

## ORIGINAL ARTICLE

# Diurnal and tidal patterns of carbon uptake and calcification in geniculate inter-tidal coralline algae

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Calcification; coralline algae; diurnal; inter-tidal; tidal.

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**Abstract**

Research on coralline algal responses to ocean acidification and other environmental stressors has increased in recent years as coralline algae is thought to stand a higher chance of being affected by acidification stress than other macroalgae. To provide context and enhance the existing eco-physiological framework for climate change studies, it is important to understand the effects of non-extreme stressors experienced regularly by inter-tidal coralline algae. In this study, we tested the potentially interacting effects of diurnal and tidal treatments on calcification in the geniculate coralline algae *Corallina frondescens* and *Corallina vancouveriensis* using <sup>13</sup>C-labeled bicarbonate. Both species deposited more calcium carbonate during the day than at night, and also when submerged (high tide) compared with when emerged (low tide) in their apical and mature segments (intergenicula). These results indicate that inter-tidal coralline algae do in fact pay a cost for living inter-tidally at the edge of an adaptive zone.

**Introduction**

The role of macroalgae as a foundational ecological group and as carbon and nutrient cyclers has attracted much attention recently in the context of changing climate. While it is generally agreed that macroalgae play important roles in ecological and chemical dynamics of near-shore ecosystems, interactions with the carbon cycle remain relatively unknown in many marine macroalgae (Koch *et al.* 2013). Indeed, algae are taxonomically and metabolically more diverse than terrestrial plants (Larkum & Vesik 2003), which complicates our understanding of their use of carbon, as well as our ability to make generalizations across groups.

In particular, coralline algae (Rhodophyta, Corallinales) have received renewed interest as potential indicators of the effects of ocean acidification (McCoy & Kamenos 2015). Beds of geniculate coralline algae along temperate, rocky shores are major players in coastal carbonate deposition (Fisher & Martone 2014). As coralline algae are both calcifiers and phototrophs, changes in available dis-

solved inorganic carbon (DIC) may reveal conflicting responses; calcification is predicted to become more costly while photosynthesis might be enhanced due to the increased availability of CO<sub>2</sub> caused by pH decline (Borowitzka 1979; Smith & Roth 1979). Isotope tracer techniques can provide a more detailed understanding of calcification within this group and yield insights to the roles of coralline algae in near-shore carbon cycling.

Many marine plants and algae use carbon concentrating mechanisms (CCMs) to elevate CO<sub>2</sub> concentrations around ribulose-1,5 biphosphate carboxylase-oxygenase, the enzyme responsible for CO<sub>2</sub> fixation (Raven 1997). While there exists a variety of CCMs, most rely on a large pool of bicarbonate, HCO<sub>3</sub><sup>-</sup>, which makes up approximately 90% of the DIC pool in the pH range of natural seawater (Zou *et al.* 2004), which ranges 7–9 at our study site (Wootton *et al.* 2008; Wootton & Pfister 2012). The most common mechanism for bicarbonate use is the conversion of HCO<sub>3</sub><sup>-</sup> to CO<sub>2</sub>, catalysed by carbonic anhydrase outside of the cell (CA<sub>ext</sub>), which allows the generated CO<sub>2</sub> to enter the cell by either diffusive or

active transport (Raven 1997). *Corallina* spp. are able to use bicarbonate for photosynthesis as well as dissolved CO<sub>2</sub> (Koch *et al.* 2013), although there is mixed evidence supporting the use of CA<sub>ext</sub> as the mechanism across this genus (Kremer & Küppers 1977; Giordano & Maberly 1989; Moulin *et al.* 2011).

Algal photosynthesis can be affected by time spent out of seawater and exposed to air. Among some inter-tidal macroalgae, the ability of a species to sustain photosynthesis when exposed is related to the vertical tidal height at which it is found (Johnson *et al.* 1974; Murru & Sandgren 2004). In other words, low inter-tidal macroalgae spend more time underwater, and those found exclusively in the low zone are less efficient photosynthesizers when exposed to air compared with those living higher in the inter-tidal. These macroalgae have developed the ability to photosynthesize efficiently when exposed (Johnson *et al.* 1974). Patterns of carbon use in seaweeds with depth suggest that those species that spend time emerged are more likely to have CCMs that allow bicarbonate use (Axelsson & Uusitalo 1988; Murru & Sandgren 2004). The absence of both of these abilities in subtidal macroalgae points to a potential cost of emergence *via* the maintenance of a broader range of metabolic mechanisms or pathways.

Why, then, do macroalgae inhabit the inter-tidal zone? Due to the effects of desiccation on invertebrate grazers and reduced exposure times to herbivorous fish, the inter-tidal zone offers an escape from grazers or grazing intensity (Lubchenco 1980; Moreno & Jaramillo 1983). However, in addition to desiccation, temperature stress and UV stress (Mathieson & Burns 1971; Johnson *et al.* 1974; Smith & Berry 1986; Davison & Pearson 1996; Martone *et al.* 2010), reduced photosynthetic potential may be included in the energetic costs of occurring inter-tidally as compared with subtidally. For example, geniculate coralline algae living higher in the inter-tidal have lower photosynthetic rates during low tide, even among those inhabiting tide pools and therefore not exposed to desiccation (Guenther & Martone 2014). There may be further interacting effects of diurnal and tidal cycles on algal photosynthesis because the timing of low tide changes seasonally and may occur either during day or night.

Many recent studies of ocean acidification have focused on inter-tidal organisms, in which adaptations to the large fluctuations in the physical environment experienced daily may be present (Harley *et al.* 2012; Noisette *et al.* 2013). Studies with animals have shown that stress-induced increases in metabolic rate can reduce energy allocated to growth, development and calcification (Stumpp *et al.* 2011; Hiebenthal *et al.* 2012). If there is indeed a cost to emergence, then inter-tidal organisms may already be living on the edge of an adaptive zone

and could instead be more vulnerable to further stressors rather than more resistant.

