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Photophysiology and hydrogen peroxide generation of the dinoflagellate and chlorophyte symbionts of the sea anemone *Anthopleura elegantissima*

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ABSTRACT

Associating with algal symbionts is considered largely beneficial for cnidarians such as corals and sea anemones, yet there are potential costs of hosting symbionts, such as the production of reactive oxygen species. We compared the photophysiology and H_2O_2 production rates of *Symbiodinium muscatinei* and *Elliptochloris marina*, the dinoflagellate and chlorophyte symbionts, respectively, of the temperate sea anemone *Anthopleura elegantissima*. Analyses of photosystem II (*PSII*) function in the two symbionts, including maximum quantum yield and relative electron transport rates, were consistent with prior studies indicating that *E. marina* has lower photosynthetic performance than *S. muscatinei* at high temperature and irradiance. The efficiency of *PSII* in both symbionts was positively affected by the addition of exogenous catalase, suggesting that both symbionts experience H_2O_2-mediated declines in *PSII* efficiency. We found that *S. muscatinei* produced more H_2O_2 than *E. marina* across all treatments, with the highest production under high light and temperature. Results were similar in experiments involving both isolated symbionts and symbionts residing within intact tentacles, indicating that the potential stress of symbiont isolation was not a significant factor biasing our results. Despite the lower typical densities of *S. muscatinei* relative to *E. marina*, extrapolations of cell-specific H_2O_2 production rates to the intact symbiosis suggest that *S. muscatinei* imposes a greater H_2O_2 burden on *A. elegantissima*. Even with this burden, however, the considerably higher productivity and fitness of *S. muscatinei*-bearing anemones documented in prior studies suggests that the net benefit of hosting *S. muscatinei* exceeds that of *E. marina*.

1. Introduction

Animal-algal symbioses are widespread in shallow-water marine environments, especially among the Cnidaria, which typically host dinoflagellates of the genus *Symbiodinium* within gastrodermal cells (Venn et al., 2008). Although endosymbiosis is largely advantageous for symbiotic cnidarians due to the acquisition of photosynthetically-fixed carbon, photosynthesis by the algal symbionts also generates disproportionately high quantities of reactive oxygen species (ROS) relative to those produced in host tissues (Dykens et al., 1992). If not effectively scavenged by enzymes and other antioxidant defenses, ROS can inflict cellular damage (Lesser, 2006). Production of ROS by algal symbionts is further exacerbated when temperature or irradiance extremes overburden or damage symbiont photosynthetic processes (Suggett et al., 2008), which can ultimately lead to the stress response known as bleaching, involving rapid loss of symbionts and/or symbiont pigments (Lesser, 1996, 1997; Weis, 2008).

Symbiotic dinoflagellates are a diverse group exhibiting a range of physiological optima, with physiological attributes and stress tolerances often reflecting symbiont ecological niches with respect to prevailing thermal and irradiance conditions (Iglesias-Prieto and Trench, 1994, 1997; Rowan et al., 1997; Tchernov et al., 2004; Robison and Warner, 2006). Likewise, the propensity to generate ROS varies among symbiont types and may be related to heat and light stress tolerance (Tchernov et al., 2004; Suggett et al., 2008; McGinty et al., 2012). While some recent studies suggest that host physiology plays a significant role in the stress tolerance of the holobiont (Hawkins et al., 2015; Krueger et al., 2015), for a given host, differences in ROS production between symbiont types could influence host bleaching susceptibility and the ecology of host-symbiont associations.

