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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

vials with the same reaction cocktail described above. All other experimental conditions were the same, with the exception that a 30 min incubation was used instead of a one hour incubation; initial trials indicated that a shorter incubation time would be sufficient for the excised tentacles and would prevent Amplex Red saturation. Tentacles were extracted from vials immediately following incubations and prior to spectrophotometric measurement of the incubation medium. Tentacles were frozen at -80 °C to allow for subsequent normalization of the H_2O_2 production data via homogenization of tentacles in seawater followed by hemacytometer counts of symbionts as described above.

A three-way ANOVAR with symbiont type and catalase treatment as factors and day as the repeated measure was used to assess the effect of H_2O_2 scavenging by catalase on relative F_v/F_m of the two symbionts. Isolated symbiont H_2O_2 production was analyzed using a three-way ANOVAR with symbiont and temperature as factors and light as the repeated measure. The analysis of H_2O_2 production in whole tentacles was similar, except that both temperature and light were repeated factors. We opted to make temperature a fixed factor in the isolated symbiont experiment due to the separate sets of isolated symbionts that were prepared for 10 °C and 20 °C treatments.

3. Results

Isolated symbiont rapid light curves illustrate the very different ability of *PSII* to tolerate short-term increases in light intensity in the two symbionts (Fig. 1). rETR of *E. marina* was limited even at low light intensities, eventually falling to zero at 1115 μmol quanta m^{-2} s^{-1}. In contrast, *S. muscatinei* was able to sustain comparatively high rETR up to the light curve endpoint of 1656 μmol quanta m^{-2} s^{-1}. Nonphotochemical quenching showed the opposite pattern between symbionts, with *E. marina* exhibiting substantially greater NPQ than *S. muscatinei*.

Further differences in *PSII* function, including the effects of H_2O_2 scavenging by catalase, were observed in the two-day outdoor experiment, in which anemones were brought outdoors following a two-month indoor acclimation period (Fig. 2). Relative F_v/F_m declined from pre-exposure levels in both symbionts over the two day period, with a significant effect of day ($F_{1,32} = 24.39$, $P < 0.001$). Green anemones hosting *E. marina* underwent significantly greater declines in F_v/F_m than brown anemones hosting *S. muscatinei* ($F_{1,32} = 12.76$, $P = 0.001$). Regardless of symbiont type, F_v/F_m was significantly higher in anemones that were incubated with catalase ($F_{1,32} = 7.29$, $P = 0.011$). There were no significant interaction effects in the analysis.

H_2O_2 production of isolated symbionts at three light levels and two temperatures showed significant effects of all three factors (Fig. 3). Across all treatments, *S. muscatinei* produced more H_2O_2 per cell than did *E. marina* ($F_{1,28} = 119.62$, $P < 0.001$). H_2O_2 production was significantly elevated under high light compared to low light or darkness ($F_{2,28} = 40.95$, $P < 0.001$), and also significantly higher at 20 °C than at 10 °C ($F_{1,28} = 36.95$, $P < 0.001$). There were no significant interactions. In excised whole tentacles, the results differed slightly, and were dominated by a significant symbiont × light interaction. Again, *S. muscatinei* produced significantly more H_2O_2 per cell than did *E. marina*, but this was true only at high irradiance, as indicated by the significant symbiont × light interaction ($F_{2,8} = 5.38$, $P = 0.048$). Surprisingly, there was no significant temperature effect in this experiment ($F_{1,8} = 0.019$, $P = 0.894$).

4. Discussion

The first direct evidence of ROS production in a symbiotic cnidarian came from work by Dykens et al. (1992) on the *A. elegantissima*/*S. muscatinei* symbiosis. Together with earlier indirect studies documenting that antioxidant enzyme concentrations were induced by symbiont presence and light exposure (Dykens and Shick, 1982, 1984), these studies highlighted the physiological significance of symbiont-generated ROS and their potentially adverse consequences for host cnidarians. More recently, a broader understanding of the phylogenetic and physiological diversity of algal symbionts has led to investigations of the relative benefits and costs these diverse symbionts bring to their hosts. For hosts capable of maintaining flexible associations with different symbionts, such as *Anthopleura* spp., the relative benefits of hosting one symbiont over another are often measured in terms of the quantity or quality of nutritional contributions (Verde and McCloskey,

