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SYMBIONT PHYSIOLOGY AND POPULATION DYNAMICS BEFORE AND DURING SYMBIONT SHIFTS IN A FLEXIBLE ALGAL-CNIDARIAN SYMBIOSIS[1]

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For cnidarians that can undergo shifts in algal symbiont relative abundance, the underlying algal physiological changes that accompany these shifts are not well known. The sea anemone *Anthopleura elegantissima* associates with the dinoflagellate *Symbiodinium muscatinei* and the chlorophyte *Elliptochloris marina*, symbionts with very different tolerances to light and temperature. We compared the performance of these symbionts in anemones maintained in an 8–11.5 month outdoor common garden experiment with simulated intertidal conditions and three levels of shading (2, 43, and 85% ambient irradiance). Symbiont densities, mitotic indices, photophysiology and pigments were assessed at three time points during the summer, a period of high irradiance and solar heating during aerial exposure. Whereas *S. muscatinei* was either neutrally or positively affected by higher irradiance treatments, *E. marina* responded mostly negatively to high irradiance. *E. marina* in the 85% irradiance treatment exhibited significantly reduced P_{max} and chlorophyll early in the summer, but it was not until nearly 3 months later that a shift in symbiont relative abundance toward *S. muscatinei* occurred, coincident with bleaching. Symbiont densities and proportions remained largely stable in all other treatments over time, and displacement of *S. muscatinei* by *E. marina* was not observed in the 2% irradiance treatment despite the potentially better performance of *E. marina*. While our results support the view that rapid changes in symbiont relative abundance are typically associated with symbiont physiological dysfunction and bleaching, they also show that significant temporal lags may occur between the onset of symbiont stress and shifts in symbiont relative abundances.

Key index words: Anthopleura; **bleaching; cnidarian-algal symbiosis;** *Elliptochloris*; **photophysiology;** *Symbiodinium*; **symbiont shuffling**

***Abbreviations*: DPM, disintegrations per minute; FSW, filtered seawater; F_v/F_m, maximum quantum yield of photosystem II; MI, mitotic index; PAM, pulse-amplitude modulated; P-I, photosynthesis-irradiance; P_{max}, maximum photosynthetic rate; PVC, polyvinyl chloride; α, photosynthetic light utilization efficiency**

Algal endosymbionts of cnidarians typically provide significant photosynthetic nutrition to their host (reviewed by Davy et al. 2012). Symbiont health is vital to the host because of these energetic contributions, and because physiologically impaired symbionts can harm the host (Weis 2008). Symbionts stressed by environmental perturbations such as temperature and irradiance extremes can produce excessive oxygen radicals that inflict damage on both the symbiont and its host (Lesser 1996, 1997, Lesser and Farrell 2004). This oxidative stress can trigger bleaching, a general paling of the host resulting from some combination of mass expulsion of symbionts and/or loss of pigments from the symbionts themselves. Although sensitivity to bleaching is determined by both symbiotic partners, the physiological tolerance of the alga appears to play a particularly important role (van Oppen et al. 2009). There is some support for the hypothesis that bleaching may be a mechanism for hosts to replace stressed symbionts with less stress-susceptible symbiont taxa (Buddemeier and Fautin 1993), but symbiont shifts can also occur without bleaching or before its onset (Berkelmans and van Oppen 2006, Thornhill et al. 2006, LaJeunesse et al. 2009). Symbiont shifts remain poorly understood, in part because of the predominantly stable nature of

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cnidarian symbioses (Thornhill et al. 2006, Dimond et al. 2011, McGinley et al. 2012). Furthermore, while there are many studies showing how different symbiont types and/or host-symbiont combinations respond physiologically to thermal stress and bleaching (e.g., Berkelmans and van Oppen 2006, Robison and Warner 2006, Abrego et al. 2008), to date there have been no detailed analyses of symbiont physiology together with their population dynamics during symbiont shifts.

While most symbiotic cnidarians associate exclusively with dinoflagellates (*Symbiodinium* spp.), sea anemones in the genus *Anthopleura* along the Pacific coast of North America are notable exceptions. These abundant intertidal species engage in flexible symbioses with the unicellular chlorophyte *Elliptochloris marina* (Letsch et al. 2009) in addition to *Symbiodinium* spp. (LaJeunesse and Trench 2000, Sanders and Palumbi 2011) over their central latitudinal range (approximately Oregon to British Columbia; Secord and Augustine 2000). *Symbiodinium muscatinei* is typically associated with anemones occurring in warmer, high irradiance habitats, such as lower latitudes and the upper intertidal zone, while the opposite is true of *E. marina* (Secord and Augustine 2000, Dimond et al. 2011). Correspondingly, the two symbionts exhibit distinctly different physiological responses to temperature and light. Whereas elevated temperature and high irradiance typically enhance photosynthesis, MI, and population density of *S. muscatinei*, they have the opposite effect on *E. marina* (Saunders and Muller-Parker 1997, Engebretson and Muller-Parker 1999, Verde and McCloskey 2001, 2002). In conjunction with these short-term physiological studies, longer term field transplantation experiments showing that relative abundances of the two symbionts can change and are environmentally determined (Bates 2000, Secord and Muller-Parker 2005) suggest links between symbiont physiology, environmental conditions, and the dominant symbiont taxon within a host.

