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# CALCIFICATION IN THE PLANULA AND POLYP OF THE HYDROID *HYDRACTINIA SYMBIOLONGICARPUS* (CNIDARIA, HYDROZOA)

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## Summary

**This study examines calcification in planulae and polyps of the hydroid *Hydractinia symbiolongicarpus*. We observed that established colonies produce a crystalline mat on their substratum and that crystals visible by polarized light microscopy occur in the vacuoles of the gastrodermal cells of both polyps and planulae. The crystalline mat was found by infrared spectroscopy to contain calcium carbonate in the form of aragonite. The composition of the vacuolar crystals and the cellular mechanisms for manufacturing them were explored by alteration of calcium levels in the environment and by the use of pharmacological agents (acetazolamide, caffeine, DIDS, diltiazem, nifedipine, procaine, Ruthenium Red, ryanodine and verapamil) that affect cellular uptake and transport of calcium and bicarbonate. The results indicated that the crystals in the vacuoles contained calcium carbonate. The gastrodermal cells are hypothesized to serve as a physiological sink for excess calcium that enters the organism during motility, secretion and metamorphosis of the planula, and to create a crystalline substratum for the colony of polyps.**

Key words: aragonite, biomineralization, calcium, calcium carbonate, coral, larva, spicule.

## Introduction

Calcitic biominerals are found in the majority of metazoans, where they provide structural support and protection. These calcitic biominerals range from hydroxyapatite crystals in vertebrates to calcium carbonate in invertebrates, particularly in members of the phylum Cnidaria (Rupert and Barnes, 1994). This includes the hermatypic, scleractinian corals, belonging to a subclass of the Anthozoa. The scleractinian, or stony, corals utilize calcium carbonate to create massive exoskeletons, which serve both for protection and as a uniform substratum. The coral reefs that are produced primarily by the skeletons of scleractinian coral are important in carbon cycling and support unique marine ecosystems (Rupert and Barnes, 1994). In these corals, the calicoblastic epidermis secretes an organic matrix on which calcium carbonate crystals grow (Simkiss and Wilbur, 1989).

In another subclass of Anthozoa, the ahermatypic, non-reef building gorgorian corals, calcium carbonate is in the form of spicules that create an internal skeleton. These spicules are formed within vacuoles of sclerocytes located in the mesoglea. Subsequently, the sclerocytes migrate to, and deposit the spicules into, the supporting axis (Simkiss and Wilbur, 1989).

Although calcification has been studied for several decades, early studies were limited to the physiology and ecology of calcification, and to structural studies for taxonomic purposes. More recently, several studies have attempted to answer questions concerning the underlying cellular mechanisms of calcification. The kinetics of calcium uptake and the incorporation of calcium into calcium carbonate skeletons have been described for *Leptogorgia virgulata* (Lucas and Knapp, 1997), *Stylophora pistillata* (Tambutte et al., 1996; Allemand et al., 1998), *Corallium rubrum* (Allemand and Tambutte, 1996), *Tubastrea faulkneri* and *Galaxea fascicularis* (Marshall, 1996). These studies suggest a 'transcellular' pathway for calcium to reach cells involved in calcification (Tambutte et al., 1996), involving voltage-gated, L-type calcium channels (Tambutte et al., 1996), and $Ca^{2+}$-ATPases in the plasma membrane (Marshall, 1996; Kingsley and Watabe, 1984).

In addition, Lucas and Knapp (Lucas and Knapp, 1997) studied the incorporation of bicarbonate, the other ion required for calcium carbonate formation, into coral skeletons of *L. virgulata*. They proposed a model for the mechanism by which calcium carbonate crystals are formed. According to their model, bicarbonate ions may be made available for the production of calcium carbonate in three ways. The first is by diffusion of dissolved $CO_2$ from the extracellular fluid into the cell. Once inside the cell, $H_2O$ and $CO_2$ combine in the presence of carbonic anhydrase to form carbonic acid ($H_2CO_3$), which spontaneously loses a hydrogen ion to form the bicarbonate ion ($HCO_3^-$). The second method is through metabolically generated $CO_2$. Carbon dioxide generated from oxidation of metabolites in the citric acid cycle is converted by carbonic anhydrase into carbonic acid, from which a hydrogen ion dissociates to form a bicarbonate ion. The final method is

simply the transport of dissolved bicarbonate from the extracellular fluid into the cell by bicarbonate transporters in the cell membrane.

In addition to biomineralization, calcium plays a major role in second messenger systems, where it controls exocytosis, cortical reactions in eggs and muscle contraction (Alberts et al., 1994). In the marine hydroid *Hydractinia symbiolongicarpus*, calcium is required for larval motility (Ball, 1996), induction of metamorphosis (Thomas et al., 1997) and secretion of adhesive material during metamorphosis (Ball, 1996).

The hydrozoan *H. symbiolongicarpus* forms polymorphic colonies that occur on the shells of pagurid hermit crabs, usually *Pagurus longicarpus*. The life cycle of organisms in the genus *Hydractinia* has been described (reviewed by Hyman, 1940). Within 24 h of fertilization, the embryo develops into a free-swimming larva, the planula, capable of undergoing metamorphosis. Following receipt of a metamorphic stimulus, typically a bacterial product in nature or other agents, including cesium, administered experimentally (reviewed by Thomas and Edwards, 1997), the planula shortens, attaches to a substratum, and undergoes biochemical and morphological changes to become a primary polyp. Apical tentacles grow out around a newly formed mouth and stolons grow out along the substratum. As these stolons extend new polyps form at intervals and eventually differentiate into specialized polyps that are responsible for feeding, reproduction and defense. Colony growth continues until the substratum, typically the surface of the shell of a hermit crab, is covered.

We observed that a crystalline mat occurred beneath the stolons of colonies growing on microscope slides. The existence of this crystalline material raised the possibility that this hydroid might share with corals the ability to produce calcium carbonate. Lucas and Knapp (Lucas and Knapp, 1997) showed that calcium carbonate spicules in vacuoles of sclerocytes of gorgonian corals were visible by polarized light microscopy. When colonies of *H. symbiolongicarpus* were examined by polarized light and differential interference contrast (DIC) microscopy, which employs polarized light, crystalline structures of varying sizes were observed moving, probably by Brownian motion, within the vacuoles of the gastrodermal cells. Crystals were also observed in gastrodermal cells of planulae. We hypothesized that these crystals were also a calcium salt, possibly calcium carbonate.

The purpose of this study was threefold: (1) to examine to occurrence of the crystalline structures throughout the life cycle of *H. symbiolongicarpus*; (2) to determine the composition of the crystalline mat beneath the colony and the crystals within the vacuoles of gastrodermal cells; and (3) to explore the cellular mechanisms that underlie calcification in this species through use of various inhibitors and modulators.