To explore the calcification responses of coralline algae to diurnal cycles in light and tidal conditions, we asked whether individuals of the sister species *Corallina frondescens* and *Corallina vancouveriensis* incorporated different amounts of <sup>13</sup>C-labeled bicarbonate (H<sup>13</sup>CO<sub>3</sub><sup>-</sup>) as a proxy for calcium carbonate deposition under these conditions. We measured the <sup>13</sup>C-label incorporation into calcium carbonate tissue and calculated estimates of new calcified tissue accretion for both apical and mature segments. By quantifying carbon fixed into the carbonate, we provide a metric of algal growth. As indeterminate growers where size is related to reproductive investment (Samson & Werk 1986; Pfister & Wang 2005), growth rates in phototrophs such as *Corallina* are a proxy for fitness. We predicted that both *C. frondescens* and *C. vancouveriensis* would incorporate the most H<sup>13</sup>CO<sub>3</sub><sup>-</sup> during daylight at high tide, and that calcification rates would be greater in apical segments than in mature segments.

## Material and Methods

### Study system

*Corallina frondescens* Postels & Ruprecht and *Corallina vancouveriensis* Yendo are geniculate (articulated) coralline algae (Corallinales, Rhodophyta) living in the inter-tidal zone in the temperate East Pacific Ocean, ranging from Baja California to Alaska. According to recent phylogenetic work, *C. frondescens* and *C. vancouveriensis* are sister species within the *Corallina* genus (Hind & Saunders 2013). Both grow articulated fronds up to approximately 15 cm in height from a basal crust that serves as a holdfast.

At our sampling site, Tatoosh Island, WA, USA (48.4° N, 128.7° W), *Corallina frondescens* and *Corallina vancouveriensis* are found in the low inter-tidal zone under the cover of a thick macroalgal canopy primarily consisting of the kelps *Saccharina sessile* and *Alaria marginata*, and also in tide pools throughout the inter-tidal zone. In both locales, *C. frondescens* and *C. vancouveriensis* grow in a mixed assemblage. During low tide, algae in the low inter-tidal zone are emergent from seawater. The macroalgal canopy shades algae living in the understory from UV stress and potentially limits photosynthesis, and also protects the understory from desiccation (Dayton 1975).

We collected 70 individuals of each species near 0 m mean lower low water in Hedophyllum Cove on Tatoosh Island on 5 July 2012. Individuals were gently cleaned of obvious epiphytes with a soft nylon brush. Specimens were stored overnight in chilled seawater, and subsequently wrapped in kelp blades, moistened paper towels and ice packs for transport by airplane to experimental

facilities in Chicago, IL. Specimens were allowed to acclimate in the experimental tanks for 10 weeks to reduce transplant stress prior to the experiment. During the acclimatization phase, specimens were fully submerged in artificial seawater and otherwise experienced experimental ambient conditions as described below.

### Experimental design

To determine how an inter-tidal environment affects bicarbonate use and calcification patterns in geniculate coralline algae, we set up laboratory experiments to explore responses to diurnal and tidal treatments. *Corallina frondescens* and *Corallina vancouveriensis* were grown in a climate-controlled growth chamber at 12 °C in artificial seawater (Instant Ocean; Spectrum Brands, Inc. Blacksburg, VA, USA) at pH 8.1–8.4. Seawater was mixed to a salinity of  $32 \pm 1$ , concordant with salinity at the collection site (Wootton & Pfister 2012). Emergent (low-tide) conditions were simulated by spraying with seawater every 15 min using timed vegetable sprayers into self-draining tanks and submerged (high tide) conditions were achieved by keeping fronds underwater. Photosynthetically active radiation (PAR) was measured with a light meter (LICOR LI-1000) and ranged from 27 to 30  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  in air (Quantum Q-90 PAR sensor) and 20–30  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  underwater (Underwater Quantum UWZ-192 PAR sensor), measured at the position of exposed and submerged algal fronds in the experimental tanks. These low light levels were selected intentionally to mimic the naturally high-density *Corallina* beds that occur beneath the kelp canopy at higher latitude, which was necessary to prevent algal bleaching over the experimental duration. All treatments experienced seasonally appropriate diurnal light cycles (lights on from 06:00–20:00 h) USA Central Time, UTC –06:00.

There were eight separate 2-l tanks within each treatment, each containing one frond (individual) of each species. To achieve a 2000 per mil enrichment of  $\text{H}^{13}\text{CO}_3^-$ , 0.36 ml of bicarbonate labeled with a heavy stable isotope of carbon (99%  $^{13}\text{C}$ ; Cambridge Isotope Laboratories, Andover, MA, USA), was introduced to each 2-l tank for the submerged treatments. In the case of emergent treatments, the isotope spike was introduced to a 16-l reservoir to supply the spray treatments. The addition of tracer did not appreciably alter the total DIC concentration of the water, which remained at  $2633 \pm 27 \mu\text{mol}\cdot\text{kg}^{-1}$  (two replicate samples of stock artificial seawater). Night experiments (11 September 2012) were started after a 60-min incubation in the dark at the start of the night (20:00 h), following a full regular diurnal cycle. Subsequently, daylight experiments (13 September 2012) were started after a 60-min incubation in

the light at the start of the day (06:00 h), following a full regular diurnal cycle.

