CELL MIGRATION OF ZOOXANTHELLAE

IN THE CORAL *MONTIPORA CAPITATA*

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ABSTRACT

Reef-building corals have evolved an elaborate symbiotic relationship with zooxanthellae which plays an important role in supporting the productivity and diversity of coral reef ecosystems. It is important to know how the population of symbionts is maintained in the healthy symbiotic relationship, since such information may be helpful for understanding coral bleaching, that is, an abnormally low density of zooxanthellae.

In plate-shaped colonies of *Montipora capitata*, the dividing cells were selectively labeled by exposing live colonies to 5-bromo-2'- deoxyuridine (BrdU) to see where the cell division take place and to test the hypothesis that cells may move within the colony. When visualized with immunohistology, the dividing cells were found in higher proportion in the shaded part of colony. Long-term tracking of the labeled cells indicated that there is a vertical migration of zooxanthellae in an upward direction through the plate. This might be the key to protecting the cells from high solar radiation and could facilitate recovery of corals from bleaching.

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INTRODUCTION

Coral reefs as threatened ecosystems

Coral reef ecosystems are characterized by high biodiversity and productivity (Sorokin 1990). However, there currently is a perception that coral reefs worldwide may be declining and threatened by a number of causes (Souter *et al.* 2000, McClanahan 2002). These threats include both natural and anthoropogenic causes, but anthropogenic disturbances are of greater concern. Examples of such anthropogenic threats are marine pollution, increased sedimentation, eutrophication, destructive fishing practices, and mass bleaching, perhaps caused by climate changes such as global warming (Hatcher *et al.* 1989, Done 1992, Hoegh-Guldberg 1999, Souter and Linden 2000, McClanahan 2002). The predictions, in general, do not promise a bright future for coral reefs (Brown 1987, Hoegh-Guldberg 1999, Wilkinson 1999, Kleypas *et al.* 1999, Knowlton 2001). According to Hoegh-Guldberg (1999), the frequency of mass coral bleaching may increase up to nearly annual by 2020. McClanahan (2000) predicts that the combination of multiple threats with bleaching may cause local and global extinction of some coral species.

Bleaching is a disruption of the normal symbiotic relationship, in which symbiotic algae are expelled from the host tissue or lose their photosynthetic pigments (Hays & Bush 1990, Brown *et al.* 1995). This has a detrimental effect on the affected corals. They become visibly pale and may suffer from reduced energy supply, resulting in mortality in some cases (Stimson *et al.* 2002). The occurrence of bleaching is frequently reported from many ocean regions throughout the world, including here in Hawaii (reviewed in Brown 1997). When bleaching occurs at the scale of an entire reefs, the reef environment is strongly disturbed. While some reefs may show recovery within six months (Hayes and Bush 1990, Le Tissier and Brown 1996, Brown *et al.* 2002), in other cases the corals fail to recover and the reef is colonized by macroalgae, resulting in a major benthic community shift (McClanahan 2000, Diaz-Pulido & McCook 2002).

High sea surface temperature is most widely believed to be the proximate cause of mass bleaching (Edwards *et al.* 2001). Evidence suggests that increased seawater temperature stresses corals and results in bleaching (Brown 1997, Jones 1997). With the global warming trend of atmospheric and sea surface temperatures, bleaching has recently started to affect even the most remote and undisturbed reefs in the world (McClanahan 2000). Bleaching seems to have increased in both frequency and intensity during the last

two decades (Gates *et al.* 1992, Brown *et al.* 1994 McClanahan 2002). While there had been no reports of bleaching before the 1870's, now worldwide bleaching events seem to be occurring in a 3-4 year cycle, often coinciding with El Nino temperature anomalies (Huppert & Stone 1998, Hoegh-Guldberg 1999).

From the ecological and conservation points of view, it is also noteworthy that coral species differ in the tolerance to and/or capacity to recover from bleaching (Marshall & Baird 2000, Stimson *et al.* 2002). A mass bleaching event in Maldives in 1998 killed 98% of branching corals (Families Acroporidae, Pocilloporidae), while the majority of massive species such as *Porites* survived (Edwards *et al.* 2001). Thus, less tolerant species are more prone to extinction should bleaching grow in frequency and severity (Hoegh-Guldberg 1999, Souter & Linden 2000).

The pressing concerns with these mass bleaching events brought attention of scientists and conservationists to research on the symbiosis between corals and their intracellular photosynthetic dinoflagellates, called zooxanthellae. It has been studied by researchers worldwide, but there still remain many questions to be answered.

Coral-algal symbiosis

The symbiosis between zooxanthellae and anthozoans is intracellular; the algal cells are enclosed in a vacuole in host endodermal cells (Figure 1). One host endodermal cell normally contains one zooxanthella cell, but occasionally can harbor two or more (Muscatine *et al.* 1998). Zooxanthellae occupy the gastrodermis (endoderm) of both polyp and coenosarc tissue (connecting sheet between polyps) exhibiting a range of densities (Ralph *et al.* 2002).

Figure 1: A zooxanthella cell observed under a confocal microscope. The zooxanthellae cell appears red due to chlorophyll autofluorescence and the host endodermal cell appears in green due to naturally-occuring green fluorescent pigments..

Early studies on the symbiosis focused on the mutualistic benefits to both partners of the symbiosis. It was found that as much as 90% of the algae's photosynthetic products were translocated to the host in the form of glycerol, making the budget of coral-algal association as a whole, autotrophic (Davy *et al.* 1996). The translocation of

glycerol has been reported to be induced by 'host factor', a low molecular-weight chemical substance released by the coral cells (Grant *et al.* 1998, Cook & Davy 2001). Nutrients such as nitrogen can be obtained by heterotrophic feeding of coral polyps on plankton. Nitrogenous wastes from the coral cells are taken up by zooxanthellae. This recycling of materials contributes to the success of the hermatypic corals in oligotrophic tropical oceans.

The symbiosis between coral and zooxanthellae is dynamic in the sense that algal cells are proliferating constantly in host tissue (Steele 1976). In normal coral tissues, the density and distribution of zooxanthellae appear to be well-regulated, and the internal density of algal cells is approximately $10^5 - 10^6$ cells per square centimeter of coral surface area (Jones & Yellowlees 1997) although there are some seasonal fluctuations (Fagoonee *et al.* 1999). Factors that can affect the density of the zooxanthellae include depth (Masuda *et. al.* 1993, Kaiser *et al.* 1993), light intensity (Falkowski & Dubinsky 1981), and nutrient availability (Dubinsky & Jokiel 1994). Steele (1976) documented that cell density is directly proportional to light intensity in sea anemone *Aiptasia tagetes*. The same trend was observed in *Montastrea annularis* (Dustan 1979) and in the solitary coral, *Fungia* spp. (Masuda *et al.* 1993). Apparently, however, there is interspecies variation in the response

to the light intensity. Although most species show a decrease in zooxanthellae density with depth, some species have more zooxanthellae in reduced light (Fitt and Cook 2001, Titlyanov *et al.* 1980, Falkowski & Dubinsky 1981). In *Stylophora pistilata,* the zooxanthellae population decreased with depth, while the cell division frequency increased (Titlyanov *et al.* 2000). The lower population density of zooxanthellae under low light may be beneficial because it reduces self-shading (Titlyanov *et al.* 2000). Titlyanov *et al.* (2000) also reported that cell division of zooxanthellae was phased in shallow corals but not in deep corals, so lowered light intensity may result in asyncronous division of zooxanthellae.

Density regulation of zooxanthellae in corals

Several processes are possibly involved in the regulation of zooxanthellae density in coral tissue. The density of zooxanthellae can increase by either cell division of zooxanthellae in the host tissue (McAulley & Cook 1994) or the phagocytotic uptake of the free-living algal cells from the surrounding water column (Colley & Trench 1985). On the other hand, the number of algal cells can decrease by degradation or digestion by the

host (Jones & Yellowlees 1997, Titlyanov *et al.* 1996), or release of zooxanthellae by the host into the environment (Stimson & Kinzie 1991, Hoegh-Guldberg *et al.* 1987, Fang *et al.* 1998). If the density of the symbiotic algae is constant, then these four processes must be kept in balance.

Since the intrinsic division rate of zooxanthellae in culture is much higher than that in the symbiotic state, it is thought that the algal cell division is somehow suppressed by the coral host (Suharsono & Brown 1992). However, the mechanism by which the zooxanthellae division is regulated is not known although there have been several hypotheses. Possible explanations include limited CO_2 availability for carbon fixation (Fitt and Cook 2001), limited supply of inorganic nitrogen or phosphorus (Cook & D'Elia 1987, Hoegh-Guldberg and Smith 1989, Falkowski & Dubinsky 1993, Ambariyanto and Hoegh-Guldberg 1996), or by both nutrients. A study done by Smith and Muscatine (1999) on sea anemones demonstrated premitotic control of algal cell division by the animal. According to their study, the algal cell division rate was affected by the host nutritional status rather than the nutrient availability to the symbionts (Smith & Muscatine 1999).

