EFFECTS OF PHOTOOXIDATIVE STRESS AND ALGAL GROWTH RATES ON ALGAL EXPULSION BY THE SYMBIOTIC SEA ANEMONE ANTHOPLEURA ELEGANTISSIMA

By

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Abstract

Many cnidarians contain endosymbiotic zooxanthellae (*Symbiodinium* sp.). These algae translocate photosynthetic products to their host. However, in high light conditions the algae may produce reactive oxygen species which can damage the host, potentially triggering algal expulsion. The host may also preferentially expel dividing algae in order to maintain a constant algal density.

In this study, algal expulsion was measured in the symbiotic sea anemone *Anthopleura elegantissima.* When the anemones were exposed to high light and high oxygen treatments, their algal density remained constant but their algal expulsion increased. Many of the expelled algae were dead, suggesting that the host may expel algae which have been damaged by reactive oxygen species.

To determine if the host preferentially expels dividing algae, samples of algae that had been either expelled or artificially isolated from the host were incubated for various time periods. The mitotic index of the expelled algae initially dropped but began to increase during incubation, suggesting that the host may preferentially expel recently-divided algae. The mitotic index of the artificially isolated algae also appeared to increase during incubation, suggesting that the host may limit algal growth *in hospite*.

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Introduction

Overview of the Anthopleura elegantissim*a symbiosis*

Anthopleura elegantissima is a small green anemone commonly found in the intertidal zone of the Pacific coast from Baja California to Alaska. It reproduces both sexually and asexually, and may form large mats of asexual clones (Ricketts *et al* 1985). Where two different clones encounter one another, they will attack each other with specialized tentacles known as acrorhagi and establish a border between the two clones (Francis 1973). *A. elegantissima* is carnivorous, using stinging cells (cnidocytes) in its tentacles to trap small organisms. This anemone is often found in areas where sand and shell fragments accumulate (Kozloff 1993), and may attach such debris to tubercles on its body, presumably for shading (Dykens and Shick 1984).

A. elegantissima obtains its coloration from endosymbiotic algae that live within vacuoles in its gastrodermal tissue. These algae are from two different phyla: green chlorophyte algae (zoochlorellae) and olive brown dinoflagellates from the genus *Symbiodinium* (zooxanthellae) (Secord and Augustine 2000). The anemones may contain one or both types of algae, or they may be aposymbiotic, containing neither alga. Aposymbiotic individuals are colored a pale off-white.

In the *A. elegantissima* symbiosis the algae supply the host with photosynthetically fixed carbon (Verde and McCloskey 1996; Engebretson and Muller-Parker 1999). The translocated carbon from symbiotic zooxanthellae is primarily comprised of glycerol (Trench 1971; Muscatine 1967; Grant *et al* 1997). When symbiotic *A. elegantissima* individuals were incubated with $H^{14}CO_3^-$, up to 65% of the carbon fixed by the zooxanthellae was translocated to the host (Engebretson and Muller-Parker 1999),

potentially providing an energy source for the host. Additionally, zoochlorellae may supply essential amino acids to the anemone (Minnick 1984).

It appears that *A. elegantissima* may control the photosynthetic rate, release of photosynthate, and population levels of its symbionts. The photosynthetic rate and release of photosynthate appear to be regulated by a "host factor," a substance found in homogenates of symbiotic host tissue (Muscatine 1967; Trench 1971; Gates *et al* 1999; Sutton and Hoegh-Guldberg 1990). When incubated in a homogenate of host tissue, zooxanthellae from *A. elegantissima* fixed 10 times more carbon than when incubated in seawater alone (Trench 1971).

The release of photosynthetically fixed carbon is affected by the host. Zooxanthellae from *A. elegantissima* released 15 times more photosynthate when incubated in a homogenate of symbiotic host tissue than when incubated in seawater. When the algae were incubated in a homogenate of aposymbiotic anemone tissue, their photosynthate release was only one-fifth that of algae incubated in the symbiotic homogenate, indicating that the release of photosynthate was not an artifact of the host tissue itself (Trench 1971).

In some invertebrate—algal symbioses, algal growth appears to be regulated by nutrient limitation of the algae by the host. When the *Hydra* symbiosis was grown in a nutrient-rich medium, the algae overgrew the host (Blank and Muscatine 1987). In some corals, limitation of the supply of nitrogen compounds to the algae by the host appears to limit the growth of symbiotic zooxanthellae. The low levels of nitrogen in the algae presumably limit their protein synthesis and thus limit algal growth. However, the algae can continue to photosynthetically fix carbon, much of which is translocated to the host.

In this way, the host can maintain a limited algal population yet receive large amounts of photosynthate from the algae (Falkowski *et al* 1993).

These examples of host regulation suggest that hosts may control their symbionts in order to maximize the benefits for the host. When algae cease to provide an advantage to the host or become a liability, is seems reasonable that the hosts would expel them. Algal expulsion has been observed in a variety of marine cnidarian symbioses, including corals (Lesser 1997; Jones and Yellowlees 1997), zoanthids (Lesser *et al* 1990), and anemones (O'Brien and Wyttenbach 1980; Suharsono and Brown 1992; McCloskey *et al* 1996; Baghdasarian and Muscatine 2000). Since the loss of symbiotic algae often causes the host to lose its coloration, this event is known as bleaching. There are several conditions in which symbiotic algae might no longer be beneficial to the host: (a) algae undergoing high levels of photooxidative stress, (b) dead or senescent algae, or (c) rapidly growing algae.

Photooxidative stress

During photosynthesis, light energy is absorbed by chlorophyll in the chloroplasts of plant cells. Usually, this energy is used to create a proton gradient and reduce NADP+. The proton gradient drives the synthesis of ATP via ATP synthase while the NADPH is used for biosynthesis in the Calvin cycle. Water is oxidized to O_2 to provide electrons for reducing the NADP+. Under high light conditions large amounts of energy are absorbed by the chlorophyll and high levels of O_2 are produced. This combination of factors also leads to increased production of reactive oxygen species (ROS) as byproducts, such as singlet oxygen, hydrogen peroxide, superoxide anions, and hydroxide radicals (Buchanan 2000). These highly reactive molecules can potentially damage cells by inappropriately

oxidizing cell components. Other factors, such as high temperatures and UV light, may also cause oxidative stress (Buchanan *et al* 2000). The production of reactive oxygen species by symbiotic zooxanthellae might trigger expulsion of the algae by their hosts (Lesser 1997).

There is extensive evidence that high light and oxygen levels can cause oxidative stress for both the host and the symbiont. Many studies have focused on the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase, and ascorbate peroxidase, under the assumption that these reflect levels of photooxidative stress. In zooxanthellae cultures, exposure to high temperatures caused increased activities of SOD and ascorbate peroxidase (Lesser 1996). In zooxanthellae isolated from the tropical anemone *Aiptasia pulchella*, high light levels and UV levels triggered increases in SOD and catalase activity (Lesser and Shick 1989). Symbiotic *A. elegantissima* individuals with greater chlorophyll levels had correspondingly higher SOD activities in the animal tissue, suggesting that photosynthesis by the symbiotic algae was the primary source of photooxidative stress. Oxygen pressure also plays a role, as anemones exposed to elevated pO_2 demonstrated increased SOD activities (Dykens and Shick 1982). In fact, light-dependent oxyradical production has been directly observed in *A. elegantissima* using electron paramagnetic resonance. Symbiotic anemones had higher levels of methane sulfinic acid (a direct measure of hydroxyl radical levels) than did aposymbiotic anemones, and symbiotic anemones exposed to UV light had the highest levels (Dykens *et al* 1992).

Despite the abundance of evidence showing increased antioxidant enzyme activity, little work has been done concerning the role of photooxidative stress in algal

expulsion in *A. elegantissima*. In the coral *Agaricia tenuifolia*, individuals exposed to elevated temperatures had an increase in algal expulsion, which was reduced by the addition of the exogenous antioxidants ascorbate and catalase (Lesser 1997). This suggests that oxidative stress triggers algal expulsion. While several studies have measured the effects of light levels and temperature on algal expulsion in *A. elegantissima*, the only study to look at the effect of oxygen levels was somewhat limited. Dykens and Shick (1984) observed that anemones exposed to elevated light or increased pO_2 had reduced levels of chlorophyll. However, chlorophyll level does not necessarily correlate to algal population (Falkowski *et al* 1993; Jones and Yellowlees 1997) so these results cannot be unambiguously interpreted as evidence of algal expulsion.

Dead zooxanthellae

When cnidarians lose symbiotic algae in high stress conditions, some of the expelled algae appear to be dead cells. Franklin *et al* (2004) measured the expulsion of algae from the coral *Stylophora pistillata*. They exposed the coral to different light and temperature regimens, isolated symbiotic zooxanthellae from the coral tissue, and stained the zooxanthellae with Sytox-green stain to measure the number of dead algae. They found that less than 10% of the zooxanthellae from the control group were dead, while up to 60% of the zooxanthellae from the high light/high temperature group were dead. When the zooxanthellae were stained with H_2DCFDA (a stain indicating oxidation), they found that over 30% of the symbiotic algae from the high light/high temperature treatment contained oxidant activity. Only 10-15% of the controls had oxidant activity. Franklin *et al* (2004) also looked at the expelled algae and found that over 40% of the expelled zooxanthellae were dead, and up to 88% were chlorotic (lacking chlorophyll).

To further test if the zooxanthellae death is related to oxidative stress, Franklin *et al* (2006) incubated pieces of *S. pistillata* in seawater containing the antioxidants ascorbic acid and mannitol and exposed the coral pieces to different light and temperature regimens. Strangely, they found that the antioxidants did not affect the percentage of dead zooxanthellae. Perhaps the light and temperature treatments were not extreme enough for the antioxidants to make a difference.

Dunn *et al* (2002) investigated zooxanthellae from the anemone *Aiptasia* sp. to determine if the algae die from necrosis or from programmed cell death (PCD). They triggered zooxanthellae death by exposing the anemones to elevated temperatures and measured several indicators of PCD, including changes in cell size and ultrastructure and an increase in DNA fragmentation. They found that zooxanthellae appear to undergo both processes, with some cells dying by PCD and others by necrosis. Dunn *et al* (2002) speculated that free radicals from oxidative stress conditions might interfere with calcium signals in the cell and trigger PCD.

Algal growth rates

In some corals, it appears the host limits algal growth in order to maintain a high flux of photosynthate into the host. When algae exhibit balanced growth, a lower percentage of photosynthate is available for translocation since it is being used for growth (Falkowski *et al* 1993). Rapidly growing algae are less beneficial for the host. The host might control net algal growth by limiting nutrient flux to the algae. However, *A. elegantissima* is a temperate anemone growing in nutrient rich waters. It is unclear whether nutrient limitation of algae by the host is a viable mechanism for controlling algal numbers under these conditions. Graham (1989) found that the mitotic index of

zooxanthellae from *A. elegantissima* increased when the anemones were raised in nutrient-enhanced water. However, there was no increase in algal density within the animal tissue, suggesting that more algae may have been expelled. Strangely, no algal expulsion was observed in the anemones, though only one individual from each treatment was examined for algal expulsion (Graham 1989). Research based on the tropical anemone *Aiptasia pulchella* suggests that hosts may limit algal growth through some mechanism other than nutrient limitation. Using flow cytometry, cultured zooxanthellae were compared to symbiotic zooxanthellae. Symbiotic zooxanthellae spent much more time in the G_1 phase of the cell cycle than did cultured zooxanthellae, though the zooxanthellae from well-fed anemones did spend less time in G_1 than zooxanthellae from starved anemones. This seems to indicate that the host is controlling details of the cycle of algal cell division, and probably not simply by nutrient limitation of the algae. Additionally, the duration of mitosis itself was quite constant and independent of the generation time (Smith and Muscatine 1999).

