

## Localization and Quantification of Carbonic Anhydrase Activity in the Symbiotic Scyphozoan *Cassiopea xamachana*

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**Abstract.** The relationship between density and location of zooxanthellae and levels of carbonic anhydrase (CA) activity was examined in *Cassiopea xamachana*. In freshly collected symbiotic animals, high densities of zooxanthellae corresponded with high levels of CA activity in host bell and oral arm tissues. Bleaching resulted in a significant loss of zooxanthellae and CA activity. Recolonization resulted in full restoration of zooxanthellar densities but only partial restoration of CA activity. High levels of CA activity were also seen in structures with inherently higher zooxanthellar densities, such as oral arm tissues. Similarly, the oral epidermal layer of bell tissue had significantly higher zooxanthellar densities and levels of CA activity than did aboral bell tissues. Fluorescent labeling, using 5-dimethylaminonaphthalene-1-sulfonamide (DNSA) also reflected this tight-knit relationship between the presence and density of zooxanthellae, as DNSA-CA fluorescence intensity was greatest in host oral epithelial cells directly overlying zooxanthellae. However, the presence and density of zooxanthellae did not always correspond with enzyme activity levels. A transect of bell tissue from the margin to the manubrium revealed a gradient of CA activity, with the highest values at the bell margin and the lowest at the manubrium, despite an even distribution of zooxanthellae.

Thus, abiotic factors may also influence the distribution of CA and the levels of CA activity.

### Introduction

The reciprocal translocation of carbon between cnidarian hosts and their endosymbiotic dinoflagellates (zooxanthellae) is essential for maintaining this keystone obligate mutualism. Zooxanthellae require inorganic carbon ( $C_i$ ), in the form of molecular  $CO_2$ , as the substrate for Rubisco-catalyzed C3 photosynthesis, while the host benefits from organic carbon synthesized and secreted by the symbionts (Streamer *et al.*, 1993). However, neither  $CO_2$  produced by host respiration nor molecular  $CO_2$  dissolved in seawater can support maximal rates of photosynthesis (Al-Moghrabi *et al.*, 1996; Gattuso *et al.*, 1999). Instead, the zooxanthellae must somehow gain access to the carbon present as  $HCO_3^-$ , the most abundant form of inorganic carbon in seawater (Allemand *et al.*, 1998; Benazet-Tambutte *et al.*, 1996). Accessing the seawater  $HCO_3^-$  pool, however, is a problem, since  $HCO_3^-$  does not readily diffuse across biological membranes and the uncatalyzed conversion of  $HCO_3^-$  to  $CO_2$  is too slow to support maximal photosynthetic rates (Gutknecht *et al.*, 1977; Muscatine *et al.*, 1989; Gattuso *et al.*, 1999). These obstacles to sufficient carbon transport can be overcome by carbonic anhydrase (CA), an enzyme that catalyzes the reversible dehydration of  $HCO_3^-$  to  $CO_2$ , the freely diffusible form of  $C_i$ . A growing body of evidence suggests that CA plays an important role in supplying inorganic carbon to the symbiont by providing zooxanthellar photosynthesis with a virtually unlimited supply of  $CO_2$  through the catalyzed dehydration of seawater  $HCO_3^-$  (Weis, 1993; Benazet-Tambutte *et al.*, 1996; Sültemeyer *et al.*, 1998; Furla *et al.*, 2000).

CA activity has been found in 28 species of marine algae and phytoplankton (Graham and Smillie, 1976) and in more

Received 17 May 2002; accepted 8 April 2003.

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**Abbreviations:** AZ, acetazolamide; CA, carbonic anhydrase;  $C_i$ , inorganic carbon; DMF, dimethyl formamide; DNSA, 5-dimethylaminonaphthalene-1-sulfonamide;  $E_o$ , total concentration of free enzyme; EZ, ethoxycarbonyl;  $i$ , fractional inhibition of enzyme activity at  $I_o$ ;  $I_o$ , zero concentration of inhibitor;  $K_i$ , inhibition constant.



than 40 species of symbiotic marine invertebrates, including 22 cnidarian species (Weis *et al.*, 1989). Furthermore, CA activity is highest in host tissues and apparently depends upon the animal's symbiotic state, as tissues of symbiotic organisms have been shown to have levels of CA activity 29 times greater than tissues of aposymbiotic animals (Weis *et al.*, 1989). In anthozoans such as *Aiptasia*, the presence of algae induces CA activity. Light intensity, water flow, and the photosynthetic rate of the zooxanthellae were also proposed as other factors potentially affecting CA induction (Weis, 1991; Weis *et al.*, 1989). Polyclonal antibodies made against mammalian CA II have been used to localize CA to regions on or near the membrane surrounding the zooxanthellae. Such labeling is consistent with the role of CA in supplying the symbiont with CO<sub>2</sub> for photosynthesis (Weis, 1993). However, because of differences in isozyme structure between organisms, especially across broad phylogenetic lines, antibodies are not ideal for localizing CA (Suzuki *et al.*, 1994).

Despite significant advances, two central questions concerning the role of CA in C<sub>i</sub> transport remain: (1) is the enzyme localized in the host tissues where it would potentially facilitate CO<sub>2</sub> transport from the surrounding seawater, and (2) what are the factors that control the levels of CA activity in symbiotic animals? This investigation examines these questions in *Cassiopea xamachana*, the upside-down jellyfish, which is an ideal organism for studying physiological mechanisms of CA-mediated carbon cycling in symbiotic cnidarians. Members of the species are densely populated with the zooxanthella, *Symbiodinium* sp., and they have a large bell that allows repeated sampling of individuals over time. Their soft body makes it easy to separate ectodermal epithelium from associated mesoglea to further localize enzyme activity to specific tissues. They also exhibit a unique behavioral adaptation in which the oral portion of the bell is oriented upward and thus receives direct sunlight while the aboral portion of the bell is positioned against the substratum. Furthermore, the tissue of the bell margin receives the highest light intensity and water flow, whereas bell tissue closer to the manubrium is sheltered. Thus, *C. xamachana* provides a unique opportunity to examine the relationship between algal density and CA activity by exploring the microenvironment of its different tissue layers and structures. Localization of CA activity to body region and epithelial surface, under various states of symbiosis, was examined by DNSA (dansylamide, or 5-dimethylaminonaphthalene-1-sulfonamide)-CA fluorescence and by direct enzyme assay.

## Materials and Methods

### Animal collection and maintenance

Specimens of *Cassiopea xamachana* ranging in bell diameter from 10 to 14 cm were collected from Marathon

Key, Florida (mile marker 57) and transported to Auburn University, Auburn, Alabama, in buckets or coolers. The medusae were held in shallow 20-gallon aquaria containing 32 ppt artificial seawater (Reef Crystals, Aquarium Systems, Mentor, OH) with trickle filter systems, undergravel filtration, and protein skimmers. Animals residing on the crushed coral substrate of the aquaria were illuminated with 80  $\mu\text{E m}^{-2} \text{s}^{-1}$  actinic light on a 12:12 light:dark photoperiod at  $\sim 25\text{--}30^\circ\text{C}$ . Animals were fed newly hatched *Artemia* nauplii three times a week.