Although it is speculated that some species of sea anemones regulate the density

of symbionts primarily by expulsion, generally the rates of expulsion in hermatypic corals are much lower than the algal division rate, and the density may be regulated by degradation of the algal cells (McCloskey *et al.* 1996, Titlyanov *et al.* 1996). When zooxanthellae are expelled many of them appear to be healthy and capable of photosynthesis (Ralph *et al.* 2001). Also the expelled zooxanthellae may contain a varying proportion of dividing cells (Steele 1976, Baghdasarian & Muscatine 2000) as well as degraded cells (Titlyanov *et al.* 1996). In corals exposed to high and low temperature shock, the expelled cells still had intact host endodermal cells surrounding them (Gates *et al.* 1992), although no data were available for cells that were naturally expelled. Titlyanov and coworkers did extensive studies on the regulation of algal population density in hermatypic corals, and concluded that symbiont density is regulated mainly by the rates of division and degradation, with expulsion of normal algal cells occurring only under extreme conditions such as high temperature and starvation (Titlyanov *et al.* 2000). Titlyanov *et al.* (1996) also reported that the number of degraded zooxanthellae was approximately the same as the number of the cells dividing in the coral tissue.

Cellular and molecular biology of bleaching

Bleaching is now understood more generally as a part of the physiological response of corals to various kinds of stressful conditions, such as temperatures, irradiances, and salinities that are outside tolerable ranges. High temperature stress might impair photosynthetic enzymes of zooxanthellae (Jones *et al.* 1998), and cause host corals to expel the damaged zooxanthellae (Brown *et al.* 1995, Fang *et al.* 1998, Huang *et al.* 1998). The process of bleaching involves exocytosis of algal cells from the host which requires calcium ions (Huang *et al.* 1998). Le Tissier and Brown (1996) observed *in situ* degradation of both zooxanthellae and host endodermal cells in intertidal colonies of *Goniastrea aspera* that were bleached by solar radiation.

Recently it has been speculated that oxidative stress plays a major role in the mechanism of bleaching. Studies on photoprotection suggest that high irradiance and ultraviolet radiation can cause significant damage to zooxanthellae. Zooxanthellae produce larger amounts of superoxide dismutase, an antioxydant, under higher irradiance to prevent damage from photosynthetically produced oxygen radicals (Lesser & Shick 1989). Lesser and Shick (1989) suggested that high irradiance, in combination with high

temperature, may induce bleaching. More specifically, high temperatures may compromise the ability of the coral-algal association to cope with high irradiance, resulting in bleaching (Fitt *et al.* 2001). Low temperature, as well as high temperature, caused bleaching in corals suggesting that the malfunction of temperature-sensitive enzymes involved in PSII might lead to increased oxidative damage (Saxby *et al.* 2003).

Observations that different species of corals exhibit different degrees of bleaching under the same environmental conditions, and the discovery of great genetic diversity of zooxanthellae using molecular techniques, led to the 'adaptive bleaching hypothesis' proposed by Buddemeier *et al.* (1993). They argued that corals that bleach can subsequently take up different strains of zooxanthellae which are better adapted to the affected environment, and therefore bleaching may be adaptive to corals. Some evidence showed that the different 'clades' of zooxanthellae have different levels of resistance to high temperature (Warner *et al.* 1996, Kinzie *et al.* 2001) However, there is some uncertainty about the underlying assumptions that the different 'clades' of zooxanthellae have different photosynthetic and acclimatory capacities (Savage et. al. 2002). The adaptive bleaching hypothesis is still controversial and its assumptions still need more testing (Kinzie *et al.* 2001).

Many questions still remain about the causes and mechanisms of bleaching. Research on the subject is largely challenged by the lack of baseline knowledge about the normal symbiotic process. Since bleaching is a disruption of the natural symbiotic system, it cannot be fully understood without understanding natural symbioses in healthy corals.

Objectives of this Study

Not only does the density of zooxanthellae appear to be regulated, but the location of zooxanthellae within a coral colony also appears to be under some controlling mechanism since the observed distribution of zooxanthellae in coral tissues is not uniform. In plates of the coral *Montipora capitata,* a species with a perforate skeleton (i.e., porous skeleton with an open 3-dimentional mesh-like structure), the tissue extends into the skeleton and can connect the upper and lower surfaces of the plate (Figure 2). The density of zooxanthellae is highest in the gastrodermis of the upper surface. The bottom gastrodermal cell layer has lower concentrations of zooxanthellae, while the reticulate tissue in between has the lowest density (Kinzie 1993). Generally, microhabitat conditions for zooxanthellae vary significantly in different locations in a coral colony (Helmuth *et al.*

1997). Thus, different mechanisms of regulation may be working in different parts of a colony.

In addition to mechanisms of density regulation, another possibility is that the zooxanthellae may be transferred from one part of the tissue to another, suggested by a previous study by Santos and Kinzie (unpublished data). They observed in overturned coral plates the restoration of the density gradient on both surfaces but no change in algal mitotic index (MI). This suggested an upward vertical migration of zooxanthellae from the old top to the new top. They also found that, in controls that were not overturned, the MI was higher on the under surface. Together these results indicate that the cells might proliferate on the shaded bottom side and migrate to the top side where more light is available for photosynthesis.

As a part of my investigation into the mechanism underlying the distribution of zooxanthellae inside the coral host, I determined the relative importance of cell division and migration in the spatial regulation of zooxanthellae in coral tissue. I used *Montipora capitata,* a species of coral that can be found in abundance in Kaneohe Bay, to test this hypothesis.

My specific research questions were:

1) Is zooxanthellae division localized to the shaded regions of coral plates?

This is to verify the previous results obtained by Santos and Kinzie. I would expect more cell division in the bottom gastrodermis (endoderm) compared to other regions.

2) Do zooxanthellae migrate from the bottom to the top in coral plates?

I would expect to see evidence of cells migrating from the bottom gastrodermis to the top gastrodermis through the tissue in the perforate middle of the plate.

A possible adaptive advantage of this hypothesized mechanism would be the protection of dividing cells on the underside of the plate from the potential harm of UV radiation. The detrimental effect of UVR can be intense (Litchman et. al. 2002). It is known that corals have a photoadaptation mechanism using UV-absorbing compounds (Kinzie 1993, Dunlap & Shick 1998, Banaszak & Trench 1995). Exposure to UV stopped cell division and increased cell size in some kinds of phytoplankton (Garde & Cailliau 2000). Cells undergoing cell division may benefit from shading because their DNA might be more susceptible to UV damage. In this scenario, algae on the upper tissues would be responsible for most the photosynthesis but radiation would be strong enough to cause DNA damage.

Experimental approach

To answer these questions, it was necessary to label dividing zooxanthellae cells in living corals and follow their movement. The easiest way to observe the movement of the cells in live tissues would be to use a vital stain to label cells. For this purpose the stain must 1) be recognizable for a long enough period to follow the migration of the cells, and 2) not reduce viability of the cells or impede natural processes, especially cell movement. After testing several different methodologies, BrdU immunohistological techniques were found to be most useful for this purpose.

5-bromo-2-deoxyuridine, also known as BrdU, can selectively label proliferating cells and is used for a wide range of developmental research projects, including those using marine organisms (Carnevali *et al.* 1997, Ladurner *et al.* 2000). This thymidine analog is incorporated into DNA during S-phase when nuclear DNA is duplicated prior to cell division (Gratzner 1982). After fixing the tissue, BrdU can be visualized using monoclonal antibodies (Martinon *et al.* 1987). BrdU immunochemistry has replaced traditional ^{3}H(tritium)-thymidine methods as a faster, safer, and easier way to label proliferating cells (Maghni *et al.* 1999). Although this method has been applied to a

number of marine organisms, it has, to my knowledge, never been tested on either corals or zooxanthellae. This study is the first attempt to utilize BrdU immunochemical labeling methods on cnidarian-zooxanthellae associations, although ^{3}H(tritium)-thymidine method has been successfully used by other researchers on sea anemones (Baghdasarian & Muscatine 2000).

Figure 2: A cross section of a plate of *Montipora capitata* showing upper and lower epidermis as well as the reticulate tissue ramifying throughout the perforate skeleton.