To investigate the relationship between symbiont physiology and potential changes in symbiont relative abundance, we evaluated *E. marina* and *S. muscatinei* physiology after long-term acclimation of the clonal sea anemone *Anthopleura elegantissima* hosting each respective symbiont in an outdoor common garden experiment with a simulated tidal regime and a gradient of three irradiance treatments. After more than 8 months (October to June) in the experimental treatments, anemones were sub-sampled at three time points over the course of the summer when irradiance and solar heating during aerial exposure impose some of the most extreme physiological challenges of the year (Dimond et al. 2011). In addition to measuring a suite of physiological characteristics in the two symbiont species, symbiont population changes over the summer were tracked and related to symbiont physiology.

MATERIALS AND METHODS

Specimen collection and experimental conditions. Zooxanthellate (brown-colored anemones hosting 99.9 ± 0.1% *S. muscatinei*, determined from tentacle clips post-collection), zoochlorellate (green-colored anemones hosting 98.4 ± 5.5% *E. marina*), and asymbiotic (white-colored anemones hosting no or very low numbers of symbionts) *A. elegantissima* were collected from a single outcrop on Tatoosh Island, Washington (48°23′ 24″ N, 124°44′ 24″ W) on September 3, 2009. It was possible that these anemones constituted a single clone, thus limiting the host and symbiont diversity tested in this study. Anemones were transported in a cooler back to Shannon Point Marine Center (SPMC) in Anacortes, Washington on the day of collection and immediately placed into flow-through seawater tables receiving natural lighting through large north-facing windows. Anemones attached themselves to numbered slate tiles (4.8 × 4.8 × 0.8 cm), which allowed them to be individually identified.

The animals were transferred to a round (3 m diameter, 0.9 m depth, 6,400 L volume) outdoor flow-through seawater tank on October 5, 2009. Unfiltered seawater was pumped into the tank from adjacent Guemes Channel (6 m depth) at a rate of $\geq$38 L · min^{-1}, equivalent to a tank turnover time of $\leq$2.8 h, with additional circulation provided by a submersible pump (Danner Mag Drive, 113 L · min^{-1}). Low tides were simulated in the tank using an electronic ball valve (Electromni Series 83, Asahi America, Malden, MA, USA) operated by a programmable relay timer (Zelio SR2B121FU, Schneider Electric, Rueil Malmaison, France) that drained the tank to the level of a lower standpipe for programmed low-tide durations. The relay timer was programmed to be synchronized with local (Burrows Bay, Washington) high and low tides corresponding to a level of 0.3 m above mean lower low water. In the tank, anemones on their slate tiles were placed on artificial turf-covered PVC platforms that rested just above the low-tide standpipe level, such that the anemones were immersed during simulated high tides (16 cm water depth above platforms) and aerially exposed during simulated low tides for periods of up to 6 h. The six PVC platforms (60 cm diameter) were distributed into three treatments: 85% irradiance, 43% irradiance, and 2% irradiance (two platforms per treatment). Irradiance levels in the treatments were modified by placing round sheets (64 cm diameter, 4.8 mm thickness) of either UV-transmitting acrylic (85% irradiance), UV-transmitting acrylic plus a layer of black window screen (43% irradiance), or opaque gray PVC (2% irradiance) over the platforms. These shields were supported 14 cm above the anemone platforms with a single PVC rod in the center of the platform, and were submerged during simulated high tides. Attenuation of ambient light within the experimental treatments during emersion conditions was determined repeatedly with a Biospherical Instruments QSL-100 4π quantum sensor (San Diego, CA, USA). Average attenuation was obtained by taking multiple readings at different time points.

Each anemone fed on plankton supplied by the flow-through seawater, and was also given a supplemental ration of one frozen squid pellet (~0.1 g wet mass) biweekly to account for the lack of natural large prey items in the experimental system. We reasoned that since *A. elegantissima* may rely heavily on benthic prey in natural settings (Sebens 1981), it was necessary to supplement food provided by the flow-through seawater system. Although most anemones lost mass over the course of the study, many produced gonads and underwent fission, indicating that they were largely healthy (B. B., J .D., G. M.-P., and L. Francis, in review). Frequent tank maintenance included keeping each anemone associated with its respective tile and the tank, platforms, and light shades free of excessive sediment and fouling organisms.

Anemone processing. Over the course of summer 2010, after the sea anemones had been maintained under the three light treatments in the tank for over 8 months, they were sampled on three dates: June 21, August 3, and September 13. At each sampling, we removed approximately one-third of the anemones (n = 5–7 each of zooxanthellate, zoochlorellate and asymbiotic anemones; asymbiotic anemones were studied for other purposes and their data are not included in this article). Anemones were homogenized in 5 μm FSW in a small blender, and aliquots of homogenate were frozen at −70°C for later protein analysis. Symbiont chlorophyll was collected by vacuum filtration of fresh anemone homogenate onto Whatman GF/C filters. Filters were wrapped in foil and frozen at −70°C until analysis. Fifteen milliliters of remaining fresh anemone homogenate was centrifuged (1,500g for 3 min) to pellet the algal symbionts. The remaining algal pellet was washed three times by repeated resuspension and centrifugation in FSW (1,500g for 3 min), then filtered through 100 μm Nitex mesh to remove any clumps. Within 1 h, the final algal suspensions were used for the measurement of photosynthetic carbon fixation, as described below. A subsample of each suspension was frozen at −70°C for later cell counts.