The host might control algal growth rates by preferentially expelling dividing algae. In one study of *A. elegantissima*, the mitotic index of expelled algae was higher than that of algae isolated from the intact symbiosis (McCloskey *et al* 1996). In the temperate anemone *Anemonia viridis*, Suharsono and Brown (1992) found that algae released into the coelenteron (i.e. expelled algae) had a higher mitotic index than did algae sampled from the endoderm. These higher mitotic indices seen in expelled algae may have been caused by (a) preferential expulsion of dividing algae or (b) an increase in the algal growth rate when the algae were released from possible limitation by the host. Baghdasarian and Muscatine (2000) tested these possible mechanisms in the tropical

anemone *Aiptasia pulchella*. The mitotic index of the expelled algae was almost twice that of algae in the host tissue. In contrast, the mitotic index of algae that had been isolated from the host and incubated for 15.5 hours was not significantly different from the mitotic index of algae recently isolated from the intact symbiosis. This suggests that release from limitation by the host is not the cause of the higher mitotic indices in expelled algae, at least in *A. pulchella* (Baghdasarian and Muscatine 2000). Further, when the symbiotic anemones were incubated in ^{3}H-thymidine, the levels of ^{3}H-thymidine in the expelled algae were significantly higher than in the retained algae, suggesting a preferential expulsion of recently-divided, replicating algae (Baghdasarian and Muscatine 2000).

In *A. elegantissima*, it remains to be determined whether preferential expulsion of dividing algae or release from host limitation is the cause for the higher mitotic indices observed in expelled algae. In previous research, expelled algae were collected only every 48 hours (McCloskey *et al* 1996). Expelled algae released from limitation in the host could quickly exhibit increased mitotic indices, and this might not be detected if samples were collected only once in 48 hours. No other research on preferential algal expulsion has been conducted on *A. elegantissima*.

This study explored the roles of photooxidative stress and algal growth rates on algal expulsion by *A. elegantissima*. Two hypotheses were tested: 1) High light levels and high oxygen levels trigger an increase in zooxanthellae expulsion from *A. elegantissima* and 2) *A. elegantissima* preferentially expels algae with a higher mitotic index. To test the first hypothesis, anemones were exposed to different light and oxygen regimens to determine the effects of photooxidative stress on algal expulsion. To test the second

hypothesis, algae were collected from the host and from expelled algae samples and incubated. The mitotic index was measured before and after incubation to determine if the host preferentially expels dividing algae.

Materials and Methods

Anemone collection and maintenance

On July 5, 2007 (day 1), 60 zooxanthellate *A. elegantissima* anemones were collected from a single tide pool on Sares Head, Fidalgo Island, WA (N 48°25.389', W 122°40.340', WGS 84). All debris and pebbles attached to the tubercles were removed, and the anemones were randomly assigned to a photooxidative stress group (POS; N=36) or a preferential algal expulsion group (PAE; N=24) (Figure 1). Each anemone was placed in a labeled 50 mL beaker covered with polyester screening and the screen was secured with a rubber band. The screening helped retain any expelled algal clumps and also kept the anemones from migrating out of the beakers. The beakers were submerged in one of two flowing seawater tanks (Table 1). A constant flow of seawater was maintained in the tanks at a depth of 10 cm, covering the beakers. The seawater was drawn directly from the ocean, so the temperature in the tanks (12-14°C) increased with the ocean seawater temperature over the summer, as it would for the anemones in the field. Diatom growth periodically fouled the tanks, so the tanks were cleaned several times during the experiment. Both tanks were cleaned at the same time.

Light and oxygen regimens

The anemones were maintained in one of two tanks: a high oxygen tank and a normal oxygen tank. The high oxygen tank was supplemented with extra oxygen using an oxygenation column (Figure 2). As much as possible, the oxygen saturation in the high oxygen tank was maintained at 200% of air saturation. This saturation level was representative of oxygen saturation values observed in the gastrodermal tissues of zooxanthellate *A. elegantissima* anemones that were exposed to moderate light levels

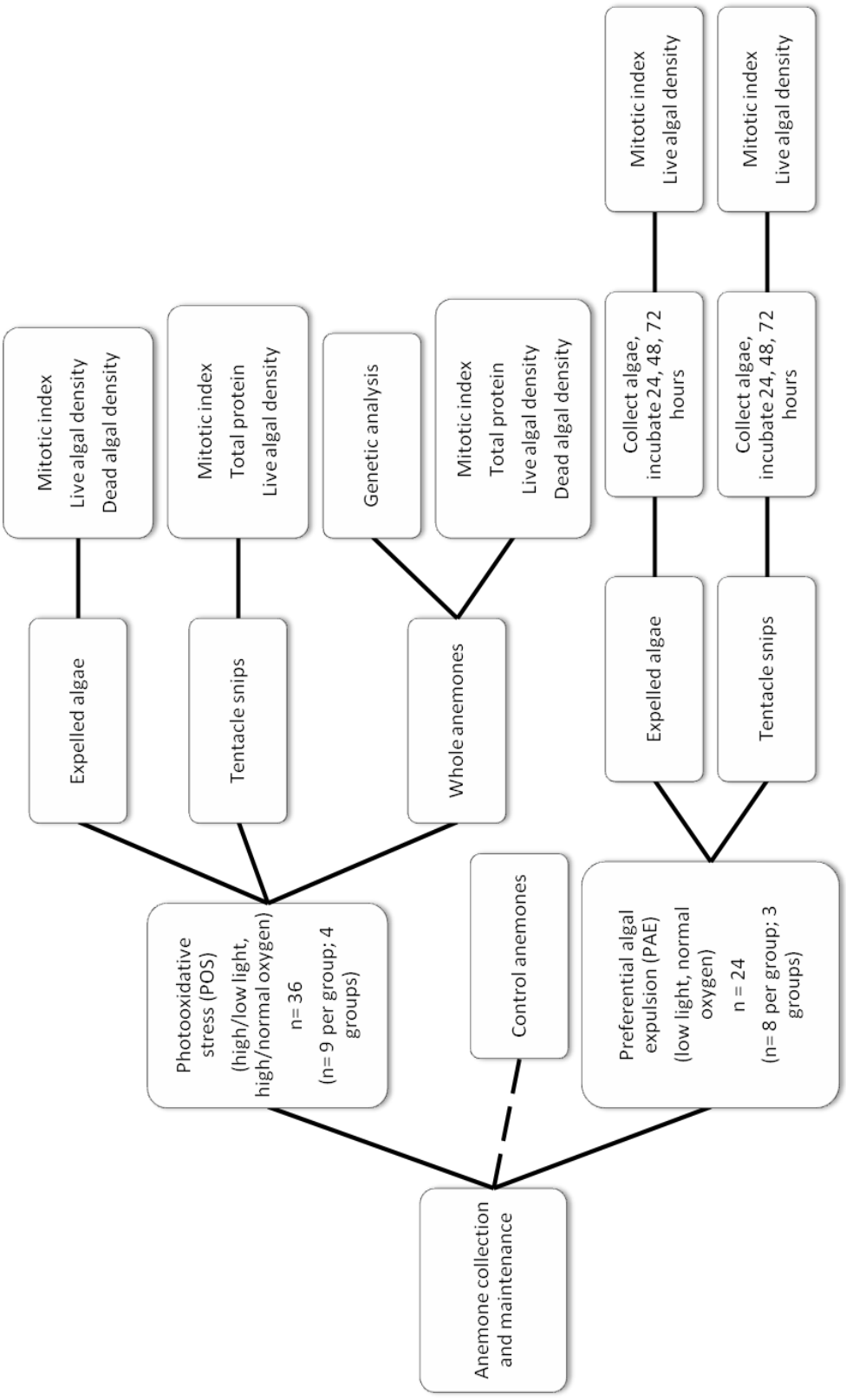

Figure 1. Flow chart of methods used.

Table 1. Arrangement of anemones within treatment groups. "POS" refers to the photooxidative stress group and "PAE" refers to the preferential algal expulsion group. The anemones were labeled by number, with nine anemones in each of the POS groups and 24 anemones in the PAE group.

	High oxygen (table 1)	Normal oxygen (table 2)
High light	POS #1-#9	POS #10-#18
Low light	POS #19-#27	POS #28-#36; PAE #1-#24

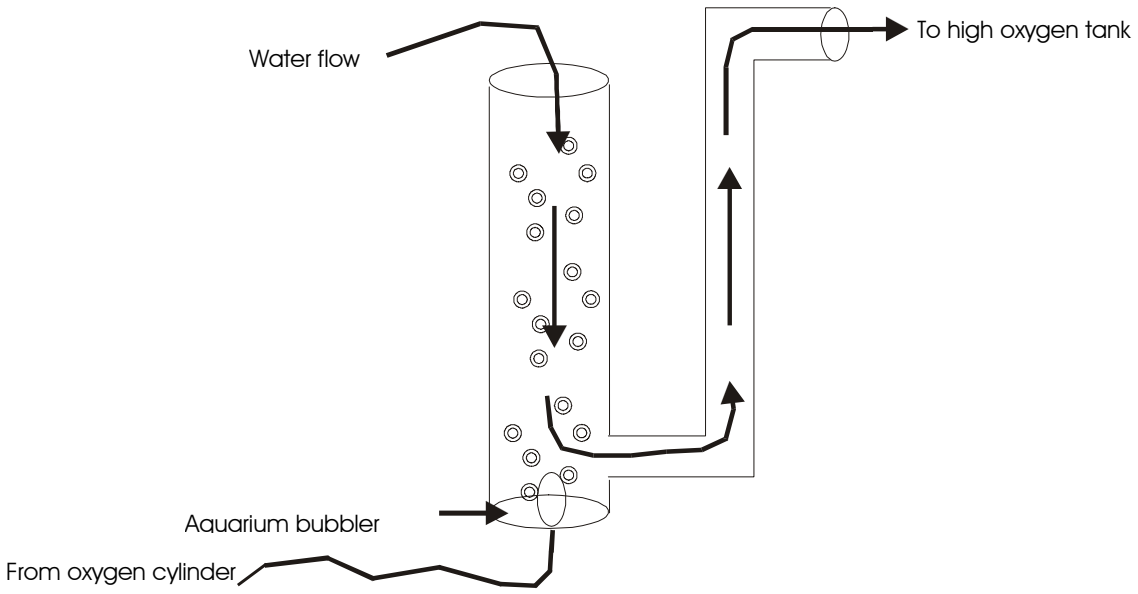

Figure 2. Diagram of the oxygenation column. Seawater flowed through the column, past the aquarium bubbler, and into the high oxygen tank. The bubbler was attached to an oxygen cylinder and bubbled pure oxygen through the column.