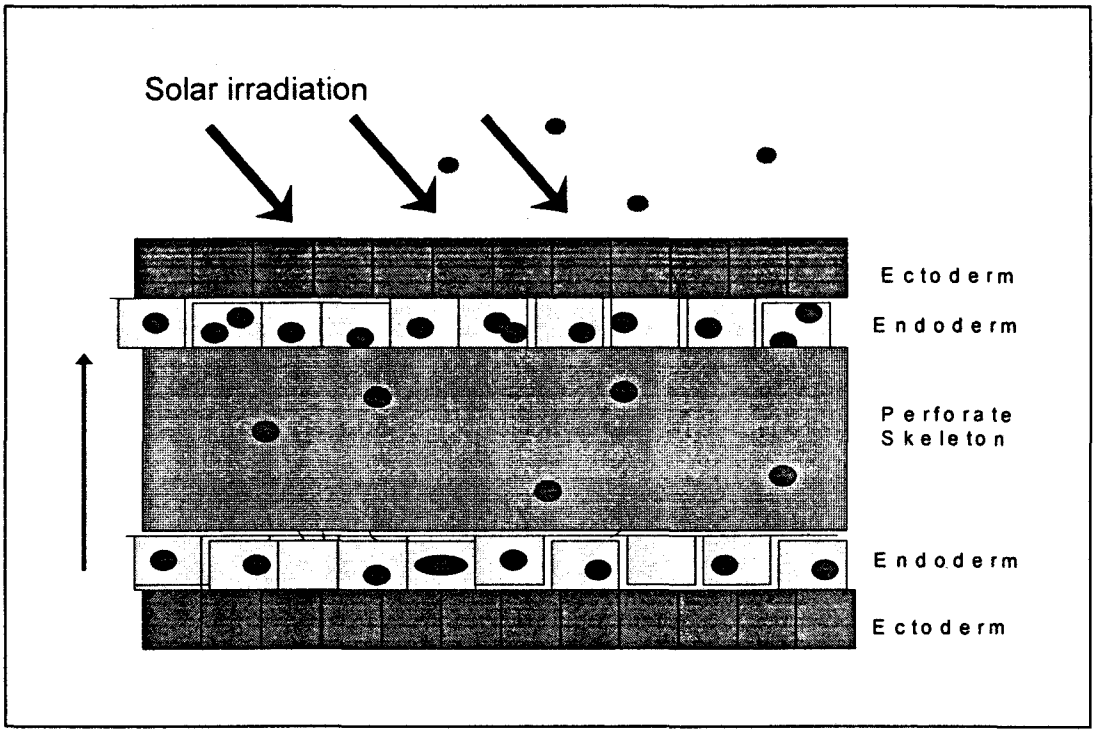


Figure 3: A proposed model for cell migration. Zooxanthellae cells make cell division in the endodermal layer which covers lower surface of the colony, and migrate upward through tissue covering perforate skeleton. The cells reach to the top endodermal layer where most photosynthesis takes place. As they photosynthesize, the damage from solar irradiation may accumulate in the cells and eventually the cells may be expelled from the coral.

MATERIALS AND METHODS

This study consisted of three main parts. First, preliminary tests were done to see if the zooxanthellae in symbiosis are able to incorporate BrdU, and under what conditions the best labeling results are obtained. Next, I exposed corals to BrdU to label the dividing zooxanthellae, and followed the subsequent movement of the cells over 3 weeks. In the third part, I compared the movement of the cells in corals in the natural orientation and in the overturned position using BrdU.

Animal collection and maintenance

All corals used for the experiments were plate-shaped *Montipora capitata,* collected from reefs around Coconut Island, Kaneohe Bay at approximately 3-5m depth. Corals were collected using SCUBA two weeks to two months prior to the experiments. Corals were visually inspected and only those that had undamaged live tissue on the both top and bottom surfaces of the plate were selected for use in the experiments. Corals were transported in natural seawater to the Hawaii Institute of Marine Biology (HIMB) within

an hour of collection.

The plate-like colonies were immediately cut into squares, with 3-cm sides using a hack saw while supporting the coral plate on a piece of neoprene wetted with seawater to prevent breaking and drying. Then the squares were kept on plastic-covered metal mesh in water tables with running seawater for two to three weeks until the cut sides were completely covered with regenerated tissue. This conveniently divided coral colonies into experimental coral units entirely covered in living tissue that can be manipulated. A constant flow of natural seawater into the tables was provided by the HIMB seawater system. The sea tables were located outside a lab under a roof, so the coral fragments were exposed to daily light/dark cycle and to natural but shaded sunlight. The intensity of the light in the sea water tables was 1.6×10^{15} quanta/sec/cm^2 at noon in the summer months of July-August (mean of three days) as measured by a quantum meter with a 4π sensor (model QSL-100, Biospherical Instruments Inc.). However, relative intensity of light at the seawater table, as measured by QSL-100 (Biospherical Instruments Inc.) may have been more variable than the intensity experienced by the corals in the field, and average light intensity may have been much lower than the field.

Effect of sea water on BrdU

I did a preliminary study to determine how long BrdU can remain effective in seawater. After the recovery and acclimation period, 6 squares of coral were transferred to 6 separate small glass jars, each with 150 ml of seawater and 1.5 ml of BrdU stock solution. The stock solution was prepared by dissolving 10 mg/ml BrdU (Sigma B-9285) in deionized water and stored in the refrigerator at 4°C for a few days before use. The final concentration of BrdU, when mixed with seawater, was 0.1 mg/ml. The coral squares were held about 1 cm from the bottom of the glass jars by small metal wire stands. Each coral piece was introduced to BrdU solution at 0, 1, 3, 6, 9, or 24 hours after the stock solution had been mixed with seawater. The coral squares were left in the glass jars for 72 hours. During this period, the jars were kept in a table with flowing seawater to control temperature, but there was no circulation in the jars.

After the incubation, the coral squares were fixed in 3.7% formaldehyde in seawater for 72 hours, and decalcified in 1:1 mixture of 5% formaldehyde and 5% formic acid solution with refrigeration (Permata *et al.* 2000). Complete decalcification normally took 2 to 4 weeks with the solution changed every 2 –3 days.

When the skeleton was completely dissolved, the remaining tissue was divided

into smaller pieces (~5 mm X 5 mm) with a razor blade and embedded in paraffin. The embedded tissue was sectioned vertically at 5 μm thickness and attached to glass slides, which were previously coated with poly-L-lysine (Sigma cat#P8920) following the vender's instruction.

The sections were deparaffinized and rehydrated in a graded series of methanol. The procedure for immunochemical detection of BrdU that I used was modified from Oinuma *et al.* (1992) and Uchida and Kaneko (1996). The sections were treated with 0.6% H_2O_2 for 30 minutes, then 0.5% trypsin in 0.19M sodium phosphate buffered saline (pH 7.4) at 37°C for 15 minutes, and 0.01% Tween-20 (Bio-Rad, cat#170-6531) in PBS for 5 minutes. Sections were then denatured in 2N HCl at 37°C for 1 hour, and neutralized in 0.05M Sodium Borate Buffer (pH8.5) for 5 minutes. The sections were rinsed with PBS after each treatment. They were then treated with 2% normal goat serum (GIBCO cat#16210-064) in PBS for 30 minutes, rinsed 3 times in PBT (0.1% Triton X-100, 0.1% bovine serum albumin in PBS), and exposed to anti-BrdU monoclonal antibody (Becton Dickinson cat#347580) at a dilution of 1:3000 in PBT, overnight at 4°C. The next morning, the slides were rinsed in PBS 3 times, and exposed to goat anti-mouse biotinylated antibodies for 5 hours at room temperature. The antibody solution was rinsed off with

PBT 3 times, and visualized with Sigma Fast 3'-3'-diaminobenzidine tablet sets (Cat#D-4293). Each tablet set, containing 3,3'-diaminobenzidine and urea hydrogen peroxide, was dissolved in 5 ml of deionized water, mixed with the same amount of PBT, applied onto the slides, and left to react for 10 minutes. Some slides were designated as controls and were not exposed to anti-BrdU antibodies but received all the other procedures. All the slides were dehydrated and sealed with coverslips and permanent mounting medium (VectaMount™, Vector Laboratories).

