in shallow waters along the northwestern Atlantic. *A. poculata* tolerates an annual temperature range of −1°C to more than 25°C near its northern range limit in Rhode Island, USA. This coral provides a previously unexplored opportunity to examine zooxanthellae division and expulsion at widely different temperatures and along a range of natural symbiont densities. Furthermore, an understanding of these processes in *A. poculata* provides further insight into its seasonal symbiosis trends (Dimond and Carrington 2007).

Materials and methods

A. poculata colonies with a wide variety of symbiont densities were collected between 6 and 8 m depth at Ft. Wetherill State Park in Jamestown, RI (41°28′40″ N, 71°21′34″ W), in September 2004 ($N = 20$) and March 2005 ($N = 15$; but see change to sample size below). Colonies of similar size (approx. 4 cm × 3 cm) were carefully removed from rock surfaces, placed in individual plastic bags, and immediately brought back to the laboratory for experimentation. After being rinsed with 1 μm filtered seawater to remove any debris or free zooxanthellae, corals were placed into individual 200 ml glass beakers filled with 100 ml of 1 μm filtered seawater. An incubator maintained the corals for 24 h at either 20°C (September) or 3°C (March) with a 12:12 light:dark regime, with 60 μmol m^{-2} s^{-1} supplied by cool white fluorescent bulbs. Temperatures were chosen to be relevant to sea surface temperatures at the time of collection, and the light level was representative of the collection depth based on published extinction coefficients for Narragansett Bay, RI (Oviatt et al. 2002). Each beaker was supplied with an airstone to aerate and circulate the water and covered with Parafilm to prevent evaporation.

After 24 h incubation, corals were gripped with forceps and shaken in their incubation water to encourage dislodgement of any expelled zooxanthellae on the coral surface. Upon removal of the coral, the incubation water was stirred vigorously in an attempt to free any zooxanthellae that might have adhered to the glass, then poured into 50 ml tubes for centrifugation. The majority of incubation water was then pipetted off to leave the pellet and 2 ml of incubation water, and 1 ml of 2% formalin solution was added to preserve the sample for later counting. Ten replicate counts of expelled zooxanthellae were made at 400× using a hemacytometer. Only fully round cells with intact cell walls and pigmentation were counted. Zooxanthellae expulsion was expressed as the zooxanthellae specific expulsion rate, μ_x (Hoegh-Guldberg et al. 1987):

$$\mu_x = 1/N \times \Delta E/\Delta t \quad (1)$$

where N is the standing stock of zooxanthellae and ΔE is the number of zooxanthellae exported over Δt (24 h). Corals from which no expelled zooxanthellae were found were excluded from the analysis.

Immediately following incubations, coral tissue was removed from incubated corals using a Waterpik (Johannes and Weibe 1970) with 1 μm filtered seawater, then homogenized in a blender. An aliquot was preserved with formalin and refrigerated for later cell counts. Zooxanthellae were counted (ten replicates) at 400× with a hemacytometer, and the mitotic index (MI), the percentage of mitotic cells in a sample, was determined from counts of 400 zooxanthellae. Coral surface area was measured with aluminum foil (Marsh 1970). Since no zooxanthellae were detected in 4 of the 15 March corals, these samples were excluded and thus the sample size for March 2005 was 11 ($N = 11$).

Due to the low sample size (400 zooxanthellae compared to the typical count of 1,000 cells) of the MI counts for the first set of experiments, MI was also determined for corals collected from 8–10 m depth in Woods Hole, MA (41°31′27″ N, 70°40′15″ W) during March 2007 ($N = 12$) and August 2007 ($N = 6$). Coral tissue was removed within a few hours of collection, and samples were preserved for later cell counts. Zooxanthellae density counts were performed as described above, and the number of dividing cells in a sample of 1,000 cells were enumerated.

Finally, it was also of interest to determine whether zooxanthellae were undergoing division even in darkness at low temperatures. For three weeks during March/April 2007, three corals ($N = 3$) were held in darkened trays in the flow-through seawater system at the Marine Resources Center at the Marine Biological Laboratory (MBL), Woods Hole, MA. This seawater system provided a steady flow of unfiltered seawater at ambient temperature. Corals and other suspension-feeding invertebrates are held in this system for months at a time requiring no supplemental feeding. Temperatures during this period averaged 4°C. After the three week period, MI was determined as described above.

Due to the wide range of symbiont densities in the corals measured, analysis of covariance (ANCOVA) was performed on MI and expulsion data using JMP 5.1, with zooxanthellae density as the covariate. If this covariate was significant, corrected means for the response variable were

used to express the mean in absence of significant co-variability in zooxanthellae density.

Results and discussion

Summary statistics for MI and expulsion rates are shown in Table 1. Analyses of covariance (ANCOVA) revealed that, while zooxanthellae MI was independent of symbiont density, expulsion rates were significantly density dependent (Tables 2, 3; Fig. 1). These analyses also indicated that both MI and expulsion rates differed significantly between months, where summer was characterized by higher MI and expulsion rates than winter. However, although the mean MI of zooxanthellae in the summer corals was nearly three-fold greater than the mean MI measured in the winter corals, expulsion rates between summer and winter did not differ as drastically (Table 1).