The frond tips (apical segments or intergenicula) of each individual of *Corallina frondescens* and *Corallina vancouveriensis* were sampled after 0 (prior to tracer addition), 4 and 12 h for the enriched carbon signal. Samples of each algal frond were also taken from intergenicula below the apical segment, referred to as mature segments, to determine the extent of incorporation of the enriched carbon signal into calcified tissue. Although carbon fixed *via* photosynthesis is added as biomass in multiple ways in phototrophs, we quantified carbon added to the calcified portion of the alga, given that *Corallina* gains both mass and surface area *via* the addition of calcium carbonate. Thus, our estimates quantify the carbon fixed into new algal biomass. Mature segments were always sampled directly below the sampled apical segment. Apical and mature segment samples were taken using forceps, and samples were subsequently rinsed three times with unlabeled seawater followed by three times with MilliQ purified water (Millipore Corp., Billerica, MA, USA) to remove labeled  $^{13}\text{C}$ -labeled DIC from the sample. Samples were placed in 0.5-ml clean microcentrifuge tubes and placed in a drying oven for 48 h at 60 °C. Sample tubes were then capped and stored for isotopic analysis.

### Algal isotope and seawater DIC analyses

We quantified the amount of carbon, as  $\text{H}^{13}\text{CO}_3^-$ , taken up by each segment by analysing  $\delta^{13}\text{C}$  values of the carbonate component of the mineralized coralline algal samples. Individual algal segments were powdered and weighed into Exetainer tubes with sample masses generally around 300  $\mu\text{g}$ . Exetainer tubes were flushed with helium and treated with 100% to 103% phosphoric acid at 26 °C for 12 h in an automated Thermo Gasbench II device coupled to a Thermo Delta V mass spectrometer. Samples were then examined, and incompletely reacted samples were re-suspended in the acid. Isotopic analysis was initiated once all samples were completely reacted (within 24 h). Isotopic compositions were corrected to standards run with the samples (National Bureau of Standards carbonate [NBS 18], National Bureau of Standards limestone [NBS 19] and lithium carbonate [LSVEC]). Results were corrected for sample size effects where appropriate by applying a linear correction factor determined by averaging linear regressions between peak intensity and  $\delta^{13}\text{C}$  offset for the analytical standards within each sample run.

We analysed all available apical segment samples for  $\delta^{13}\text{C}$  ( $n = 8$  per factorial treatment) from the 0-, 1-, 4- and 12-h collections. Sample loss occurred in rare cases when individual sample weights were too small for iso-

tope analysis. Three replicates of mature segments were analysed from the 0-, 1- and 12-h collections. Mature segments were generally too large to analyse the entirety of the segment; therefore, we ran subsamples obtained after homogenizing and powdering the entire segment. We ran additional replicates of 12-h samples from submerged daytime treatments (*Corallina frondescens*  $n = 6$ , *Corallina vancouveriensis*  $n = 7$ ), which showed the greatest variability in  $\delta^{13}\text{C}$ .

Seawater samples for DIC analysis were collected in sealed Exetainer tubes from the stock artificial seawater used in both submerged and emergent treatments (Labco Limited, Lampeter, Wales, UK). Sample tubes were prepared with 100  $\mu\text{l}$  of 85% phosphoric acid and flushed with helium gas prior to seawater sampling. One milliliter of artificial seawater was injected directly into the prepared tubes using a syringe. Samples were incubated at 26 °C for 16 h in an automated Thermo GasBench II device prior to analysis on a Thermo Delta V mass spectrometer as above.

#### Quantifying algal calcification

We determined the new deposition ( $G$ ) of calcium carbonate minerals during the incubation interval using measured  $\delta^{13}\text{C}$  values and isotope mass balance calculations [equations (1) and (2) below; Table S1]. The  $\text{CO}_2$  released during phosphoric acid digestion derived from two sources: new algal  $\text{CaCO}_3$  and previously accreted skeletal carbonate mineral. The new carbon acquired in each measured algal segment ( $\delta^{13}\text{C}_\text{N}$ ) was assumed to have the same carbon isotope composition as the labeled seawater DIC,  $\delta^{13}\text{C} = 2000\text{‰}$ . This assumption is supported by our measurements of natural carbon isotope ratios in coralline algae from this field site. Both tissue  $\delta^{13}\text{C}$  ( $-2.3\text{‰}$  to  $-2.7\text{‰}$  Vienna Pee Dee Belemnite [VPDB]; Table S2) and seawater  $\delta^{13}\text{C}$  ( $-0.7\text{‰}$  to  $-0.5\text{‰}$  VPDB; Bian 2013) is indicative of the slight fractionation expected to be generated by algal respiration and typical variations in seawater DIC that is detectable with naturally occurring isotopes as opposed to isotope labels. The previously accreted skeletal material in a segment was assumed to have a  $\delta^{13}\text{C}$  value equal to the measured value for the  $t = 0$  sample from the corresponding frond ( $\delta^{13}\text{C}_\text{P}$ ). The measured  $\delta^{13}\text{C}$  value ( $\delta^{13}\text{C}_\text{M}$ ), representing the mixture of new calcified tissue and previously accreted carbonate mineral, is then represented by the mixing equation:

$$\delta^{13}\text{C}_\text{M} = f_\text{G} \delta^{13}\text{C}_\text{N} + (1 - f_\text{G}) \delta^{13}\text{C}_\text{P} \quad (1)$$

where  $f_\text{G}$  is the fraction of the calcium carbonate mineral precipitated during the experiment, and  $\delta^{13}\text{C}_\text{N}$  and  $\delta^{13}\text{C}_\text{P}$

are the carbon isotope compositions of the newly precipitated and previously accreted calcium carbonate, respectively. We solve equation (1) for  $f_\text{G}$ , and then determine calcium carbonate accretion during the incubation interval using:

$$G = f_\text{G} \cdot M_\text{I} \quad (2)$$

where  $M_\text{I}$  is the mass of the calcium carbonate component of the segment. We used the  $\text{CO}_2$  yield from the phosphoric acid digestions to determine  $M_\text{I}$ . These yields were determined mass spectrometrically by converting the measured sample peak area on the  $m/z$  44 signal (the dominant  $\text{CO}_2^+$  ion beam) to an equivalent  $\text{CaCO}_3$  mass using a calibration curve fitted to our  $\text{CaCO}_3$  standards.