Diurnal pattern of cell division

The diurnal pattern of cell division was assessed to determine if there were peaks in cell division at a particular time of the day. The purpose was to find out what time of the day the zooxanthellae might most actively take up BrdU. If there was any daily periodicity, it would influence timing of my experimental protocols. To do this, samples of zooxanthellae were taken from the bottom of a single *M. capitata* colony at every 2 hours for 24 hours by waterpicking. The sampling started at 15:00 and ended at the same time of the following day. The colony I used was approximately 600 cm^2. At each sampling, cells

in a 1-cm² area were collected by waterpicking the area until the white calcium carbonate skeleton was exposed. The areas to be sampled were randomly selected and were at least 2 cm apart from each other to minimize effects of the damage caused by earlier sampling.

Samples were fixed and stained in Lugol's solution (Burnham 1967). Cells were concentrated by centrifugation, suspended in 1 ml of deionized water, mounted on a glass slide, and the mitotic index was determined with a microscope at 400X. Mitotic index was calculated as the number of cells with a division furrow, divided by the total number of the cells observed. Approximately 1000 cells were counted for each data point.

Measurment of algal cell division rate with a single pulse exposure to BrdU

To test the effect of BrdU on coral tissue, coral squares were exposed to BrdU solution in sea water for different time periods; 24, 48, and 72 hours. BrdU was added only once at the beginning of the experiment and coral squares were removed at these time intervals. The experimental corals and BrdU solution were prepared as described above. After the exposure, the corals were fixed and processed as described above. The cell division rates were measured by mitotic index and the percentage of cells labeled with BrdU.

Cell division and movement

Seven 3 cm X 3 cm squares of coral were taken from a single colony of *Montipora capitata* collected from Kaneohe Bay. The squares were prepared as described above. These corals were exposed to 50 μM BrdU solution in seawater for 48 hours starting at 3 PM. During this period, seawater was changed every 4 hours and fresh BrdU stock solution was added at each water change. All coral squares and the solution were kept in a single flat glass bowl with gentle stirring provided by a magnetic stir bar. Coral squares were held off the bottom by plastic mesh. The glass bowl was kept in a flowing seawater tank outside under the same conditions coral squares were acclimated in. The experiment was conducted in the winter of 2002 (February – March). Water temperature during this period was 21 - 23°C. After 48 hours in BrdU, one piece of coral was sampled immediately (Day 0), followed by sampling on days 2, 4, 7, 10, 14, and 21. The samples were randomly selected, and the treatment was as described above.

Cell division and movement in normal and overturned orientation

In the summer of 2002 (July – August), the previous experiment was repeated with an increased number of treatments. Two *M. capitata* colonies were used. Fourteen coral squares were taken from each colony, and allowed to heal from the cut as described above. Then 14 pieces from one colony were flipped over, and other pieces were left in the natural orientation. Half of the pieces from each group were randomly selected and exposed to BrdU as described above. The other pieces were incubated in a glass bowl as well but received only natural seawater as a control. Water temperature of the sea water table at this time was between 26°C and 27°C.

Treatment groups were exposed to BrdU for 48 hours with seawater and BrdU changed every 4 hours, then they were returned to the running sea water table with their orientation as at the beginning of the experiment. One piece from each of the 4 groups (BrdU/control: natural/flipped) was randomly sampled on days 0, 2, 4, 7, 10, 14, and 21. The samples were processed for immunohistology as described above. A last sampling was planned on day 42, but only the control coral which was not turned over and did not receive BrdU survived to 42 days. All other groups had lost tissue and skeleton was exposed.

Data Collection and Statistical Analysis

Slides were observed at 400X under a compound light microscope (Olympus BX-51). The labels of the all slides were masked to avoid observer bias. Cell density and percentage of BrdU-labeled cells were assessed in the upper and lower gastrodermal layers of each section on the slides. Only alternate sections were counted to avoid counting the same cells twice, because the diameter of the cells was approximately 10 μm while the thickness of the sections was 5 μm.

Length of the endodermal tissue layer in the sections was measured by the number of fields of view that covered the entire length and multiplied by the diameter of the field of view. "Linear cell density" was calculated by dividing the total number of cells along the endodermal layer by the length of the tissue layer observed. The gastrodermis of the coral *M. capitata* which contains the zooxanthellae is essentially a single thin layer of tissue, and appeared as linear array of single cells in histological sections. The nuclei of both coral and algal cells that had incorporated BrdU appeared dark brown (Figure 4). Mitotic index was calculated as the number of cells that were in the duplex stage (Figure

5) divided by the total cell number counted. The percentage of BrdU-labeled cells was expressed as the number of cells with brown nuclei over total number of the cells counted.

Standard error for mitotic index and percentage of BrdU cells were calculated using the following equation for sample proportion (Moore & McCabe 1998):

$$SE\,p = \sqrt{p\,(1\text{-}p) / n}$$

where p is the observed proportion of the cells and n is the total number of the cells counted. Statistical tests were performed using the statistical software Minitab 12 for Windows (Minitab Inc.).

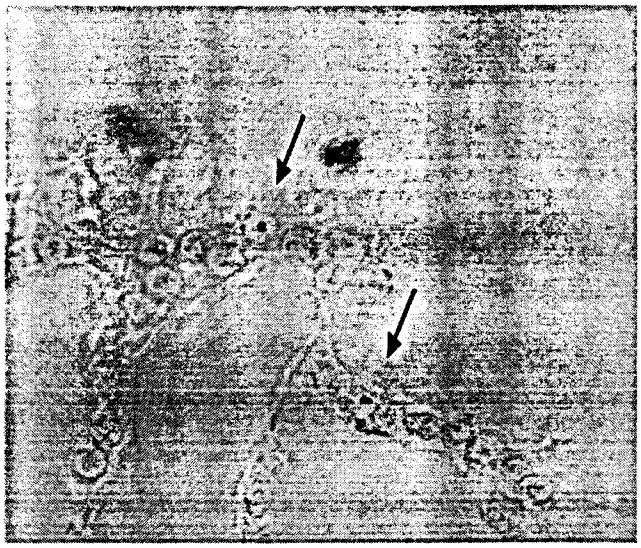


Figure 4: Nuclei of zooxanthellae labeled with BrdU in a cross section of *Montipora capitata*. The coral was exposed to BrdU for 48 hours, then fixed and exposed to anti-BrdU (see text for details).

Figure 5: A zooxanthellae cell in cell division. A division furrow and two daughter nuclei are visible.

RESULTS

The effect of seawater on BrdU

BrdU-labeled cells were found only in the treatments that were mixed 0, 1, and 3 hours before the coral was introduced to the BrdU solution. No BrdU-labeled cells were found in corals that were exposed to BrdU seawater solutions older than 6 hours. I concluded that BrdU remains active in seawater for between 3 to 6 hours, and determined that BrdU would be replenished every 4 hours in further experiments. This is consistent with results obtained from another lab (McFall-Ngai, personal communication).

The diurnal pattern of cell division

The mitotic index of zooxanthellae from the tissue of the underside of a plate was measured over a 24-hour period (Figure 6), fluctuated throughout the day, but showed no clear peak. Generally MI seems to be higher in the morning than the afternoon. Since there was no clear peak in the cell division rate of zooxanthellae, BrdU was administered periodically throughout the day in all subsequent experiments.

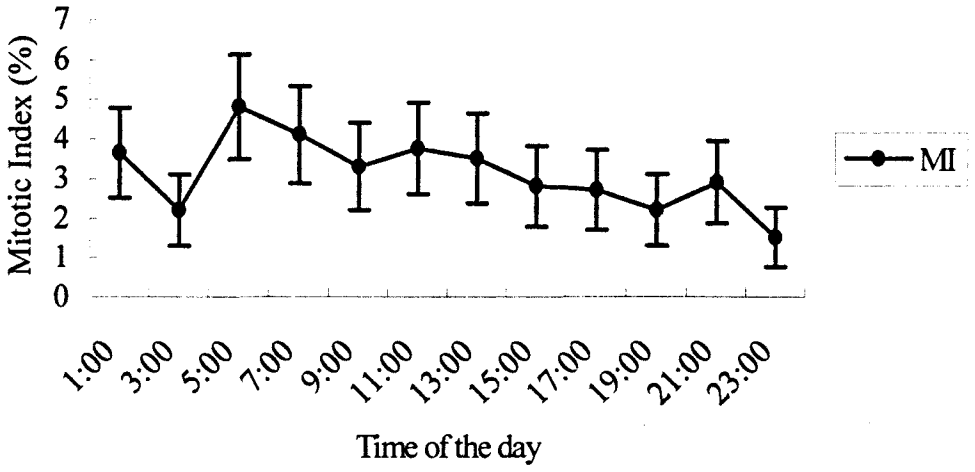


Figure 6: Diurnal pattern of zooxanthellae mitotic index in 24-hour period. Data points represent mitotic index of zooxanthellae and error bars indicate 95% confidence intervals. The graph was arranged to start at midnight although the actual experiment started at 15:00.

