Coral reefs, one of Earth’s most beautiful ecosystems, are home to an astonishing array of biodiversity with 32 of the 34 recognized animal phyla present (Wilkinson, 2002). This diversity is being utilized to find new cancer fighting drugs such as sarcophytols A and B (Fujiki, Suganuma and Suguri, 1989), and eleutherobin (Long, Carboni and Wasserman, 1998).

Reefs are also essential to the communities which surround them, providing the approximately 450 million people that live within 60 kilometers of a coral reef with a source of livelihood (Clive, 2006). Coral reefs provide large economic benefits for these communities with a total net benefit of approximately 30 billion USD per year and a net present value of approximately 800 billion USD (Cesar, Burke and Pet-Soede, 2003).

Although reefs are highly valued, they are in danger. Already 27% percent of the reefs worldwide have been destroyed (Cesar, Burke and Pet-Soede, 2003), and 58% of the remaining reefs are at risk due to human activity (Bryant, Laurretta and McManus, 1998). Currently, two main threats to coral reefs are overexploitation and coral bleaching. Research was conducted to address these problems using ascorbic acid as the method of remediation.

**Overexploitation**

Overexploitation is the unsustainable collection practice of local fishermen. This may cause an unnatural predator-prey relationship resulting from fishermen collecting a certain type of fish, or by collecting coral at a faster rate than it can naturally reproduce; such practices are destroying the coral reefs. It is estimated that 36% of reefs worldwide are affected by this problem (Bryant, Laurretta and McManus, 1998). Annually, approximately 11-12 million pieces of coral are exported from natural reefs (Wabnitz, Taylor and Green, 2003). This leaves a deficit of natural coral, thus harming the overall reef health.

When studying soft corals, marine scientists are faced with a predicament. Since propagating corals in a captive environment has been largely unsuccessful, the only way to obtain coral for research is to take from the reefs. An alternative to collecting from natural reefs is captive propagation, but this comes with its own set of problems. In a captive environment, sexual propagation of coral is nearly impossible due to the need to mimic the exact conditions that exist on a natural reef (Borneman, 2001). An alternative is to encourage asexual propagation using a technique called fragmentation (Highsmith, 1982). This is a process in which part of the parent coral is cut off and both the fragment and parent regenerate into full colonies. Although fragmenting is easily conducted with stony corals, the soft corals often do not survive due to a long recovery time and high risk of deadly infection.

**Coral Bleaching**

Rising seawater temperatures are one of the largest threats to coral reefs. 32.8% of corals are at elevated risk of extinction due to the increased sea surface temperatures, a drastic increase from previous decades (Carpenter, Abral and Aeby, 2008). Increased water temperatures are a threat to a coral as it causes “bleaching,” the release of the coral’s symbiotic algae zooxanthellae (Symbiodinium sp.). This often causes the death of the coral since the zooxanthellae is responsible for up to 90% of the coral’s energy requirements (Marsh and Schuttenberg, 2006); thus, the expulsion of zooxanthellae results in a mortality rate for the coral of over 90% (Global Coral Reef Monitoring Network, 2004). Bleaching events have proved devastating for coral reefs, most notably in the mass bleaching event of 1998, which severely damaged 16% of the world’s reefs and killed approximately half of the reefs in the Indian Ocean and surrounding South Asia (Global Coral Reef Monitoring Network, 2004).

From 1876-1979 only three bleaching events have occurred (Wellington, Glynn and Strong, 2001), but in the following 38 years, dozens of minor events have been reported (Lesser, 2007). Unfortunately, bleaching is only likely to increase in frequency in coming years due to the rising global temperatures (Hansen, Sato and Ruedy, 2006). Unless coral can build up an increased thermal tolerance of 0.2-1.0˚C per decade, mass-bleaching events will occur annually (Donner, Skirving and Little, 2005).

**Experimental Purposes**

This research had two goals. The purpose of the first experiment was to determine the effects of ascorbic acid on the rate of tissue regeneration and the survival rates of fragmented soft corals. The purpose of the second experiment was to determine the effects of ascorbic acid on the rate of zooxanthellae expulsion in coral exposed to increased water temperatures and to monitor the concentration of zooxanthellae present in the coral throughout the bleaching process.

**Methodology**

**Experiment I**

The experiments were conducted in 45-liter aquariums filled with artificial seawater. This offered increased consistency between tests, and allowed for consistency in the ascorbic acid concentration added to the
water. The following parameters were tested and maintained at the following levels in all experiments to ensure consistency and mimic the properties of natural seawater: specific gravity was maintained between 1.024-1.025, the nitrate level must remain below 5 parts per million (ppm), pH was kept between 8.40-8.50, carbonate hardness was held in a range of 9-11 degrees carbonate hardness (dKH), and temperature was kept between 25 and 27 degrees Celsius. The two experiments were run in series, starting with the testing on coral tissue regeneration rates, and moving to the tests on coral bleaching.