### Terminology and experimental protocol

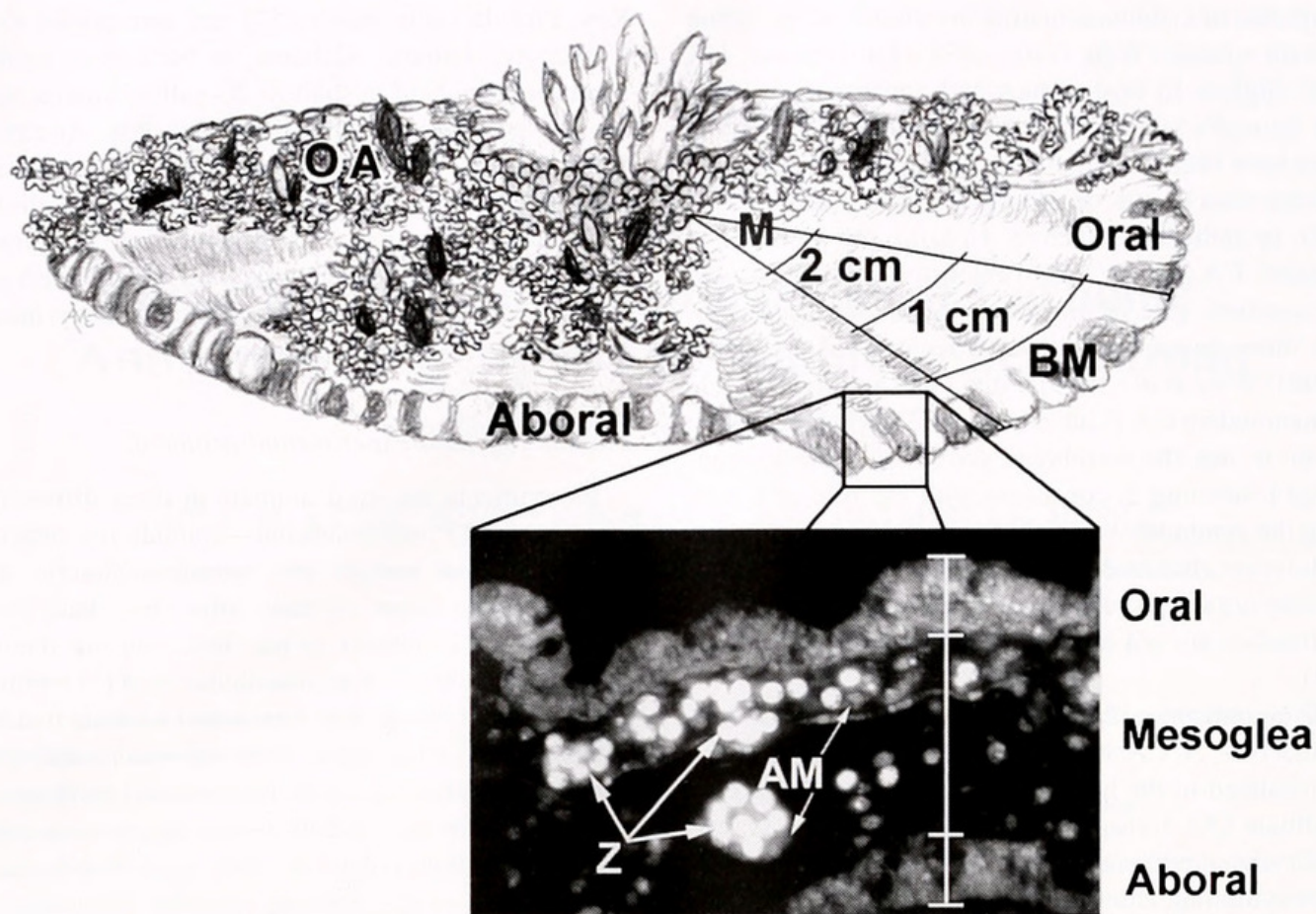
Experiments involved animals in three different symbiotic states: (1) field-condition—animals recently collected from the field and in the natural symbiotic state; (2) bleached—the same animals after they had been cold-shocked and incubated in the dark, causing them to lose significant numbers of zooxanthellae; and (3) repopulated—the same cold-shocked and bleached animals that had been placed under actinic light in the laboratory and allowed to undergo repopulation by their remaining symbionts.

Baseline samples of bell margin tissue were taken from eight freshly field-collected symbiotic jellyfish (acclimated in the field to a light intensity of about 1000  $\mu\text{E}$ ) and were fixed or frozen as described below for determination of zooxanthella density and CA activity and localization. These eight symbiotic individuals were then cold-shocked for 1 h at  $4^\circ\text{C}$  and incubated in the dark for 1–3 weeks to bleach. Cold-shocked animals expelled 67%–70% of their zooxanthellae and were considered to be bleached. After 3 weeks, bleached animals were exposed to the actinic light (80  $\mu\text{E}$ ) of the aquarium for 1–5 weeks to allow zooxanthellae remaining in their tissues to mitotically divide and repopulate bell tissue to algal densities similar to those found in field-collected animals. During bleaching and recolonization, bell margin tissue samples of about 0.5 g were taken every 7 days and treated as described below.

### Anatomy and tissue sampling protocol

Several regions of *C. xamachana* tissue, determined by the animal's unique morphology and orientation in its environment, were assayed for carbonic anhydrase (CA) activity and algal density (Fig. 1): (1) bell margin tissue—a concentric ring about 1 cm in width, running around the perimeter of the bell; (2) oral arm tissue—the outpocketings of the gastrovascular cavity and overlying tissue layers that extend up into the water column; (3) the epithelial layer from the oral (subumbrellar) and aboral (exumbrellar) regions of the bell; (4) isolated mesogleal tissue; and (5) samples of intact bell tissue along a radial transect extending from the bell margin to the manubrium (the gastrovascular cavity located at the center of the bell).





**Figure 1.** Drawing of *Cassiopea xamachana* showing oral arms (OA) and bell tissues. The left half of the animal is shown in the midst of beating the bell margin, so that the markings of the aboral epithelial layer are evident. The right half of the animal is drawn prostrate, and a transect of the bell is shown illustrating the regions that were used in the various assays described in the methods section. BM—tissue collected at the bell margin, 1 cm—tissue collected 1 cm in from the bell margin, 2 cm—tissue collected 2 cm in from the bell margin, M—manubrium—bell tissue collected from adjacent to the manubrium. The inset is an epi-fluorescent micrograph of a cross section taken through the bell margin. Diffuse fluorescence in the oral and aboral epithelial layers is due to DNSA-CA labeling. The cell membranes of amoebocytes in the mesoglea also exhibit DNSA-CA fluorescence. The bright circles within the amoebocytes are autofluorescing zooxanthellae (Z). In a color micrograph, the DNSA-CA fluorescence would be blue and the algal fluorescence red. Am: amoebocyte.

Tissue samples (~0.5 g) were excised for algal cell counts and determination of CA activity for individuals in different symbiotic states. Samples of intact oral arm tissue from field-condition and bleached animals were also excised from the animal and analyzed for CA activity and algal density. The primary oral arms branch into smaller secondary arms, and these are further subdivided into smaller tertiary arms. Since most zooxanthellae are found in the mouths of the secondary and tertiary oral arms, the large, primary oral arms were removed to eliminate large amounts of mesoglea, which lacks significant numbers of zooxanthellae. Unlike many typical cnidarian-algal symbioses in which the algal symbionts are located in the gastrodermis, in *C. xamachana*, the algal symbionts are present in amoebocytes, mobile cells that migrate through the animal's mesoglea (Fitt and Trench, 1983; see Fig. 1). These amoebocytes also cluster close to the epithelial layer of the oral surface of the jellyfish. All excised *C. xamachana* tissues

used for the CA assays were frozen in liquid nitrogen and stored at  $-95^{\circ}\text{C}$  until analyzed.