Measurement of algal cell division rate with a single pulse exposure to BrdU

The mitotic index and the percentage of cells labeled with BrdU were compared in the tissues from the underside of plates which had been exposed to BrdU for different durations (Figure 7). For all the treatments, the proportion of cells labeled with BrdU was higher than the mitotic index. In the samples that were fixed 24 and 48 hours after BrdU exposure, the BrdU stain appeared only in single cells whereas the samples at 72h had

some BrdU-labeled cells that were in the duplex stage. Some duplex and BrdU-labeled algal cells were also observed in the coral tissue covering the perforate skeleton. The nearly doubled increase in the percentage of labeled cells at 72 hours is probably due to the creation of two daughter cells per each labeled cell after completing cell division.

The specific cell division rate measured by mitotic index was compared between the top and bottom tissue layer (Table 1). "Top" refers to the gastrodermal layer attached to the epidermis of upper surface of the coral whereas "bottom" refers to the gastrodermal layer attached to the epidermis of lower surface of the colony. For all of the treatments, mitotic index was higher in the bottom tissue (Table 1), although a statistically significant difference was only found in treatment at 72 hours after exposure to BrdU.

Bottom cells at 72 hours after BrdU exposure had higher division rates as indicated by both mitotic index and BrdU results (Figure 7). Although it is possible that higher cell division rate was caused by experimental conditions, such as oxygen depletion, the increase in these indices was not statistically significant, except for BrdU for 72 hours. I concluded that BrdU itself has neither a stimulatory nor an inhibitory effect on cell division in the short term (Maghni *et al.* 1999). The variation in mitotic index among the treatments may reflect the difference in the cell division rate of individual coral fragments.

Thus I felt that use of BrdU would not result in unrepresentative artifacts in division rates.

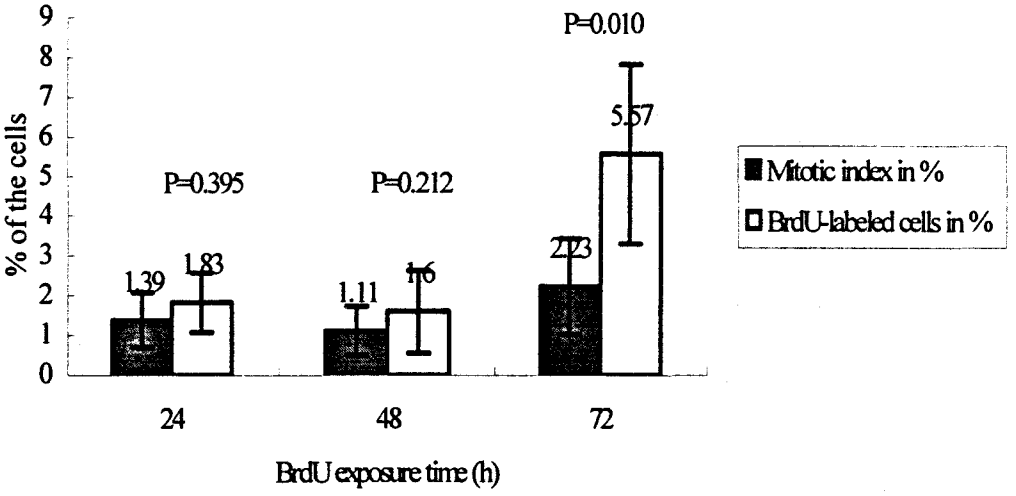


Figure 7: Test of the effects of BrdU on cell division rates measured by two different methods in the bottom tissue of *Montipora capitata.* Vertical lines indicate 95% confidence intervals. P-value indicates statistical significance of Hα: top ≠ bottom.

Table 1: Specific cell division rate in the top and bottom endodermal layer of 3 plates of *Montipora capitata.* P-value indicates statistical significance of Hα: top < bottom.

Time after BrdU exposure (h)	Mitotic index in % in the top (±SE)	Mitotic index in %in the bottom (±SE)	P-value
24	1.00 (±0.18)	1.39 (±0.35)	P=0.155
48	0.70 (±0.15)	1.11 (±0.31)	P=0.118
72	0.53 (±0.16)	2.23 (±0.61)	P=0.005

Cell division and movement

Cell density in the both top and bottom endodermal layers showed great changes during the first few days after the end of 48 hours of exposure to BrdU (Figure 8A), but then remained stable from the fourth day until the end of the experiment. In the coral that was flipped but not exposed to BrdU, the increase of the cell density in the new top layer and decrease in the new bottom layer were also seen, but not the initial increase (Figure 9).

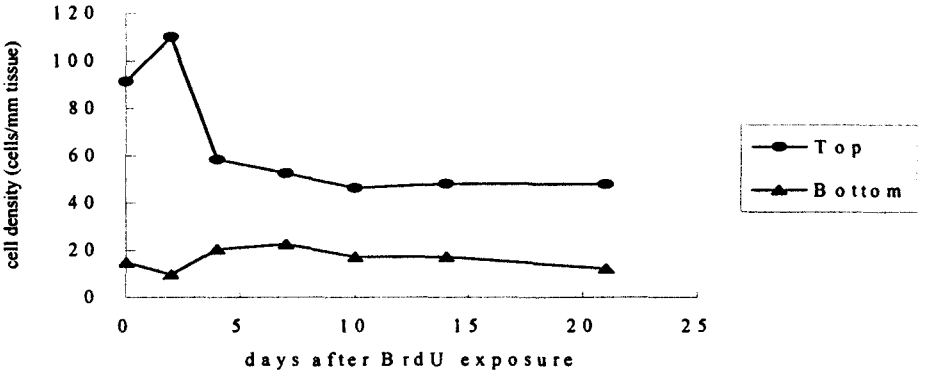
Fourty-eight hours of continuous exposure to BrdU (Fig 8) increased the percentage of labeled cells by about 2.5-fold (by comparison with single pulse exposure, Fig 7). Since 4.5 – 1.83 = 2.67 % of the cells entered S-phase during 48 hours, the length of the entire cell cycle would be 48 / 0.0267 = 1798 hours (assuming that cells have complete asynchronous division). At a given time, 1.83 % of the cells were in S-phase as measured by a single pulse exposure. Thus, the length of S-phase is about 1798 X 0.0183 = 33 hours. Because the duration of S-phase was longer than the time during which BrdU remains active, less frequent addition of BrdU would have been enough to get the same level of labeling at 48 hours.

Initially the bottom tissue had a slightly higher percentage of BrdU-labeled cells (Fig 8B). In both upper and lower gastrodermal layer the number of BrdU-labeled cells

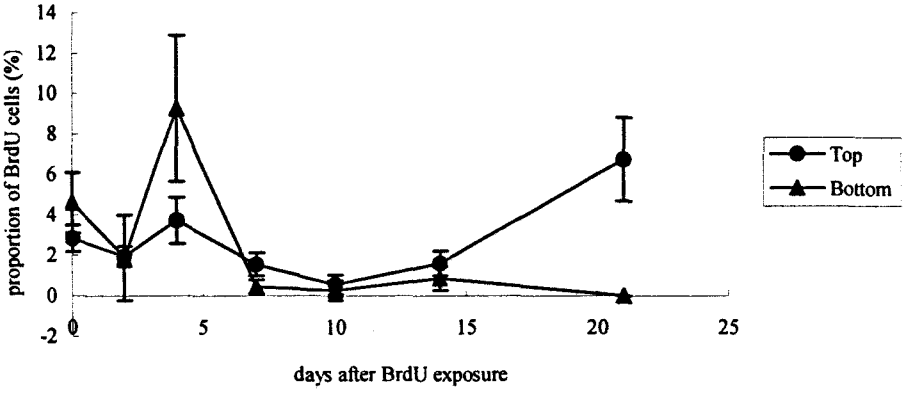
went down by day 10. However, on day 21, the proportion of BrdU cells in top layer showed a large increase (Fig 8B).

When the experiment was repeated with corals in both normal orientation and overturned in the summer, data for normally oriented corals showed a very similar pattern (Figure 10). The cell density in the new top slowly increased while it decreased in the new bottom. However, the percentage of the cells labeled with BrdU remained relatively low in overturned corals, indicating there was not much cell division going on during the BrdU exposure period, which was immediately after the corals were overturned.

A.



B.



C.