Symbiont photosynthetic rates. Photosynthetic rates of isolated symbionts were measured using the ^{14}C bicarbonate protocol of Bergschneider and Muller-Parker (2008), with the following modifications. P-I measurements were obtained with 11 irradiance levels from 0 to 1,400 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}. DPM of samples were determined using a Perkin-Elmer TriCarb 2910TR liquid scintillation counter (Waltham, MA, USA). To obtain cell-specific carbon fixation rates, the concentration of isolated symbionts was determined by four replicate counts of at least 80–100 algal cells using a hemocytometer under a compound microscope. The maximum light-saturated photosynthetic rate (P_{max}) and the light utilization efficiency (α; the initial slope of the P-I curve) were derived from a hyperbolic tangent function (Jassby and Platt 1976) applied to P-I data in SigmaPlot 11.0. June P-I curves did not have sufficient resolution at low irradiances to accurately compute light utilization efficiencies (α), so only α from August and September were included in the analyses.

Anemone protein, symbiont chlorophyll, symbiont density, and MI. Anemone soluble protein content was determined by the spectrophotometric method of Lowry et al. (1951) with BSA as the standard. Symbiont chlorophyll was assessed as described in Dimond et al. (2011) using the spectrophotometric equations of Jeffrey and Humphrey (1975) for *S. muscatinei* samples and those of Holden (1976) for *E. marina* samples. Briefly, washed algal suspensions were vacuum filtered onto GF/C glass fiber filters, stored at −70°C, then ground to a pulp in a motorized tissue grinder with either 100% acetone (*S. muscatinei* samples) or 100% methanol (*E. marina* samples) prior to centrifugation and spectrophotometric analysis of the supernatant. Symbiont density in anemone homogenates was determined using a hemocytometer as described above for isolated symbiont cell counts. MI was determined by counting the number of cells with well-defined division furrows in a sample of 1,000 cells. Time of collection was assumed to have no bearing on MI, as MI measurements have not revealed any regular diel pattern of division in either of these symbionts (Wilkerson et al. 1983, Verde and McCloskey 1996).

PAM fluorometry. For September samples only (due to instrument availability), the dark-adapted maximum quantum yield of photosystem II (F_v/F_m) was determined using a PAM fluorometer (Walz DIVING-PAM, Effeltrich, Germany). Maximum quantum yield is a measure of the efficiency of photochemistry; reduced efficiency may reflect either photoprotection or photodamage (Fitt et al. 2001). Symbionts within intact host tissues were tested prior to anemone processing by taking F_v/F_m measurements of anemone oral disks in expanded posture with a 1 cm fiber optic probe. Anemones were held in darkness for 30 min before obtaining F_v/F_m measurements.

Environmental data. Temperatures in each irradiance treatment were monitored with Hobo WaterTemp Pro dataloggers encased in white rubber protective boots and mounted on the platforms next to the anemones. Photosynthetically active radiation (PAR; 400–700 nm) data collected at sea level at 15 min intervals were acquired from nearby Padilla Bay National Estuarine Research Reserve (D. Bulthuis, N. Burnett, and H. Bohlmann unpubl. data, Padilla Bay National Estuarine Research Reserve Monitoring Program; http://cdmo.baruch.sc.edu/). The percentage of ambient light attenuation within experimental treatments was determined as described above.

To permit a comparison of the light and temperature environment in the three experimental irradiance treatments with natural conditions on a rocky shore, we computed temperature and light statistics for a 3.5 month summer period spanning the anemone sampling dates (June 1 through September 13). The Hobo WaterTemp Pro dataloggers we used in the experimental tank had been deployed for a prior study on a rocky shore with abundant *A. elegantissima* populations on San Juan Island, Washington (Dimond et al. 2011). For the field study, loggers were mounted to bedrock at 0.2 and 1.8 m above mean lower low water (MLLW) from July 2008 through July 2009. We used field data from June 1, 2009 to July 22, 2009 and merged it with data from July 23, 2008 to September 13, 2008 to compare field data and experimental treatment data over an equivalent time period (but different years). Attenuation of light at the shore heights of the field dataloggers was determined by estimating mean water depth during July 2008 at these heights (0.2 and 1.8 m) from published tide levels (NOAA; http://tidesandcurrents.noaa.gov/), followed by application of published extinction coefficients for local waters (Nelson et al. 2003) to the estimated mean water depths.

Statistical analyses. For each dataset except F_v/F_m, we used two-way ANOVA to test the effects of irradiance treatment and sampling date. PAM fluorometry (F_v/F_m) data were analyzed with a one-way ANOVA testing the effect of irradiance treatment. Significant month or treatment effects were followed with Tukey post hoc tests, and significant interactions were evaluated by tests of simple main effects. If Levene's test of equality of variances showed significant heteroscedasticity, data were either square root or inverse transformed. One dataset (*S. muscatinei* MI) could not be corrected with transformation, so we used a lowered significance threshold (α = 0.025) for this test to reduce the probability of type I error (Keppel and Wickens 2004). All other tests were evaluated using α = 0.05. We used SPSS version 18 (IBM, Armonk, NY, USA) for all statistical analyses.

For each analysis, sample sizes were usually five to seven anemones, but varied from as few as one to as many as 17 for several reasons. First, some mortality occurred as a result of severe heating and desiccation during two aerial exposure episodes that coincided with warm, high irradiance conditions. Second, in September, most zoochlorellate anemones in the 85% irradiance treatment had shifted their dominant symbiont population from *E. marina* to *S. muscatinei* and had to be excluded from all analyses except symbiont density (only one zoochlorellate anemone maintained a dominant *E. marina* population). Third, some anemones underwent asexual reproduction (fission) and the two daughter anemones were treated as separate individuals, with some sampled on different dates.