## Materials and methods

### *Experimental organisms*

Colonies of *Hydractinia symbiolongicarpus* (Buss and Yund, 1989) were purchased from Cape Fear Biological Supply Company, South Port, NC, USA. The colonies were reared in the laboratory in a marine aquarium exposed to a diurnal cycle of 14 h:10 h L:D. Colonies were maintained in artificial sea water (ASW; Instant Ocean™, Burlington, NC, USA) at a temperature of 22–23 °C and a salinity of 34–36 ppt and fed daily with nauplii of *Artemia salina* (Carolina Biological Supply Company, Burlington, NC, USA). Fertilized eggs were obtained by exposing male and female colonies to light following a period of darkness (Ballard, 1942) and embryos were collected approximately 1.5 h later. Embryos were reared in ASW; to prevent bacterial induction of metamorphosis, the ASW was filtered through 45 μm and 22 μm filters with 100 μg $ml^{-1}$ streptomycin sulfate added to the filtrate. Transplanted colonies were established by explanting a group of 3–4 polyps from a colony of *H. symbiolongicarpus* on a hermit crab shell, affixing them to glass microscope slides with quilting thread (Buss et al., 1984), and allowing them to grow for weeks or months, according to the protocol described above for colonies.

### *Analysis of composition of crystalline mat by infrared spectrometry*

To determine the composition of the crystalline mat below the stolons of *H. symbiolongicarpus*, polyps were transplanted to glass microscope slides (Buss et al., 1984) and allowed to grow for 20 months. After 20 months, the tissues of the transplanted colonies were removed by immersing the colonies in 5.25 % sodium hypochlorite (Clorox bleach; Kingsley and Watabe, 1984; Lucas and Knapp, 1997; Marshall, 1996) for 24 h. Afterwards, loose particles of the crystalline mat were vacuum-filtered from the bleach solution onto 22 μm biological filters and rinsed 5 times with glass-distilled water. The crystals were oven-dried at 125 °C for 48 h.

The crystals were analyzed on a BioRAD FT-IR Spectrometer 175C using the KBr press method. A sample of commercially available $CaCO_3$ (Sigma) was analyzed as a control. Samples were mixed with KBr and a pure KBr press was used as a reference. The spectra obtained were analyzed using BioRad FTS software and compared to spectra previously obtained by others (Adler and Kerr, 1962; Kikuchi and Tamiya, 1984).

### *Analysis of composition of vacuolar crystals and mechanisms of formation*

To elucidate the composition and examine the cellular mechanisms involved in the formation of the crystals located in the vacuoles of the gastrodermal cells of *H. symbiolongicarpus*, we manipulated the ions in the environment and used various inhibitors to determine the effect of altering the amount of available calcium and carbonate on the formation of crystals. These quantitative experiments were performed on planulae, rather than polyps, because of the enhanced ability to control the levels of available calcium and carbonate and the relative ease in maintaining the specimens in the treatments.

For all experiments testing the effects of different treatments

on the number of crystals per planula, embryos were reared in 35×10 mm plastic Petri dishes. It was necessary to rear planulae in ASW alone for the first 6 h, since planulae 0–6 h old exhibit high sensitivity to treatment solutions, particularly those manipulating calcium levels, which often results in mortality or extreme deformities. To maintain a consistent experimental protocol, planulae were transferred to all treatment solutions 6–8 h after fertilization and were then reared in treatment solutions until 48–50 h post-fertilization. Treatment solutions were changed daily, except for nifedipine, which was changed every 8 h owing to its instability in aqueous solution. Planulae can creep along a substratum by both ciliary and muscular motility or attach themselves to the substratum with mucus secreted from the anterior end, and remain motionless; left undisturbed, they tend to assume the latter behavior. Thus, to reduce behavioral variation, both experimental and control planulae were kept undisturbed in the dark.

### *Quantification of crystals*

At 48 h, a squeeze preparation of 3–6 planulae from each treatment group were observed with a Zeiss Axioskop using polarized light optics and video-recorded. Only planulae of normal size and exhibiting normal development were video-recorded. Still photos were captured (in JPEG file format) using Zipshot™ and Arc PhotoImpression™ software on a Hewlett Packard Pavilion PC (8370) or Hewlett Packard Pavilion Notebook (N3250). Since no significant difference was found in the size distribution of crystals between treatments, the number of crystals in each planula was counted to quantify calcification. Three replicates of each treatment were performed.

Data from each of the three replicates were analyzed for significance using two-way ANOVA without correlation. In all cases, no significant difference ($P$=0.312) was found among the replicates of the treatments. However, a significant difference ($P$<0.001) was found between the treatments. To determine which treatments were significantly different from the appropriate controls, several pairwise multiple comparison procedures were used, including Tukey's Test, the Student–Newman–Keuls Method, and the Bonferroni $t$-test. In addition, Student's $t$-test was also used to demonstrate significance. A $P$-value of less than 0.05 was considered to be significant. All statistical analyses were performed on SigmaStat 2.03 software. Results are presented in the text as mean ± S.D. of the pooled group; in graphs, values are means ± S.E.M. Significance is shown as the results of the Tukey's test.

### *Chemicals used*

Acetazolamide, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), diltiazem, nifedipine, ryanodine and verapamil were dissolved in undiluted dimethylsulfoxide (DMSO) to produce 10 mmol $l^{-1}$ stock solutions. The stock solutions were diluted in ASW for use. The final concentration of DMSO never exceeded 1 %. Caffeine readily dissolves in ASW and a 10 mmol $l^{-1}$ stock solution was prepared that was then diluted in ASW for use. Procaine and Ruthenium Red require vigorous agitation to dissolve in ASW. All chemicals were purchased from Sigma except for ryanodine, which was purchased from Calbiochem.

Sea water with elevated calcium (50 mmol $l^{-1}$ $Ca^{2+}$) was prepared by mixing 52 ml of 0.34 mol $l^{-1}$ $CaCl_2$, 17 ml of 0.53 mol $l^{-1}$ KCl, 62 ml of 0.37 mol $l^{-1}$ $MgCl_2$, 28 ml of 0.90 mol $l^{-1}$ $MgSO_4$, 3.98 ml of 0.54 mol $l^{-1}$ $NaHCO_3$, and then adding dry NaCl and water to bring the final volume to 1000 ml with a salinity of 35 p.p.t. (modified from Woods Hole Formulae and Methods, 5th edition, 1964, Marine Biological Laboratory, Woods Hole, MA, USA). $Ca^{2+}$-free sea water was prepared by omitting $CaCl_2$ from the previous formula.

## Results

### *Occurrence of crystals during the life cycle*

The crystalline structures of interest occurred in planulae, primary polyps, and polyps from established colonies of *H. symbiolongicarpus*. Fig. 1 shows a comparison of micrographs of the same planula taken with brightfield optics (Fig. 1A) and polarized light (Fig. 1B), revealing that the refractile crystals were limited to the endodermal cells. The crystals were located in the large vacuoles of the gastrodermal cells (Fig. 1C), where they moved freely by Brownian motion. The largest crystals were morphologically similar consisting of an ovoid spindle, often with an indention along the long axis (Fig. 1C). These crystals were not refractile nematocysts (Fig. 1C), as confirmed by examination of crystals and nematocysts by polarized light and DIC microscopy.