(315 μmol quanta • m^{-2} • s^{-1}) (Dykens and Schick 1982). Unfortunately, changes in the oxygen cylinder pressure caused some fluctuations in the oxygen flow rate. Because of this, the oxygen saturation briefly dropped as low as 155% and occasionally overshot above 200% during the course of the summer. These fluctuations occurred over the span of minutes to a few hours before they were corrected. As illustrated in Figure 3, the normal oxygen tank was set up parallel to the oxygenated tank and maintained at the ambient seawater oxygen level of 90-95% saturation. Oxygen levels were determined using a YSI model 550A oxygen electrode. The oxygen levels were measured at the beginning of the experiment and several times per week after that, and also after making any adjustments to the oxygen or water flow rates. An opaque divider was erected perpendicular to the two tanks, partitioning both into a low light region and a high light region. Both regions were illuminated with high-intensity, broad-spectrum metal halide lights (MH-400, 400 watts, Hubbel Lighting, Christianburg, VA). The low light region had an intensity of 200 μmol quanta • m^{-2} • s^{-1} and the high light region had an intensity of 2,000 μmol quanta • m^{-2} • s^{-1}. Light levels were measured at the beginning of the experiment using a Li-Cor LI-1400 light meter with a cosine sensor. For each treatment group, a ring of equivalent light intensity was marked on the bottom of the tank using a wax pencil. The beakers were placed on these marks and randomly replaced to a position somewhere on the ring after each collection period. The anemones were maintained on a 16 h light: 8 h dark cycle to simulate environmental conditions in June and July. Timers were used to turn the lights on at 0500 h and off at 2100 h. For three hours after dawn (0500 h to 0800 h) and for three hours before dark (1800 h to 2100 h), the light intensity of all treatment groups was reduced approximately 50% using fiberglass window screen

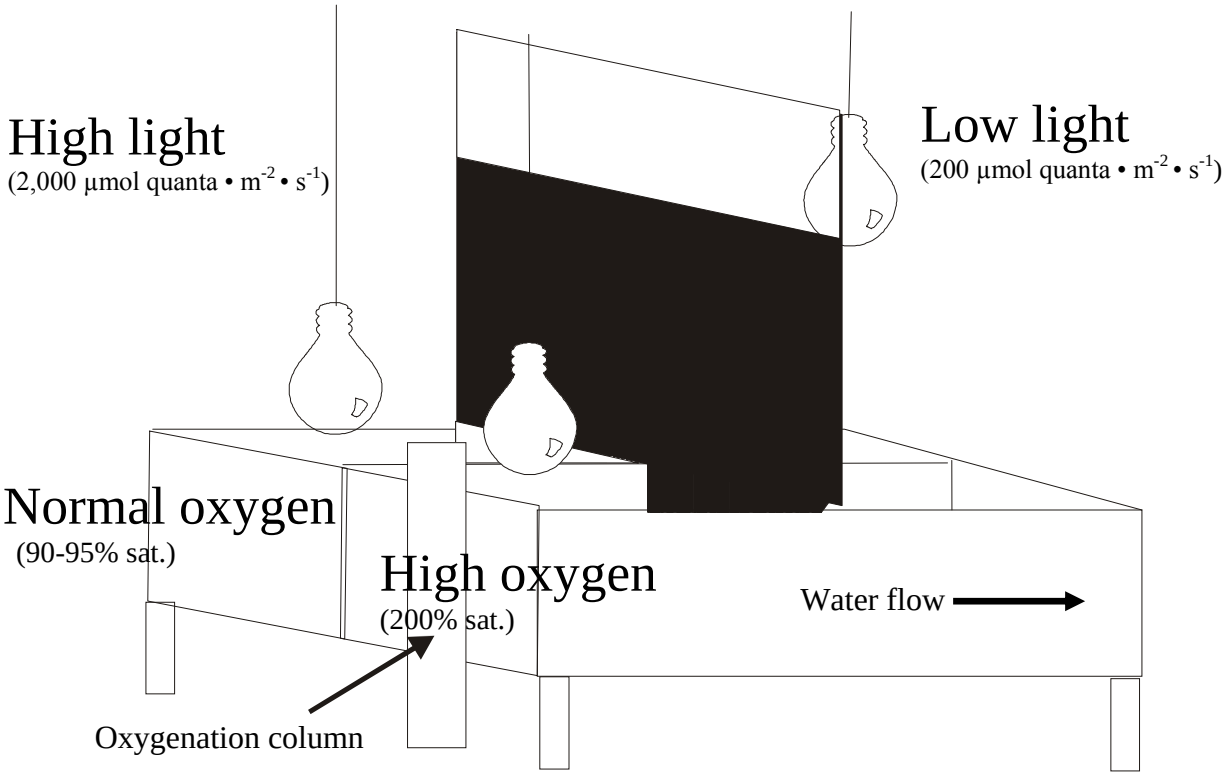

Figure 3. Diagram of the experimental setup. The oxygenation column oxygenated the incoming water for the high oxygen tank, while the normal oxygen tank received plain seawater. The divider between the high light and low light treatments extended down into the tanks, ending at the water level. Seawater flow passed from the high light ends of the tanks to the low light ends and exited via drains.

to mimic natural light cycles. This resulted in integrated daily light exposures of 93.6 mol quanta • m^{-2} • day^{-1} and 9.36 mol quanta • m^{-2} • day^{-1} for the high light and low light treatments, respectively. For comparison, the integrated daily light intensity at Rosario Beach Marine Lab was about 65 mol quanta • m^{-2} • day^{-1} during summer and 5 mol quanta • m^{-2} • day^{-1} during winter (Verde and McCloskey 2007).

Photooxidative Stress (POS): expelled algae

To test the effects of photooxidative stress on algal expulsion, anemones in the POS group were maintained in one of four treatment groups: (1) high light, high oxygen; (2) high light, normal oxygen; (3) low light, high oxygen; and (4) low light, normal oxygen (Table 1). Each treatment group in the POS group contained nine anemones and was subdivided into groups A-D (Table 2). Expelled algae were collected from anemones in the POS group four times per day on collection days (according to the schedule in Table 3) to account for any diel patterns in mitotic index or algal expulsion. Though samples were collected in weeks 1, 3 and 5, only samples from weeks 1 and 5 were analyzed for the sake of time. Expelled algae were collected by flushing the anemone's gastrovascular cavity with filtered seawater (seawater filtered through Whatman number 1 filter paper) and collecting any free algae which were washed out. The beaker and screen were also rinsed with filtered seawater and the algae were collected. To pellet the expelled algae, the samples were centrifuged for 15 minutes at 2,000 rpm using a tabletop centrifuge. This model of centrifuge did not have a dial indicating the centrifuge speed in units of gravity (g), so the speed was calculated using the rpm and the centrifuge radius: 2,000 rpm was equivalent to 313 x g at the top of the sample and 693 x g at the bottom (Thermo Scientific 2008). It was observed that some algae stuck to the sides of the

Table 2. Anemone treatment group assignments (POS). Each anemone was labeled with a number and a group letter (A-D). The group letters were used to determine the time of day for collecting algae (Table 3).

High light, high oxygen	High light, normal oxygen	Low light, high oxygen	Low light, normal oxygen
1A	10A	19A	28A
2A	11A	20A	29A
3B	12B	21B	30B
4B	13B	22B	31B
5C	14C	23C	32C
6C	15C	24C	33C
7D	16D	25D	34D
8D	17D	26D	35D
9D	18D	27D	36D

Table 3. Schedule for collection of expelled algae from the POS group. For each time period, all anemones with that letter designation had their expelled algae collected. Day 1 was July 5, 2007. Week 1 was days 4-7, week 5 was days 32-35. *On day 35, groups B and C were accidentally exchanged. However, there was no significant difference between the exchanged groups and the other groups in the same time period (1-way ANOVA; $p>0.05$).

	0400 h	1000 h	1600 h	2200 h
Days 4, 32	A	B	C	D
Days 5, 33	B	C	D	A
Days 6, 34	C	D	A	B
Days 7, 35	D	A	B (C*)	C (B*)

centrifuge tubes and failed to pellet completely, so SDS was added to the centrifuge tubes to make a final concentration of 0.05% SDS. This concentration of SDS has been used to successfully isolate viable chlorophyte algae (McAuley 1986). This treatment helped prevent the cells from sticking to the sides of the centrifuge tubes and reduced the loss of algae. After centrifugation, the supernatant was discarded and the algal pellet was frozen for later analysis. Later, the samples were thawed and the algal pellet was homogenized in a 7 mL Wheaton glass hand tissue homogenizer for 50 strokes to break up algal clumps and disperse diatoms. The sample was washed two times by resuspending the pellet in artificial seawater (Artificial Ocean; 30-34 ppt) and centrifuging it for five minutes at 2,000 x g in a microcentrifuge. The pellet was then resuspended in artificial seawater by vortex mixer to allow viewing of the algae. A 30 µL drop was placed on a hemocytometer and viewed with a light microscope (Nikon Eclipse E200) at 100X. If the sample was contaminated with many diatoms or if the zooxanthellae were difficult to distinguish, the sample was viewed at 400X. Zooxanthellae in all nine regions of the hemocytometer grid (equaling a total of 1 µL) were counted, with a minimum of 100 zooxanthellae counted whenever possible. Occasionally, the samples were so contaminated with diatoms and debris that they had to be diluted down to fewer than 100 zooxanthellae to allow viewing. The numbers of live zooxanthellae, dead zooxanthellae, and dividing zooxanthellae were observed and recorded. Zooxanthellae that appeared transparent and that lacked the normal chlorophyll color (i.e. chlorotic cells) were classified as dead zooxanthellae, while zooxanthellae containing a definite cell plate were classified as dividing zooxanthellae. The algal counts were done on three separate aliquots for each sample, with an average of 122 cells counted per aliquot.

Photooxidative Stress (POS): tentacle snips

Tentacle snips were collected from the anemones on days 9, 23, and 36. Dissecting scissors were used to clip the tips of several tentacles from each anemone. The tips were frozen in filtered seawater for storage. Later, the tips were thawed and homogenized in a 7 mL Wheaton glass hand tissue homogenizer for 25 strokes. The homogenate was centrifuged for five minutes at 2,000 x g in a microcentrifuge to pellet the algae. The supernatant was removed and its volume measured. To determine the protein content of the supernatant, a modification of the Bio-Rad colorimetric protein assay (Bio-Rad Laboratories, Inc., Hercules, CA) was used (400 µL of sample and 1.6 mL of dye reagent that had been diluted 1:4 with dH_2O). Bovine gamma globulin was used as the standard. Both standards and samples were assayed in triplicate. The algal pellet was washed and resuspended as described previously. The volume was measured and the numbers of live zooxanthellae and dividing zooxanthellae were determined as described previously. The numbers of dead zooxanthellae were not recorded because their density was so low that they were not observed initially.