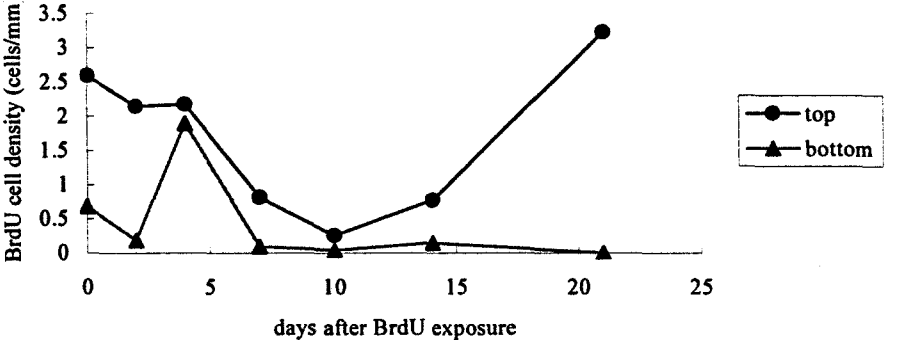


Figure 8: Cell density (A), percentage of cells that were labeled with BrdU (B), and number of the BrdU-labeled cell per linear millimeter (C) in a normally oriented M. capitata after 48 hours of exposure to BrdU. The error bars indicate 95% CI.

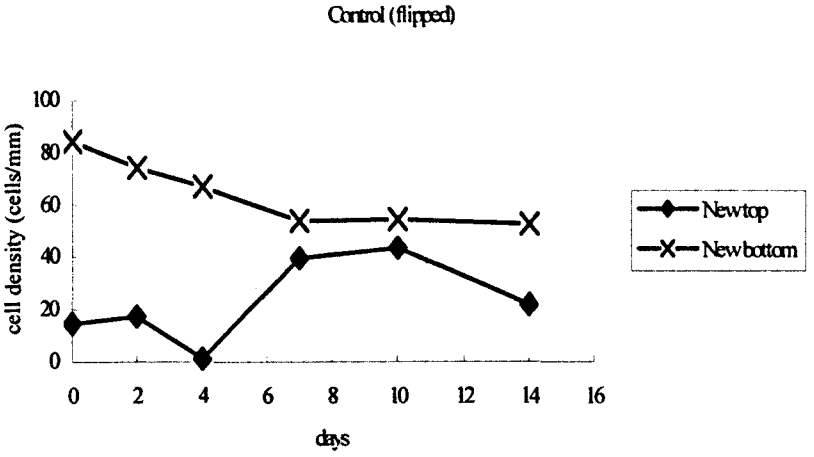


Figure 9: The time-course of cell density in overturned *Montipora capitata* not exposed to BrdU. Top and bottom refer to new orientation.

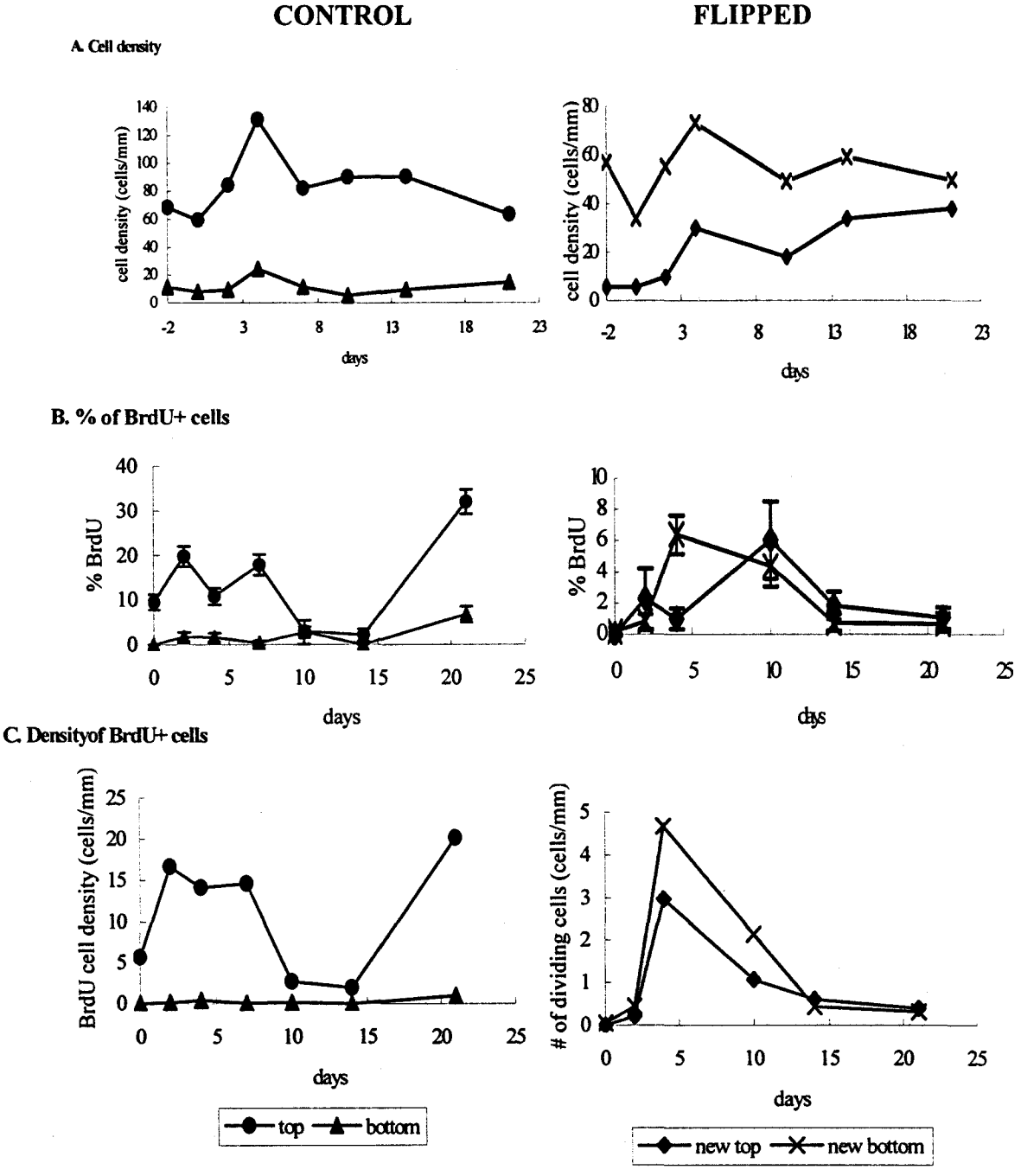


Figure 10: Cell density (A), percentage of cells that were labeled with BrdU (B), and number of the BrdU-labeled cell per linear millimeter (C) in normally oriented ("control", left) and overturned ("flipped", right) *M. capitata* after 48 hours of exposure to BrdU. The error bars indicate 95% CI. Note different Y axis scale for all flipped coral graphs.

References

Ambariyanto and Horgh-Guldberg O. (1996) Nutrient enrichment and the ultrastructure of zooxanthellae from the giant clam *Tridacna maxima*. Marine Biology 125:359-363.

Baghdasarian G. and Muscatine L. (2000) Preferential expulsion of dividing algal cells as a mechanism for regulating algal-cnidarian symbiosis. Biol. Bull. 199:278-286.

Banaszak A. T. and Trench R. K. (1995) Effects of ultraviolet (UV) radiation on marine microalgal-invertebrate symbioses. II. The synthesis of mycosporine-like amino acids in response to exposure to UV in *Anthopleura elegantissima* and *Cassiopeia xamachana.* J of Exp. Mar. Biol. Ecol. 194: 233-250.

Brown B. E., Clark K. R. & Warwick R. M. (2002) Serial patterns of biodiversity change in corals across shallow reef flats in Ko Phuket, Thailand, due to the effects of local (sedimentation) and regional (climatic) perturbations. Marine Biology 141: 21-29.

Brown B. E., Le Tissier M. D. A. & Bythell J. C. (1995) Mechanism of bleaching deduced from histological studies of reef corals sampled during a natural bleaching event. Marine Biology 122: 655-663.

Brown B. E. (1987) Worldwide death of corals – natural cyclical events or man-made pollution? Marine Pollution Bulletin 18(1): 9-13.

Brown B. E. (1997) Coral bleaching: causes and consequences. Coral Reefs 16:S129-S138.

Brown B. E., Dunne R. P., Ambarsari I., Le Tissier M. D. A. and Satapoomin U. (1999) Seasonal fluctuations in environmental factors and variations in symbiotic algae and chlorophyll pigments in four Indo-Pacific coral species. Marine Ecology Progress Series 191: 53-69.

Brown B. E., Dunne R. P., Scoffin T. P. and Le Tissier M. D. A. (1994) Solar damage in

intertidal corals. Marine Ecology Progress Series 105: 219-230.