#### Statistical analysis

Due to unequal variance across our data set (heteroskedasticity), we square-root transformed our data. We used a two-sample  $t$ -test to test for differences in calcification between apical and mature segments. Subsequent tests were carried out separately for apical and mature segments.

To test whether calcium carbonate accretion occurred over time, we used a linear mixed effects model with sample time nested within algal individual as a random effect using the lme4 package in the statistical language R (version 2.14.2; R Development Core Team, 2012). We compared models with and without sample time as an explanatory variable using the likelihood ratio test.

Next, we asked whether species identity, given the effects of diurnal and tidal treatments, affected calcification rates using a three-way analysis of variance (ANOVA) for samples taken after 4 and 12 h. For cases for which species identity was not significant, we subsequently pooled data from both species and looked for diurnal and tidal treatment effects using a two-way ANOVA for samples taken after 4 and 12 h. For cases in which species identity was statistically important, we performed the two-way ANOVA separately by species.

#### Light:dark calcification ratios

Another metric used for quantifying diurnal calcification patterns is a light:dark ratio (Ikemori 1970; Pentecost 1978; El Haikali *et al.* 2004; Martin *et al.* 2013), which offers a metric of comparison with other studies of algal growth and calcification. We calculated the light:dark calcification ratios for apical and mature segments by dividing the average amount of new calcified tissue ( $\mu\text{g}$  of calcium carbonate) after 12 h in the dark ( $G_\text{D}$ ) by the average amount of new calcified tissue after 12 h in the light

( $G_L$ ).  $G_L:G_D$  ratios in *Corallina frondescens* and *Corallina vancouveriensis* were calculated separately for each treatment (diurnal, tidal) and for apical and mature segments.

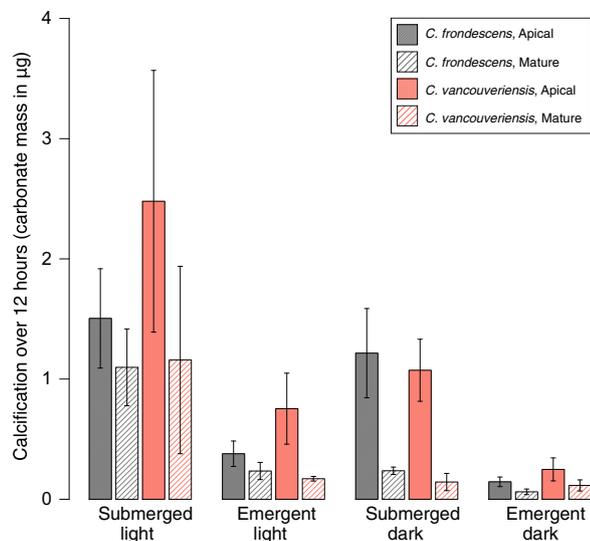
## Results

### Differences between apical and mature segments

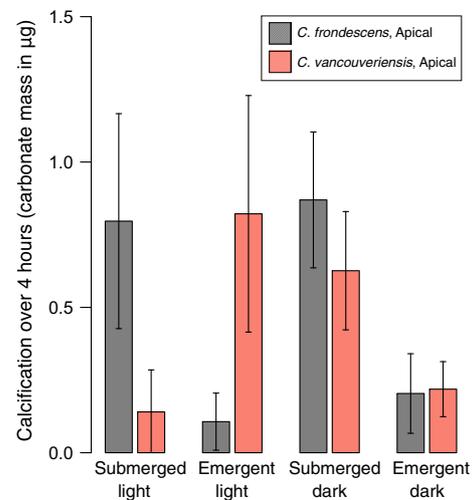
Overall, apical segments accreted 1.7 times more calcium carbonate than did mature segments when we pooled data from all treatments ( $t$ -test,  $P = 0.002$ ). Differences between apical and mature segments were strongly driven by the submerged night-time treatment ( $t$ -test;  $P < 0.001$ ), in which apical segments grew six times more than mature segments after 12 h. Due to these differences, we analysed drivers of calcification in apical and mature segments separately.

### Calcification in apical segments

In apical segments, calcium carbonate accretion occurred over the 12-h experiment (Figs 1 and S1). We fitted the data to a linear mixed model of calcification ( $G$ ) that included species identity, diurnal treatment, tidal treatment, sample time and a random effect of sample time nested within each algal individual. This model provided a better fit than one that excluded the fixed effect of sample time, indicating that calcification occurred over time in apical segments (likelihood ratio test,  $P < 0.001$ ).



**Fig. 1.** Calcium carbonate precipitation after 12 h in each treatment for the apical and mature segments of *Corallina frondescens*, in gray, and *Corallina vancouveriensis*, in pink. Bars representing apical segments are fully shaded and bars representing mature segments are marked with diagonal lines. Vertical lines show SE.



**Fig. 2.** Calcium carbonate precipitation after 4 h in each treatment for the apical segments of *Corallina frondescens*, in gray, and *Corallina vancouveriensis*, in pink. Bars representing apical segments are fully shaded and bars representing mature segments are marked with diagonal lines. Vertical lines show SE.