RESULTS

Environmental conditions. Aerial exposure, light and temperature in the outdoor, flow-through seawater tank reflected seasonal changes in the local Salish Sea environment (Fig. 1; compare with conditions shown in Dimond et al. 2011). Experimental shading altered treatment temperatures through their influence on solar heating during aerial exposure, with maximum temperatures inversely proportional to the amount of shading (Fig. 1; Table 1). Estimated irradiance conditions at 0.2 and 1.8 m above MLLW at the rocky shore on San Juan Island were similar to the 43% and 85% irradiance treatments, respectively, but rocky shore mean and maximum temperatures were considerably higher than those in the experimental treatments (Table 1). This may be attributed to different thermal characteristics of bedrock versus the artificial turf-covered PVC platforms, and the longer daytime aerial exposure durations at +1.8 m.

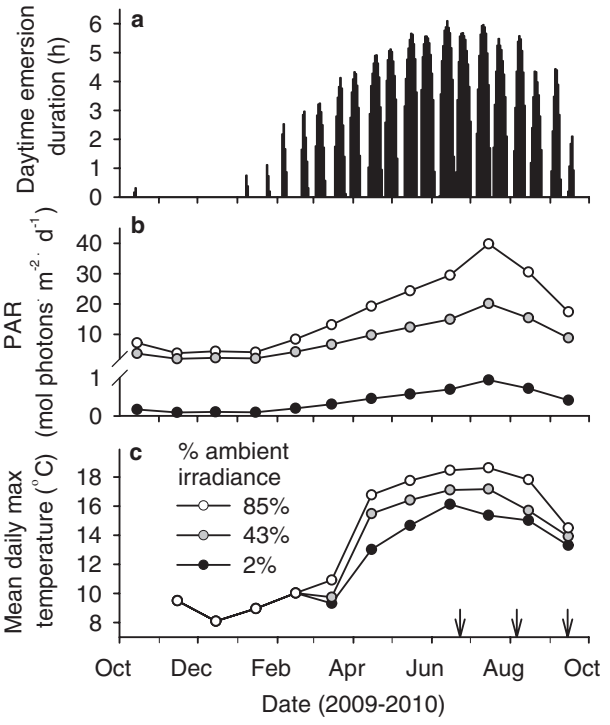


FIG. 1. Aerial exposure durations in the experimental tank (a), estimated mean monthly photosynthetically active radiation (PAR) (b) and mean daily maximum temperature (c) in the three experimental irradiance treatments. Symbols in (b) as in (c). Arrows in (c) indicate the three host anemone sampling events during summer 2010.

Symbiont densities and mitotic indices. Irradiance treatment generally had opposite effects on the density of the two symbionts (Fig. 2, a and h). The highest densities of both symbionts occurred in the 43% irradiance treatment, but whereas the lowest densities of *S. muscatinei* occurred in the 2% irradiance treatment, the lowest densities of *E. marina* occurred in the 85% irradiance treatment. ANOVA (Table 2) indicated that the effect of irradiance treatment on symbiont density was significant for *S. muscatinei*, whose densities were significantly lower in the 2% irradiance treatment than in the two higher irradiance treatments. For *E. marina* densities, a significant interaction between month and treatment resulted from a lack of significance in June, but consistently lower density in the 85% irradiance treatment in August and September.

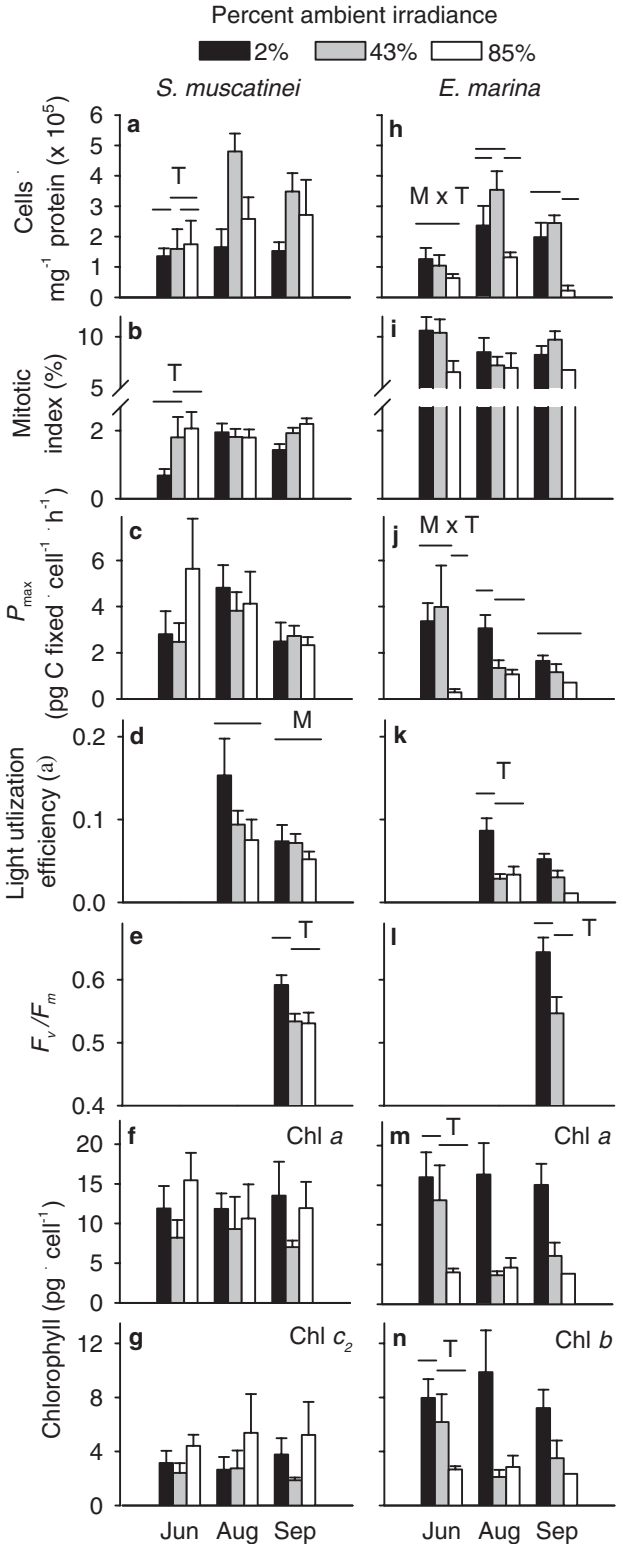
Mitotic indices of the two symbionts (Fig. 2, b and i) were also differently affected by irradiance treatment, with MI of *S. muscatinei* staying the same or increasing with irradiance treatment while MI of *E. marina* was lowest under 85% irradiance. However, ANOVA (Table 1) showed that irradiance treatment effect on MI was significant for *S. muscatinei* and not for *E. marina* MI.