In polyps of established colonies, the crystals, identifiable in polarized light micrographs (Fig. 1D), were located in the pigmented gastrodermal cells of the body wall, as observed by DIC microscopy (Fig. 1E). Crystals also appeared in the endodermal cells that line the gastrovascular tubes of the stolons (Fig. 2A). Few crystals were observed in the endodermal cells of the hypostome or tentacles (Fig. 1D,E). Furthermore, no crystals were observed in the ectoderm of the body column (Fig. 1D,E), tentacles (Fig. 1D,E) or stolons (Fig. 2A).

In addition to the crystals observed in the endodermal cells, a crystalline layer was observed in association with the stolonal mat (Fig. 2B), between the surface of the substratum and the stolons and, to a certain degree, intermingled with the living stolons. Two components make up the crystalline mat: dead, crystallized stolon tubes (Fig. 2C,D) and inorganically precipitated crystals (Fig. 3A). The dead, hardened stolon tubes (Fig. 2C), the walls of which are refractile (Fig. 2D), were intertwined with living stolon tubes. Crystal growth that was separate from the crystallized stolon tubes (Fig. 3A) occurred in patches. In addition, upon the death of a colony, crystalline structures resembling polyps remained on the surface of the shell of the hermit crab (Fig. 3B).

### *Analysis of the compostion of the crystalline mat by infrared spectrometry*

The crystalline mat beneath a colony accumulates to the

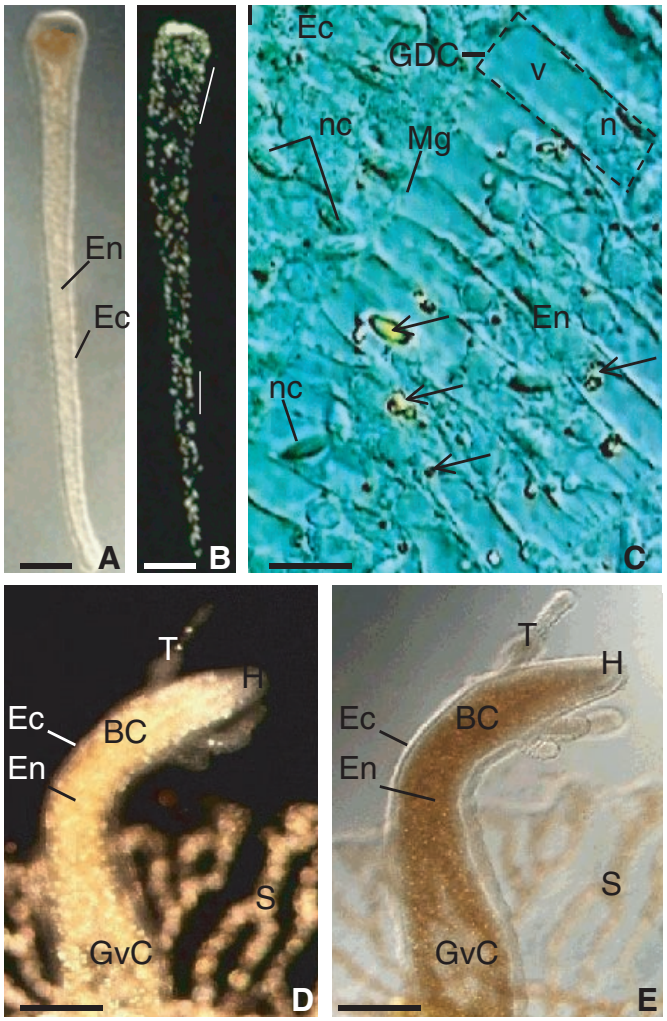


Fig. 1. Location of crystals in the planulae and polyps. (A,B) Comparison of the same live planula (slightly different because of movement) viewed by (A) brightfield and (B) polarized light microscopy. The crystals are located in the endoderm (En); the ectoderm (Ec) is devoid of crystals. The white bars in B indicate the margin of the ectoderm. Scale bar, 100 µm. (C) Posterior end of planula as seen with DIC microscopy. The mesoglea (Mg) forms the boundary between the endoderm (En) and ectoderm (Ec). The nucleus (n) of the gastrodermal cell (GDC; cell area indicated by a dashed line) is suspended in a large vacuole (v) by thin strands of cytosol. Arrows indicate crystals within the large vacuoles. The position of crystals do not correspond to those of nematocysts (nc). Scale bar, 10 µm. (D,E) A living polyp from a colony growing on a slide, viewed by (D) polarized light and (E) DIC microscopy. The mouth, located at the tip of the hypostome (H), opens into the gastrovascular cavity (GvC). Crystals are located in the endodermal cells of the body column (BC) and stolons (S) of the polyps; few, however, are located in the hypostome or tentacles (T). The ectoderm (Ec) is devoid of crystals. Scale bar, 200 µm.

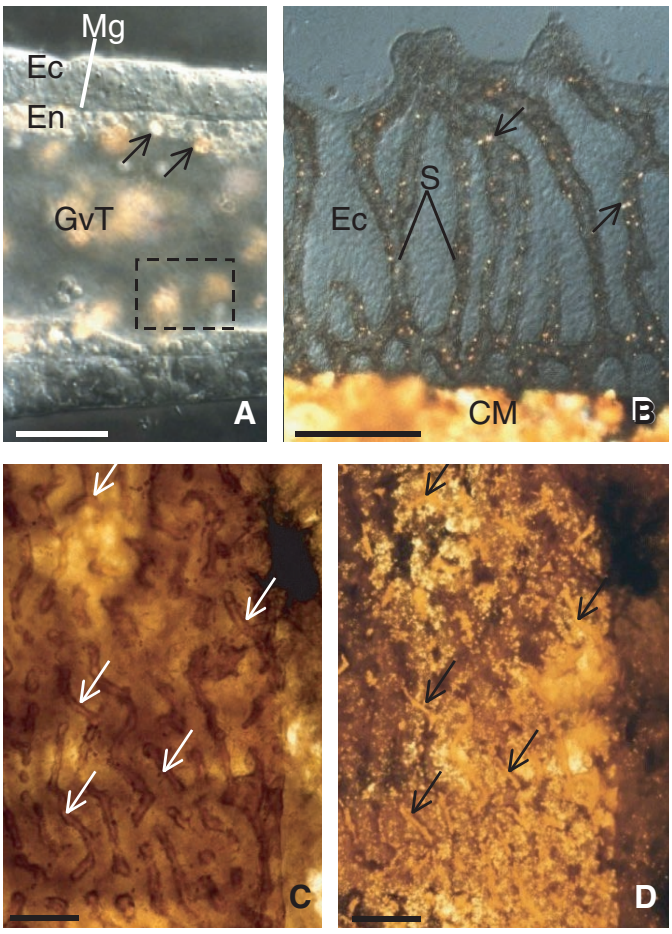


Fig. 2. Stolons and stolonal mat of established colonies. (A) Section of a new stolon from a portion of a colony growing on a slide. The stolon is a tubular structure composed of an outer layer of ectoderm (Ec) surrounding an inner layer of endoderm (En) that lines the gastrovascular tube (GvT). The crystals (arrows) are confined to the endoderm and are intracellular rather than free in the gastrovascular tube. The dashed box encloses two crystals that are out of the plane of focus. Scale bar, 20 µm. (B) New stolons growing from a portion of a colony recently transplanted to a microscope slide. New stolonal tubes (S) are separated by a layer consisting only of ectoderm (Ec). The crystals (arrows) are located within the endodermal cells that form the walls of the tube; the ectodermal cells that separate the tubes are devoid of crystals. The lower part of the section shows a crystalline mat (CM) below the living stolons, just out to the plane of focus. Scale bar, 200 µm. (C,D) Comparison of the same section of the stolonal mat, grown on a coverglass and viewed from the underside by (C) brightfield and (D) polarized light microscopy. What appear to be dead, hardened stolon tubes (arrows) in C run throughout the stolonal mat. The dead tubes are refractile when observed by polarized light (arrows in D correspond to arrows in C). Scale bar, 200 µm.