At the 4-h collection, we observed some variation across species and individuals (Fig. 2). Interestingly, light treatment alone did not significantly affect calcification in either species (two-way ANOVA,  $P > 0.25$ ), while emergent conditions reduced calcium carbonate accretion in *Corallina frondescens* (two-way ANOVA,  $F_{1,24} = 7.790$ ,  $P = 0.010$ ) and emergent conditions combined with darkness interacted to reduce calcification in *Corallina vancouveriensis* (two-way ANOVA,  $F_{1,22} = 4.453$ ,  $P = 0.047$ ). Calcification patterns between the two species became more consistent over a longer experimental duration. Over the 12-h experimental period, *C. frondescens* and *C. vancouveriensis* did not differ significantly in their apical calcium carbonate accretion (Fig. 1; two-way ANOVA after 4 h,  $F_{1,53} = 0.027$ ,  $P = 0.870$ ; two-way ANOVA after 12 h,  $F_{1,56} = 1.029$ ,  $P = 0.315$ ). Submergence greatly increased calcification (two-way ANOVA, species pooled,  $F_{1,52} = 25.08$ ,  $P < 0.001$ ) and daylight had a positive effect (two-way ANOVA, species pooled,  $F_{1,52} = 5.727$ ,  $P = 0.020$ ).

### Calcification in mature segments

As in apical segments, calcium carbonate accretion occurred in mature segments over the 12-h experiment (Fig. S2). We fitted the data to a linear mixed model of calcification ( $G$ ) that included species identity, diurnal treatment, tidal treatment, sample time and a random effect of sample time nested within each algal individual. This model provided a better fit than one that excluded the fixed effect of sample time, indicating that calcium

carbonate accretion did occur over time in mature segments (likelihood ratio test,  $P < 0.001$ ). While our analysis cannot differentiate definitively between isotopic exchange and new skeletal tissue accretion in mature segments, isotopic exchange would require recrystallization, which is unlikely to occur in detectable amounts (here up to 2.6%, Table S1) on a 12-h timescale. Recent work supports evidence of calcification in mature segments of *Corallina* and other geniculate corallines (Martone 2010; Fisher & Martone 2014).

We analysed mature segments sampled at  $t = 0, 1$  and 12 h (Fig. S2), but present the results for 12-h samples only (Fig. 1). We found that both daylight and submergence increased calcification in mature segments (two-way ANOVA light,  $F_{1,27} = 8.357$ ,  $P = 0.007$ ; two-way ANOVA tide,  $F_{1,27} = 5.155$ ,  $P = 0.031$ ). Interestingly, our results indicate a larger effect of daylight on calcium carbonate skeletal accretion in mature segments than we observed in apical segments (Fig. 1).

#### Light:dark calcification ratios

Mean light:dark calcification ratios ( $G_L:G_D$ ) in apical segments were 2.6 and 1.2 in exposed and submerged *Corallina frondescens* and 3.0 and 2.3 in exposed and submerged *Corallina vancouveriensis*, respectively (Fig. 3A). Mean light:dark calcification ratios in mature segments were 3.8 and 4.6 in exposed and submerged *C. frondes-*

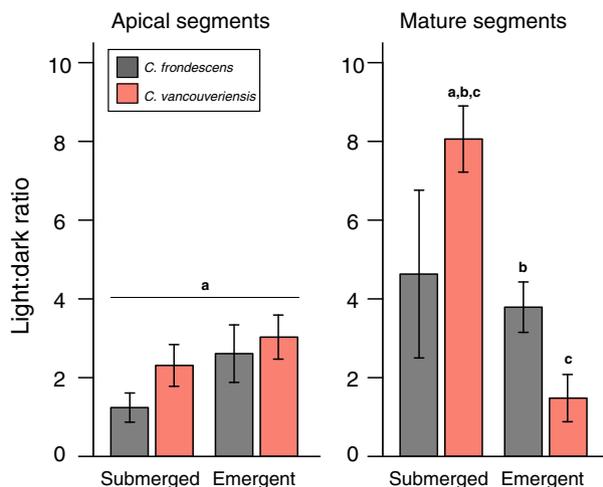
*cens* and 1.5 and 8.1 in exposed and submerged *C. vancouveriensis*, respectively (Fig. 3B). Despite differences in means,  $G_L:G_D$  were not significantly affected by species identity within any treatment (two-sample  $t$ -tests, all  $P > 0.05$ ; Fig. 3). *Corallina vancouveriensis* in the submerged treatment had elevated  $G_L:G_D$  in mature segments relative to apical segments [two-sample  $t_{10} = -5.77$ ,  $P = 0.0002$ ]. Light:dark calcification was affected by tidal conditions only in mature segments of *C. vancouveriensis* [two-sample  $t_8 = -6.38$ ,  $P = 0.002$ ].

## Discussion

### Diurnal patterns

Calcification in geniculate coralline red algae is generally directly related to photosynthetic rate (Pentecost 1978) and likely occurs by the 'trans calcification' enzymatic mechanism, which was originally documented in the green freshwater alga *Chara corallina* (McConnaughey & Whelan 1997). In trans calcification, seawater  $\text{HCO}_3^-$  is taken up and converted to  $\text{CO}_2$  for photosynthesis via disproportionation, which subsequently produces the carbonate ( $\text{CO}_3^{2-}$ ) used in algal calcification (McConnaughey & Whelan 1997). Reduced  $\text{CO}_2$  fixation has been documented in the dark (Kremer & Küppers 1977; Pentecost 1978; Borowitzka 1979; Ramus & Rosenberg 1980; Coutinho & Zingmark 1987; Hanelt *et al.* 1993), leading to lower rates of calcium carbonate precipitation due to tight links between algal photosynthetic activity (the greatest source of  $\text{CO}_2$  fixation) and calcification. Notably, however, some species of high-latitude coralline algae in the genus *Clathromorphum* are able to sustain calcification in the dark for several months, pointing to mechanisms of carbon storage (Adey *et al.* 2013). In light of what is known about the physiology of calcification, it is not surprising that we found that, overall, both *Corallina frondescens* and *Corallina vancouveriensis* deposited more calcium carbonate during daylight than in the dark (Figs 1 and 3).