Photosynthetic parameters. Light-saturated P_{max} (Fig. 2, c and j) of *S. muscatinei* remained relatively high regardless of irradiance treatment, while those of *E. marina* tended to be depressed by higher irradiance treatments. ANOVA (Table 2) showed no effect of month or treatment for *S. muscatinei*, but there was a significant month × treatment interaction for *E. marina* that was due to an absence of significant differences in September, but much lower Pmax among the 85% and 45% irradiance treatments in August, and the 85% irradiance treatment in June.

Light utilization efficiencies (Fig. 2, d and k) of both symbionts tended to decline with increasing irradiance treatment. However, ANOVA (Table 2) indicated that irradiance treatment was significant

TABLE 1. Environmental characteristics of experimental treatments and a nearby rocky shore for comparison (San Juan Island, Washington), for the period June 1 through September 13.

	Experimental treatments (% ambient irradiance)			Rocky shore (height above MLLW)	
	2%	43%	85%	+0.2 m	+1.8 m
Mean daytime aerial exposure duration (h)	4.2	4.2	4.2	3.9	8.7
Mean daily max PAR (μmol photons · m^{-2} · s^{-1})	30	643	1272	895	1420
Mean daily total PAR (mol photons · m^{-2} · d^{-1})	0.7	16.1	31.8	22.5	35.6
Mean daily max temperature (°C)	15.3	16.4	18.0	17.5	25.6
Mean temperature (°C)	12.5	12.7	12.9	11.3	14.5
Mean daily min temperature (°C)	11.4	11.4	11.4	9.8	9.7



only for *E. marina*; algae in the 2% irradiance treatment had significantly greater light utilization efficiency than algae from the two higher irradiance treatments. There was a significant effect of month for *S. muscatinei,* with lower light utilization efficiency in September than in August.

FIG. 2. Population density, mitotic index, maximum photosynthetic rate (P_{max}), light utilization efficiency (α), maximum quantum yield of photosystem II (F_v/F_m), and chlorophyll concentration for *Symbiodinium muscatinei* (a–g) and *Elliptochloris marina* (h–n) in the three experimental irradiance treatments. Data shown are means (± SE) from three sampling events in June, August, and September 2010 (n = 1–17 for each treatment; n = 1 for the September 85% irradiance treatment in panels i, j, k, m, and n). Horizontal bars above graphs denote results of Tukey post hoc tests or simple main effects comparing factors and interactions ($P < 0.05$), with significant factors denoted by letters above the bars (M = month, T = irradiance treatment, M × T = month × irradiance interaction; T and M apply to all bars within a graph, while M × T apply to each set of bars).

F_v/F_m declined significantly at higher irradiance treatments for both symbionts (Fig. 2, e and l; Table 2). *S. muscatinei* F_v/F_m was significantly higher in the 2% irradiance treatment, and comparable for anemones maintained at 43% and 85% irradiance levels. F_v/F_m of *E. marina* was significantly higher in the 2% irradiance treatment than in the 43% irradiance treatment. F_v/F_m data for *E. marina* from the 85% irradiance treatment were omitted because of mixed-symbiont populations and unreliable fluorescence signals from the largely bleached individuals.

Chlorophyll. Irradiance treatment had no significant effect on *S. muscatinei* chlorophyll content, while *E. marina* chlorophyll content was strongly affected (Fig. 2, f, g, m, and n). Chlorophylls *a* and *b* in *E. marina* were significantly lower in the two higher irradiance treatments than in the 2% irradiance treatment; the 43% and 85% irradiance treatments were statistically indistinguishable (Table 2). Pigment ratios (data not shown) in both symbionts were unaffected by irradiance regime (Table 2).

Symbiont changes in anemone hosts. Over the course of the three summer sampling points, sea anemones in all treatments except for zoochlorellate anemones in the 85% irradiance treatment had stable dominant symbiont complements (Fig. 3). On the final sampling event in September, all but one zoochlorellate anemone in the 85% irradiance treatment had shifted from an *E. marina* -dominated to an *S. muscatinei* -dominated symbiont population. The one anemone that remained predominantly zoochlorellate had an *S. muscatinei* complement of 0.5%, while two anemones increased to 53%–58% *S. muscatinei,* and three others increased to 93%–97% *S. muscatinei.* Our standard hemocytometer count procedure had a background symbiont detection threshold of ~0.1%, and we found that 41% of all zoochlorellate anemones sampled during the experiment (excluding those in the September 85% irradiance sample) had background populations of 0.1%–12% *S. muscatinei.* The shift to *S. muscatinei* dominance in the originally zoochlorellate anemones resulted from an increase in *S. muscatinei* densities and a decrease in *E. marina* densities from prior levels in August (Fig. 4).