#### *Tissue fixation for fluorescence localization of carbonic anhydrase*

In tandem with tissue collection for measurement of CA activity, portions of *C. xamachana* bell margin tissue were excised and fixed for CA localization with 5-dimethylaminonaphthalene-1-sulfonamide (DNSA, dansylamide) (Husic and Hsieh, 1993). About  $1\text{ cm}^2$  of intact bell tissue was fixed in a 2% paraformaldehyde solution with 0.07 M NaCl and 0.3 M Millonig's phosphate buffer (pH 7.4) for 4 h at room temperature. Mouths from oral arm branches were also fixed as described above. Fixed tissue was initially stored in a solution consisting of 50% Tissue Freeze Media (TFM) (Electron Microscopy Science, Fort Washington, PA.) at  $4^{\circ}\text{C}$  for up to 1 month. If fixed tissue was stored for



longer than 3 months, subsequent attempts to label it with DNSA showed little or no fluorescence.

Before being sectioned, tissue samples were infiltrated overnight in 100% TFM and subsequently oriented for sectioning such that both oral and aboral epithelial layers would be seen when frozen sections (14  $\mu\text{m}$ ) were cut using a Reichert-Jung Frigocut 2800N cooled to  $-30^\circ\text{C}$ . Sections were transferred to slides coated with poly-L-lysine to thaw and were immediately labeled with DNSA. These sections allowed for easy comparison of fluorescence in the epithelial layers. Orientation of the oral epithelial was confirmed by placing the oral side of the tissue on Whatman filter paper and then embedding and sectioning filter paper and tissue together.

#### *K<sub>i</sub> values of sulfonamide inhibitors of Cassiopea carbonic anhydrase*

Inhibition constant ( $K_i$ ) values for DNSA were determined to help ascertain the minimum useful concentration for the fluorescent labeling studies described in the next sections (e.g., Husic and Hsieh, 1993). DNSA (10 mM), dissolved in dimethyl formamide (DMF), as well as DMF alone, and two other inhibitors—acetazolamide (AZ) and ethoxzolamide (EZ)—were titrated against about 200  $\mu\text{mol CO}_2 \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  of supernatant *C. xamachana* CA activity, which was approximately a 29-fold increase in the uncatalyzed rate. Inhibition constants were determined using double reciprocal inhibition plots (Easson and Stedman, 1937), according to the following equation:

$$I_o/i = K_i/(1 - i) + E_o$$

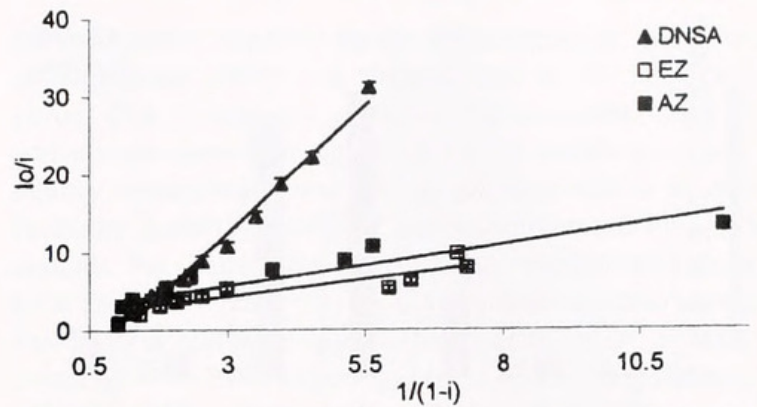
where  $E_o$  is the total concentration of the free enzyme and  $i$  is the fractional inhibition of enzyme activity at an inhibitor concentration of  $I_o$ .

AZ and EZ had  $K_i$  values of 1.04 and 0.86 nM, respectively, and these values were typical for these strong inhibitors. However, DNSA was a much weaker inhibitor, with a  $K_i$  of 6.5  $\mu\text{M}$  (Fig. 2). Based on the  $K_i$  value for DNSA and the relative intensity of tissue fluorescence observed in incubations at different concentrations of DNSA (see below), 50  $\mu\text{M}$  DNSA (about 10 times the  $K_i$ ) was used for the subsequent experiments involving the visualization and localization of CA in jellyfish tissue. Furthermore,  $K_i$  values for AZ and EZ were used to determine the concentrations of these inhibitors for use in the negative controls.

#### *DNSA labeling of carbonic anhydrase*

Final concentrations of all sulfonamide inhibitors of CA activity were diluted in 0.2 M potassium phosphate buffer (pH 7.5) from 10 mM stock solutions dissolved in 100% DMF (Dermietzel *et al.*, 1985; Husic and Hsieh, 1993).

CA was localized in frozen tissue sections of *C. xamachana* by incubating thawed tissue sections in a graded



**Figure 2.** Easson-Stedman plots for dansylamide (DNSA), acetazolamide (AZ), and ethoxzolamide (EZ) titrated against carbonic anhydrase activity from homogenates of oral arm tissue of field-condition *Cassiopea xamachana*. Mean  $\pm$  SEM ( $n = 4$ ). Regression equations and correlation coefficients for each inhibitor are as follows:  
 DNSA in  $\mu\text{M}$ :  $y = 6.52x - 7.09$ ,  $R^2 = 0.98$   
 AZ in nM:  $y = 1.04x + 2.6$ ,  $R^2 = 0.81$   
 EZ in nM:  $y = 0.87x + 1.75$ ,  $R^2 = 0.81$

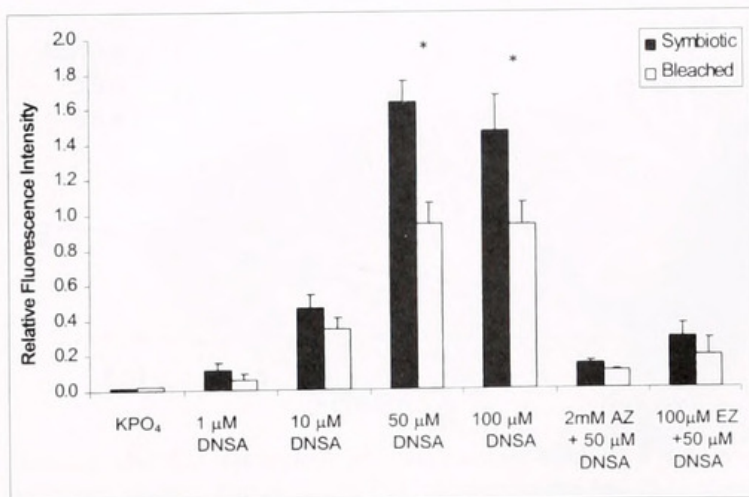
concentration series of DNSA (1–100  $\mu\text{M}$ ). Sections were incubated for 30 min to allow DNSA-CA binding to occur. A comparison of bound DNSA fluorescence to background indicated that 50  $\mu\text{M}$  DNSA provided optimum contrast for subsequent comparisons (Fig. 3). Negative control sections were incubated in either 0.2 M potassium phosphate buffer + 200 ml 100% DMF or in 2 mM AZ or 100  $\mu\text{M}$  EZ, for 30 min followed by a second 30-min incubation in 50  $\mu\text{M}$  DNSA with 2 mM AZ or 100  $\mu\text{M}$  EZ, respectively. Microspheres of 1% intensity (In-Speck Blue calibration kit I-722I, Molecular Probes, Eugene, OR) were added to each slide as an internal standard for fluorescence. Because unbound DNSA does not fluoresce, it was not necessary to rinse the sections after incubation. Incubated sections were placed under coverslips, sealed with fingernail polish, and viewed immediately as described below.