extent that it can be analyzed directly using infrared (IR) spectrometry. To determine if it is composed of calcium carbonate, the IR spectra for the crystalline mat and commercially available $CaCO_3$ (Sigma) were analyzed. These substances yielded similar spectra (Fig. 4), but with slightly different peaks in transmittance, consistently within 50 $cm^{-1}$ wave numbers. This analysis confirmed the crystals to be calcium carbonate. The transmittance peak in the 1430–1480 $cm^{-1}$ wave number range ($V_3$, Fig. 4) represents the

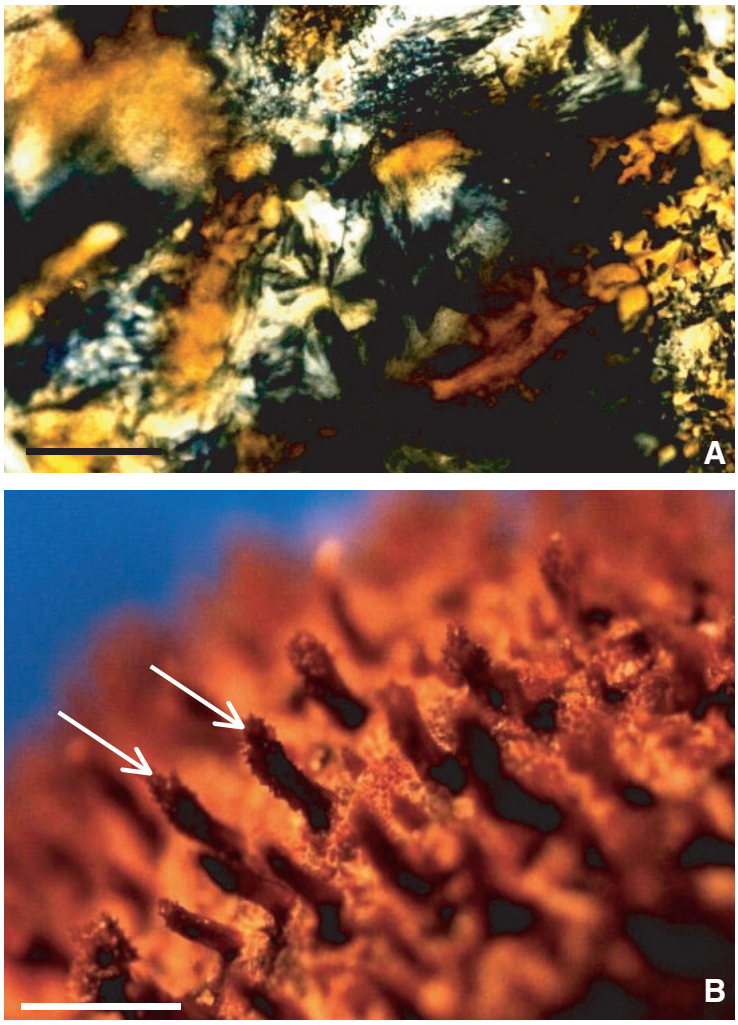


Fig. 3. Crystals of stolonal mat and a portion of the skeleton of a colony. (A) Evidence of inorganic precipitation of crystals in the crystalline mat. Scale bar, 50 µm. (B) Skeleton of a dead colony of *H. symbiolongicarpus*. Arrows indicate mineralized remains of polyps on a shell of a hermit crab. Bar, 250 µm.

doubly degenerate asymmetric stretching of the $CO_3^{2-}$ radical. Peaks in the 860–880 $cm^{-1}$ wave number range ($V_2$, Fig. 4) correspond to out-of-plane bending, and peaks in the 690–720 $cm^{-1}$ wave number range ($V_4$, Fig. 4) represent doubly degenerate planular bending. The sample has peaks at approximately 1190 and 1800 $cm^{-1}$ wave number that are characteristic of aragonite. A comparison of the IR spectra obtained in the present study with the IR spectra of calcium carbonate obtained in studies by Adler and Kerr (Adler and Kerr, 1962) and Kikuchi and Tamiya (Kikuchi and Tamiya, 1984) indicates that the crystalline mat is calcium carbonate in the aragonite form, whereas the commercially purchased calcium carbonate is a mixture of the calcite and aragonite forms.

*Requirement for calcium in vacuolar crystal formation*

Vacuolar crystals could not be obtained in sufficient quantity for direct analysis. It was therefore necessary to manipulate the availability of ions and determine their effect on crystal formation. To illustrate the role of calcium in crystal formation, two sets of experiments were conducted: (1) exposure of larvae to different concentrations of calcium, and (2) exposure of larvae to inhibitors or activators of a variety of calcium