TABLE 2. Results of ANOVA for the suite of parameters measured for *Symbiodinium muscatinei* and *Elliptochloris marina*.

	Month			Irradiance treatment			Month × irradiance treatment		
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
S. muscatinei									
Symbiont density	2	2.81	0.07	2	4.08	**0.02**	4	1.38	0.25
Mitotic index	2	1.26	0.29	2	3.99	**0.02***	4	1.85	0.13
P_{max}	2	3.12	0.05	2	0.37	0.70	4	0.93	0.45
Light use efficiency (α)	1	5.53	**0.02**	2	2.48	0.10	2	1.08	0.35
F_v/F_m	na			2	3.74	**0.04**	na		
Chl *a*	2	0.11	0.89	2	1.84	0.17	4	0.29	0.88
Chl c_2	2	0.02	0.98	2	1.71	0.19	4	0.11	0.98
Chl *a*:Chl c_2	2	0.11	0.90	2	1.75	0.18	4	1.00	0.41
E. marina									
Symbiont density	2	8.74	**<0.001**	2	16.53	**<0.001**	4	3.03	**0.03**
Mitotic index	2	1.44	0.25	2	1.74	0.19	4	0.96	0.44
P_{max}	2	1.00	0.38	2	11.25	**<0.001**	4	4.26	**0.01**
Light use efficiency (α)	1	3.09	0.09	2	11.37	**<0.001**	2	1.94	0.16
F_v/F_m	na			1	7.62	**0.02**	na		
Chl *a*	2	1.88	0.16	2	13.92	**<0.001**	4	1.30	0.28
Chl *b*	2	1.27	0.29	2	10.00	**<0.001**	4	0.76	0.56
Chl *a*:Chl *b*	2	0.93	0.40	2	0.78	0.47	4	0.79	0.54

*α = 0.025.
Significant *P* values shown in bold.

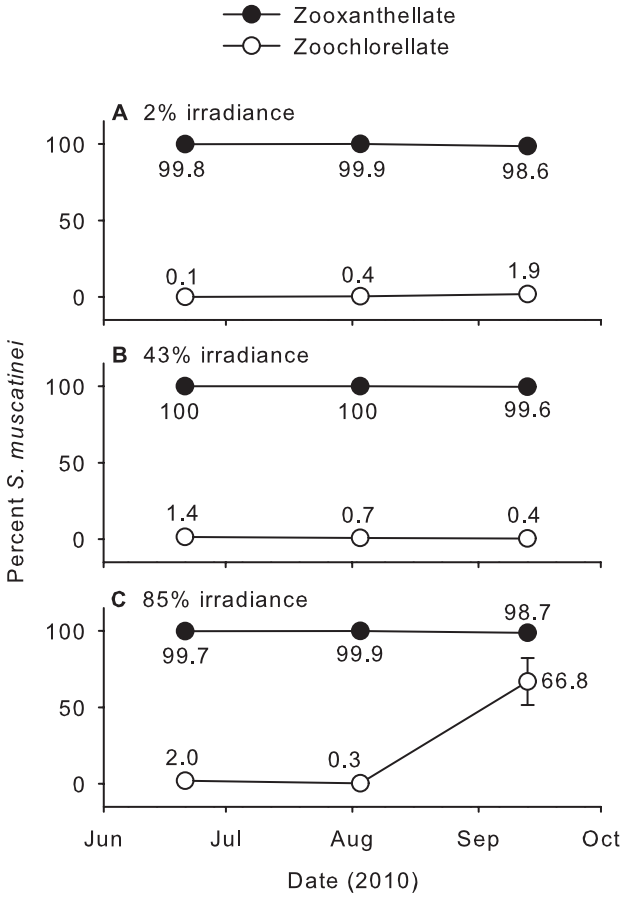


FIG. 3. Symbiont relative abundances in *Anthopleura elegantissima* over time in the three experimental treatments, expressed as the relative abundance of *Symbiodinium muscatinei* (mean ± SE) in anemones that were initially dominated by either *S. muscatinei* (zooxanthellate) or *Elliptochloris marina* (zoochlorellate). For each treatment, $n = 7–11$.

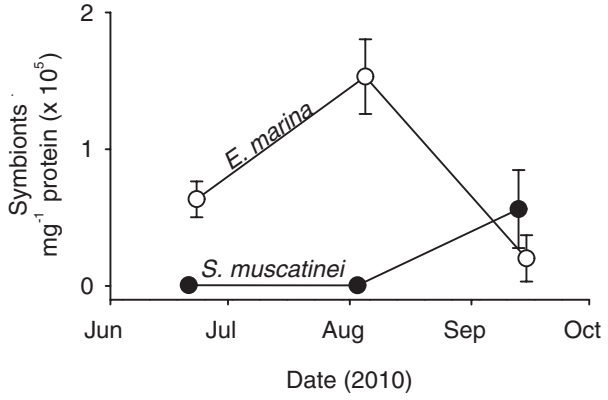


FIG. 4. Symbiont population densities within zoochlorellate anemones in the 85% irradiance treatment (mean ± SE; $n = 6–7$).