Buddemeier R. W. and Fautin D. G. (1993) Coral bleaching as an adaptive mechanism: A testable hypothesis. Bioscience 42: 320-326.

Burnham, C. R. (1967) Cytological smear techniques. Mimeograph. Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul.

Carnevali, M. D. C., Bonasoro, F and Biale, A. (1997) Pattern of bromodeoxyuridine incorporation in the advances stages of arm regeneration in the feather star *Antedon mediterranea.* Cell Tissue Research 289: 363-374.

Colley N. J & Trench R. K. (1985) Cellular events in the reestablishment of a symbiosis between a marine dinoflagellate and a coelenterate. Cell and Tissue Research 239: 93-103.

Cook C. B. and Davy, S. K. (2001) Are free amino acids responsible for the 'host factor' effects on symbiotic zooxanthellae in extracts of host tissue? Hydrobiologia 461: 71-78.

Cook C. B. and D'Elia C. F. (1987) Are natural populations of zooxanthellae ever mutrient-limited? Symbiosis 4: 199-212.

Davy S. K., Lucus, I. A. N. amd turner J. R. (1996) Carbon budgets in temperate anthozoan-dinoflagellate symbiosis. Marine Biology 126: 773-783.

Diaz-Pulido, G. and McCook, L. (2002) The fate of bleached corals: patterns and dynamics of algal recruitment. Marine Ecology Progress Series 232: 115-128.

Done T. J. (1992) Phase shifts in coral reef communities and their ecological significance. Hydrobiologia 247: 121-132.

Dubinsky Z. & Jokiel P. L. (1994) Ratio of energy and nutrient fluxes regulates symbiosis between zooxanthellae and corals. Pacific Science 48(3): 313-324.

Dunlap W. C. and Shick J. M. (1998) Ultraviolet radiation-absorbing mycosporine-like amino acids in coral reef organisms: A biochemical and environmental perspective. J. Phycol. 34: 418-430.

Dustan, P. (1979) Distribution of zooxanthellae and photosynthetic chloroplast pigments of the reef-building coral *Montastrea annularis*. Ellis and solander in relation to depth on a West Indian coral reef. Bulletin of Marine Science 29: 79-95.

Edwards S. E. (1999) Using in situ substratum sterilization and fluorescence microscopy in studies of microscopic stages of marine macroalgae. Hydrobiologia 398/399: 253-359.

Edwards A. J., Clark S., Zahir H., Rajasuriya A., Naseer A and Rubens J. (2001) Coral bleaching and mortality on artificial and natural reefs in Maldives in 1998, sea surface temperature anomalies and initial recovery. Marine Pollution Bulletin 42(1): 7-15.

Fagoonee I., Wilson H. B., Hassell M. P. and Turner J. R. (1999) The dynamics of zooxanthellae populations: A long-term study in the field. Science 283: 843-845.

Falkowski P. G. & Dubinsky Z. (1981) Light-shade adaptation of *Stylophora pistillata*, a hermatypic coral from the Gulf of Eilat. Nature 289: 172-174.

Falkowski P. G. & Dubinsky Z. (1993) Population control in symbiotic corals. Bioscience 43(9) 606-611.

Fang L., Wang J. & Lin K. (1998) The subcellular mechanism of the release of zooxanthellae during coral bleaching. Proc. Natl. Sci. Counc. ROC(B) 22:150-158.

Fine M., Oren U. and Loya Y. (2002) Bleaching effect on regeneration and resource translocation in the coral *Oculina patagonica*. Marine Ecology Progress Series 234: 119-125.

Fitt W. K. (2000) Cellular growth of host and symbiont in a cnidarian-zooxanthellar symbiosis. Biological Bulletin 198: 110-120.

Fitt, W. K., Brown, B. E., Warner M. E. and Dunne, R. P. (2001) Coral bleaching: interpretation of thermal tolerance limits and thermal thresholds in tropical corals. Coral Reefs 20: 51-65.

Fitt W. K. and Cook C. B. (2001) Photoacclimation and the effect of the symbiotic environment on the photosynthetic response of symbiotic dinoflaggellates in the tropical marine hydroid *Myrionema amboinense*. J. of Exp. Marine Biology and Ecology 256: 15-31.

Fitt W. K., McFarland F. K., Warner M. E. and Chilcoat G. C. (2000) Seasonal patterns of tissue biomass and densities of symbiotic dinoflagellates in reef corals and relation to coral bleaching. Limnology and Oceanography 45(3): 677-685.

Gateno D., Israel A. Barki Y. and Rinkevich B. (1998) Gastrovascular circulation in an octocoral: Evidence of significant transport of coral and symbiotic cells. Biol. Bull. 194: 178-186.

Garde K. and Cailliau C. (2000) The impact of UV-B radiation and different PAR intensities on growth, uptake of ^{14}C, excretion of DOC, cell volume, and pigmentation in the marine prymnesiophyte, *Emiliania huxleyi*. J. of Exp. Marine Biology and Ecology 247: 99-112.

Gates R. D., Baghdasarian G. and Muscatine L. (1992) Temperature stress causes host cell detatchment in symbiotic cnidarians: Implications for coral bleaching. Biological Bulletin 182: 324-332.

Grant A. J., Remond M. and Hinde R. (1998) Low molecular-weight factor from *Plesiastrea versipora* (Scleractinia) that modifies release and glycerol metabolism of isolated symbiotic algae. Marine Biology 130: 553-557.

Gratzner G. H. (1982) Monoclonal antibody to 5-bromo and 5-iodo-deoxyuridine: a new reagent for detection of DNA replication. Science 218: 474-475.

Hayes R. L. and Bush P. G. (1990) Microscopic observations of recovery in the

reef-building scleractinian coral *Montastrea annularis*, after bleaching on a Cayman reef. Coral Reefs 8: 203-210.

Helmuth B. S. T., Timmerman B. E. H. and Sebens K. P. (1997) Interplay of host morphology and symbiont microhabitat microhabitat in coral aggregations. Marine Biology 130: 1-10.

Hoegh-Guldberg O. (1994) Population dynamics of symbiotic zooxanthellae in the coral Pocillopora damicornis exposed to elevated ammonium [$(NH_4)_2SO_4$] concentrations. Pacific Science. 48: 263-272.

Hoegh-Guldberg O. (1999) Climate change, coral bleaching and the future of the world's coral reefs. Mar. Freshwater Res. 50: 839-866.

Hoegh-Guldberg O., McCloskey L. R. and Muscatine L. 1987. Expulsion of zooxanthellae by symbiotic cnidarians from the Red Sea. Coral Reefs 5: 201-204.

Hoegh-Guldberg O. and Smith G. J. (1989) Influence of the population density of zooxanthellae nad supply of ammonium on the biomass and metabolic characteristics of the reef corals *Seriatopora hystrix* and *Stylophora pistillata.* Marine Ecology Progress Series 57: 173-186.

Huang S., Lin K. & Fang L. (1998) The involvement of calcium in heat-induced coral bleaching. Zoological studies 37(2): 89-94.

Huppert A. and Stone L. (1998) Chaos in the Pacific's coral bleaching cycle. The American Naturalist 152(3): 447-459.

Hutton D. M. C. and Smith V. J. (1996) Antibacterial properties of isolated amoebocytes from the sea anemone *Actinia equina.* Biol. Bull. 191: 441-451.

Jones R. J. (1997) Changes in zooxanthellar densities and chlorophyll concentrations in corals during and after a bleaching event. Marine Ecology Progress Series 158: 51-59.

Jones R. J., Hoegh-Guldberg O., Larkum A.W. D. & Schreiber U. (1998) Temperature-induced bleaching of corals begins with impairment of the CO2 fixation mechanism in zooxanthellae. Plant, Cell and Environment 21: 1219-1230.

Jones R. J. & Yellowlees D. (1997) Regulation and control of intracellular algae (=zooxanthellae) in hard corals. Philosophical Transactions of the Royal Society of London, Biological Sciences 352(1352): 457-468.

Kaiser P. Schlichter D. and Fricke H. W. (1993) Influence of light on algal symbionts of the deep water coral *Leptoseris fragilis*. Marine Biology 117: 45-52.

Kinzie R. A. (1993) Effects of ambient levels of solar ultraviolet radiation on zooxanthellae and photosynthesis of the reef coral *Montipora verrucosa*. Marine Biology 116: 319-327.

Kinzie R. A., Takayama M, Santos S. R. and Coffroth M. A. (2001) The adaptive bleaching hypothesis: experimental tests of critical assumptions. Biol. Bull. 200: 51-58.