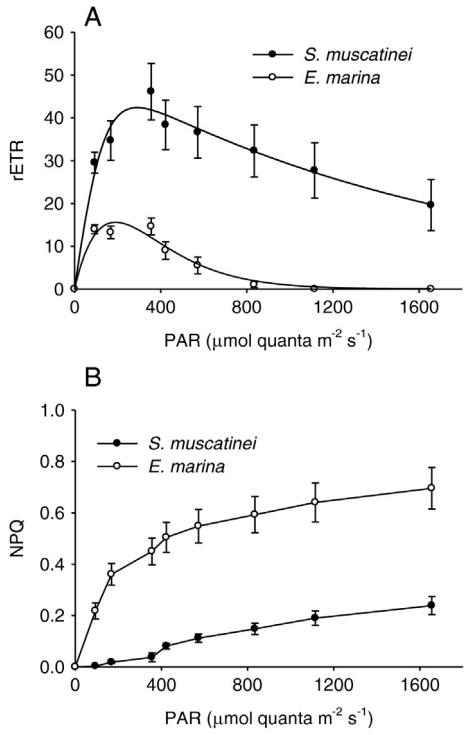

Fig. 1. Relative electron transport rates (rETR; A) and nonphotochemical quenching (NPQ; B) in *S. muscatinei* and *E. marina*, as determined by rapid light curves performed on symbionts extruded from excised *A. elegantissima* tentacles. Data shown are means ± SE.

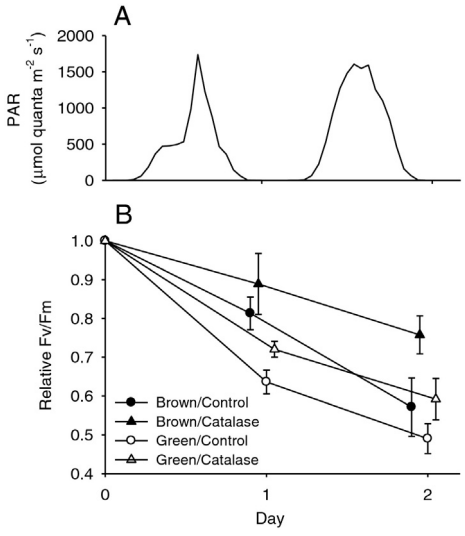

Fig. 2. Outdoor experiment testing the response of *PSII* maximum quantum yield (F_v/F_m) to two days of exposure to full sunlight, with and without the addition of exogenous catalase to the medium. (A) Photosynthetically active radiation (PAR) during the two-day period. (B) Relative F_v/F_m of *S. muscatinei* and *E. marina* over the two-day period. Data were normalized to pre-exposure values and are shown as means ± SE, with slight offsetting for better visualization of error bars. Brown refers to *S. muscatinei*-bearing anemones, while green refers to *E. marina*-bearing anemones.