#### *Carbonic anhydrase localization using DNSA-CA fluorescence*

DNSA was chosen, rather than polyclonal antibodies to vertebrate CA II (Kingsley and Watabe, 1987; Weis, 1993), because (1) it binds 1:1 with all isoforms of CA and thus it lends itself to direct quantification of CA; (2) once bound to CA, its resultant fluorescence is extremely stable, unlike that of fluorescent markers conjugated to antibodies; (3) it fluoresces only in a bound state with CA, so there is no background fluorescence; (4) the relative fluorescent intensity of DNSA-CA is easily quantified by comparing DNSA-CA fluorescence to that of standard 1% intensity fluorescent microspheres; and (5) DNSA-CA fluorescence can be quenched by the nonfluorescent sulfonamide inhibitors acetazolamide and ethoxzolamide.

Sections were viewed and photographed using a Zeiss





**Figure 3.** Relative fluorescent intensity values of the DNSA-CA complex in bell margin tissue sections from field-condition and bleached animals. Mean  $\pm$  SEM ( $n = 5$ ). An asterisk indicates a significant difference ( $P < 0.0001$ ) in the fluorescence value obtained with 50  $\mu$ M DNSA between field-condition and bleached animals.

ICM 405 inverted microscope illuminated by a mercury arc lamp and equipped with Zeiss Neofluar quartz objectives. An Omega excitation filter of  $360 \pm 20$  nm and emission filter of  $460 \pm 25$  nm provided proper fluorescent excitation of the DNSA-CA complex while preventing interference from algal autofluorescence. For each data point, images of both oral and aboral epithelial layers of five sections were captured with an 8-bit CCD Sony XC-75 video camera and accumulated using a frame accumulation program written by Dr. Thomas Pitta (Department of Biological Sciences, Auburn University) using the Lab-View program (National Instruments; Austin, TX). Only 2–5 frames were necessary to accumulate DNSA-labeled sections; however, to compensate for the low fluorescence of the negative controls, 10–50 frames of those sections were accumulated. Concurrent with each image of DNSA-labeled animal tissue sections, two frames of 1% fluorescence intensity microspheres were accumulated. The intensity of microsphere fluorescence was used to determine the relative fluorescence intensity of labeled cnidarian tissue.

Fluorescent intensity values of sections of *C. xamachana* bell tissue were determined with Sigma Scan 2.0 (Jandel Scientific; San Rafael, CA) using the following procedure. For each section of bell tissue examined, the oral and aboral epithelial layers were each meticulously outlined, and the intensity of each region's area was calculated by Sigma Scan to produce an overall fluorescence intensity score for each epithelial layer examined. The number of frames accumulated for each region varied between DNSA-labeled tissue and negative controls, due to differences in their fluorescence intensity. Therefore, the overall score for each epithelial layer was divided by the number of frames accumulated to yield a final standardized fluorescence intensity score. Likewise, microsphere fluorescence was also calcu-

lated by dividing the raw intensity score by the number of frames accumulated. Standardized fluorescent intensity scores of cnidarian tissue were then divided by the In-Speck microsphere's fluorescence intensity to produce a relative fluorescent intensity for each epithelial layer.

The Sigma Scan program was also used to determine the percentage of each labeled section that was epithelial tissue or mesogleal tissue.

#### *Delta pH electrometric assay of carbonic anhydrase activity*

Chemicals for the following procedures were purchased from Fisher Scientific (Norcross, GA) or Sigma (St. Louis, MO) unless otherwise noted. Frozen tissue samples were thawed and homogenized in 1 ml of ice-cold reaction buffer (10 mM Tris, 225 mM mannitol, and 75 mM sucrose adjusted to pH 7.4 with phosphoric acid) (Henry, 1991), using 40 complete passes of a 15-ml Wheaton ground glass mortar and Teflon-coated pestle on a Fisher Sted Fast Stirrer Model SL300. Homogenized samples were centrifuged in an Eppendorf 5804R tabletop microfuge at  $10,000 \times g$ , 2 min, 4 °C to separate cytosolic host CA from animal tissue debris and algae.

CA activity in the supernatant was analyzed using the electrometric delta pH assay (Henry, 1991). Microscopic analysis of the pellet, performed to determine algal densities (see below), revealed that the algal symbionts were not lysed by the homogenization procedure. This suggests that the supernatant CA activity was primarily, if not exclusively, of animal host origin. Briefly, 50  $\mu$ l of supernatant was added to 6 ml of buffer in a water-jacketed reaction vessel cooled to 4 °C. The reaction was started by the addition of 100  $\mu$ l of CO<sub>2</sub>-saturated water, and the catalyzed hydration reaction was measured by following the drop in pH of approximately 0.15 units as a result of the production of H<sup>+</sup>. CA activity was expressed as  $\mu$ mol CO<sub>2</sub>  $\cdot$  min<sup>-1</sup>  $\cdot$  g wet weight of tissue<sup>-1</sup>.

#### *Algal density determinations*

Zooxanthellar population densities were immediately ascertained for each 1-ml sample of homogenized tissue by using a Fisher Scientific Neubauer ruled, Ultraplane hemocytometer. Algal densities were determined as the mean of five aliquots of homogenized samples from five animals. Algal concentrations were standardized per gram of tissue wet weight.

#### *Statistical analysis of data*

CA activity and zooxanthellar density were either square root or log transformed as necessary to satisfy the *F* test for homogeneity. A repeated-measures ANOVA was run to compare changes in CA activity and algal number between field-condition, bleached, and repopulated states in individ-



uals over an 8-week period. Paired Student's *t* tests were used to compare algal density and CA activity in field-condition, week-3 bleached, and week-5 repopulated samples. A one-way ANOVA was run for algal number and CA activity in the following comparisons: of bell margin *versus* arm tissue; between and among oral, aboral, and mesogleal tissue; and of tissue samples across a transect from bell margin to manubrium. Scheffé PLSD post hoc tests were run on all ANOVAs (Sokal and Rohlf, 1995). All statistical analyses were performed using Stat-View (SAS Institute, Cary, NC).