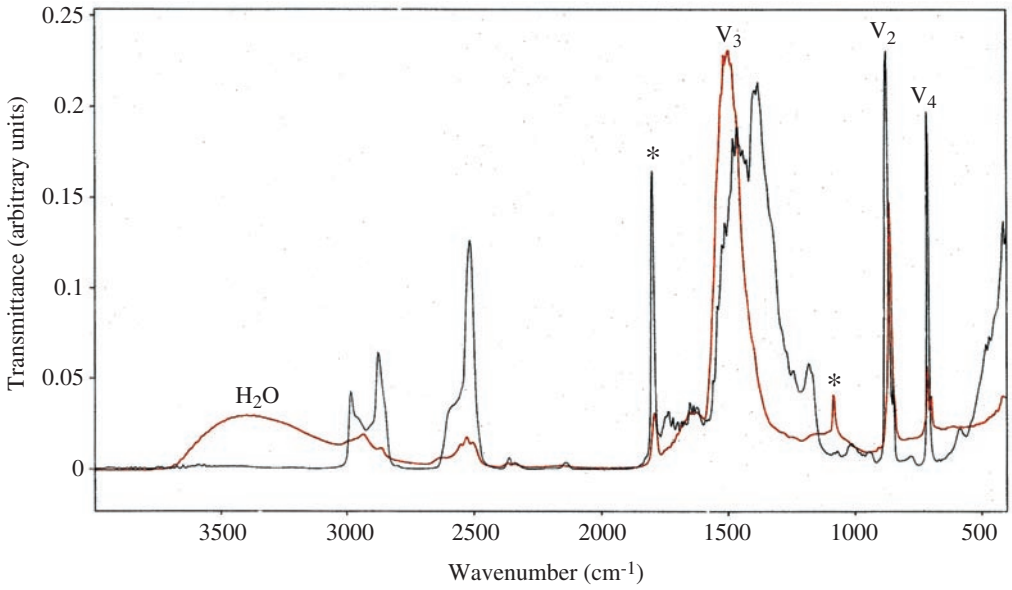


Fig. 4. Analysis of crystalline mat and commercially available calcium carbonate by infrared (IR) spectrometry. The infrared spectra were obtained for the crystalline mat (red line) and commercially available calcium carbonate (black line). The graph shows transmittance (arbitrary units) at each wavenumber. By comparing the IR spectra obtained in this study with those of calcium carbonate polymorphs obtained in previous studies (Adler and Kerr, 1962; Kikuchi and Tamiya, 1984), the crystalline mat was determined to be aragonite, whereas, the commercially available calcium carbonate was determined to be a mixture of aragonite and calcite, accounting for the wide and double headed $V_3$ band. The peaks in transmittance are due primarily to the $CO_3^{2-}$ radical of calcium carbonate. $V_3$, the characteristic transmittance peak for calcium carbonate, represents doubly degenerate asymmetric stretching of the $CO_3^{2-}$ radical. $V_2$ corresponds to out-of-plane bending and $V_4$ to doubly degenerate planular bending. Both sample have other notable peaks (asterisk) found in the spectra of aragonite. The peak in the range of approximately 3250–3750 wavenumber $cm^{-1}$ is due to the presence of water ($H_2O$) in the samples.

transporting proteins. The results, presented below, are summarized in Table 1.

*Exposure of larvae to different concentrations of calcium*

Fig. 5 shows the number of crystals formed in planulae exposed to different concentrations of calcium. The mean number of crystals formed at 48 h in larvae reared in ASW (9 mmol $l^{-1}$ $Ca^{2+}$ control) was 493±121 (± S.D.). Crystal production in larvae reared in calcium-free sea water was significantly reduced ($P$<0.001; $N$=15) to 213±89, or 43±18 %, of the ASW control group. Additionally, the number of crystals formed in planulae reared in 50 mmol $l^{-1}$ $Ca^{2+}$ sea water increased significantly ($P$<0.05) to 634±93, or 133±19 %, of control values.

*Exposure of larvae to modulators of calcium transporters*

To determine the effects of the L-type voltage-gated calcium channel blocker nifedipine on crystal production, embryos were exposed to 0.1 mmol $l^{-1}$ nifedipine in 1 % DMSO in ASW and reared to 48 h (Fig. 6). Groups of embryos reared in 1 % DMSO and ASW alone served as controls. The mean number of crystals formed in planulae reared in 0.1 mmol $l^{-1}$ nifedipine in 1 % DMSO was significantly reduced to 327±54, or 68±11 % of the

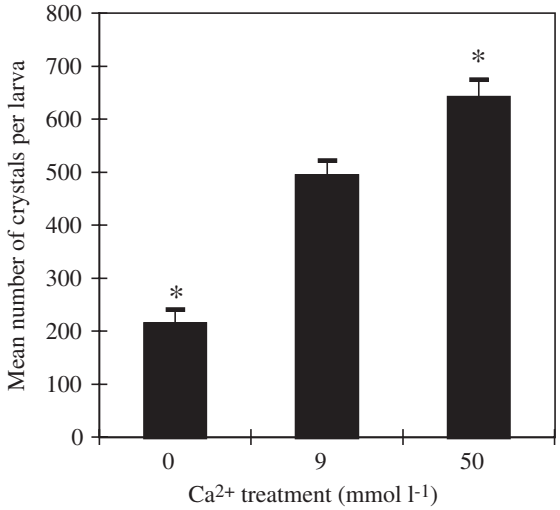


Fig. 5. The effects of extracellular calcium levels on crystal formation in planulae. Planulae were exposed to calcium-free SW (0 mmol $l^{-1}$; $N$=15), 9 mmol $l^{-1}$ $Ca^{2+}$ SW (9 mmol $l^{-1}$; $N$=17) and 50 mmol $l^{-1}$ $Ca^{2+}$ SW (50 mmol $l^{-1}$; $N$=10). *Significant difference compared with 9 mmol $l^{-1}$ $Ca^{2+}$ SW (for comparison of 0 mmol $l^{-1}$ $Ca^{2+}$ SW with 9 mmol $l^{-1}$ $Ca^{2+}$ SW, $P$<0.001; for 50 mmol $l^{-1}$ $Ca^{2+}$ SW with 9 mmol $l^{-1}$ $Ca^{2+}$ SW, $P$<0.05). Values given as mean ± S.E.M.

Table 1. *Summary of the statistical analysis performed to determine whether the treatments caused a significant difference in crystal production compared to the appropriate controls*

| | | | | Probability ($P$) value | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Two-way ANOVA | | | | | | | |
| | | | | Tukey | | SNK method | | Bonferroni | | Student's $t$-test | |
| Treatments | $N$ | Mean | S.E.M. | ASW | DMSO | ASW | DMSO | ASW | DMSO | ASW | DMSO |
| ASW 9 | 17 | 493.41 | 29.425 | NA | NA | NA | NA | NA | NA | NA | NA |
| ASW 9-M | 12 | 665.75 | 31.642 | 0.001 | NA | <0.001 | NA | 0.002 | NA | 0.9995 | NA |
| 50 | 10 | 633.5 | 33.289 | 0.034 | NA | 0.001 | NA | 0.062 | NA | 0.995 | NA |
| 50-M | 12 | 1034.8 | 30.773 | <0.001 | NA | <0.001 | NA | <0.001 | NA | 0.9995 | NA |
| 0 | 15 | 213.2 | 26.932 | <0.001 | NA | <0.001 | NA | <0.001 | NA | 0.9995 | NA |
| Caff | 15 | 201.8 | 26.631 | <0.001 | NA | <0.001 | NA | <0.001 | NA | 0.9995 | NA |
| Proc | 12 | 331.25 | 14.163 | 0.004 | NA | <0.001 | NA | 0.008 | NA | 0.9995 | NA |
| RR | 12 | 294.75 | 26.924 | <0.001 | NA | <0.001 | NA | <0.001 | NA | 0.9995 | NA |
| DMSO | 9 | 533.33 | 38.319 | 1 | NA | 0.341 | NA | 1 | NA | 0 | NA |
| AZ | 13 | 273.08 | 36.762 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.9995 | 0.9995 |
| DIDS | 14 | 234.43 | 24.1 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.9995 | 0.9995 |
| Dz | 14 | 145.14 | 14.774 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.9995 | 0.9995 |
| Nif | 10 | 326.8 | 17.188 | 0.01 | 0.002 | <0.001 | <0.001 | 0.018 | 0.005 | 0.9995 | 0.9995 |
| Ry | 11 | 243.18 | 15.293 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.9995 | 0.9995 |
| Vp | 16 | 248.75 | 19.344 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.9995 | 0.9995 |

$N$, number of planulae per treatment group; Mean, mean number of crystals per treatment group; S.E.M., standard error of the mean; Tukey, Tukey's test; SNK method, Student–Neuman–Keuls method; Bonferroni, Bonferroni $t$-test.