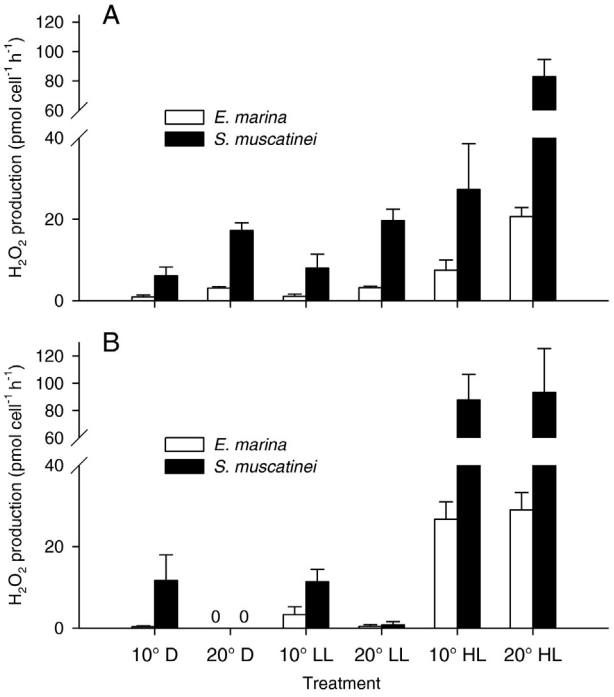

Fig. 3. Production of H_2O_2 by *S. muscatinei* and *E. marina*. (A) Incubation with isolated symbionts. (B) Incubation with excised whole tentacles. Light treatments are abbreviated as D = dark, LL = low light, HL = high light. Data shown are means ± SE. Note that the average cell volume of *S. muscatinei* is 2.5 times greater than *E. marina*.

2001, 2002; Loram et al., 2007; Cantin et al., 2009). Aside from contributing less or lower-quality energy to their hosts, under sub-optimal conditions stress-susceptible symbionts may incur costs to their hosts associated with oxidative stress (Suggett et al., 2008; McGinty et al., 2012), potentially contributing to observed patterns of symbiont distribution, abundance, and specificity. We hypothesized that the lower light and temperature tolerance of *E. marina* would also be manifest by an increased capacity to produce ROS under elevated temperature and irradiance. Although our comparative assessment of *PSII* function in the two symbionts was consistent with prior studies indicating that *E. marina* is more physiologically sensitive to high light and temperature than *S. muscatinei*, the hypothesis that *E. marina* produces greater quantities of H_2O_2 was not supported.

Our hypothesis was based on several studies showing that *E. marina* is comparatively incapable of photoacclimation to increased light and temperature (Verde and McCloskey, 2001, 2002; Secord and Muller-Parker, 2005; Bergschneider and Muller-Parker, 2008; Dimond et al., 2013). Baseline photosynthetic activity, as shown here using chlorophyll fluorescence-based techniques, and as documented in earlier studies with oxygen evolution and carbon fixation methods (e.g. Verde and McCloskey, 2001, 2002; Bergschneider and Muller-Parker, 2008), is consistently higher in *S. muscatinei*. When temperature and irradiance are elevated, *S. muscatinei* responds by increasing its photosynthetic rates, while photosynthetic rates of *E. marina* either remain the same over short time periods (minutes to days) or decline to eventual collapse over longer scales (days to months) (Verde and McCloskey, 2002; Dimond et al., 2013). We hypothesized that a limited ability of *E. marina* to process increased photon fluxes photochemically would make it more disposed to ROS production relative to *S. muscatinei*, but this was not the case in our experiments. Rapid light curve data instead suggest that *E. marina* may be capable of effective nonphotochemical quenching, involving the dissipation of excess absorbed energy as heat. Carotenoids, such as the xanthophylls, play a key role in nonphotochemical quenching. Compared to *S. muscatinei*, *E. marina* contains high levels of carotenoids despite its smaller size (Verde and McCloskey, 2007), perhaps explaining its nonphotochemical quenching capabilities. However, given that our experiments tested only short-term exposure to heat and light stress, it is possible that the mechanisms used by *E. marina* to dissipate excess light energy are not robust to chronic stress, as prior studies suggest (Verde and McCloskey, 2002; Dimond et al., 2013).

A *S. muscatinei* cell averages 2.5 times the volume of an *E. marina* cell (Verde and McCloskey, 1996; Bergschneider and Muller-Parker, 2008), so it is perhaps not surprising that *S. muscatinei* has both higher photosynthetic rates and produces more H_2O_2. However, H_2O_2 production rates were over three times higher in *S. muscatinei*, indicating that even though *S. muscatinei* is larger, it still produces slightly more H_2O_2 on a volume-specific basis. Likewise, light-saturated oxygen production rates of *S. muscatinei* are typically over three times greater than those of *E. marina* (Verde and McCloskey, 2002; Secord and Muller-Parker, 2005). The higher production of H_2O_2 by *S. muscatinei* may therefore simply be related to its higher photosynthetic rates. The particularly hyperoxic conditions created by *S. muscatinei* photosynthesis would favor ROS generation since, as a strong oxidant, O_2 has a natural tendency to generate ROS as intermediates in a series of univalent electron transfers (Fridovich, 1998). On the other hand, Suggett et al. (2008) found that a stress-susceptible *Symbiodinium* phylotype produced comparable to slightly higher levels of H_2O_2 than a more tolerant type in spite of its lower gross O_2 production rates, illustrating that H_2O_2 production is not necessarily coupled with O_2 production.