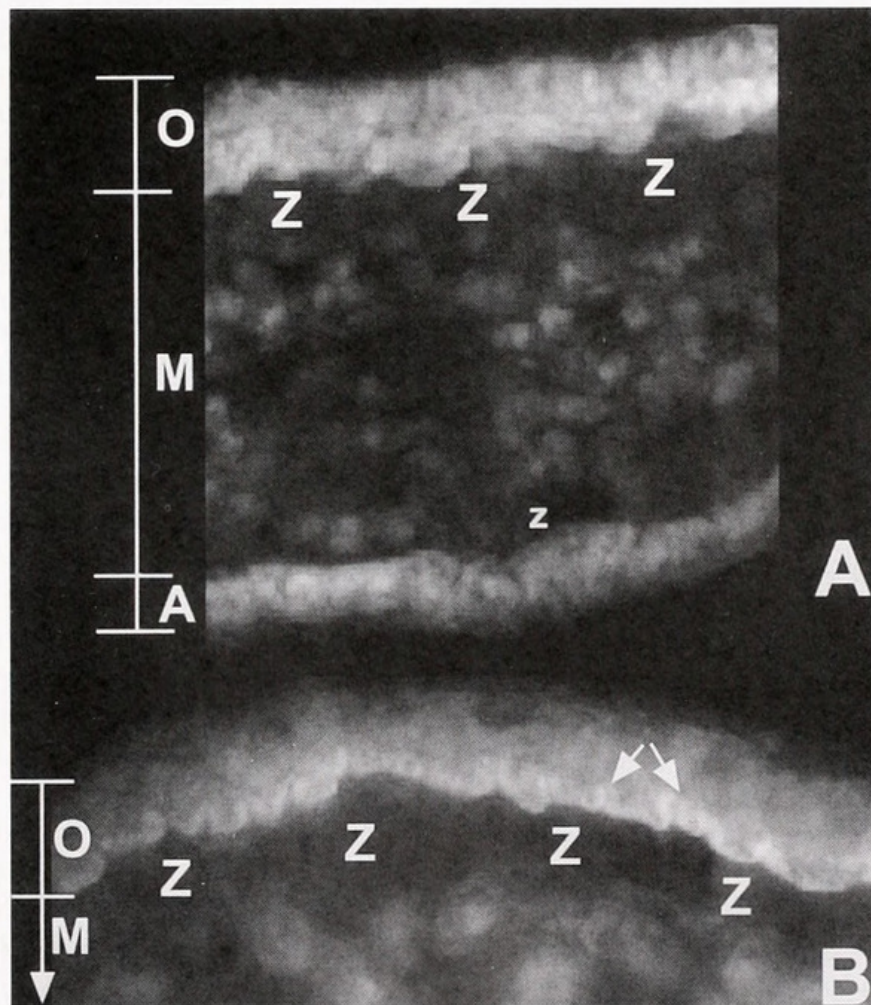
## Results

### *Localization of carbonic anhydrase via DNSA-CA fluorescence*

Frozen sections of *Cassiopea xamachana* bell margin incubated with 50  $\mu$ M DNSA showed intense labeling of

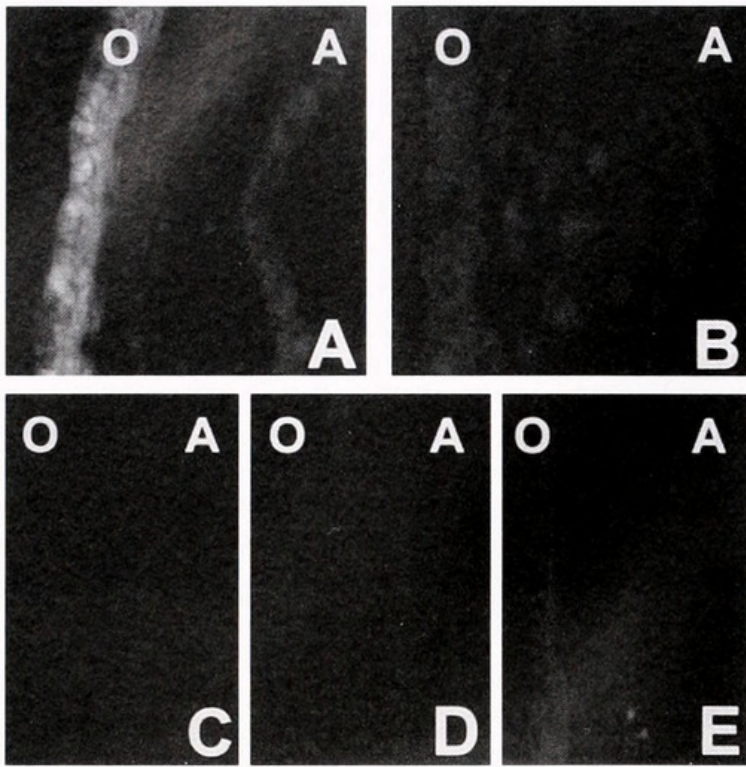
oral and aboral epithelial layers. By comparison, there was no significant DNSA-CA fluorescence in the mesogleal tissues (Figs. 1 and 4A). A distinct banding pattern, which was always associated with a cluster of amoebocytes containing zooxanthellae, was also occasionally seen in the oral epithelial layer (Fig. 4B). In field-condition and bleached animals, the oral epithelial layers had, respectively, about 27% and 32% greater DNSA-CA fluorescence than aboral bell margin epithelial layers (field-condition:  $P = 0.02$ ; bleached:  $P = 0.03$ ) (e.g., Figs. 4A, 5A, and 5B). The sum of oral and aboral epithelial labeling of field-condition jellyfish sections had 44% greater DNSA-CA fluorescence than the sum of oral and aboral layers from sections of bleached animals ( $P < 0.0001$ ).

Sectioned tissues of field-condition and bleached animals incubated in 50  $\mu$ M DNSA alone had respectively 13 and 8 times more DNSA-CA fluorescence than when these tissues were incubated in 2 mM AZ + 50  $\mu$ M DNSA ( $P < 0.0001$



**Figure 4.** DNSA-CA fluorescence in sectioned tissue of *Cassiopea xamachana* bell margin. Abbreviations: O, oral surface; A, aboral surface; M, mesoglea; Z, zooxanthellae. (A) Image of bell margin tissue incubated in 50  $\mu$ M DNSA, showing the localization of CA to the oral and aboral epithelial tissue layers. The majority of the zooxanthellae are adjacent to the oral epithelial layer. Comparatively few zooxanthellae are found adjacent to the aboral epithelial layer. (B) In some instances, a distinct band of DNSA-CA fluorescence (arrow) occurred in the oral epithelial layer directly adjacent to underlying aggregations of zooxanthellae (not visible) within amoebocytes (not visible).





**Figure 5.** DNSA-CA fluorescence in sectioned tissue of *Cassiopea xamachana* bell margin: O indicates the oral surface and A the aboral surface of the bell. Bell margin tissue from recent field-collected symbiotic jellyfish (A) had higher intensity fluorescence than that from bleached jellyfish (B) when incubated in  $50 \mu\text{M}$  DNSA. Negative controls incubated in potassium phosphate buffer + dimethylformamide (C), 2 mM acetazolamide (AZ) followed by  $50 \mu\text{M}$  DNSA + 2 mM AZ (D), and  $100 \mu\text{M}$  ethylzolamide (EZ) followed by  $100 \mu\text{M}$  EZ +  $50 \mu\text{M}$  DNSA (E) had little or no fluorescence.