Whole anemones: genetic analysis

On day 36, one anemone from each POS treatment group was frozen on dry ice and mailed to Mike Morgan (Assistant Professor, Berry College, Mount Berry, GA) for analysis of genes induced during exposure to high light and high oxygen. The RNA was isolated from each anemone and a Northern blot was performed for each sample to determine the relative abundance of the target RNA. The anemone RNA samples were probed for catalase using a DIG-labeled probe. The probe's chemiluminescence was detected on x-ray film and the resulting image was analyzed by densitometry to

determine the relative abundance of catalase transcripts. The densitometry data were log 2 transformed, statistically analyzed, and graphed.

Whole anemones: analysis of algal density and mitotic index

In the afternoon of day 36, the remaining POS anemones were frozen. These whole anemones were used to measure baseline algal counts and mitotic indices in algae from intact anemones for comparison with expelled algae and for comparison between POS treatment groups. Also, the total protein content was determined and used for standardizing algal expulsion levels to mg animal protein. For the analysis, the anemones were thawed and homogenized for five minutes in 80 mL artificial seawater, using a Hamilton Beach® blender on high speed. The resulting homogenate was filtered through fiberglass window screen to remove non-solubilized material. The filtered homogenate was centrifuged for 15 minutes at 2,000 rpm in the tabletop centrifuge to pellet the algae. The supernatant was removed, its volume was measured, and an aliquot was frozen. The supernatant aliquot flocced upon thawing, so it was centrifuged an additional 15 minutes at 2,000 rpm in the tabletop centrifuge to remove the debris. To determine protein content, the supernatant was then analyzed with the Bio-Rad protein assay using bovine gamma globulin as a standard. Both standards and samples were analyzed in triplicate. The algal pellets were homogenized in a 15 mL Kontes Glass Co. hand tissue homogenizer for 50 strokes to break up algal clumps, the volume was measured, and an aliquot was frozen. This aliquot was later thawed, resuspended in artificial seawater, and the live, dead, and dividing zooxanthellae were counted.

Control anemones

Several groups of control anemones were analyzed to determine if there was any "tank effect" on the experimental anemones, and also to determine the effects of freezing on algal counts and mitotic indices. Twelve anemones were collected July 2, 2008 from the original tide pool. Of these anemones, eight were frozen and later thawed and analyzed as described previously for whole anemones, while the remaining four were homogenized and analyzed for protein content without first freezing them. Counts of live and dead algae and mitotic indices were made after freezing and thawing the samples, however. Eight more anemones were collected on July 23. These were divided in half, with half of each anemone frozen before analysis and the other half analyzed immediately, as described for the July 2 samples.

Preferential Algal Expulsion (PAE): expelled algae

The PAE group was used to determine if the anemones preferentially expelled dividing algae. The PAE anemones were maintained in the low light, normal oxygen treatment group for at least 25 days to allow the anemones to adjust to the conditions. On days 26 to 34 the experiment was performed. The PAE anemones were subdivided into groups based on how long the algae samples were to be incubated and at what time of day the samples were to be collected (Table 4). As with the POS group, the collection times were staggered (0400 h, 1000 h, 1600 h, and 2200 h) to account for any diel patterns. Twenty-four hours before a collection of expelled algae from a group was to be taken, the anemones in that group were thoroughly flushed with seawater to remove diatoms and old expelled algae. Then the anemones were returned to the low light, normal oxygen tank for 24 hours further incubation. At the end of the 24 hour period, all the algae which had been expelled during the 24 hours were collected using methods

Table 4. Anemone assignments for the PAE group. Each anemone was labeled with a number and a group letter (A-D). The group letters were used to determine the time of day for collecting algae. Group A was collected at 0400 h, group B at 1000 h, group C at 1600 h, and group D at 2200 h.

24 hour incubation	48 hour incubation	72 hour incubation
9A	1A	17A
10A	2A	18A
11B	3B	19B
12B	4B	20B
13C	5C	21C
14C	6C	22C
15D	7D	23D
16D	8D	24D

described previously, with the exception that no SDS was used. Thus, each algal pellet contained only zooxanthellae expelled in the previous 24 hours. After these preparatory steps, the algal pellets were used in the incubation stage of the experiment.

For the incubation stage, the volume of the algal pellet was measured and the pellet was divided into two aliquots. A 250 µL aliquot was resuspended in 10 mL of filtered seawater, placed in a 15 mL centrifuge tube, and incubated for the appropriate amount of time (24, 48, or 72 hours) in the low light, normal oxygen tank. After incubation, the algae were pelleted by centrifuging 15 minutes at 2,000 rpm with the tabletop centrifuge and frozen for later analysis. This was the "post-incubation" sample (incubation time = 24, 48 or 72 hours). The remainder of the initial expelled algae sample (total minus the 250 µL aliquot) was frozen for later analysis without further incubation. This was the "pre-incubation" sample (incubation time = 0). For analysis, both pre- and post-incubation pellets were thawed, their volumes were measured, and the numbers of live and dividing zooxanthellae were determined as described previously.

Preferential Algal Expulsion (PAE): tentacle snips

To determine if removal from the host affects the mitotic index of zooxanthellae, algae were isolated from tentacle snips and incubated in a manner similar to that of the expelled PAE algae. On days 27 to 30, tentacle snips were collected from the PAE anemones using the same schedule described in Table 4. The algae were isolated from the tentacle snips using the methods described previously for POS tentacle snips. The resulting algal pellet was resuspended in 500 µL of filtered seawater. This sample was divided into two aliquots and incubated as described previously for the PAE expelled algae.

Data analysis

The data were analyzed using GraphPad Prism and SPSS 11.0. The data were tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests. If the data failed to pass either test, they were square root, cube root, or rank transformed until they passed at least one of the normality tests. In a few cases even ranked data failed both normality tests, but these data had a normal distribution based on visual inspection of their Q-Q plots. Percentage data were arcsine transformed and then rank transformed if necessary to achieve normality.

Comparisons between two groups were analyzed with t tests. If both groups represented repeated measurements of the same individuals, a paired t test was used. Comparisons between several groups were analyzed with ANOVAs and Tukey post hoc tests. If each group represented repeated measurements of the same individuals, an ANOVA with repeated measures was used. If multiple treatment combinations were used (e.g. high and normal oxygen levels vs. high and low light levels), a two-way ANOVA was used. If the results from the two-way ANOVA were significant, a one-way ANOVA and a Tukey post hoc test were used to clarify the significant relationships. Finally, comparisons between multiple groups were analyzed with a one-way ANOVA and a Tukey post hoc test. Significant differences were indicated by letters above the columns.

Results

Photooxidative Stress (POS): expelled algae

To simplify comparisons, the numbers of expelled algae for all samples were standardized to zooxanthellae cells expelled • mg animal protein^{-1}. Samples from weeks 1 and 5 showed similar trends, so they were pooled to simplify comparisons and smooth variability in the samples. The anemones expelled significantly more algae, both live and dead, in the evening than in the morning (Figure 4).

The light and oxygen treatments did not have a consistent effect on the expulsion of live algae (Figure 5A). However, both light and oxygen levels significantly affected the number of dead algae expelled, with more dead algae expelled in high light and in high oxygen conditions (Figure 5B). The total number of algae expelled (live and dead combined) was higher for high light treatments than for low light treatments (Figure 5C), though oxygen did not show an effect here.

The mitotic index of the expelled zooxanthellae showed a diel pattern. The mitotic index tended to be high in the morning and decrease in the afternoon, though this trend was not significant when each collection was considered separately (Figure 6A). When the expelled algae were pooled into A.M. (0400 h and 1000 h) and P.M. (1600 h and 2200 h) groups, the mitotic index was significantly higher in the morning than in the evening (Figure 6B). The light and oxygen levels affected the mitotic index as well, with higher mitotic indices in high light, normal oxygen conditions (Figure 7).

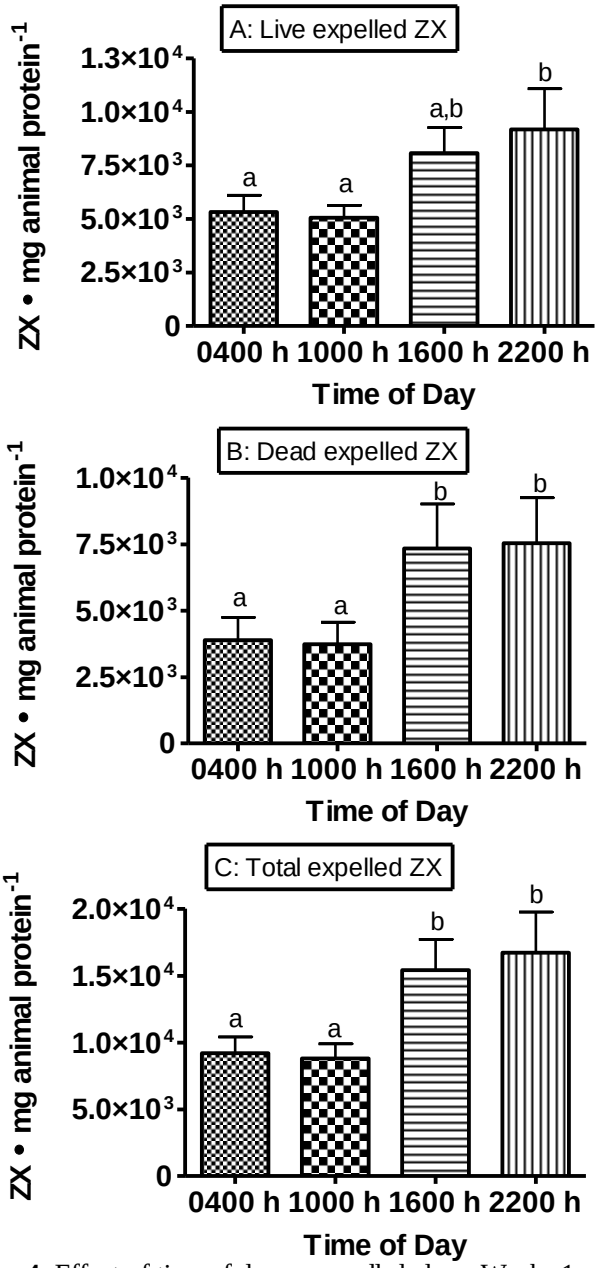

Figure 4. Effect of time of day on expelled algae. Weeks 1 and 5 were pooled. Error bars represent standard error. N=32 for each time of day. **A: Live expelled zooxanthellae (ZX).** Data were cube root transformed prior to analysis. The indicated times were significantly different (ANOVA with repeated measures; $p<0.01$). **B: Dead expelled ZX.** Data were rank transformed prior to analysis. The indicated times were signficantly different (ANOVA with repeated measures; $p<0.001$). **C: Total expelled ZX** (live+dead). Data were square root transformed prior to analysis. The indicated times were signficantly different (ANOVA with repeated measures; $p<0.001$).