DISCUSSION

Although the physiology and population shifts of *E. marina* and *S. muscatinei* in their anemone hosts have been examined in prior studies, they have never been studied in combination. To our knowledge, our study represents the most comprehensive investigation to date of symbiont physiology and population dynamics leading up to symbiont shifts in a cnidarian-algal symbiosis. The results corroborate the widely held view that, when they occur at all, rapid changes in symbiont relative abundances are most closely associated with symbiont physiological dysfunction and bleaching. Interestingly, however, we found that shifts from *E. marina* to *S. muscatinei* did not directly coincide with initial indications of stress in *E. marina*; physiological stress was apparent in *E. marina* for at least 2 months

before there was any evidence of growing *S. muscatinei* prevalence.

Our nearly 1-year study with simulated intertidal conditions under seasonally variable natural light and temperature fluctuations yielded results that generally support those of short-term studies conducted under controlled experimental conditions (Saunders and Muller-Parker 1997, Engebretson and Muller-Parker 1999, Verde and McCloskey 2001, 2002). All of these studies, despite differences in duration and experimental light and temperature conditions, show the effects of increased irradiance and temperature to be either positive or neutral for *S. muscatinei* and mostly negative for *E. marina*. In our experiment, the simulated intertidal regime combined with the shading treatments exposed anemones to a gradient of irradiance and temperature similar to what they would naturally experience in sunlight-exposed microhabitats along a vertical gradient in the intertidal zone (Bingham et al. 2011, Dimond et al. 2011). The coupling of irradiance and temperature resulting from aerial exposure conditions creates important synergistic effects of the two factors on symbiont physiology that are especially relevant to conditions naturally encountered by intertidal *A. elegantissima* (Dimond et al. 2011). These synergistic effects, as well as the extended duration of our experiment, explains the tendency for stronger treatment effects in our study in comparison to previous shorter term studies where the effects of light and temperature were studied in isolation (Saunders and Muller-Parker 1997, Verde and McCloskey 2001, 2002). Moreover, the longer term nature of our study allowed us to observe a considerable lag in symbiont shifts following the onset of environmental and physiological stress, highlighting the importance of sampling over longer time scales to capture potential lags in biological responses.

Prolonged exposure of zoochlorellate anemones to high irradiance conditions led to a shift in symbiont dominance from *E. marina* to *S. muscatinei,* with this shift lagging at least 2–3 months behind measureable physiological stress, including reduced P_{max} and reduced chlorophyll content, in *E. marina* in June. This lag between symbiont stress and shifts in population density may explain why similar shifts from *E. marina* to *S. muscatinei* in *A. xanthogrammica* transplanted from low to high intertidal pools on Vancouver Island, British Columbia in the late spring did not occur until 2–4 months later, in late summer (Bates 2000). The widespread shift in symbiont dominance from *E. marina* to *S. muscatinei* among zoochlorellate anemones in the 85% irradiance treatment was apparently a consequence of reduced photosynthetic performance in *E. marina* combined with the relative high photosynthetic performance of *S. muscatinei*. We assume that these shifts occurred via proliferation of background populations (i.e., "shuffling"; Baker 2003) of *S. muscatinei* that were present within zoochlorellate anemones on prior sampling dates, however uptake of exogenous *S. muscatinei* (i.e., "switching"; Baker 2003) cannot be ruled out. Overgrowth of *E. marina* by *S. muscatinei* was likely a consequence of the opposite effects of higher irradiance treatments on mitotic indices in the two symbionts. Based on average treatment mitotic indices and estimated durations of cytokinesis for the two symbionts (*E. marina* = 69 h; *S. muscatinei* = 28 h; McCloskey et al. 1996), calculated specific growth rates were nearly twice as high for *E. marina* as for *S. muscatinei* under the 43% irradiance regime (*E. marina* = 0.030 d^{-1}; *S. muscatinei* = 0.016 d^{-1}), but very similar under the 85% irradiance regime (*E. marina* = 0.019 d^{-1}; *S. muscatinei* = 0.018 d^{-1}), suggesting that growth of *S. muscatinei* may have been able to catch up with that of *E. marina*. It is also noteworthy that the shift to *S. muscatinei* dominance coincided with a drop in *E. marina* density, suggesting that bleaching via *E. marina* expulsion facilitated the shift to *S. muscatinei*. Symbiont expulsion in *A. elegantissima* is highly responsive and positively related to irradiance regime (McCloskey et al. 1996), and it could be augmented by the presence of dysfunctional symbionts through feedbacks such as oxidative stress or lack of photosynthate transfer. Oxidative stress is likely to play a role in eliciting symbiont shifts since it has been well documented to result from symbiont photophysiological damage and is the proximal cause of mass symbiont expulsion and resultant bleaching (Lesser 1996, 1997, Weis 2008). Clearly, the physiological and cellular processes that mediate symbiont stability and shifts through growth, expulsion and retention are important areas for further study.