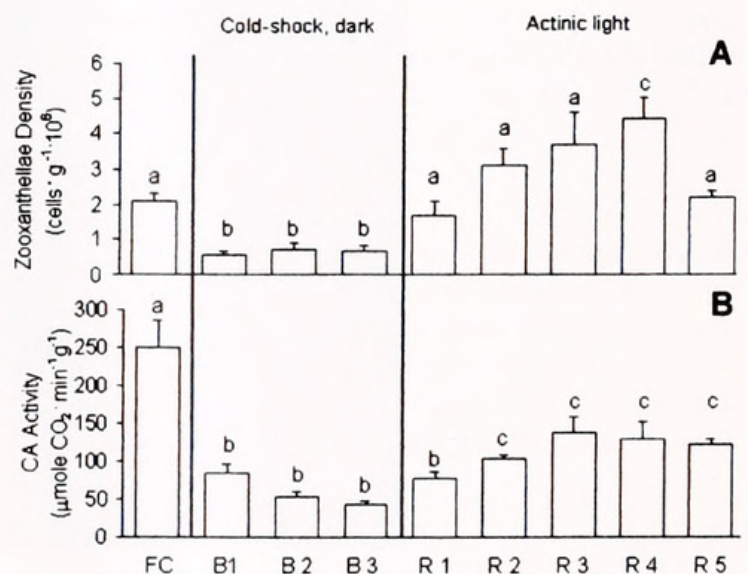
in both field-condition and bleached comparisons). Also, field-condition and bleached tissues incubated in  $50 \mu\text{M}$  DNSA alone had 5 times brighter DNSA-CA fluorescence than when  $100 \mu\text{M}$  EZ was added to the incubation medium ( $P < 0.0001$  in both field-condition and bleached comparisons) (Figs. 3 and 5). Similarly, tissues of field-condition and bleached animals incubated in  $50 \mu\text{M}$  DNSA exhibited fluorescence values that were, respectively, 124 and 37 times greater than those for tissues incubated in  $\text{KPO}_4$  +  $200 \mu\text{l}$  DMF ( $P < 0.0001$  in both field-condition and bleached comparisons) (Figs. 3 and 5). The differences in the quantitative DNSA-CA fluorescence values between tissues of jellyfish in the field-condition *versus* bleached states were similar to those differences in CA activity found using the electrometric delta pH assay (see below).

#### *Carbonic anhydrase activity and algal densities in field-condition, bleached, and repopulated jellyfish*

CA activity was directly related to the presence of zooxanthellae in *C. xamachana*. Field-condition symbiotic jellyfish initially housed algal densities of approximately  $2 \times 10^6$  cells  $\cdot \text{g}^{-1}$  wet weight of host tissue and had CA activities of  $250 \mu\text{moles CO}_2 \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  (Fig. 6). These

symbiotic animals were bleached by cold shock and dark incubation and subsequently lost about 71% of their initial algal densities and 66% of their initial CA activities after one week of treatment. Bleached animals had a translucent beige or blue hue depending on the concentration of the pigment Cassio blue in host tissues and were easily distinguished from the dark brown color typical of field-condition and repopulated jellyfish. Throughout the 3 weeks that jellyfish were in the bleached condition, zooxanthellar density did not decrease after the initial decline, remaining at approximately  $6.7 \times 10^5$  cells  $\cdot \text{g}^{-1}$  wet weight of host tissue, a value that was significantly lower than that for algal densities in field-condition symbiotic *C. xamachana* ( $P < 0.0001$ ) (Fig. 6A). However, CA activities in bleached animals did continue to decrease during this 3-week period. Bleached animals lost an additional 16% of CA activity between weeks 2 and 3 resulting in an overall decrease of 83% compared to initial CA activity from field-condition symbiotic animals ( $P < 0.0001$ ) (Fig. 6B).

Exposure to actinic light for 7 days resulted in the repopulation of host tissue by zooxanthellae. Within the first week of light incubation, algal densities in repopulated individuals more than doubled compared to values for animals bleached 3 weeks (Fig. 6A). Algal densities at this point were not different from those in field-condition jellyfish ( $P = 1.0$ ). As animals made the transition from the bleached to the repopulated condition, increased CA activities directly corresponded to increased zooxanthellar densities. CA activity in animals repopulated for one week increased by about 80%, from 43 to  $77 \mu\text{moles CO}_2 \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  (Fig. 6B). Despite this increase, CA activity in



**Figure 6.** Fluctuation of zooxanthellar density (A) and carbonic anhydrase activity (B) monitored over 8 weeks in the same group of *Cassiopea xamachana* in three symbiotic states: field-condition (S); bleached (cold-shocked and dark-treated; B1–B3); and repopulated (incubated in actinic light; R1–R5). Mean  $\pm$  SEM ( $n = 8$ ). Differing lowercase letters above bars indicate conditions that were significantly different at  $P \leq 0.01$ .

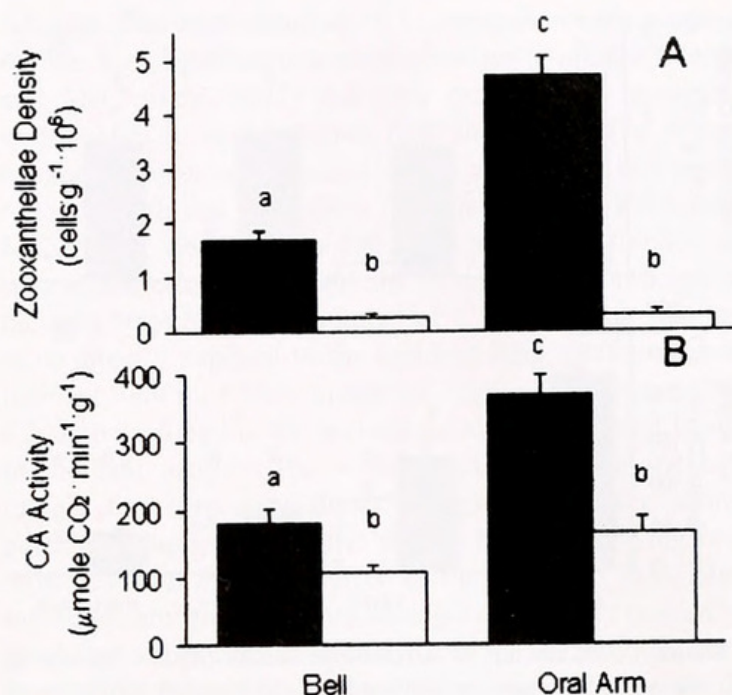


animals repopulated for one week remained at only 30% of the CA activity in field-condition animals, even though algal densities had fully recovered to values found in field-condition animals (Fig. 6). In animals repopulated for 4 weeks, zooxanthellar densities increased to twice those found in field-condition animals. However, at week 5, densities of zooxanthellae decreased to  $2.2 \times 10^6$  cells  $\cdot$  g $^{-1}$ , which was approximately equivalent to the algal densities in field-condition animals ( $P = 0.6$ ). During weeks 3 through 5 of the repopulation state, *C. xamachana* CA activity remained constant at about 129  $\mu$ moles CO $_2$   $\cdot$  min $^{-1}$   $\cdot$  g $^{-1}$ , only half the activity found in field-condition jellyfish ( $P < 0.003$ ) (Fig. 6B). Therefore, during the initial repopulation period, CA activities directly corresponded to increased algal densities of repopulated animals. However, enzyme activities stabilized within 3 weeks, whereas zooxanthellar density continued to fluctuate. CA activities of repopulated animals never returned to the values found in field-condition animals, although algal densities from animals repopulated for 5 weeks and field-condition animals were approximately equal (Fig. 6A, B).