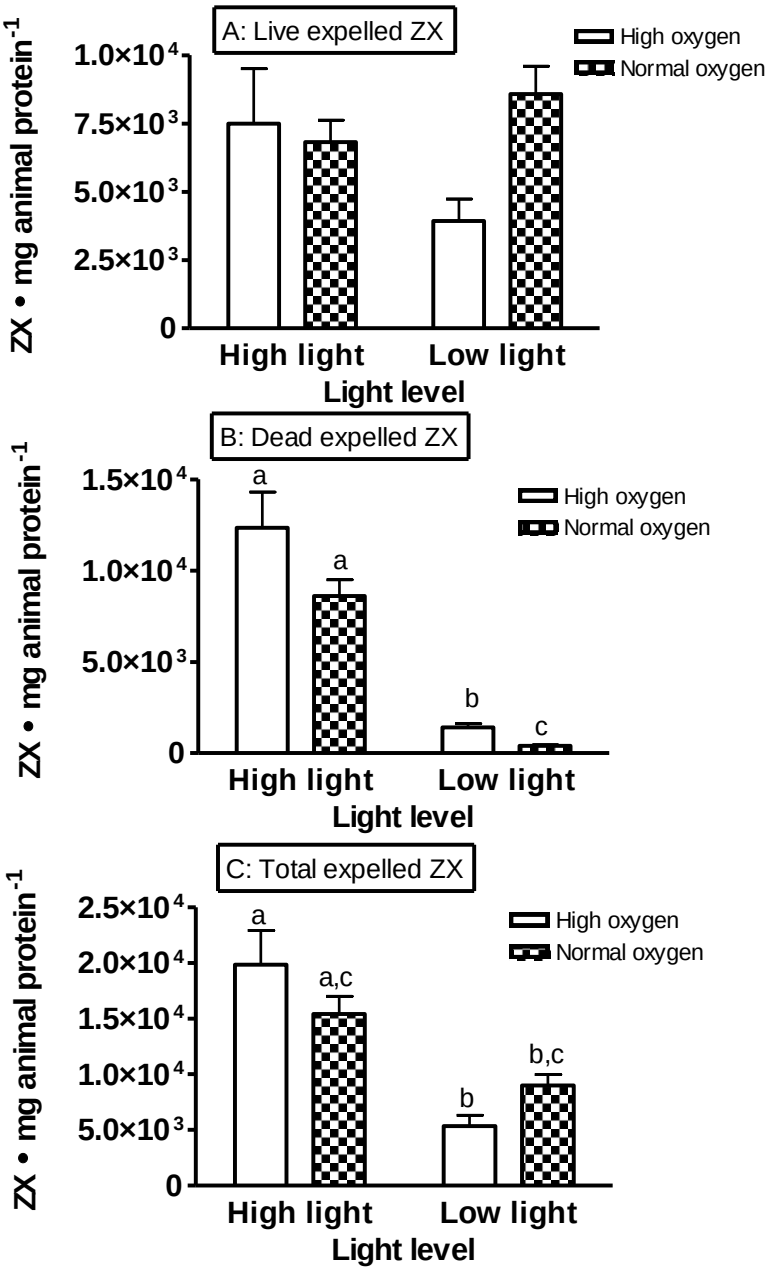

Figure 5. Effects of light and oxygen levels on expelled algae. Weeks 1 and 5 were pooled. N=8 for each treatment group. Error bars represent standard error. **A: Live expelled zooxanthellae (ZX).** There was a significant interaction between light and oxygen (two-way ANOVA; $p<0.05$), but none of the groups were significantly different (one-way ANOVA; $p>0.05$). **B: Dead expelled ZX.** Data were rank transformed prior to analysis. Light and oxygen both showed a significant effect (two-way ANOVA; $p<0.001$ [light], $p<0.001$ [oxygen]). The indicated groups were significantly different (one-way ANOVA; $p<0.05$). **C: Total expelled ZX** (live+dead). Both light and the interaction of light and oxygen were significant (two-way ANOVA; $p<0.001$ [light], $p<0.05$ [interaction]). The indicated groups were significantly different (one-way ANVOA; $p<0.001$).

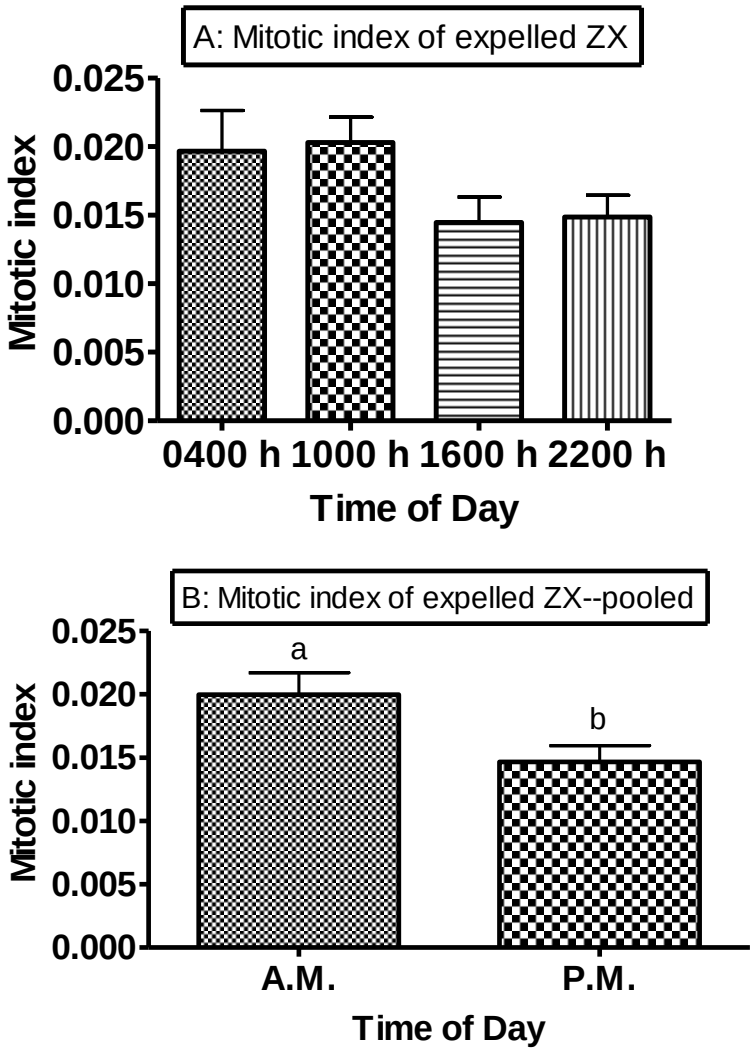

Figure 6. Effect of time of day on mitotic index of expelled algae. Weeks 1 and 5 were pooled. N=36 for each time of day. Error bars represent standard error. **A: Mitotic index of expelled zooxanthellae (ZX).** Data were rank transformed prior to analysis. There was no significant difference among times of day (ANOVA with repeated measures; $p>0.05$). **B: Mitotic index of expelled ZX--pooled.** Data were pooled into A.M. and P.M. groups and rank transformed. There was a significant difference between the groups (paired t-test, $p<0.05$).

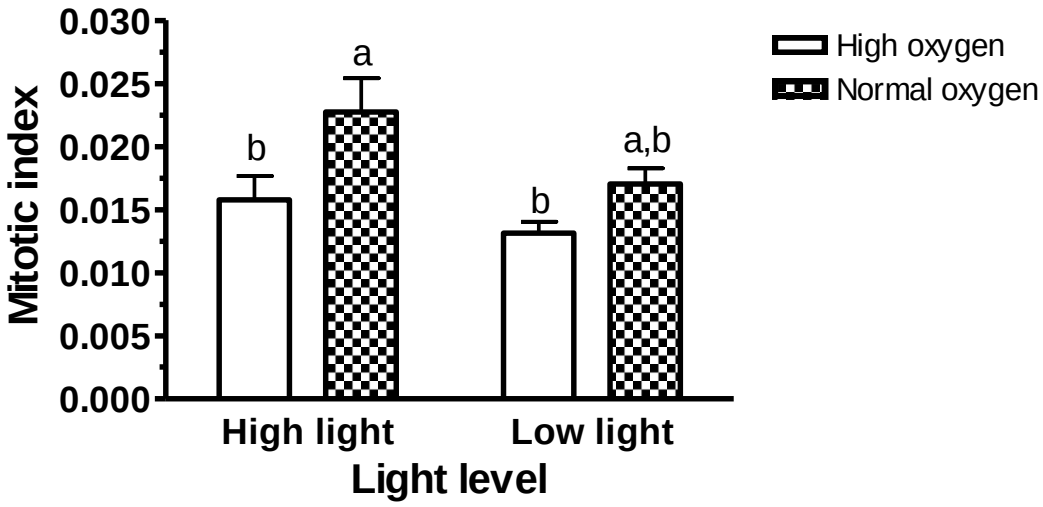

Figure 7. Effects of light and oxygen levels on mitotic index of expelled zooxanthellae. Weeks 1 and 5 were pooled. N=9 for each treatment group. Error bars represent standard error. Both light and oxygen levels showed significant effects (2-way ANOVA; $p<0.05$ [light], $p<0.01$ [oxygen]). The indicated groups were significantly different (one-way ANOVA; $p<0.01$).

Photooxidative Stress (POS): tentacle snips

Overall, the algal counts from the tentacle snips showed a high degree of variability. The size of the tentacle snips (as reflected by total protein content) varied greatly (Figure 8A) due to the anemones' rapid withdrawal response during the sampling process. This size variability may have affected the other parameters measured in the tentacle snips, such as algal density. The algal density per unit animal protein varied widely among treatments, weeks, and individual anemones (Figure 8B). The mitotic index was quite variable as well (Figure 8C).

Whole anemones: genetic analysis

Preliminary results from the RNA analysis suggested that the anemones in the high light treatments showed an increased expression of catalase.

Whole anemones: analysis of algal density and mitotic index

The whole anemones were somewhat similar in size, as reflected by their total protein content (Figure 9A). The control anemones had a significantly higher protein content than did the POS anemones. The algal density per unit animal protein of the whole anemones was quite constant, with the exception of the high light, high oxygen treatment group, which was significantly higher than the high light, normal oxygen group and the control anemones (Figure 9B). There were significantly more dead zooxanthellae in the high light treatments than in the low light treatments (Figure 9C). The mitotic index was similar in all treatments as well, with no significant difference in mitotic index among the four treatment groups and the anemone control group (Figure 10). However, the fresh control anemones had a significantly higher mitotic index than the frozen control anemones (Table 5).