$P$ values obtained are given for treatment comparisons with both the artificial sea water (ASW) control and the dimethylsulfoxide (DMSO) control.

NA, indicates treatments that were not dissolved in DMSO and therefore were not compared to the DMSO control.

ASW, artificial sea water with 9 mmol $l^{-1}$ $Ca^{2+}$ (nonmotile planulae); ASW 9-M, artificial sea water with 9 mmol $l^{-1}$ $Ca^{2+}$ (motile planulae); 50, 50 mmol $l^{-1}$ $Ca^{2+}$ sea water (nonmotile planulae); 50-M, 50 mmol $l^{-1}$ $Ca^{2+}$ sea water (motile planulae); 0, calcium-free sea water; Caff, caffeine; Proc, procaine; RR, Ruthenium Red; DMSO, dimethylsulfoxide; AZ, acetazolamide; DIDS, 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid; Dz, diltiazem; Nif, nifedipine; Ry, ryanodine; Vp, verapamil.

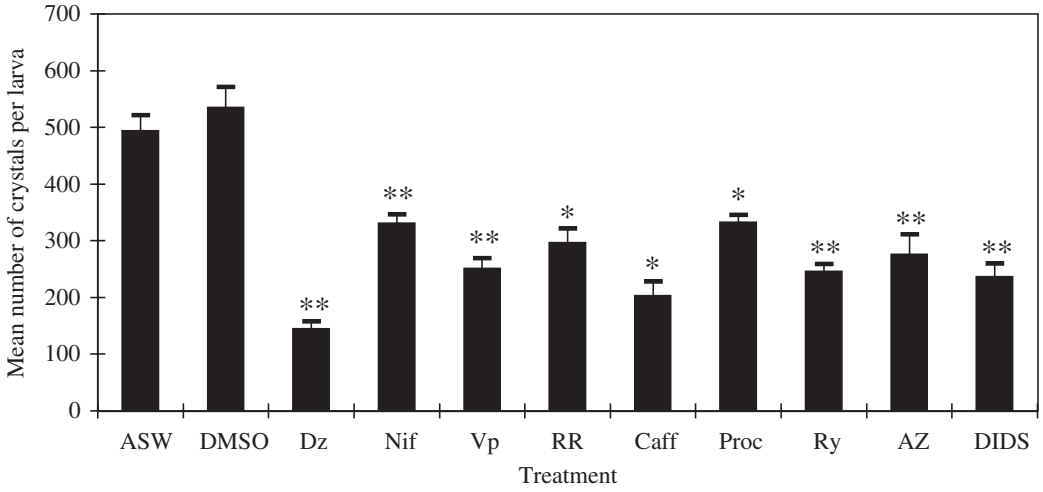


Fig. 6. The effects of treatments with pharmacological agents on crystal formation in planulae. Artificial sea water (ASW; *N*=17) and 1 % dimethyl sulfoxide in ASW (DMSO; *N*=9) serve as controls. Diltiazem (Dz; 0.001 mmol $l^{-1}$; *N*=14), nifedipine (Nif; 0.1 mmol $l^{-1}$; *N*=10) and verapamil (Vp; 0.001 mmol $l^{-1}$; *N*=16) are inhibitors of L-type calcium channels. Ruthenium Red (RR; 0.1 mmol $l^{-1}$; *N*=12) is an inhibitor of $Ca^{2+}$-ATPases. Caffeine (Caff; 0.1 mmol $l^{-1}$; *N*=15), procaine (Proc; 0.001 mmol $l^{-1}$; *N*=12), and ryanodine (Ry; 0.001 mmol $l^{-1}$; *N*=11) are modulators of ryanodine-sensitive calcium stores. Acetazolamide (AZ; 0.01 mmol $l^{-1}$; *N*=13) is an inhibitor of carbonic anhydrase, and DIDS (0.001mM; *N*=13) is an inhibitor of anion transport. Crystal production in the presence of inhibitors is compared with that in ASW (*N*=17) and 1 % DMSO (*N*=9) controls. *Significant difference compared to ASW; **significant difference compared to ASW and 1 % DMSO for treatments requiring DMSO. Values are means ± S.E.M.

ASW control (*P*=0.01; *N*=10) and 61±10 % of the DMSO control (*P*<0.01; *N*=10). The number of crystals formed in planulae exposed to 1 % DMSO, 533±115, or 112±23 % of the ASW control, was not significantly different (*P*=1.000; *N*=9) from the number of crystals formed in planulae reared in ASW alone.

To examine the effect of other inhibitors of L-type calcium channels, planulae were exposed to 0.001 mmol $l^{-1}$ verapamil and 0.001 mmol $l^{-1}$ diltiazem (Fig. 6). Verapamil significantly reduced the number of crystals produced in larvae to 249±77, or 50±16 % of the ASW control (*P*<0.001; *N*=16) and 47±15 % of the 1 % DMSO control (*P*<0.001; *N*=16). Similarly, diltiazem significantly reduced the number of crystals produced in larvae to 145±55, or 29±11 % of the ASW control (*P*<0.001) and 27±10 % of 1 % DMSO control (*P*<0.001; *N*=14).

The effects of an inhibitor of $Ca^{2+}$-ATPases was explored by rearing larvae in 0.1 mmol $l^{-1}$ Ruthenium Red (Fig. 6). Ruthenium Red significantly reduced the number of crystals produced in larvae to 295±93, or 60±19 % of the ASW control (*P*<0.001; *N*=12).

To determine the effects of modulators of ryanodine-sensitive intracellular calcium stores on crystal production, planulae were treated with 0.1 mmol $l^{-1}$ caffeine, 0.001 mmol $l^{-1}$ ryanodine, or 0.001 mmol $l^{-1}$ procaine (Fig. 6). Caffeine has been shown to induce the release of calcium from the sarcoplasmic reticulum through ryanodine receptors (RyR; Dutro et al., 1993; Iizuka et al., 1998; Shimoda et al., 2000). In this study, caffeine reduced the mean number of crystals produced in larvae to 202±103, or 41±38 % of the ASW control (*P*<0.001; *N*=15). Ryanodine and procaine, both inhibitors of the release of calcium from intracellular stores through RyR receptors in vertebrate cells (Dutro et al., 1993; Iizuka et al., 1998; Shimoda et al., 2000), also significantly reduced crystal production in the planulae of *H. symbiolongicarpus*. The mean number of crystals formed in planulae reared in 0.001 mmol $l^{-1}$ ryanodine was 243±51, or 49±10 % of the ASW control (*P*<0.001; *N*=11) and 46±10 % of DMSO control (*P*<0.001; *N*=11). The mean number of crystals formed in planulae reared in 0.001 mmol $l^{-1}$ procaine was 331±49, or 67±10 % of the ASW control (*P*<0.01; *N*=12).