Ignoring other potential regulatory processes such as zooxanthellae digestion or pre-mitotic control, these results at least partially explain the increase in chlorophyll density observed in azooxanthellate *A. poculata* colonies during the warm season (late summer/early autumn) and the subsequent decrease during the cold season (Dimond and Carrington 2007). The ability of zooxanthellae to divide at temperatures as low as 3°C and even at 4°C in prolonged darkness (Table 1) is remarkable given that photosynthesis in *A. poculata* symbionts is nearly shut down at 6.5°C (Jacques et al. 1983). Zooxanthellae in *A. poculata* specimens from Woods Hole, MA were identified by Rowan and Powers (1991) to be *Symbiodinium* clade B, which appears to be a cold-tolerant genotype found in other high-latitude symbioses, including *Anthopleura elegantissima* along the North American west coast (Baker 2003). Facultative heterotrophy may allow these zooxanthellae to persist and divide, but probably not without cost to the host coral (Steen 1986), especially since *A. poculata* is inactive with contracted polyps and tentacles, and does not appear to feed at temperatures below approximately 5°C (Dimond and Carrington 2007). In spite of this inactivity, winter incubation corals were able to expel symbionts at rates comparable to those at 20°C. Maintenance of high

Table 2 ANCOVA results for mitotic index (MI). All four MI sampling dates are included in the analysis

Source	DF	MS	*F*	*P*
Season	3	27.32667	15.3642	<.0001
log(ZD*)	1	0.001084	0.0006	0.9804
log(ZD) × Season	3	0.44058	0.2477	0.8625

* ZD = zooxanthellae density

Table 3 ANCOVA results for zooxanthellae expulsion (log transformed)

Source	DF	MS	*F*	*P*
Season	1	9.897505	18.3131	0.0002
log(ZD*)	1	77.644122	143.6632	<.0001
log(ZD) × Season	1	1.207757	2.2347	0.1465

* ZD = zooxanthellae density

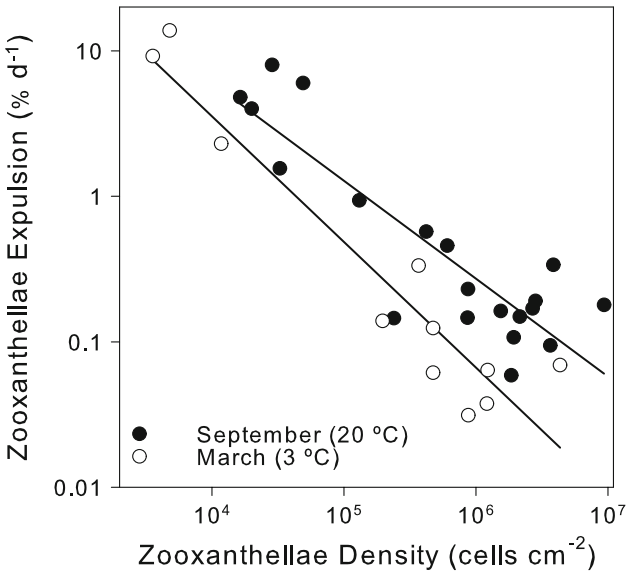

Fig. 1 *Astrangia poculata* zooxanthellae expulsion rates as a function of zooxanthellae density for September (*closed circles*) and March (*open circles*) incubations. Temperatures were chosen based on ambient temperatures in Narragansett Bay, RI, at the time of collection. Both axes are log scales. Values of zero were excluded from the analysis

Table 1 Summary of zooxanthellae Mitotic Index (MI) and expulsion rate for each experiment (mean ± SE)

Experiment	Temp (°C)	MI (%)	Expulsion (% d^{-1})	ANCOVA-corrected expulsion (% d^{-1})
September '04	20	4.40 ± 0.48	1.41 ± 0.52	1.27 ± 0.42
August '07	22	4.42 ± 0.50		
March '05	3	1.46 ± 0.31	1.74 ± 1.06	0.89 ± 0.46
March '07	3	1.58 ± 0.21		
Mar/Apr '07 Dark	4	2.87 ± 0.44		

expulsion rates relative to symbiont growth during winter and spring could help curb zooxanthellae exploitation of host resources. This may also occur under other conditions of stress; for example, Peters and Pilson (1985) noted loss of zooxanthellae when *A. poculata* colonies were stressed with sedimentation. During the warm season, low expulsion rates relative to zooxanthellae division appear to facilitate controlled increases in symbiont density, possibly via positive feedback from zooxanthellae translocation of photosynthate to the host.

Examination of zooxanthellae export as a function of zooxanthellae density offers further insight into *A. poculata* symbiont regulation (Fig. 1). Expulsion of zooxanthellae was negatively related to zooxanthellae density in both incubations, and the lack of a significant interaction term shows that this function is similar in both seasons (Table 3). These results indicate that corals with symbiont densities $<10^5$ cm^{-2} export a greater proportion of their standing stock of zooxanthellae than corals with higher densities of zooxanthellae. Notably, this is also the approximate threshold at which symbiont pigmentation becomes difficult to discern with the naked eye, making corals appear essentially azooxanthellate (e.g., Thieberger et al. 1995; Dimond and Carrington 2007). The disproportionate expulsion of zooxanthellae from these corals could be analogous to the short-term loss of zooxanthellae observed during experimental infection of some aposymbiotic hosts (e.g., Davy et al. 1997); the fundamental explanation for both phenomena may be that key cellular processes for symbiosis have not yet been activated by symbiosis-dependent gene regulation (Rodriguez-Lanetty et al. 2006). For example, a prolonged host cell cycle and suppression of apoptosis appears to be a prerequisite for successful symbiosis (Rodriguez-Lanetty et al. 2006), so high rates of expulsion in azooxanthellate/aposymbiotic hosts may reflect high rates of host cell apoptosis that are natural under non-symbiotic conditions. In any case, these expulsion trends help explain the presence of small populations of zooxanthellae in many so-called azooxanthellate *A. poculata* colonies that are largely stable over long periods of time (at least 15 months; Dimond and Carrington 2007).

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