As a ratio,  $G_L:G_D$  (calcification in light:calcification in dark) can be difficult to interpret without availability of raw calcification data in both light and dark. The ratio provides less detailed results than raw calcification data, and can be ambiguously interpreted. However, to facilitate comparison with previous studies of algal calcification, we calculated  $G_L:G_D$  for each treatment (Table 1). Comparison of  $G_L:G_D$  between submerged apical segments of *Corallina frondescens* and *Corallina vancouveriensis* in this study and those of other *Corallina* spp. from the literature indicate lower proportions of calcification in the dark in *C. frondescens* and *C. vancouveriensis* than in their congeners (Table 1). Cooler water temperature



**Fig. 3.** Light:dark calcification ratios ( $G_L:G_D$ ) at 12 h for (A) apical and (B) mature segments. All bars in (A), labeled a, are statistically different from mature segments of *Corallina vancouveriensis* in (B), labeled a (two-sample  $t$ -tests, all  $P < 0.0004$ ). In (B), bars labeled b are statistically different from one another [two-sample  $t_7 = -4.04$ ,  $P = 0.005$ ] and bars labeled c are statistically different from one another [two-sample  $t_8 = -6.38$ ,  $P = 0.0002$ ]. In both panels, data for *Corallina frondescens* are in grey and *C. vancouveriensis* in pink. Vertical lines show SE.

**Table 1.** Light:dark calcification ratios ( $G_L:G_D$ ) from the literature for congeners in the genus of articulated corallines *Corallina* and a crustose coralline *Lithophyllum cabiochae* for reference. Data from this study refer to submerged experimental treatments.

light: dark ratio	species	location	temp. (°C)	source
1.2:1	<i>Corallina frondescens</i>	Temperate E. Pacific	12	This study
2.3:1	<i>Corallina vancouveriensis</i>	Temperate E. Pacific	12	This study
3.8:1	<i>Corallina pilulifera</i>	Temperate W. Pacific	15	Ikemori (1970)
3–4:1	<i>Corallina officinalis</i>	Temperate E. Atlantic	20	Pentecost (1978)
3.6:1	<i>Corallina elongata</i>	Mediterranean	13–17	El Haikali <i>et al.</i> (2004)
3.5:1	<i>Lithophyllum cabiochae</i>	Mediterranean	16	Martin <i>et al.</i> (2013)

in our study could be driving these small differences in  $G_L:G_D$ , which could be linked to slower overall growth. Although we observed differences between calcium carbonate precipitation in *C. frondescens* and *C. vancouveriensis* using the  $G_L:G_D$  calcification metric, we did not observe differences in other analyses that account for calcification in the light and dark separately, perhaps reflecting both the phylogenetic and niche similarity of these species.

In temperate settings, algal respiration in the dark can lower the pH of the diffusive boundary layer (Hurd *et al.* 2011), which may be responsible for observations of lower calcification rates of coralline algae in the dark than during daytime (Gao *et al.* 1993; Martin *et al.* 2013; Fig. 3). Conversely, a lower pH would make  $CO_2$  and  $HCO_3^-$  more available in the boundary layer for photosynthesis as reduction in pH changes the relative abundances of DIC forms. In warmer tropical settings, measurements from red calcareous crusts reveal very low calcification rates that can lead to dissolution of calcium carbonate in the dark (Chisholm 2000). A possible explanation is increased respiration rates overnight in warm, tropical settings, which lower pH in the diffusion boundary layer at the algal surface. Metabolic processes, including photosynthesis, calcification and respiration, change the water chemistry within this boundary layer and can affect the passage of ions between seawater and the algal surface (Hurd 2000). For example, some tropical green algae in the genus *Halimeda*, known to alter surface pH on daily cycles (de Beer & Larkum 2001), grow non-calcified tissue at night, and calcify during the day when respiration-induced lower pH is less significant (Hay *et al.* 1988).

Accordingly, our results do show decreased calcification at night and when plants are emergent, but we nevertheless observed net calcification rather than evidence of net dissolution (Figs 1 and 2).

### Tidal patterns

Tidal treatment was also an important driver of algal calcification, with both *Corallina frondescens* and *Corallina vancouveriensis* depositing more calcium carbonate when submerged than emerged (Fig. 1). This was perhaps due to amplified boundary layer effects; emergent individuals remained moist but disconnected from surrounding seawater and replenished sources of bicarbonate. Given that boundary layer effects are likely to be important (Hurd *et al.* 2011; Cornwall *et al.* 2013a, 2014), our experiments may provide a conservative estimate of the effects of emergence on calcification. By spraying emergent individuals at regular intervals, our experimental conditions may have provided greater seawater exchange and bicarbonate supply to the algal surface than experienced by emergent individuals in nature. However, we do note that articulated corallines in nature can be characterized by turf-like assemblages that reduce evaporation and retain surface moisture. Thus, coralline algae in emergent field conditions may interact with some seawater during a period of emergence. While it is not possible to perfectly mimic field conditions in a laboratory setting, our experimental conditions did nevertheless provide stress in the form of a limited DIC pool, greater connectivity to the atmosphere (and atmospheric  $CO_2$ ) and periodic drying as compared with submerged conditions. Therefore, these laboratory conditions capture the stressors that accompany emergence in the field, and enable us to trace a small-scale process that usually occurs in an open marine system.