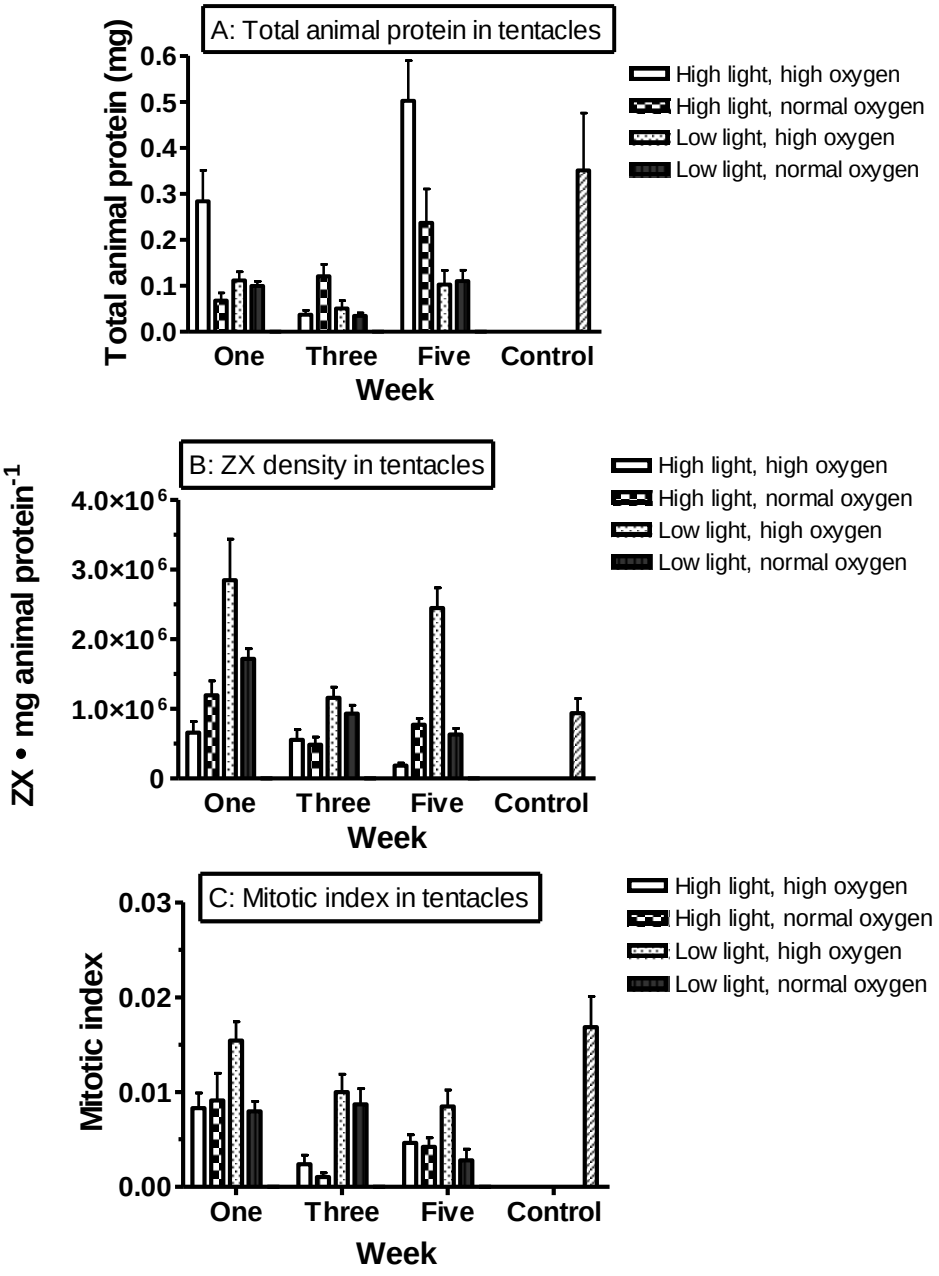

Figure 8. Comparison of tentacle snips. Error bars represent standard error. N=9 in weeks 1 and 3; N=8 in week 5 and controls. Controls were collected July 2, 2008. **A: Total animal protein in tentacles.** Comparison of tentacle size as reflected by total protein content. **B: Zooxanthellae (ZX) density in tentacles.** Comparison of zooxanthellae density among treatment groups and control. **C: Mitotic index in tentacles.** Comparison of mitotic indices among treatment groups and control.

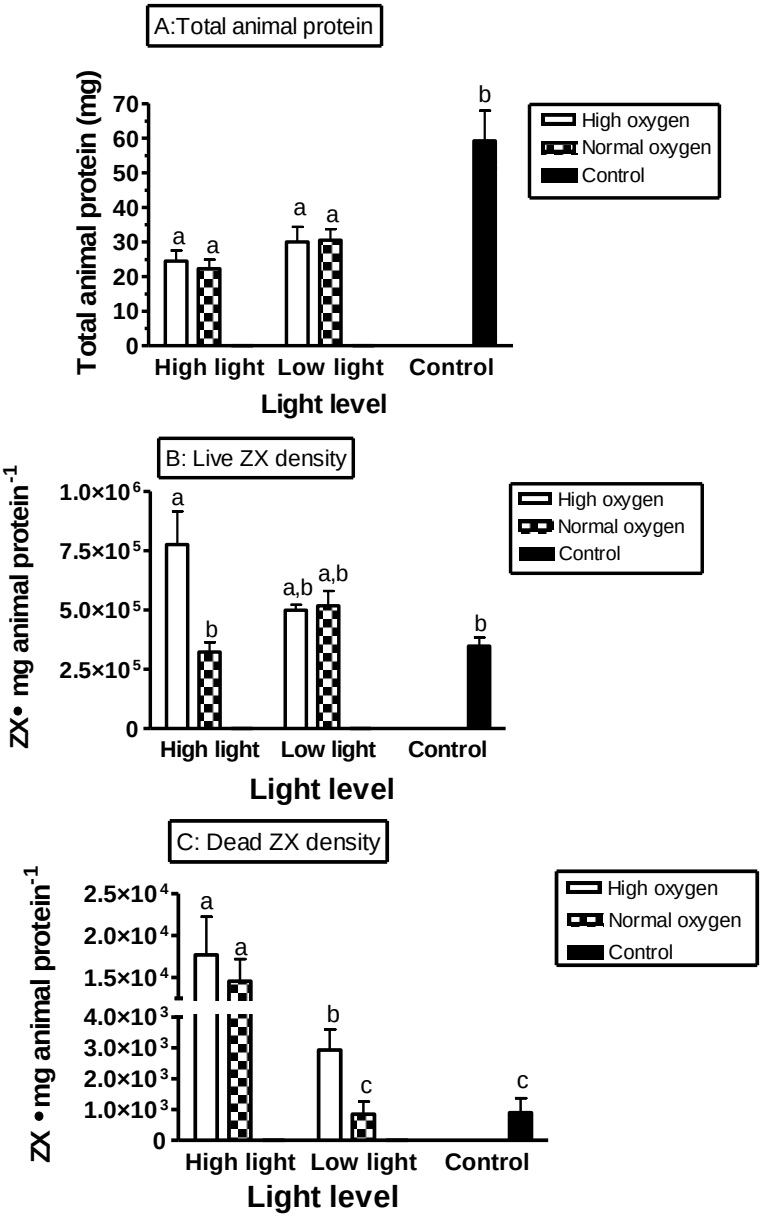

Figure 9. Comparison of whole anemones. Control anemones were collected July 2, 2008. N=8 for each treatment group and controls. Error bars represent standard error. **A: Total animal protein.** The treatments did not show a significant effect (two-way ANOVA; $p>0.05$). However, the indicated groups were significantly different (one-way ANOVA; $p<0.05$). **B: Live zooxanthellae (ZX) density.** Oxygen and the interaction of light and oxygen both had a significant effect (two-way ANOVA; $p<0.01$ [oxygen], $p<0.01$ [interaction]). The indicated groups were significantly different (one-way ANOVA; $p<0.05$). **C: Dead ZX density.** Data were rank transformed prior to analysis. Light, oxygen, and the interaction of light and oxygen were all significant (two-way ANOVA; $p<0.001$ [light], $p<0.05$ [oxygen], $p<0.05$ [interaction]). The indicated groups were significantly different (one-way ANOVA; $p<0.05$).

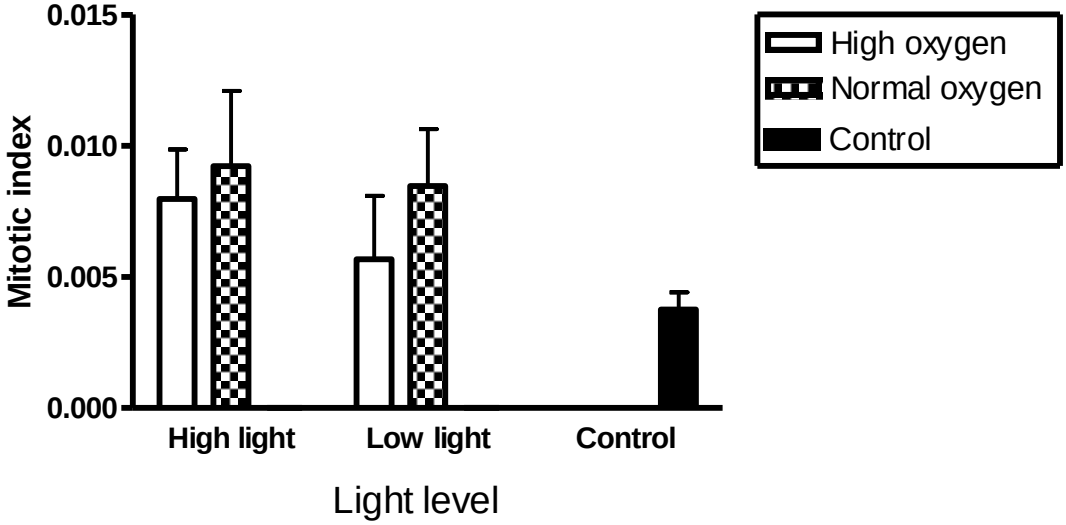

Figure 10. Comparison of mitotic index of zooxanthellae from whole anemones. N=8 for each treatment group, N=16 for controls. Control anemones were collected July 2 and 23, 2008. Error bars represent standard error. There was no significant difference among treatments and controls (2-way ANOVA; $p>0.05$).

Table 5. Comparison of fresh and frozen control anemones. Data were analyzed with an unpaired t test. Mitotic index data were rank transformed prior to analysis. The mitotic indices of the control anemones collected on July 2 and 23 were not significantly different from each other, so the mitotic index data from both dates were pooled. The "controls" referenced in other figures were frozen controls.

	Fresh	**Frozen**	
	Mean ± SEM	**Mean ± SEM**	**p value**
Protein (mg)	89.82 ± 10.16 N = 4	59.21 ± 8.78 N = 8	0.060
Live ZX density (10^3 cells/mg animal protein)	394.15± 64.74 N = 4	347.66 ± 37.08 N = 8	0.516
Dead ZX density (10^3 cells/mg animal protein)	0.407 ± 0.278 N = 4	0.901 ± 0.466 N = 8	0.497
Mitotic Index	0.00944 ± 0.00117 N = 12	0.003757 ± 0.00065 N = 16	0.001

Comparison of retained and recently expelled algae

The proportion of dead algae was much higher in the recently expelled algal pellets than in the whole anemones (Figure 11). Conversely, there was a higher proportion of live algae in the whole anemones. Additionally, the mitotic index of the recently expelled algae was significantly higher than that of the retained algae (either tentacle snips or whole anemones) (Figure 12).

Preferential Algal Expulsion (PAE)

For the incubated expelled algae, the post-incubation algal density (incubation time = 24, 48 or 72 hours) was lower than the pre-incubation (incubation time = 0) algal density, indicating a loss of zooxanthellae during the experiment or processing (Figure 13A). The post-incubation mitotic index was lower than the pre-incubation mitotic index as well (Figure 13B). However, with increased incubation time, the post-incubation mitotic index began to approach the pre-incubation mitotic index. To better illustrate this trend, the mitotic index was standardized to the mean pre-incubation mitotic index by dividing all the values by the mean pre-incubation mitotic index. This made the trend more obvious, with the 24 hour incubation period mitotic index significantly lower than the pre-incubation mitotic index (Figure 13C).