It is more difficult to explain why we did not observe shifts from *S. muscatinei* to *E. marina* among zooxanthellate anemones in the 2% irradiance treatment. In the 2% irradiance treatment, *S. muscatinei* exhibited low densities and mitotic indices, whereas conditions for *E. marina* were favorable. Estimated specific growth rates of *S. muscatinei* and *E. marina* in the 2% irradiance treatment were 0.011 and 0.030 d^{-1}, respectively, suggesting that *E. marina* had the capacity to overgrow *S. muscatinei*. However, two potential key differences between this treatment and the 85% irradiance treatment were that no bleaching was observed, and both symbionts remained photosynthetically competent. Although *S. muscatinei* may not function optimally under low temperature and irradiance, it has been shown to be physiologically tolerant of a wide range of conditions (Muller-Parker et al. 2007). This supports the idea that symbiont shifts are often associated with, or perhaps more rapidly produced by, symbiont physiological dysfunction and bleaching. Symbiont shifts do occur without bleaching in other systems, such as during post-bleaching reversion of reef coral symbiont assemblages to their pre-bleaching state (Thornhill et al. 2006, LaJeunesse et al. 2009),

and in these instances it may simply take more time for symbiont shuffling to occur through competitive displacement of one symbiont by another. Either shifts from *S. muscatinei* to *E. marina* may have eventually occurred in the 2% irradiance treatment over a longer period of time, or not at all due to the lack of bleaching and the photosynthetic competence of both symbionts under low light conditions. Bates (2000) similarly observed no symbiont shifts among zooxanthellate *A. xanthogrammica* transplanted from upper to lower intertidal pools in the field over a 4 month summer period, contrasting with the shifts observed among originally zoochlorellate anemones transplanted from lower to upper pools. Collectively, results of studies on symbiont shifts in *Anthopleura* spp. (Bates 2000, this study) are broadly similar to those of Baker's (2001) reciprocal vertical transplantation of several Caribbean reef corals, showing bleaching and symbiont shifts among most upward transplants, and lack of bleaching and symbiont shifts among most downward transplants.

The extent to which symbiotic cnidarians are flexible in their ability to associate with multiple algal symbionts has become an increasingly intriguing and complex subject. Amidst the considerable diversity of *Symbiodinium* spp. that have been detected in shallow reef environments, only a small subset appears to form viable symbioses (Coffroth et al. 2006, 2010, Pochon et al. 2010, Takabayashi et al. 2012). Although some symbiotic *Symbiodinium* spp. are generalists living with a variety of hosts over broad geographic areas, the majority are specialists associating with distinct host species, sometimes only in certain locales (Thornhill et al. 2009, LaJeunesse et al. 2010). Host fidelity to a single dominant symbiont appears to be the most common form of relationship (Goulet 2006), but increasingly sensitive molecular techniques continue to reveal background populations of other symbionts in hospite, suggesting that the potential to engage in viable symbioses with more than one symbiont may be greater than previously thought (Mieog et al. 2007, Silverstein et al. 2012). However, even well-known examples of so-called flexible symbioses are often characterized by a high degree of spatial and temporal stability of just one dominant symbiont, even when challenged with bleaching or large seasonal environmental fluctuations (Thornhill et al. 2006, 2009, Dimond et al. 2011, Levine and Muller-Parker 2012, McGinley et al. 2012). This tendency for stability over change has raised questions as to whether symbiont shifts are a viable acclimatization strategy for symbiotic cnidarians (Goulet 2006, Thornhill et al. 2006, 2009). On the other hand, experimental manipulations that simulate environmental change often elicit symbiont shifts (e.g., Rowan et al. 1997, Bates 2000, Baker 2001, this study) in symbioses that have been found to be largely stable in nature (Thornhill et al. 2006, Dimond et al. 2011, Levine and Muller-Parker 2012), suggesting that symbiont shifts require environmental perturbations of greater severity or duration than have been commonly observed in nature. Indeed, Baird et al. (2007) argue that when forced to acclimatize to enduring environmental changes, such as transplantation experiments or geographical climate gradients, hosts often have the capacity to do so by associating with different algal symbionts. *Anthopleura* symbioses provide an excellent case study of the paradoxically stable yet dynamic nature of cnidarian symbioses: typically characterized by a single dominant symbiont, they are temporally stable over short (annual) time scales (Dimond et al. 2011, Levine and Muller-Parker 2012), yet vary geographically (Secord and Augustine 2000), can be forced to shift experimentally (Saunders and Muller-Parker 1997, Bates 2000, this study), and are forecasted to shift naturally over longer time scales (Verde and McCloskey 2007, Dimond et al. 2011, Levine and Muller-Parker 2012).

Beyond their physiological effects, changes in a host's dominant symbiont type are likely to have important ecological consequences for cnidarian hosts because their symbionts are not only genetically and physiologically diverse, but also functionally diverse (Loram et al. 2007). In comparison to the highly diverse *Symbiodinium* lineages found in most cnidarians, the dinoflagellate and chlorophyte symbionts of *A. elegantissima* are especially genetically and physiologically divergent from one another, potentially accentuating the impacts of a change in the dominant symbiont. As with different types of *Symbiodinium* in tropical hosts (Loram et al. 2007), *E. marina* and *S. muscatinei* translocate different amounts of photosynthate (Engebretson and Muller-Parker 1999, Verde and McCloskey 2007, Bergschneider and Muller-Parker 2008) and possibly different quality photosynthetic products to their host (Trench 1971, Minnick 1984), which appears to affect host fitness in turn (B. B., J .D., G. M.-P., and L. Francis, in review). In a companion study, we found reproductive tradeoffs associated with hosting one symbiont over another; under favorable environmental conditions, whereas *E. marina* contributed more to anemone sexual reproduction, *S. muscatinei* appeared to facilitate the high rates of cloning by fission that make *A. elegantissima* so successful in the intertidal zone (B. B., J .D., G. M.-P., and L. Francis, in review). Therefore, as climatic warming ensues, the likely replacement of *E. marina* by *S. muscatinei* (Dimond et al. 2011, Levine and Muller-Parker 2012) may have significant ecological consequences for *A. elegantissima*.

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