#### Comparison of zooxanthellar densities and carbonic anhydrase activity in bell margin and oral arm tissue in field-condition and bleached animals

Bell margin tissue from bleached animals had zooxanthellar densities that were only 16% of those found in bell margin tissue of field-condition animals ( $P < 0.0001$ ). Similarly, zooxanthellar densities in oral arm tissue from bleached animals were only 6% of those found in corresponding tissue from field-condition animals ( $P < 0.0001$ ) (Fig. 7A). CA activity reflected these trends in zooxanthellar densities. CA activity in bell margin tissue of bleached animals was 61% of that measured in bell margin tissue of field-condition animals ( $P = 0.0003$ ), and activity in oral arm tissue of bleached animals was 45% of activity in oral arm tissue of field-condition animals ( $P < 0.0001$ ) (Fig. 7B).

Comparison of bell margin and oral arm zooxanthellar density and CA activity within groups of field-condition or bleached animals also supported this trend. Oral arm tissues of field-condition animals had 2.8 times greater algal density ( $P < 0.0001$ ) and 2 times greater levels of CA activity ( $P < 0.0001$ ) when compared to bell margin tissues from the same individual. However, bell margin and oral arm tissue from bleached animals were not significantly different in either algal density ( $P = 0.17$ ) or CA activity ( $P = 0.06$ ) (Fig. 7A, B).



**Figure 7.** Distribution of zooxanthellar density (A) and CA activity (B) in bell margin and oral arm tissue of field-condition and bleached individuals of *Cassiopea xamachana*. Field-condition animals are represented by black bars; bleached animals are represented by white bars. Mean  $\pm$  SEM ( $n = 21$ ). Differing lowercase letters above bars indicate conditions that were significantly different at  $P < 0.0001$ .

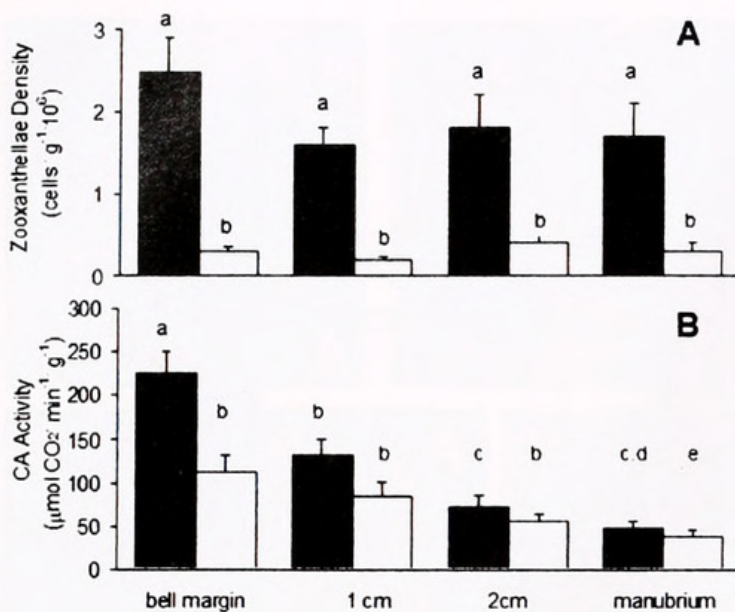
#### Distribution of zooxanthellar density and carbonic anhydrase activity across the bell radius

Algal counts revealed no significant difference in zooxanthellar ( $P = 0.74$ ) density across the bell radius within field-condition ( $P = 0.14$ ) or bleached groups of animals (Fig. 8A). However, CA activity varied along the bell radius, with the outer bell margin of field-condition animals containing significantly higher levels of CA activity than any of the samples from the inner sections of the bell ( $P < 0.0001$ ) (Fig. 8B). CA activity decreased progressively in the tissue samples collected at 1 cm and 2 cm in from the margin, but it was not significantly different between the 2-cm sample and that from the innermost sample, the manubrium ( $P > 0.05$ , Fig. 8B). There was no significant difference in CA activity in any of the samples from the bleached jellyfish, except for the sample from the manubrium.

#### Discussion

The regulation of induction and deinduction of CA activity in various structures and tissues of cnidarian hosts has been proposed to be related to changes in zooxanthellar density (Weis, 1991; Weis and Reynolds, 1999). Our analysis of CA activity in *Cassiopea xamachana*, as it relates to zooxanthellar density, anatomical location of the algae, and light or dark treatment of animals, supports this hypothesis. Previous work with the anemones *Aiptasia pulchella* and





**Figure 8.** Distribution of zooxanthellar density (A) and CA activity (B) across the bell radius in field-condition and bleached individuals of *Cassiopea xamachana*. See Figure 1 for an explanation of where each tissue sample was taken from across the diameter of the bell. Field-condition animals are represented by black bars; bleached animals are represented by white bars. Mean  $\pm$  SEM ( $n = 13$  for field-condition and 9 for bleached animals). Differing lowercase letters above bars indicate conditions that were significantly different at  $P \leq 0.03$ .

*Anthopleura elegantissima* confirmed that the symbiotic state of the host influences the levels of CA activity in the host tissues (Weis, 1991; Weis and Reynolds, 1999). Likewise, the presence and levels of CA activity reported here correspond to algal densities in the bell margin tissue of individual *C. xamachana* under different symbiotic states. Animals, whether freshly collected or repopulated, housed greater numbers of symbionts and had correspondingly higher CA activity within their bell margin tissues than when these same animals were bleached.

The correlation between algal density and CA activity extends to other tissues in *C. xamachana* as well. Oral arm tissue has been shown to contain 3-fold higher densities of zooxanthellae than whole bell tissue (Blanquet, 1979). This corresponds to our results that show oral arm tissues possess 2-fold higher CA activity. A similar relationship between algal density and CA activity has also been found in symbiotic anemones and giant clams (Weis *et al.*, 1989; Verde and McCloskey, 1998; Baillie and Yellowlees, 1998).

The underlying reasons for tissue-specific differences in zooxanthellar density are not known, but they may be related to differences in the microenvironments of different tissue types. Higher algal densities found in oral arms may be the result of increased colonization area provided for endosymbionts in the sinuous folding of the oral arm tertiary branches (as compared to the uniform plane of the bell) and higher amounts of incident light (see below). Zooxanthellae are also randomly dispersed throughout the thinner mesoglea of the arms (Blanquet and Phelan, 1987) instead

of being concentrated at the epidermal-mesogleal interface as is the case with the thicker mesoglea of the bell tissue. Thus, differences in the distribution of zooxanthellae, and therefore, corresponding differences in CA activity, could be due to the structure of these tissues that compose the surrounding microenvironment of the algal symbiont.