### *Requirement for carbonate in crystal formation*

Manipulation of bicarbonate in seawater is difficult because of the effects of atmospheric $CO_2$. To test whether or not the crystals contained bicarbonate, an attempt was made to reduce the intracellular concentrations of bicarbonate by treatment of larvae with acetazolamide, a carbonic anhydrase inhibitor (Maren, 1977), and by treatment with DIDS, an inhibitor of bicarbonate transport (Madshus, 1988). The results of these studies are included in Fig. 6 and Table 1.

Embryos were treated with 0.01 mmol $l^{-1}$ acetazolamide in 0.1 % DMSO or 0.001 mmol $l^{-1}$ DIDS in 0.01 % DMSO and reared to 48 h. Groups of embryos reared in 1 % DMSO and in ASW served as controls. The number of crystals formed in 0.01 mmol $l^{-1}$ acetazolamide was significantly reduced to 273±133, or 55±27 % of the ASW control (*P*<0.001; *N*=13) and 51±25 % of the DMSO control (*P*<0.001; *N*=13). The number of crystals formed in planulae exposed to 0.001 mmol $l^{-1}$ DIDS was also significantly reduced to 234±90, or 48±18 % of the ASW control (*P*<0.001; *N*=14) and 44±17 % of the DMSO control (*P*<0.001; *N*=14).

## Discussion

### *Occurrence of crystals during the life cycle*

Crystals were found in the vacuoles of gastrodermal cells of both the planula and the polyp of *H. symbiolongicarpus*. They occurred exclusively in gastrodermal cells. In the planulae, the crystals occurred in gastrodermal cells along the entire length of the organism. In polyps, they occurred in gastrodermal cells of the body column and stolons, with few occurring in the gastrodermis of the tentacles or hypostome.

### *The crystalline mat contains calcium carbonate*

Analysis of the crystalline mat using IR spectroscopy identified the crystals as calcium carbonate. Calcium carbonate can occur in several different crystalline forms, the two most common being calcite and aragonite, which can be distinguished by their IR spectra (Adler and Kerr, 1962; Kikuchi and Tamiya, 1984). Calcite and aragonite have very similar crystal structures; the calcium ions of both are located in almost the same lattice positions in layers, alternating with layers of carbonate ions. The major difference between the two is in the arrangement of the carbonate ions (Falini et al., 1996). Although calcite is the more stable form of calcium carbonate at normal temperatures and pressures (Falini et al., 1996), aragonite is more 'popular' in biological structures. Aragonite is the prevalent form in coral reefs and in the shells of mollusks (Kikuchi and Tamiya, 1984). By comparing the IR spectra obtained in this study with those obtained in previous studies (Adler and Kerr, 1962; Kikuchi and Tamiya, 1984), we determined that the crystals in *H. symbiolongicarpus* are calcium carbonate in the form of aragonite.

### *Vacuolar crystals of gastrodermal cells contain calcium carbonate*

Our data suggest that the free-floating vacuolar crystals contain calcium. Alteration of calcium levels in surrounding sea water and manipulation of calcium levels by the use of inhibitors of calcium transport yielded the results expected if the crystals are made of calcium, that is they either significantly increased or reduced crystal production.

Methods to demonstrate the presence of carbonate are not as readily available. There are few, if any, cytochemical methods for staining bicarbonate or carbonate, and it is difficult to reliably alter carbonate levels in surrounding sea water, owing to the exchange of $CO_2$ between sea water and the atmosphere. In this study, therefore, we attempted to alter bicarbonate levels available for calcium carbonate production by using acetazolamide, an inhibitor of carbonic anhydrase activity (Maren, 1977), and DIDS, an inhibitor of anion transporters (Madshus, 1988). Both of these inhibitors significantly reduced crystal production in planulae of *H. symbiolongicarpus*, indicating that bicarbonate is a component of the crystals.

### *Cellular mechanisms involved in calcification*

#### *Inhibitors of L-type calcium channels and $Ca^{2+}$-ATPases*

Voltage dependent L-type calcium channels have been isolated and cloned from scleractian coral, where they have been shown to be associated with the calicoblastic epithelium, the site involved in calcium carbonate precipitation (Zoccola et al., 1999). The ability of inhibitors of L-type calcium channels to inhibit crystal formation in *H. symbiolongicarpus* suggests that calcium channels are involved in calcification in this hydroid as well. Nifedipine, verapamil and diltiazem were also shown to inhibit calcium incorporation into the skeleton of *S. pistillata* (Tambutte et al., 1996). Verapamil also has been shown to inhibit calcium incorporation into the skeletons of *C. rubrum* (Allemand and Grillo, 1992) and *G. fascicularis*, but not *T. faulkneri* (Marshall, 1996). Diltiazem was the most effective in reducing crystal production in *H. symbiolongicarpus* and also exhibited the highest degree of inhibition of calcium incorporation into the skeleton of *S. pistillata* (Tambutte et al., 1996). Inhibitors of N- and T-type calcium channels were shown to be ineffective inhibitors of calcification in *S. pistillata* (Tambutte et al., 1996).

In addition to calcium channels, several studies demonstrate the role of $Ca^{2+}$-ATPases in calcification in corals as well as in *H. symbiolongicarpus*. Ruthenium Red inhibited calcium incorporation in *G. fascicularis* and *T. faulkneri* (Marshall, 1996), and vanadate, another $Ca^{2+}$-ATPase inhibitor, was shown to inhibit calcification in *L. virgulata* (Kingsley and Watabe, 1984).

#### *Modulators of ryanodine-sensitive calcium stores: caffeine, ryanodine, procaine*

Two types of intracellular calcium stores have been identified; those that are inositol trisphosphate ($InsP_3$) sensitive and those that are ryanodine sensitive (Iizuka et al., 1998; Shimoda et al., 2000). Caffeine, ryanodine and procaine, all modulators of ryanodine-sensitive calcium stores, were effective inhibitors of crystal formation in *H. symbiolongicarpus*. Caffeine, which significantly reduced crystal production, induces the release of calcium from ryanodine-sensitive intracellular stores (Iizuka et al., 1998; Shimoda et al., 2000). The reduction of crystal production by treatment with caffeine was possibly due to depletion of the calcium from the calcifying vacuole.

The inhibitory effect of ryanodine and procaine on crystal formation in *H. symbiolongicarpus* cannot be as easily explained. In vertebrate cells, ryanodine and procaine are both inhibitors of the release of calcium from ryanodine-sensitive stores (Dutro et al., 1993; Iizuka et al., 1998; Shimoda et al., 2000). It was predicted, therefore, that treatment of planulae with ryanodine or procaine would result in near normal or even increased crystal production, rather than the decrease that was observed. However, there is evidence that, in invertebrates, ryanodine has the opposite effect on ryanodine-sensitive calcium stores, so that instead of inhibiting the release of calcium, ryanodine induces the release of calcium from ryanodine-sensitive stores. Lea (Lea, 1996), who performed his studies on the shore crab *Carcinus maenas* and the lobster *Homarus vulgaris*, did not exclude the possibility of a separate ryanodine-releasable calcium store or a second isoform of the ryanodine receptor with different properties from the 'classic'

ryanodine receptor. Additionally, ryanodine has been demonstrated to release calcium from intracellular stores in sea urchin eggs (Buck et al., 1994). Unfortunately, there are no other studies of the effects of caffeine, ryanodine, or procaine on calcification in other Cnidaria for the purpose of comparison.