Sea anemones of the genus *Anthopleura* along northeast Pacific intertidal shores engage in a particularly unique symbiosis with two especially phylogenetically and physiologically different symbionts: the chlorophyte *Elliptochloris marina* (Letsch et al., 2009) and the dinoflagellate *Symbiodinium muscatinei* (LaJeunesse and Trench, 2000). Based on metrics including photosynthetic rate, mitotic index, chlorophyll content, and population density, several studies have concluded that while *S. muscatinei* is tolerant of a broad range of environmental

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conditions, *E. marina* is relatively sensitive to high light and temperature (Saunders and Muller-Parker, 1997; Verde and McCloskey, 2001, 2002; Muller-Parker et al., 2007; Bergschneider and Muller-Parker, 2008; Dimond et al., 2012, 2013). This sensitivity appears to limit *E. marina* to cooler, low light environments such as higher latitudes, the low intertidal zone, or well-shaded areas (Secord and Augustine, 2000; Secord and Muller-Parker, 2005; Dimond et al., 2011). The flexible nature of these symbioses is illustrated by studies showing that transplantation of *E. marina*-hosting anemones to warmer, brighter environments results in shifts to *S. muscatinei* (Bates, 2000; Dimond et al., 2013). In the clonal anemone *Anthopleura elegantissima*, Dimond et al. (2013) observed that shifts from *E. marina* to *S. muscatinei* under simulated high intertidal conditions were associated with depressed carbon fixation and bleaching of *E. marina*. The oxidative stress-based model for cnidarian bleaching (Weis, 2008), whereby ROS generation by algal symbionts during heat and light stress triggers their expulsion by the host, would therefore suggest that ROS production by *E. marina* may be the proximate cause for the loss of these symbionts from anemones exposed to high temperature and irradiance.

In this study, we tested the hypothesis that the photophysiological sensitivity of *E. marina* makes it more prone to ROS production than the comparatively robust *S. muscatinei*. Among the numerous forms of ROS, H_2O_2 is thought to play a particularly important role in cnidarian symbioses because of its function as a cell signaling molecule, its long lifetime, and its capacity to diffuse rapidly through cells (Smith et al., 2005; Lesser, 2006). We thus focused our study on H_2O_2 production by the two symbionts. Initially, we analyzed H_2O_2 production in isolated cells. However, given that isolated symbionts have been found to exhibit symptoms of physiological stress upon isolation, including ROS production (Goiran et al., 1997; Wang et al., 2011), we also performed similar experiments using excised tentacles housing symbionts within intact host cells. Additionally, we evaluated symbiont photophysiology with pulse-amplitude modulated fluorometry, including rapid light curve comparisons and the effect of H_2O_2 scavenging on the maximum quantum yield of photosystem II.

2. Methods

Specimens of *Anthopleura elegantissima* were collected from two different locations in the Salish Sea of northwestern Washington State, USA. Individuals hosting *Symbiodinium muscatinei* (denoted "brown anemones" due to the golden-brown color imparted by this symbiont) were collected on Chuckanut Island (48° 40.566'N, 122° 30.167'W), while individuals hosting *Elliptochloris marina* (denoted "green anemones" due to the green color imparted by this symbiont) were collected from Cone Island (48° 35.534'N, 122° 40.481'W), approximately 15 km to the southwest. Anemones were acclimated together in an indoor flow-through seawater table receiving natural light through north-facing windows for two months before experimentation. These anemones were used for rapid light curves and for analysis of H_2O_2 production in isolated symbionts. For a later, second set of experiments involving H_2O_2 production in freshly excised anemone tentacles, anemones of both symbiotic states were collected within close proximity (5 m horizontal distance at a similar tidal elevation) at Lawrence Point, Orcas Island (48° 39.684'N, 122° 44.516'W). These anemones were maintained in the same seawater table mentioned above, but were used for experiments within two weeks of collection. In all cases, symbiont identity was verified by viewing excised tentacles under light microscopy, and no mixed-symbiont populations were observed.