The algal samples isolated from the tentacle snips also lost zooxanthellae during incubation (Figure 14A). In fact, the 24 hour incubation period lost so many algae that the samples from that time period could not be analyzed. The post-incubation mitotic index seemed to increase relative to the pre-incubation mitotic index for these non-expelled algae as well, though not significantly (Figure 14B). To better illustrate this trend, for each incubation time period the post-incubation mitotic index was standardized to the mean pre-incubation mitotic index. Though the trend was toward a higher mitotic

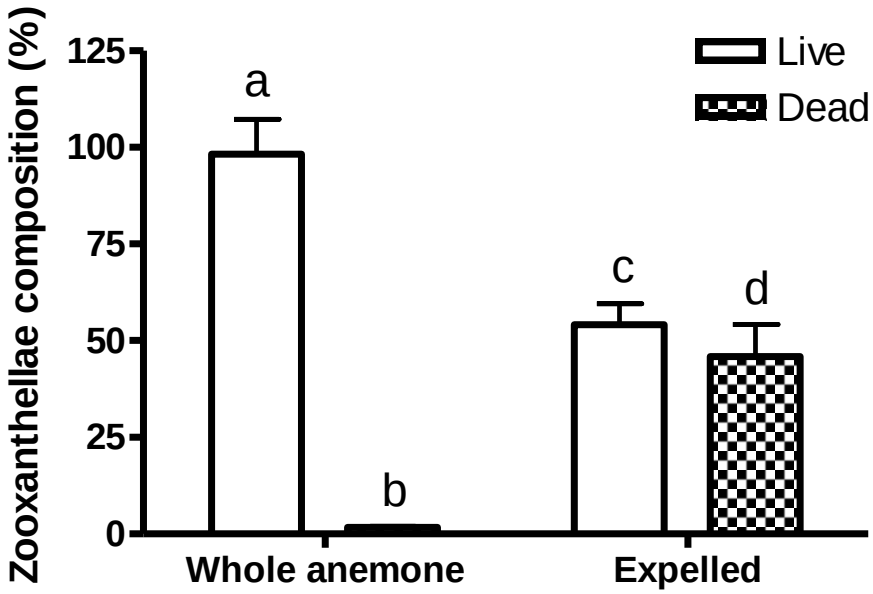

Figure 11 . Comparison of zooxanthellae composition between whole anemones and recently expelled algae. N=32 for each group. Expelled algae were pooled from weeks 1 and 5. Error bars represent standard error. Data were rank transformed prior to analysis. The indicated groups were significantly different (one-way ANOVA; $p<0.001$).

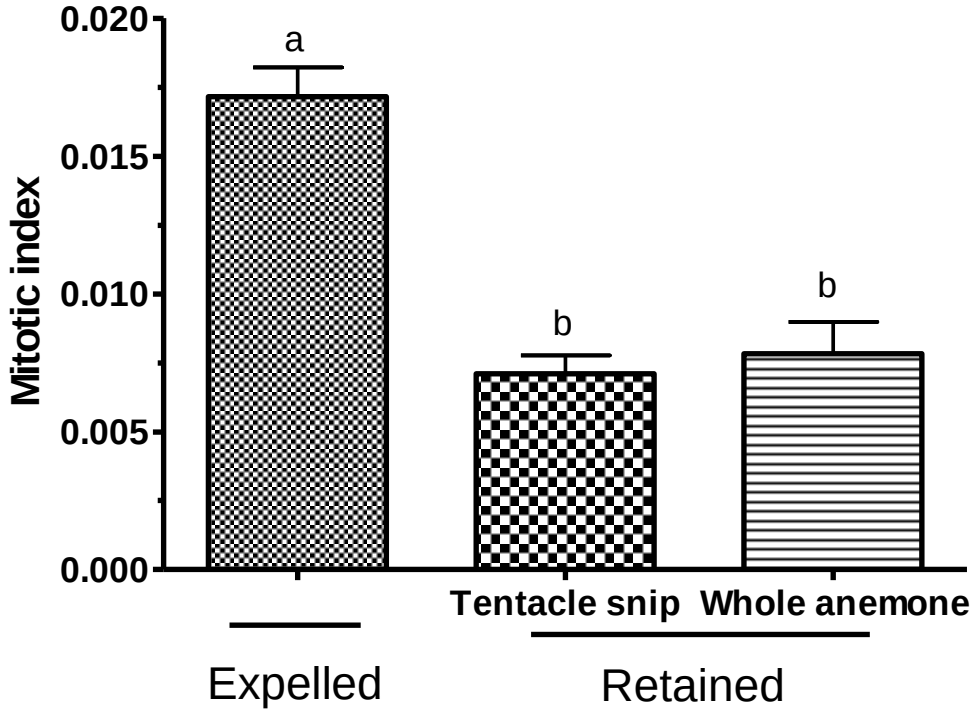

Figure 12. Comparison of mitotic index between recently expelled and retained zooxanthellae. N=32 for whole anemones; N=36 for expelled algae and tentacle snips. Weeks 1 and 5 were pooled for the expelled algae; weeks 1, 3, and 5 were pooled for the tentacle snips. Error bars represent standard error. Data were rank transformed prior to analysis. The indicated groups were significantly different (one-way ANOVA; $p<0.001$).

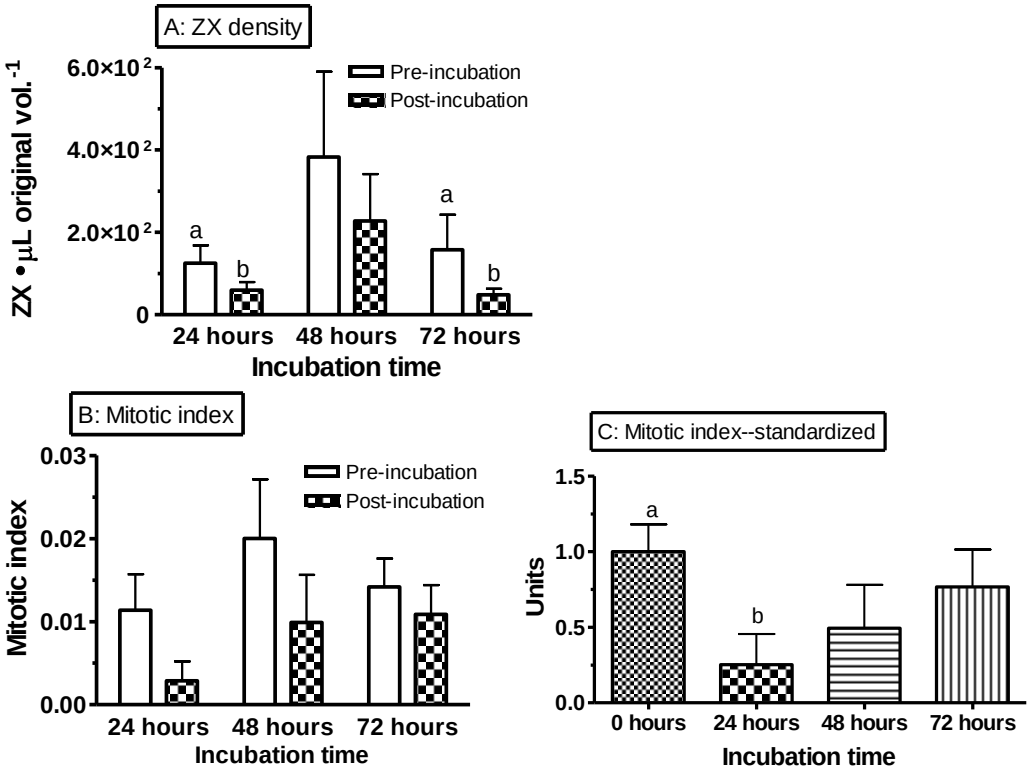

Figure 13. Comparison of expelled algae at different incubation times of the Preferential Algal Expulsion (PAE) experiments. N=24 for incubation time=0 (graph C); N=8 for all other groups. Error bars represent standard error. **A: Zooxanthellae (ZX) density.** Data were rank transformed prior to analysis. While the incubation treatments had no significant effect (two-way ANOVA; $p>0.05$), the pre- and post- incubation groups were significantly different for 24 and 72 hours (paired t-tests; $p<0.05$). **B: Mitotic index.** The incubation treatments had no significant effect (two-way ANOVA; $p>0.05$). There was no significant difference between pre- and post-incubation groups either (paired t-tests; $p>0.05$). **C: Mitotic index--standardized.** Mitotic index was standardized to mean pre-incubation mitotic index. There was a significant difference between the 0 and 24 hour incubation time periods (unpaired t-test; $p<0.05$).

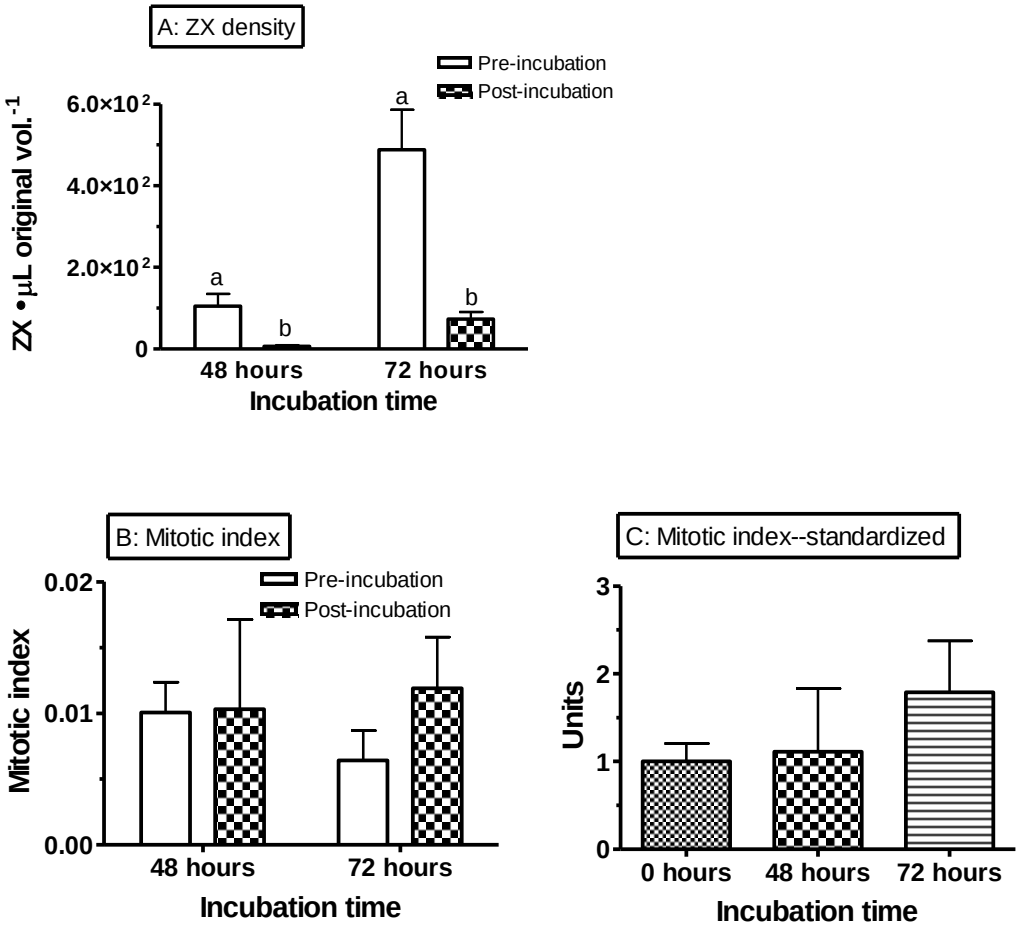

Figure 14. Comparison of algae isolated from tentacle snips at different incubation times of the Preferential Algal Expulsion (PAE) experiments. N=16 for incubation time=0 (graph C); N=8 for all other groups. Error bars represent standard error. **A: Zooxanthellae (ZX) density.** Data were square root transformed prior to analysis. Both the incubation time and the status (incubated or not) had a significant effect (two-way ANOVA; $p<0.001$). The pre- and post-incubation groups were signficantly different for both 48 and 72 hours (paired t-tests; $p<0.001$). **B: Mitotic index.** The incubation treatments had no significant effect (two-way ANOVA; $p>0.05$). There was no significant difference between pre- and post-incubation groups (paired t-tests; $p>0.05$). **C: Mitotic index--standardized.** Mitotic index was standardized to mean pre-incubation mitotic index. There was no significant difference between the incubation time periods and time = 0 (unpaired t-test; $p>0.05$).