Oxidative stress can impair photosynthetic activity by damaging thylakoid components (most notably the D1 protein of *PSII*) and inhibiting their repair, resulting in declines in *PSII* efficiency (Warner et al., 1999; Lesser, 2006). The effect of H_2O_2 on symbiont *PSII* maximum quantum yield was apparent when anemones were incubated with dissolved catalase, which scavenged H_2O_2 and significantly enhanced F_v/F_m compared to controls. Although *E. marina* underwent a greater overall decline in F_v/F_m than *S. muscatinei*, by day 2 there was a trend suggesting that *S. muscatinei* experienced a greater positive effect of the exogenous catalase than *E. marina*. Such an effect hints that H_2O_2 may play a relatively larger role in *S. muscatinei* photophysiology, which is supported by H_2O_2 production data.

Hydrogen peroxide has been implicated as a causal factor in *PSII* breakdown among freshly isolated *Symbiodinium* spp. (Wang et al., 2011), suggesting that symbiont isolation can cause unwanted physiological stress. We were concerned that the stress of isolation could bias or augment our results, so we repeated our experiments using freshly excised tentacles which house algal symbionts within intact gastrodermal tissues. Although we found no temperature effect in this experiment, the general trend of higher H_2O_2 production in *S. muscatinei* was maintained. Interestingly, we found higher basal (dark and low light) levels of H_2O_2 production at 10 °C than at 20 °C. While we can conclude that symbiont isolation appears to have had no disproportionate effect on ROS production in these symbionts, differences between the two experiments likely reflect differences between the isolated vs. *in hospite* environment. For example, the *in hospite* environment includes the photoprotective effect of the host tissues (Dimond et al., 2012) as well as both the ROS generation and scavenging capacities of these tissues; apparently, host ROS scavenging was not able to compensate for the higher H_2O_2 production by *S. muscatinei*. It is also possible that discrepancies between the two experiments reflect variation in the acclimation state of the algal symbionts as a result of differences in the time and site of collection.

Our experiments suggest that *S. muscatinei* imposes a greater H_2O_2 burden on the host than does *E. marina*. However, as with studies on bleaching in reef corals, symbiont density is at least as important a factor as symbiont identity for the health of the association (Cunning and Baker, 2014). Using symbiont density data from six studies, the average density (symbionts mg^{-1} host protein) of *E. marina* is 2.5 times higher than that of *S. muscatinei* (Verde and McCloskey, 1996, 2001, 2002; Engebretson and Muller-Parker, 1999; Bergschneider and

Muller-Parker, 2008; Dimond et al., 2011). Multiplying the highest recorded *E. marina* H_2O_2 production rate of 29 pmol cell^{-1} h^{-1} in the high temperature, high light treatment of the excised tentacle experiment by a factor of 2.5 gives a rate of 72.5 pmol cell^{-1} h^{-1}, which is closer to but still less than the rate of 93 pmol cell^{-1} h^{-1} recorded for *S. muscatinei* in the same treatment. Thus, we can conclude that when symbiont density is taken into consideration, *S. muscatinei* still imposes a greater H_2O_2 burden on its host. Nonetheless, given that *S. muscatinei*-bearing anemones exhibit higher fitness than aposymbiotic or *E. marina*-bearing individuals (Bingham et al., 2014), the benefits of hosting *S. muscatinei* clearly outweigh their costs. These benefits include carbon translocation rates at least 2–3 times higher than for *E. marina*-bearing anemones under high temperature and irradiance conditions (Engebretson and Muller-Parker, 1999; Verde and McCloskey, 2001, 2002). Meanwhile, although *E. marina* provides much less carbon to its host, it does so at a lower cost in terms of H_2O_2 production, perhaps contributing to its successful relationship with *A. elegantissima.*

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