The first fragmenting subjects were coralimorphs from the genus *Actinodiscus*. In a culturing aquarium, scissors were used to cut the column of the coralimorph. After this incision was performed the coral was removed from the aquarium and placed on a dissection mat. The coralimorph was then severed with a scalpel into two equal parts, ensuring each fragment contains portion of the mouth, which is present on the oral disk, and a portion of the stomach, which is located in the column. This was repeated on six coralimorphs until twelve fragments were obtained. The fragments were then placed into three Petri dishes filled with a reef rubble substrate. Fine mesh netting was then placed over the top of each Petri dish to keep the fragments in place, and one Petri dish was then placed in each of the aquariums. Aquarium 1 was then supplemented with 0.56 mg/L of ascorbic acid; Aquarium 2 was supplemented with 0.28 mg/L of aqueous pH balance ascorbic acid; Aquarium 3 was not supplemented and served as a control. Throughout the experiment, this supplementation continued once daily, at the same concentration. To observe the rate of tissue regeneration in the coralimorphs, measurements were taken using a caliper every other day throughout the 22-day testing period.

The next test fragments were Pulsing Xenia (*Xenia sp*). To fragment, scissors were used to cut the column of the *Xenia*. A total of ten fragments were cut, and placed in empty Petri dishes, which were placed in three new aquariums. Aquarium 1 of the *Xenia* test was supplemented with 1.0 mg/L of ascorbic acid daily, Aquarium 2 with 1.5 mg/L of ascorbic acid daily, and Aquarium 3 with no supplementation, which served as a control. Since the polyps of *Xenia* pulsate, accurate growth measurements are exceedingly difficult (Benayahu, Berner and Achiituv, 1989). For this reason, tissue regeneration was observed by measuring the speed of attachment. This was measured every four hours throughout the 120-hour trial.

The final fragmenting subject was the Finger Leather coral (*Sinularia flexibilis*). The *Sinularia* was fragmented using scissors to cut a “branch” of the coral, and this was repeated until six fragments were obtained. Again the fragments were placed in Petri dishes filled with reef rubble substrate. These were then placed in the final set of testing aquarium. Ascorbic acid dosing was the same as the *Xenia* experiment, supplementing Aquariums 1, 2 and 3 with 1.5 mg/L, 1.0 mg/L, and 0 mg/L of ascorbic acid respectively. To observe health and tissue regeneration, the fragments were again measured using a caliper every other day over the 12-day testing period.

Experiment II
The aquariums were prepared in the same manner as the previous experiment with the exception of temperature. The water temperature of the aquarium serving as the control was kept at a standard 25°C, while the temperature of the other two aquariums was raised to 32°C, the bleaching threshold for most corals (Glynn and D’Croz, 1990). Three different types of coral were used for testing: green *Montipora capricornis*, red *Montipora capricornis*, and *Montipora undata*. Each of these coral was fragmented into 24 1-cm² pieces using a dremel tool, and eight fragments of each species were placed into the three aquariums. Throughout the experiment, one of the aquariums with increased water temperatures was supplemented daily with 1.0 mg/L of ascorbic acid.

To determine the rate of zooxanthellae expulsion, a count of zooxanthellae present in the coral was conducted. To do this, the zooxanthellae needed to be isolated, and this was done following a similar procedure used to isolate guard cell protoplasts from the epidermis of *Vicia fabia* leaves (Kruse, Zeiger and Tallman, 1989). Every two hours throughout experimentation, one fragment from each aquarium was removed and placed separately in a blender with 10 mL of saltwater, and blended for 90 seconds while the blender was shaken vigorously. This process homogenized the calcium carbonate “skeleton” of the coral, while leaving the zooxanthellae cells intact. This mixture was then placed in a test tube for enumeration.

The zooxanthellae solution was shaken to avoid settling of the solid material to the bottom. A syringe was used to extract a
10 µL sample of solution, which was then placed onto a hemocytometer. The sample was observed under 100-power microscope magnification, and a photograph was taken. The zooxanthellae located in the hemocytometer grid were then counted. To determine the zooxanthellae concentration, the following formulas were used:

\[
\text{Dilution}(D) = \frac{V_{\text{New}}}{V_{\text{Old}}}
\]

\[
\text{Concentration}(C) = D \cdot \frac{N}{V}
\]

This process of homogenization and enumeration was continued bi-hourly throughout the first 12-hours of experimentation, with a final measurement at the twentieth hour.

**Results**

**Experiment I**

In the aquarium with an ascorbic acid concentration of 0.28 mg/L the four corallimorph fragments grew an average of 2.7 mm in diameter over the course of the 22-day measurement period, with all fragments surviving. The fragments supplemented with 0.56 mg/L of ascorbic acid grew an average of 2.0 mm in diameter. Again all fragments survived. The unsupplemented fragments in the control decreased in size until the 16th day upon which all four fragments had died.