Nowhere are these tissue-specific differences more obvious than in the oral *versus* aboral epithelial layers of the bell margin. *C. xamachana* exhibits an unusual behavior of residing "upside-down," with its concave exumbrellar portion of the bell on the substratum and its oral arms suspended in the water column to capture planktonic prey (Bigelow, 1900). Higher densities of zooxanthellae have been observed associated with the subumbrellar (oral) epithelial tissue of the bell than with the exumbrellar (aboral) bell tissue (Blanquet and Riordan, 1981; Blanquet and Phelan, 1987). This asymmetry in endosymbiont density may be due to a disparity in the endosymbiotic microenvironment that results from the animal's upside-down posture. The subumbrellar zooxanthellae have more direct access to light and possibly easier access to inorganic carbon in the form of dissolved bicarbonate in the water column. Concurrent with this asymmetric zooxanthellar distribution is a heterogeneous oral-aboral distribution of CA activity. Furthermore, within the subumbrellar region itself, areas of intense banding of DNSA-CA fluorescence were occasionally observed directly above amoebocytes that contained zooxanthellae. These may also be examples of differences in CA distribution due to a disparity in the microenvironment. *Symbiodinium microadriaticum*, the symbiont isolated from *C. xamachana*, is positively phototactic in culture (McLaughlin and Zahl, 1966), and the amoebocytes containing the zooxanthellae have been proposed to move about the animal (Fitt and Trench, 1983; Colley and Trench, 1983; Muscatine *et al.*, 1986; Fitt and Cook, 2001). Although a phototactic response has never been demonstrated *in situ*, some evidence indicates that the amoebocytes are more concentrated in tissue areas that receive more direct light (Blanquet and Riordan, 1981). If that proves to be the case, some sort of communication between symbiont and amoebocyte might exist in order for the amoebocytes to be directed to regions of high light intensity. This possibility deserves more systematic investigation, as such a trophism could be mediated not just by light intensity alone, but also by metabolic factors, such as oxygen production or inorganic carbon translocation on the part of the symbiont, that are sensed or recognized by the amoebocyte or perhaps even the host. Thus, just as specific structures appear to exhibit differential levels of CA activity due to differences in the density of zooxanthellae, tissues within a structure may exhibit a disparity in algal density that leads to asymmetric enzyme distributions within that structure.

The relationship between CA activity in the oral epithelial tissue, the presence of amoebocytes clustered below the



oral surface, and the high densities of algae within those amoebocytes raises another interesting possibility in the regulation of CA activity. Given our finding that CA activity is regulated in cells (the oral epithelium) remote from those that contain the symbionts (amoebocytes), a signaling pathway may exist between host and symbiont that is involved in the regulation of CA induction.

One apparent discrepancy between algal density and CA activity may point to the involvement of other factors in the regulation of CA. Algal density is approximately equal along the radius of the bell, from the outer margin to the inner manubrium, whereas CA activity is high in the margin, but decreases in tissue samples taken from areas across the bell approaching the manubrium. This suggests that factors other than the simple presence or density of zooxanthellae influence CA activity.

One such factor could be light intensity, a variable that controls the photosynthetic rate of the zooxanthellae (Weis *et al.*, 1989; Gattuso *et al.*, 1993; Verde and McCloskey, 1998). In *C. xamachana*, light intensity varies seasonally and with water depth, and it could also potentially vary across the bell surface as a function of shading by the oral arms. Variations in light intensity could result in different physiological adaptations of the endosymbionts in different regions of the animal (Patterson *et al.*, 1991; Kühl *et al.*, 1995). In this study, differences in light intensity may help to explain why repopulated animals had significantly lower CA activity than field-condition individuals despite having the same algal densities. Field-condition animals were adapted to  $1200\text{--}1500\ \mu\text{E m}^{-2}\text{ s}^{-1}$ , a light intensity that is in the range needed for maximum photosynthetic activity ( $1497.2\ \mu\text{E m}^{-2}\text{ s}^{-1}$ ; Verde and McCloskey, 1998). The actinic light source of the aquarium, under which the animals were allowed to repopulate with zooxanthellae, was  $80\ \mu\text{E m}^{-2}\text{ s}^{-1}$ , well below the intensity needed for maximal photosynthesis, even taking into account possible photoacclimation (Chang *et al.*, 1983; Muller-Parker, 1984; Prézelin, 1987; Fitt and Cook, 2001). Therefore, although algal density was high, the photosynthetic rate of the zooxanthellae may have been quite low, resulting in lower CA activities. In contrast, symbiotic and repopulated anemones (*Aiptasia pulchella*) had equivalent levels of CA activity (Weis, 1991). This may, however, have been more closely related to the laboratory conditions under which the experiment was conducted. These animals had been maintained under low light intensities ( $40\ \mu\text{E m}^{-2}\text{ s}^{-1}$ ) for an extended time before being used in the experiments. As a result, they were acclimated to photosynthesizing under constant, low light intensities. Thus, zooxanthellae from these symbiotic and repopulated anemones most likely had similar photosynthetic rates.

Although the intensity of incident light on specific tissues was not measured, the possibility that it may affect CA activity in different tissues of *C. xamachana* is worth con-

sidering. The zooxanthellae of *C. xamachana* are adapted for the high light environments their hosts inhabit (Verde and McCloskey, 1998) and may preferentially associate with tissues receiving higher light intensities. The higher zooxanthellar densities found in oral arm *versus* bell tissue reported here and elsewhere (Blanquet, 1979; Verde and McCloskey, 1998) may be due to the increased illumination of oral arm tissue, as the mouths on the tertiary branches of the oral arms are the structures of *C. xamachana* that are most directly exposed to the incident light. Differences in incident light may also explain the higher algal density and CA activity found in the oral *versus* aboral epithelial layers of the bell margin. The oral epithelial layer of the bell margin tissue receives direct sunlight, unlike the aboral portion of the bell, which is shaded by the overlying oral arms; the oral epithelial layer of the bell; and associated mucilage, amoebocytes, and mesoglea. A similar pattern of symbiont distribution in response to light intensity has been reported in species of the coral *Montastrea* (Rowan *et al.*, 1997).

Water motion over the surface of symbiotic cnidarians is another abiotic factor that could potentially affect endosymbiont photosynthesis, and thus host CA activity. Although water flow over the bell was not directly measured, other reports have presented evidence supporting the idea that changes in the rate of water flow could alter the delivery of nutrients (*e.g.*, inorganic carbon) and thus alter the metabolic rates of host and symbiont (Patterson, 1992; Shashar *et al.*, 1993), especially in areas of low water flow (Dennison and Barnes, 1988; Weis *et al.*, 1989; Lesser *et al.*, 1994; Gardella and Edmunds, 2001). Furthermore, evidence suggests that CA activity could be affected by water flow (Lesser *et al.*, 1994; Weis *et al.*, 1989).

In summary, CA activity in host tissue appears to be influenced by a number of factors. The presence and density of the algal symbiont influences the presence and level of CA activity, but that alone cannot explain all of the tissue-specific differences in CA. Abiotic factors, such as light intensity and water flow, which influence the photosynthetic rate of the symbiont, may also play a role in regulating host CA activity. The interaction between algal density and abiotic factors represents a potentially viable avenue for future research.

### Acknowledgments

We thank Dr. William K. Fitt and the Key Largo Marine Research Laboratory for providing facilities for collection of animals; S. Cashion, T. Wakefield, W. Bailey, and M. Shawkey for assistance in field collection of animals; Dr. Mary Mendonça for assistance with statistics; and two anonymous reviewers for their constructive comments. Support for this research was provided by an Auburn University graduate school grant to AME; Alabama Agricultural Ex-



periment Station funding to SCK and RPH; and NSF grants (IBN 97-27835 and IBN 02-30005) to RPH.

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