Kleypas J. A, Buddmeier R. B., Archer D., Gattuso J, Langdon C and Opdyke B. N. (1999) Geochemical censequences of increased atmospheric carbon dioxide on coral reefs. Science 284: 118-120.

Knowlton N. (2001) The future of coral reefs. PNAS 98(10): 5419-5425.

Ladurner P., Rieger R. and Baguna J. (2000) Spatial distribution and differentiation potential of stem cells in hatchlings and adults in the marine platyhelminth *Macrostomum* sp.: A bromouridine analysis. Developmental Biology 226: 231-241.

Lesser M. P & Shick J. M. (1989) Effects of irradiance and ultraviolet radiation on photoadaptation in the zooxanthellae of *Aiptasia pallida*: primary production, photoinhibition, and enzymatic defenses against oxygen toxicity. Marine Biology 102: 243-255.

Le Tissier M.D'A. A. and Brown B. E. (1996) Dynamics of solar bleashing in the

intertidal reef coral *Goniastrea aspera* at Ko Phuket, Thailand. Mar. Ecol. Prog. Ser. 136: 235-244.

Litchman E., Neale P. J. and Banazak A. T. (2002) Increased sensitivity to ultraviolet radiation in nitrogen-limited dinoflagellates: Photoprotection and repair. Limnol. Oceanogr. 47(1): 86-94.

Marshall P. A. and Baird A. H. (2000) Bleaching of corals on the Great Barrier Reef: differential susceptibilities among taxa. Coral Reefs 19: 155-163.

Martinon F., Rabian C., Loiseau P., Ternynck T., Avrameas S. and Colombani J. (1987) In vitro proliferation of human lymphocytes measured by an enzyme immunoassay using an anti-5-bromo-2-deoxyuridine monoclonal antibody. J. Clin. Lab. Immunol. 23:153.

Maghni K., Nicolescu O. M. and Martin J. G. (1999) Suitability of cell metabolic colorimetric assays for assessment of CD4+ T cell proliferation: comparison to 5-bromo-2-deoxyuridine (BrdU) ELIZA. Journal of Immunological Methods 223: 185-194.

Masuda, K., Goto, M., Maruyama, T. and Miyachi, S. 1993. Adaptation of solitary corals and their zooxanthellae to low light and UV radiation. Marine Biology 117: 685-691.

McAulley P. J. & Cook C. B (1994) Effects of host feeding and dissolved ammonium on cell division and nitrogen status of zooxanthellae in the hydroid *Myrionema amboinense*. Marine Biology 121: 343-348.

McClanahan T. R. (2000) Bleaching damage and recovery potential of Maldivian coral reefs. Marine Pollution Bulletin 40(7): 587-597.

McClanahan T. R. (2002) The near future of coral reefs. Environmental Conservation 29(4): 460-483.

McCloskey L. R., Cove T. G. and Verde E. A. (1996) Symbiont expulsion from the

anemone *Anthopleura elegantissima* (Brandt) (Cnidaria; Anthozoa). J of Exp. Mar. Biol. Ecol. 195: 173-186.

Moore D. S. and McCabe G. P. (1998) Introduction to practice of statistics, 3rd ed. W. H. Freeman and Co. New York. pp825.

Morris S. M. (1991) The genetic toxicology of 5-bromodeoxyuridine in mammalian cells. Mutaion Research 258: 161-188.

Muscatine L., Ferrier-Pages C., Blackburn A., Gates R. D., Baghdasarian G. and Allemand D. (1998) Cell-specific density of symbiotic dinoflagellates in tropical anthozoans. Coral Reefs 17: 329-337.

Oinuma T., Kawano J. and Suganuma T. (1992) Bromodeoxyuridine-immunohistochemistry on cellular differentiation and migration in the fundic gland of *Xenopus laevis* during development. Cell Tissue Res. 269: 205-212.

Olano C. T. and Bigger C. H. (2000) Phagosytotic activities of the gorgonian coral *Swiftia exserta*. Journal of Invertebrate Pathology 76: 176-184.

Permata W. D., Kinzie R. A. and Hidaka M. (2000) Histological studies on the origin of planulae of the coral *Pocillopora damicornis*. Marine Ecology Progress Series 200: 191-200.

Ralph P. J., Gademann R. and Larkum A. W. D. (2001) Zooxanthellae expelled from bleached corals at 33 degrees C are photosynthetically competent. Marine Ecology Progress Series 220: 163-168.

Ralph, P. J., Gademann, R. Larkum, A. W. D. and Kuhl M. (2002) Spatial heterogeneity in active chlorophyll fluorescence and PSII activity of coral tissues. Marine Biology 141: 639-646.

Ribes M, Coma R and Gili J. (1998) Heterotrophic feeding by gorgonian corals with symbiotic zooxanthellae. Limnol. Oceanogr. 43(6): 1170-1179.

Savage A. M., Trapido-Rosenthal H. and Douglas A. E. (2002) On the functional significance of molecular variation in *Symbiodinium,* the symbiotic algae of Cnidaria: photosynthetic response to irradiance. Marine Ecology Progress Series 244: 27-37.

Saxby T., Dennison, W. D. and Hoegh-Guldberg O. (2003) Photosynthetic responses of the coral *Montipora digitata* to cold temperature stress. Marine Ecology Progress Series 248: 85-97.

Smith, G. J. and Muscatine L. (1999) Cell cycle of symbiotic dinoflagellates: variation in G1 phase-duration with anemone nutritional status and macronutrient supply in the *Aiptasia pulchella – Symbiodinium pulchrorum* symbiosis. Marine Biology 134: 405-418.

Sorokin Y. I. (1990) Aspects of trophic relations, productivity and energy balance in coral-reef ecosystems. In: Coral Reefs Ecosystems of the World vol. 25 (eds. Falkowski P. G. and Woodhead A. D.), pp.401-410. Plenum Press, New York.

Souter D. W. and Linden O. (2000) The health and future of coral reef systems. Ocean & Coastal Management 43: 657-688.

Steele R. D. (1976) Light intensity as a factor in the regulation of the density of symbiotic zooxanthellae in *Aiptasia tagetes*. J. Zool. Lond. 179: 387-405.

Stimson J & Kinzie R. A. (1991) The temporal pattern and rate of release of zooxanthellae from the reef coral *Pocillopora damicornis* (Linnaeus) under nitrogen-enrichment and control conditions. Journal of Experimental Marine Biology & Ecology 153(1): 63-74.

Stimson J, Sakai K. and Sembali H. (2002) Interspecific comparison of the symbiotic relationship in corals with high and low rates of bleaching-induced mortality. Coral Reefs 21: 409-421.

Suharsono and Brown B. E. (1992) Comparative measurements of mitotic index in

zooxanthellae from a symbiotic cnidarian subject to temperature increase. J. Exp. Mar. Ecol. 158: 179-188.

Toller W. W., Rowan R. and Knowlton N. (2001) Zooxanthellae of the *Montastraea annularis* species complex: Patterns of distribution of four taxa of *Symbiodinium* on different reefs and across depths. Biol. Bull. 201: 348-359.

Tytlyanov, E. A., Shaposhinikova M. G. and Zvalinskii V. I. 1980. Photosynthesis and adaptation of corals to irradiance 1. Contents and native state of photosynthetic pigments in symbiotic microalga. Photosunthetica 14(3): 413-421.

Titlyanov E.A., Tsukahara J., Tytlyanova T. V., Leletkin V. A., van Woesik R. & Yamazato K. (2000) Zooxanthellae population density and physiological state of the coral *Stylophora pistillata* during starvation and osmotic shock. Symbiosis 28: 303-322.

Titlyanov E.A., Tytlyanova T. V., Leletkin V. A., Tsukahara J., van Woesik R. & Yamazato K. (1996) Degradation of zooxanthellae and regulation of their density in hermatypic corals. Marine Ecology Progress Series 139: 167-178.

Uchida K. and Kaneko T. (1996) Enhanced chloride cell turnover in the gills of chum salmon fry in seawater. Zoological Science 13: 655-660.

Warner M. E., Fitt W. K. and Schmidt G. W. (1996) The effect of elevated temperature on the photosynthetic efficiency of zooxanthellae *in hospite* from four different species of reef coral: a novel approach. Plant, Cell and Environment 19: 291-299.

Wilkerson F. P., Kobayashi D. and Muscatine L. (1988) Mitotic index and size of symbiotic algae in Caribbean reef corals. Coral Reefs 7: 29-36.

Wilkinson C. R. (1999) Global and local threats to coral reef functioning and existence: review and predictions. Mar. Freshwater Res. 50: 867-878.