Thapsigargin, which induces the release of calcium from $InsP_3$-sensitive stores, was not an effective inhibitor of calcification in *S. pistillata* (Tambutte et al., 1996), *T. faulkneri* and *G. fascicularis* (Marshall, 1996).

*Inhibitor of carbonic anhydrase: acetazolamide*

Carbonic anhydrase has been shown to play a central role in calcification in corals (Kingsley and Watabe, 1987; Lucas and Knapp, 1996). It can aid in calcification by catalyzing the hydration of $CO_2$ resulting in the production of $HCO_3^-$. It can also catalyze the conversion of $HCO_3^-$ to $CO_2$, the reverse of the above reaction, thus reducing the acidity of the environment and making it more conducive for calcification (Kingsley and Watabe, 1987). Cytochemical studies have demonstrated high levels of carbonic anhydrase activity on the membrane of spicule-forming vacuoles and in electron-dense bodies that are hypothesized to contain materials used in spicule formation in *L. virgulata* (Kinsley and Watabe, 1987).

Acetazolamide (Diamox), a specific inhibitor of carbonic anhydrase (Maren, 1977) effectively reduced crystal production in *H. symbiolongicarpus*, suggesting that carbonic anhydrase plays a role in calcification. Acetazolamide reduced the incorporation of calcium and carbonate into the skeletons of *C. rubrum* (Allemand and Grillo, 1992), *L. virgulata* (Lucas and Knapp, 1997) and *S. pistillata* (Tambutte et al., 1996).

*Inhibitor of anion transporters: DIDS*

The anion transporter inhibitor, DIDS (Madshus, 1988), was an effective inhibitor of crystal formation in *H. symbiolongicarpus*. There are several proposed methods for the action of DIDS, including the inhibition of electrogenic $Cl^-$ flux coupled with $Ca^{2+}$ flux (Yasumasu et al., 1985) or the inhibition of $Na^+/HCO_3^-$ symporters (reviewed by Madshus, 1988). The latter method has been implicated in the inhibition of calcification by DIDS in *C. rubrum* (Allemand and Grillo, 1992), *L. virgulata* (Lucas and Knapp, 1997), and *S. pistillata* (Tambutte et al., 1996).

### *Calcification in the planulae and polyps*

Rather than evolving solely as a supporting structure, it has been hypothesized that the calcium carbonate skeleton of corals developed as a calcium sink for the removal of waste calcium that was subsequently adapted for structural support (Lowenstam, 1981; Constanz, 1986). We propose that in *H. symbiolongicarpus* both functions are exhibited, but in different stages of the life cycle.

The planula utilizes calcium sequestration as a physiological sink. The data obtained from the present study, together with data obtained from previous cytochemical studies (A. M. Dandar, C. L. Rogers and M. B. Thomas, manuscript submitted for publication), suggest the following hypothetical model for calcification in the planulae of *H. symbiolongicarpus*. Septate junctions, which are tight junctions found primarily in invertebrates (reviewed by Thomas and Edwards, 1991), occur between ectodermal cells of *H. symbiolongicarpus* (C. L. Rogers and M. B. Thomas, personal observation), suggesting that calcium does not enter the planula by a paracellular route. It is more likely that calcium passively diffuses into the ectoderm cells by L-type calcium channels for use in motility, secretion of mucus or metamorphosis. Subsequently, in order to maintain a low intracellular concentration of calcium, calcium is pumped, by $Ca^{2+}$-ATPases, out of ectodermal cells into the intercellular space. Since, according to our model, gastrodermal cells take up excess calcium, the intercellular space would have a lower concentration of calcium than the surrounding sea water. It would therefore be more energy-efficient for the planula to pump calcium into the intercellular space than into calcium-rich sea water. To maintain a lower level of calcium in the intercellular spaces, calcium is then sequestered in an insoluble form in the gastrodermal cells. The cytochemical studies by Dandar et al. (A. M. Dandar, C. L. Rogers and M. B. Thomas, manuscript submitted for publication) suggest that calcium first enters and accumulates in the cytosol and nucleus of the gastrodermal cells. At a later stage, calcium that accumulates in the cytosol and nucleus is translocated into the vacuole. Bicarbonate moves by facilitated diffusion into the vacuoles from either the seawater or from bicarbonate produced intracellularly, depending on the activity of carbonic anhydrase. This process involves DIDS-sensitive anion transporters. In the vacuoles, calcium combines with bicarbonate to form calcium carbonate. This model presents a mechanism for sequestering calcium in an insoluble form, an energy-efficient means of ridding the organism of excess calcium.

After the planula metamorphoses into a polyp, the cellular mechanism for sequestering calcium is retained and adapted to form a crystalline mat. Crystals occur in the endoderm of the stolons. Whether they are produced in the stolons or reach them by migration of the epithelium, resulting from mitosis of cells of the body column, as has been shown for other hydroids (Davis, 1973; reviewed by Thomas and Edwards, 1991), is currently under investigation. These crystal-containing cells in the stolons appear to contribute to the formation of the crystalline mat underlying the colony and the mechanism of formation seems to involve death of stolons. We observed dead, crystallized remains of stolons running throughout the crystalline mat, suggesting that death of the crystal-containing cells leads to the formation of a crystalline conglomerate. This proposal is supported by the observation that when an entire colony dies, it leaves behind a crystalline skeleton of polyps and stolons. The exact cause of the death of the stolons in a living colony is unknown. It is probably due to blockage of the gastrovascular tubes, but we cannot rule out the possibility of a more controlled process such as apoptosis.

In addition to calcification in the dead gastrovascular tubes,

inorganic precipitation and growth of crystals occurs as described in the stony coral, where seed crystals, produced in specialized cells, are deposited into the skeleton mass and act as a nucleus for the deposition of additional calcium carbonate (Constanz, 1986).

The function of the crystalline mat underlying the colony is unclear. The mat may simply be the final product of excretion of excess calcium. Preliminary evidence, however, indicates that colonies that are unable to produce the crystalline mat over long periods of time lose their ability to adhere firmly to the substratum. An alternative or additional function may be to increase surface area for colony growth, which is limited for an organism whose substratum is the surface of a shell occupied by a hermit crab. The shell itself, of course, cannot grow. If, however, the hydroids release calcium carbonate onto the surface of the shell, they can increase the surface area available to them. Our calculations for a shell typical of those colonized by *H. symbiolongicarpus* (9×13 mm) indicate that if a colony created a crystalline mat 0.5 mm thick, the surface area would increase from 544 to 610 $mm^2$. Since the average density of the polyps is 1.48 polyps $mm^{-2}$, the modified substratum would accommodate 45 more polyps, which could contribute positively to the reproductive success of the colony.

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