The average attachment time of the four Xenia fragments, which were supplemented with 1.5 mg/L of ascorbic acid daily, was 28 hours. The two fragments supplemented with 1.5 mg/L of ascorbic acid daily, both attached in 60 hours. Finally, the four unsupplemented control fragments did not attach throughout the 120-hour observation period, and only two of the original four fragments survived.

The two *Sinularia flexibilis* fragments that were supplemented with 1.0 mg/L of ascorbic acid daily, grew a total of 15 mm over the 12-day trial. In the group of *S. flexibilis* supplemented with 1.5 mg/L of ascorbic acid, one showed no growth, decreasing 1 mm over the testing period, while the other fragment decreased in size until the sixth day when it died. The two fragments in the unsupplemented control, decreased in size steadily throughout experimentation until death on days eight and twelve.

From these tests on three separate species of soft coral fragments it can be concluded that ascorbic acid increased the speed of tissue regeneration, and as a result, increased the survival rate of fragmented soft corals. It was also observed that 1.0 mg/L of daily ascorbic acid supple-

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**Fig. 2** Attachment time of Xenia fragments, unsupplemented and supplemented with 1/0 mg/L and 1.5 mg/L of ascorbic acid.

**Fig. 3** Daily growth of *Sinularia flexibilis* fragments unsupplemented and supplemented with 1.0 mg/L and 1.5 mg/L of ascorbic acid.
mentation was optimal (in regards to the concentrations tested) for increasing tissue regeneration and survival rate of the fragments.

Experiment II
The zooxanthellae concentration in the unsupplemented fragments of green *Montipora capricornis*, which were subjected to bleaching temperatures, decreased steadily throughout the 20-hour testing period from the initial concentration of $4.2 \times 10^6$ cells/mL to $6.0 \times 10^5$ cells/mL, an average expulsion of $1.8 \times 10^5$ cells/mL each hour. The fragments supplemented with 1.0 mg/L of ascorbic acid and kept at bleaching temperatures of 32˚C retained much more zooxanthellae. The initial zooxanthellae concentration was $4.1 \times 10^6$ cells/mL and decreased to $3.2 \times 10^6$ cells/mL. This calculates to an average expulsion of $3.4 \times 10^4$ cells/mL hourly, an expulsion rate over four times slower than the unsupplemented coral. Finally, the zooxanthellae concentration in the fragments in the control, which were unsupplemented and kept at a natural temperature of 25˚C, remained steady throughout experimentation starting and ending experimentation with a $4.2 \times 10^6$ cell/mL concentration.

In the red *Montipora capricornis* the control again remained essentially unchanged, increasing from $4.0 \times 10^6$ cells/mL to $4.1 \times 10^6$ cells/mL. The zooxanthellae concentration of the unsupplemented group kept at 32˚C once again steadily decreased throughout testing, losing an average of $1.6 \times 10^5$ zooxanthellae cells/mL every hour. The supplemented coral had a much higher tolerance to the heat, with a loss of only $1.2 \times 10^6$ cells/mL throughout the entire test, averaging a zooxanthellae loss of $6.3 \times 10^4$ cells/mL hourly.

The purple *Montipora undata* had similar expulsion rates as the coral in previous experiments. The concentration of zooxanthellae remained steady in the control, dropping from $4.1 \times 10^6$ cells/mL to $4.0 \times 10^6$ cells/mL. The unsupplemented increased temperature group retained only 17% of its original zooxanthellae, decreasing from $4.1 \times 10^6$ cells/mL to $7.0 \times 10^5$ cells/mL at an average rate of $1.7 \times 10^5$ cells/mL hourly. The zooxanthellae concentration in the group supplemented with ascorbic acid did not decrease, but in fact increased from $4.1 \times 10^6$ cells/mL to $4.2 \times 10^6$ cells/mL, a remarkable gain when compared with the rates of expulsion in the unsupplemented fragments.

Observing the data collected on the three types of coral tested, it can be concluded that supplementation of 1.0 mg/L of ascorbic acid daily greatly decreases the rate of zooxanthellae expulsion in heat stressed coral, thus greatly reducing coral bleaching.

Discussion
The Role of Ascorbic Acid in Tissue Regeneration
Ascorbic acid was the chosen method of increasing the rate of tissue regeneration due to its key role in the production of healthy collagen (Kramer, Fillios and
Bowler, 1979). To produce collagen, the amino acid proline must become oxidized in a process called hydroxylation (Mussini, Hutton Jr. and Udenfriend, 1967). This forms hydroxyproline, an essential element of collagen. Iron is necessary to perform the oxidation, and uses ascorbic acid to return iron to its necessary oxidized state. Without ascorbic acid the hydroxylation cannot be carried out completely, leading to weakened collagen. (van B. Robertson and Schwartz, 1953). Because collagen is necessary for wound repair (van B. Robertson and Schwartz, 1953), ascorbic acid was chosen to aid tissue regeneration.