index with increased incubation time (similar to that seen in the expelled algae in Figure 13C), the trend was not significant for these non-expelled algae.

Discussion

Though there are many aspects to algal expulsion in *A. elegantissima*, this study sought to address two main questions: (1) does *A. elegantissima* expel more algae during probable photooxidative stress conditions? and (2) does *A. elegantissima* preferentially expel dividing algae?

Photooxidative Stress (POS)

The data from these experiments suggest that *A. elegantissima* maintains a relatively constant algal density, regardless of light and oxygen conditions. The high light treatment was quite intense (10 hours per day at 2,000 μmol quanta • m^{-2} • s^{-1}) and likely exceeded the light intensity that the anemones would encounter under natural conditions. The oxygen level of the high oxygen treatment was also more than twice that of the ambient seawater. These treatment conditions should have elicited a bleaching response equal to or greater than what the anemones would demonstrate in their natural habitat, yet the number of live algae expelled per unit animal protein showed no consistent pattern with either light or oxygen level (Figure 5). Perhaps the risk of photooxidative stress during the summer is insufficient to justify expelling valuable symbiotic zooxanthellae. The preliminary RNA data suggest that the anemones from the high light treatments expressed more catalase and may have been able to compensate for the increased photooxidative stress in that way. The mitotic index of the symbiotic zooxanthellae tended to be slightly higher in high light conditions also (Figure 10), so the algal growth rate may have compensated for at least a moderate increase in algal expulsion in the high light treatments.

The numbers of expelled live zooxanthellae were unaffected by light and oxygen conditions, suggesting that *A. elegantissima* may constitutively expel a small amount of live zooxanthellae regardless of the conditions. Alternatively, the anemone may expel high numbers of live algae which subsequently die after expulsion. This could result in constant numbers of live algae across the treatment groups. For example, perhaps more algae are expelled in the high light groups, but most of those algae are dead or die soon after expulsion. If a lower proportion of the expelled algae are alive in the high light groups, if more total algae are expelled, the number of live algae would remain similar to the number expelled by the low light groups. This explanation is supported by the observation that more dead zooxanthellae were expelled in high light and high oxygen conditions. The expulsion of high numbers of dead zooxanthellae during photosynthetic stress has been observed in at least one other cnidarian symbiosis (Franklin *et al* 2004; Franklin *et al* 2006). Perhaps zooxanthellae are damaged by photooxidative stress in high light and high oxygen conditions and the damaged zooxanthellae are preferentially expelled by the host. The percentage of dead zooxanthellae was much higher in the expelled algae than in the symbiotic algae. This suggests that either the anemone preferentially expels dead algae or the algae are expelled and then die at an increased rate after they leave the host.

Preferential Algal Expulsion (PAE)

Since rapidly growing zooxanthellae may be presumed to be detrimental to the host, it is of interest to determine if hosts can control the growth rates of their symbiotic algae. Zooxanthellae that had been recently expelled from *A. elegantissima* had significantly higher mitotic indices than zooxanthellae that had been isolated from tentacle

snips or whole anemones (Figure 12). This suggests that the host is controlling algal growth by some means. Perhaps the host restricts nutrient availability to the symbiotic algae, though this seems unlikely since *A. elegantissima* lives in nutrient-rich temperate waters and nutrients can diffuse through the host. A more likely explanation is host regulation of the algal cell cycle. Smith and Muscatine (1999) found that symbiotic zooxanthellae from the anemone *Aiptasia pulchella* spent much longer in the G_1 phase of the cell cycle than did free-living zooxanthellae, though the length of mitosis was quite similar for both symbiotic and free-living algae (Table 6). This suggests that hosts are able to control the algal cell cycle. An extended G_1 phase would also explain the lower mitotic index that was observed in the symbiotic zooxanthellae from *A. elegantissima*. If the G_1 phase is proportionally longer than the M phase in symbiotic algae, the symbiotic algae would exhibit a lower mitotic index since fewer cells in a sample would be undergoing mitosis at a given time.

It appears that *A. elegantissima* may preferentially expel algae in the S or G_2 phase of the cell cycle. This is supported by at least two lines of evidence. First, when expelled algae samples were incubated 24 hours, the mitotic index decreased significantly, becoming even lower than the mitotic index of retained algae (Figures 13C, 12). If the cells had been expelled without regard to the stage of the cell cycle, the pre- and post-incubation mitotic indices should have been the same. However, since the post-incubation mitotic index was lower, the cells were likely expelled shortly before the M phase. The expelled algae samples were collected within 24 hours of expulsion, so the high mitotic indices in the recently expelled algae samples represent cells that divided shortly before or just after being expelled. After 24 hours of additional incubation, the

Table 6. Comparison of cell cycle stages in zooxanthellae from *Stylophora pistillata* (from Smith and Muscatine 1999).

	Stage length (days)	
Stage	**Free-living ZX**	**Symbiotic ZX from fed anemones**
G_1	1.0	6.4
S	0.6	1.1
G_2	0.8	1.5
M	0.2	0.3

samples contained primarily non-dividing cells. It is unlikely that the decrease in mitotic index was due to a shift from asynchronous to synchronous cell division since the samples were controlled for time of day. It is also unlikely that the decrease was an artifact of the centrifugation process, since algae isolated from the tentacle snips did not exhibit this decrease in mitotic index (Figure 14C). The diel pattern of algal expulsion in *A. elegantissima* is a second line of evidence supporting the preferential expulsion of algae in the S or G_2 phase. Smith and Muscatine (1999) found that for symbiotic zooxanthellae in *Aiptasia pulchella*, entry into the S phase peaked at entry into the dark period (1800 h in their experiment). Additionally, *A. pulchella* preferentially expelled zooxanthellae that had recently passed through the S phase (Baghdasarian and Muscatine 2000). In *A. elegantissima* in this experiment, algal expulsion increased during the afternoon and peaked at 2200 h (Figure 4). If the *A. elegantissima* symbiosis is similar to the *A. pulchella* symbiosis, this suggests that *A. elegantissima* may preferentially expel algae that have entered the S phase.

In addition to preferentially expelling dividing algae, *A. elegantissima* may also control algal growth by restricting algal growth rates *in hospite*. When zooxanthellae were isolated from tentacle snips and incubated for 48 or 72 hours, their mitotic indices increased relative to the pre-incubation algae from tentacle snips (Figure 14C, D). Though this trend was not significant in these data, this suggests that the host may have restricted the algal growth rate in some way, since removal from the host appeared to allow the algae to increase their mitotic index. Again, this is probably not due to a diel

pattern or a shift from asynchronous to synchronous cell division, since the samples were controlled for time of day.

Other observations

Another interesting result was the observation of a diel pattern in the mitotic index of expelled algae (Figure 6). Since symbiotic zooxanthellae within *A. elegantissima* do not show a diel pattern in mitotic index (Wilkerson *et al* 1983), the presence of a diel pattern in the expelled algae suggests that the host may alter the cell division pattern of its symbiotic algae. When the algae are expelled from the host, they may revert to the synchronous cell division pattern observed in other free-living dinoflagellates (Weiler and Chisholm 1976). Also, since the expelled algae samples in the morning represent algae expelled over the previous 18 hours, the higher mitotic index seen in the morning samples is consistent with algae that are undergoing cell division after being expelled in the G_2 phase the previous evening.

The variability in the tentacle snips might be explained by the observation that the algal density was much higher in the tentacle snips than in the whole anemone (Figures 8B, 9B). Since the size of the tentacle snips varied widely due to the anemones' rapid withdrawal response, perhaps some samples represented tentacle tips while other samples represented the whole tentacle. If the algal density is greater in the tentacle tips, the different sizes of tentacle snips would lead to variability in the algal density.

The mitotic index of fresh control anemones was significantly higher than that of frozen control anemones (Table 5). The only difference between the fresh and frozen controls was the time of homogenization of the whole tissue: fresh anemones were homogenized immediately and frozen anemones were homogenized after being frozen.

The cell counts for both fresh and frozen control anemones were done on algal samples that had been frozen and thawed, so the freezing of algal cells before counting was not the source of the variability. Perhaps freezing the tissue samples prior to homogenization made the dividing cells more susceptible to shearing. If dividing cells were separated during the homogenization process, the mitotic index would be underrepresented. If this is true, since the tissue samples used in this study were frozen prior to homogenization, their mitotic indices may have been artificially depressed. This would explain the difference between the mitotic indices observed in this study and the two-fold higher mitotic indices observed by others (McCloskey *et al* 1996).

Another source of variability could be the loss of zooxanthellae during the PAE incubation experiments. Both the expelled algae and tentacle snip incubations lost large amounts of algae during incubation. However, this should not have affected the mitotic indices, since the mitotic index is the ratio of dividing cells to total cells. The loss was likely caused by zooxanthellae that failed to centrifuge into the pellet. During pre-experiment trials, it was found that large numbers of zooxanthellae stuck to the sides of the centrifuge tubes and failed to pellet. This problem could be alleviated by the addition of SDS. However, SDS was not used in the PAE experiments because it was feared that SDS might affect the algal growth rate during incubation.

Conclusions

It appears that photooxidative stress does not affect the algal density or algal expulsion rate in *A. elegantissima* except to increase the expulsion of dead zooxanthellae. Photooxidative stress may trigger the expulsion of live algae which then subsequently die outside the host, or the stress may kill symbiotic algae which are then expelled by the

host. *A. elegantissima* appears to control algal growth by preferentially expelling algae in the S or G_2 phase of the cell cycle and potentially restricting the growth rate within the host.

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