Rapid light curves (RLC; White and Critchley, 1999, Ralph and Gademann, 2005) were performed on isolated symbionts to compare symbiont photophysiology in a controlled setting without the influence of host tissue light attenuation (Dimond et al., 2012). Symbionts were obtained by clipping ~4 anemone tentacles per individual (n = 6 anemones per symbiotic state) and squeezing the symbiont-containing gastrodermal cells out with the flat edge of a set of forceps. The symbionts were extruded onto a glass microscope slide covered with a polycarbonate membrane filter and immersed into a petri dish filled with seawater maintained at ambient seawater temperature (10 °C). Samples were held in near darkness for 1–2 min before commencing the RLC. A pulse-amplitude modulated fluorometer (Diving-PAM, Walz, Germany) was used to generate rapid light curves at 8 actinic light levels (94, 169, 357, 423, 572, 835, 1115, and 1656 μmol quanta m^{-2} s^{-1}) as measured by a LiCor LI-190 quantum sensor. The fiber optic probe of the fluorometer was fixed at 5 mm above the sample with a clamp and stand. The relative electron transport rate, rETR, was calculated by multiplying the light intensity (photosynthetically active radiation, PAR) by the effective quantum yield ($\Delta F/F_{m'}$) of photosystem II (*PSII*) measured at each light level (rETR = $\Delta F/F_{m'} \times$ PAR). Nonphotochemical quenching (NPQ) was calculated using the initial $F_{m'}$ reading as the maximum, F_m [NPQ = $(F_m - F_{m'}) / F_{m'}$] (Ralph and Gademann, 2005).

To determine the relative effect of H_2O_2 scavenging on symbiont photophysiology, 18 anemones hosting *S. muscatinei* and 18 hosting *E. marina* were placed in 200 ml glass beakers and exposed to natural sunlight for two days in an outdoor flow-through seawater bath that maintained the jars at ambient seawater temperature. The water level of the bath was held just below the top of the beakers. Half of the anemones (n = 9 for each symbiont) received a dose of catalase at 250 U ml^{-1} in 5 μm filtered seawater (FSW) (following Lesser, 1997), while the other half received 5 μm FSW as a control. Freshly mixed catalase in FSW was added in the morning and afternoon on both days (approximately 09:00 and 16:00). To gauge symbiont photophysiology, the dark adapted maximum quantum yield of *PSII*, F_v/F_m, was measured with the Diving-PAM fluorometer before day 1 (pre-exposure), after day 1, and after day 2. All F_v/F_m values were normalized to pre-exposure values to enable relative comparisons between the two symbiont species. Anemones were dark-adapted for 30 min prior to testing.

Symbiont H_2O_2 production was measured with Amplex Red (Molecular Probes, Eugene, Oregon) following the method of Suggett et al. (2008), including the use of a temperature-controlled photosynthetron with a rose-colored light filter to exclude wavelengths that cause Amplex Red photobleaching. Experiments were performed first with freshly isolated symbionts, then with freshly excised whole tentacles. For experiments with isolated symbionts, *A. elegantissima* (n = 12, 6 with *S. muscatinei*, 6 with *E. marina*) were cut in half and one half was homogenized in a blender for the low temperature (10 °C) experiment, while the other half was saved for the high temperature (20 °C) experiment later in the day. To clean the algal cells and remove anemone tissue, the homogenate was centrifuged (1200 ×*g*) for 2 min, the supernatant discarded, and the pellet resuspended in FSW. This process was repeated twice more. The remaining pellet was suspended in 3.5 ml of FSW, a 0.5 ml sample was frozen for later cell counts, and 1 ml was placed in each of three labeled glass scintillation vials with Amplex Red at 100 μm final concentration. Vials were placed into a photosynthetron (OHPT Inc., Lewes, Delaware) for incubation at 930, 100 or 0 μmol quanta m^{-2} s^{-1} for 1 h. After incubation, the samples were centrifuged at 1200 ×*g* for 5 min and the supernatant was read with a spectrophotometer (Hewlett-Packard UV–Vis, Palo Alto, California) at 571 nm. Samples were not maintained at treatment temperatures post-incubation, but measurements were made within approximately 10 min of incubation. A standard curve from 0 to 10 μM H_2O_2 was used to calculate H_2O_2 concentrations. To normalize the measured H_2O_2 levels to symbiont cell concentration, symbionts cells were counted using a hemocytometer with four replicate counts of at least 80 cells per replicate and the H_2O_2 levels were converted to H_2O_2 per symbiont.

Similar H_2O_2 production experiments were performed with excised tentacles containing symbionts within intact host cells. For these experiments, slight modifications to the above protocol were made. Instead of using isolated cells, single tentacles from each of six replicate sea anemones of each symbiotic state were excised and placed into scintillation