Importance of Experiment I
As mentioned previously, overcollection of coral is a major threat to the reefs. This research makes fragmenting a more viable method of obtaining soft corals. Instead of collecting from a natural reef, fragmenting can now be used with a higher success rate. Many reef aquarium hobbyists have utilized this research by fragmenting their own coral instead of buying coral collected from the reefs.

This research could also aid in the healing of damaged natural reefs because wounded coral is in need of rapid tissue regeneration. Ascorbic acid could be supplemented to a natural reef to benefit the coral in their recovery process. Furthermore, because ascorbic acid increased the rate of tissue regeneration, we know that collagen is produced during wound repair.

The Role of Ascorbic Acid in Coral Bleaching
It is thought that coral bleaching is caused by oxidative stress brought about by increased temperatures (Downs, Fauth and Halas, 2002). When temperatures increase, zooxanthellae become hyperproductive. Since zooxanthellae is photosynthetic, it produces higher amounts of oxygen (O₂), which is absorbed into the coral tissues. Some of this oxygen reacts to form hydrogen peroxide (H₂O₂) which is a harmful oxidant, and in the presence of iron, a trace element in seawater at a concentration of 3 mg/L (Armstrong, 1957), breaks down into the free radical of the hydroxyl ion (OH⁻) (Goldstein, Meyerstein and Czapski, 1993). This presents a double bind for the coral. If it retains the zooxanthellae, the concentration of H₂O₂ and OH⁻ increase to levels high enough to kill the coral, and if it releases the zooxanthellae, it will often die of starvation (Jones, Hoegh-Guldberg and Larkum, 1998). Ascorbic acid was used in the experimentation because it is a natural antioxidant and greatly reduces or eliminates the free radical effects of the hydroxyl ion, and the resulting oxidative effects of hydrogen peroxide (Deutsch, 1998), thus allowing the coral to retain its zooxanthellae.

Importance of Experiment II
The significant decrease in the rate of zooxanthellae expulsion is a significant breakthrough in the prevention of the bleaching on natural coral reefs. A current proposal to stop coral bleaching by Ove Hoegh-Guldberg, a leading coral reef researcher, is the construction of structures to shade the reefs to reduce the effects of high temperatures (University of Queensland, CRC Reef Research Centre, 2008). Supplementation of ascorbic acid could be used to defend reefs that have a high risk of a bleaching event.

A further contribution of the research is the enumeration of the rate of zooxanthellae expulsion in heat stressed coral. This rate is currently unknown in many species of coral, but through this experiment the rate of zooxanthellae expulsion in green Montipora capricornis, red Montipora capricornis, and green Montipora undata are now documented.

The zooxanthellae isolation technique developed in this experiment is a novel method that is far simpler than methods currently used. This could aid in the research of coral bleaching, zooxanthellae, and the rates of zooxanthellae expulsion in heat stressed coral.

Conclusion
These findings could prove to be remarkably beneficial to the aid of threatened coral reefs. Already the method of increasing the survival rate of propagated coral is being used by many marine aquarium hobbyists throughout the United States, thus reducing the amount of coral being taken from natural reefs. As mentioned previously, the current method to slow or stop coral bleaching is to shade reefs that are at high risk of bleaching events. Supplementation of coral reefs with pH balanced ascorbic acid could be a simpler, more cost effective method of reducing coral bleaching on reefs with high bleaching risks. Reduced calcification of coral reefs has devastated natural reefs to such great extent that electrified grid structures are being constructed called biorock. Through electrolysis, the electrified grid breaks down H₂CO₃ into H₂ and CO₃⁻, the carbonate ion then binds with calcium to create CaCO₃. There are currently eight artificial reefs constructed of this biorock with sizes up to 220 meters long, but the process is obtrusive, and the artificial calcification is slow (Henderson, 2006). Ascorbic acid supplementation to these reefs to increase calcification would again be a simpler, more cost effective method to aid reefs at high risk of acidification. Supplementation of ascorbic acid to natural coral reefs would provide a threefold benefit of increased wound repair, calcification, and resistance to raised temperatures.
References


Caleb Kruse is a freshman from Colorado Springs, Colorado. He has a passion for life that is rooted in a fascination with the world. Caleb enjoys skiing and photography, and has recently uncovered the thrill of mountain biking on single-track paths in the Stanford foothills. He also enjoys recreational running, and has completed two marathons. Caleb loves learning and the study of new research that he finds from all disciplines. He loves sharing his passion with anyone who is interested, whether it be one of his eight siblings, or a fellow student. He hopes to continue his exploration of the world by majoring in biology.