### Interactions between diurnal and tidal patterns

We expected to find that the cost of growing in emergent habitats was greater at night than during the day. Despite a cost to both emergence and darkness, there was no treatment interaction to indicate an increased cost when both conditions occurred together after 12 h (Fig. S3B). Light:dark calcification ratios ( $G_L:G_D$ ) indicated proportionally reduced calcification in the dark, as they differed from 1, and revealed an effect of tidal treatment in mature segments only (Fig. 3). The different information obtained from calcification rates and  $G_L:G_D$  stems from the difficulty of interpreting calcification patterns from ratios, which here are confounded by changes occurring either during the day or night. Although  $G_L:G_D$  is a com-

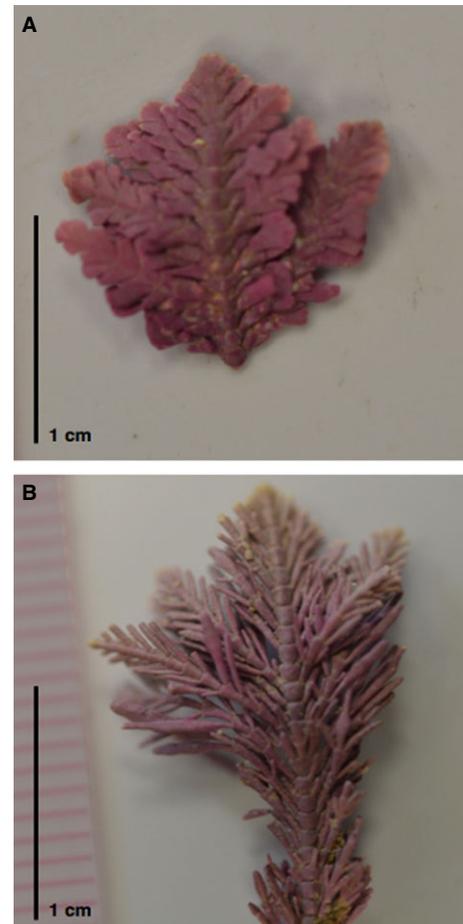
monly reported metric (Table 1), we recommend the use of calcium carbonate accretion estimates rather than  $G_L$ : $G_D$  for meaningful comparisons in future studies.

Although we did not observe synergy between diurnal and tidal effects on calcification in *Corallina frondescens* and *Corallina vancouveriensis*, this may change in a future, acidified ocean. The effects of boundary layers on protecting inter-tidal calcifiers are likely to become increasingly important in the future ocean (Cornwall *et al.* 2014), and may also interact with temperature excursions and other effects of low tide. In particular, boundary layers over inter-tidal coralline algae living beneath a kelp canopy may have great importance at low tide, when algal individuals rely solely on surface moisture. In that case, we would expect that the timing of low-tide events could determine diurnal patterns of calcification in geniculate coralline algae, particularly through interacting effects of low tide, night and acidification.

High variation in calcification rates between fronds also may have obscured our ability to detect a significant treatment interaction between darkness and emergent conditions (Figs 1 and 2). Between-frond variation in calcification rates of geniculate corallines that have been observed in nature may be due, in part, to the presence of additional meristems (locations of new growth) as frond size increases (Martone 2010; Fisher & Martone 2014). Our experimental fronds were all of similar size (1.0–1.5 cm in length), chosen to have few (two to five) branches per frond and were placed into treatments at random, all of which should have reduced biases associated with size or meristem number. Therefore, we note that apical segments of both species under dark, emergent conditions grew only 30–40% as much as they did in daytime emergent conditions, despite high variance and non-significance of this treatment interaction (Fig. 1). In order to detect a significant threshold for an interaction, the difference would have to have been about twice what we observed.

#### Patterns in mature and apical segments

Geniculate corallines lack chloroplasts in the tips of their apical segments (Pearse 1972; LaVelle 1979), which is responsible for the white color of meristematic tissue in those tips. Thus, growth in meristematic tissue depends on translocation of organic matter from more mature portions of the plant, which is a metabolically dependent process (Lee & Carpenter 2001). In coralline algae, fusions between cells enable lateral and vertical translocation of photosynthates from photosynthetically active tissue to other parts of the thallus (Steneck 1983). In *Corallina*, apical tips are on the order of 0.1–0.5 mm long and comprise the upper edge of the apical segment (Fig. 4).



**Fig. 4.** Photographs of dried (A) *Corallina frondescens* and (B) *Corallina vancouveriensis* specimens collected from Tatoosh Island on 3 July 2012.

The existence of cell fusions allows several interpretations of the patterns of calcification in mature *versus* apical segments, and we thus present two hypotheses for reduced tissue accretion in mature compared with apical segments in the dark. First, if photosynthetic rates are higher in apical segments, then one might expect higher calcification rates as well. Based on evidence supporting reduced photosynthetic pigment content in apical segments (Pearse 1972; LaVelle 1979), we discount this hypothesis. Second, if photosynthates from mature segments are driving meristematic calcification within the apical segments, then there may be fewer leftover resources in mature segments for their own use. This could lead to lesser accretion despite greater photosynthesis in mature segments, which are effectively acting as a source of resources to the apical region. The elevated  $G_L$ : $G_D$  of mature segment samples reveals a greater light-dependence of calcification in mature segments than in apical segments (Fig. 3), indicating that *Corallina* may

allocate resources to promote growth in apical segments over growth in mature segments. Calcification patterns observed in the geniculate coralline *Calliarthron cheilosporioides* indicate greater meristematic calcification in larger fronds and provide some evidence in favor of this mechanism (Martone 2010).

#### Fitness implications in the modern and future oceans

It has been postulated that inter-tidal organisms are better suited to climate-related changes in the marine environment, as they are adapted to highly fluctuating environments compared to organisms living subtidally (Harley *et al.* 2012; Raven *et al.* 2012). The existence of strong zonation patterns among inter-tidal algae and animals suggests that these organisms are instead adapted only to the particular physical limits of emergence times, *i.e.* temperature excursions and changes in seawater chemistry during periods of low tide or isolation of tide pools from the surrounding seawater that are associated with a specific tidal height (Johnson *et al.* 1974; Smith & Berry 1986; Axelsson & Uusitalo 1988; Davison & Pearson 1996; Murru & Sandgren 2004). In addition, a low tide rarely lasts longer than several consecutive hours. Thus, while inter-tidal resistance appears reasonable in the short-term, it is unknown whether this resistance extends to sustained or permanent exposure to stressful conditions.

To date, mixed evidence for increased inter-tidal resistance exists specifically for geniculate coralline algae. For example,  $p\text{CO}_2$  did not affect respiration, gross primary production and calcification rates of *Corallina elongata* from tide pools in either light or dark (Egilsdottir *et al.* 2013). This result contrasts with the inter-tidal *Corallina officinalis*, in which calcification rates showed the expected parabolic response of skeletal accretion to elevated  $p\text{CO}_2$  (Smith & Roth 1979; Ries *et al.* 2009), including in the absence of changes in photosynthesis (Hofmann *et al.* 2012). Similarly, a comparison of inter-tidal *C. elongata* to an inter-tidal crustose coralline, *Lithophyllum incrustans*, and a subtidal maerl-forming coralline, *Lithothamnion corallioides*, revealed that inter-tidal species that regularly experience large diurnal pH fluctuations are not necessarily less affected by elevated  $p\text{CO}_2$  than their subtidal counterparts (Noisette *et al.* 2013). Along these lines, one might also expect inter-tidal algae to be well adapted to solar UV radiation. The inter-tidal *Corallina sessilis* showed reduced calcification in response to UVB, which acted synergistically with increased ocean acidification (Gao & Zheng 2010). These results highlight a great variation in algal response to abiotic factors governing calcification in coralline algae, even among congeners and growth forms, and point to a need

to better quantify the physiology of calcification in geniculate coralline algae.

Diurnal patterns in coralline algal calcification are also important in an environmental context. For organisms such as macroalgae where reproductive capacity is related to size, metrics of growth serve as a proxy for fitness (Samson & Werk 1986; Pfister & Wang 2005). Large diurnal cycles in pH occur in coastal environments, especially in kelp-dominated systems where night-time pH is lower than daytime pH due to light-dependent patterns of algal photosynthesis (Wootton *et al.* 2008; Wootton & Pfister 2012; Cornwall *et al.* 2013b). In laboratory cultures of the geniculate coralline *Arthrocardia corymbosa*, diurnally fluctuating pH reduced net algal calcification (Cornwall *et al.* 2013b). In other words, while calcification was generally lower at night than during the day, reduced pH overnight further reduced night-time calcification. As algal respiration contributes to decreasing night-time calcification, it is also important to consider effects of climate change on respiration rates.

Longer-term studies of laboratory cultures over 3–10 months (Ragazzola *et al.* 2012, 2013) and changes in field specimens over 30 years (McCoy & Ragazzola 2014) have shown that ocean acidification can affect calcification in coralline algae at a larger scale, and also interact with organism physiology and energetic trade-offs in cell wall thickness and overall skeletal thickness. As we seek to understand the conflicting effects of increased carbon as a resource for these primary producers *versus* as a stressor due to changed ocean pH, studies such as the one reported here will serve to link short-term calcification processes to longer-term environmental change.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** New growth of calcium carbonate (in  $\mu\text{g}$ ) in apical segments over experimental periods that include 1, 4, and 12 h post-enrichment.

**Figure S2.** Growth of new calcium carbonate (in  $\mu\text{g}$ ) in mature segments over experimental periods that include 1 and 12 h post-enrichment. Note that y-axis range differs from Fig. S1.

**Figure S3.** Submerged individuals grew more at (A) 4 and (B) 12 h (two-way ANOVA;  $F_{1,50} = 3.410$ ,  $P = 0.071$ ,  $F_{1,56} = 25.08$ ,  $P < 0.001$ , respectively). Individuals grew more in daylight at 12 h than at night (two-way ANOVA;  $F_{1,56} = 5.727$ ,  $P = 0.020$ ), while daylight did not have a significant effect on growth after only 4 h (two-way ANOVA;  $F_{1,50} = 0.590$ ,  $P = 0.446$ ). There was no significant interaction between tidal and diurnal treatments at either time point (two-way ANOVA;  $F_{1,50} = 1.800$ ,  $P = 0.186$ ,  $F_{1,56} = 0.133$ ,  $P = 0.716$ , respectively).

**Figure S4.** Growth in the apical segment plotted against growth in the mature segment for each individual frond for which both samples were taken. Dashed line is a 1:1 line included for reference.

**Table S1.** Carbon and oxygen isotope data ( $\delta^{13}\text{C}$  in ‰ relative to VPDB and  $\delta^{18}\text{O}$  in ‰ relative to VSMOW) and calculated new growth fraction (fG) and growth (G in  $\mu\text{g}$ ) for all samples.

**Table S2.** Carbon and oxygen isotope data ( $\delta^{13}\text{C}$  in ‰ relative to VPDB and  $\delta^{18}\text{O}$  in ‰ relative to VSMOW) for 4 field specimens of *Corallina vancouveriensis* collected at Tatoosh Island, WA in June 2012.