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Experimental Increases of Zooxanthellae Density in the Coral *Stylophora pistillata* Elucidate Adaptive Mechanisms for Zooxanthellae Regulation

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Abstract

As was shown the increase in zooxanthellae population density was evoked by lowering the light intensity under experiments with hermatypic corals (Titlyanov et al., 1983) and by sufficient supply of zooplankton (Muscatine et al., 1989). The zooxanthellae density in branches of *Stylophora pistillata* corals was experimentally altered by lowering the light intensity and providing the corals with an *ad lib* supply of a zooplankton. A simultaneous influence of these factors on the physiological state of coral branches was investigated with coral fragments containing different algal population densities per polyp, ranging from high densities 24,000 ± 2,300, to medium densities 13,500 ± 1,300, to low densities 830 ± 350. Transferring the corals with low zooxanthellae numbers from high light intensity (80–90% photosynthetic active radiation, PAR_0) to dim light (20% PAR_0), and providing the corals with an *ad lib* supply of a zooplankton, evoked a significant two-fold increase in algal concentrations. Higher zooxanthellae concentrations showed significant increases in the coral's photosynthetic capacity

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and respiration rate. Acclimation to low light intensity occurred through the increase in the zooxanthellae's chlorophyll content, zooxanthellae density and an increase in the coral's photosynthetic capacities. These adaptations were only apparent in coral experiments with low initial algal populations. No physiological changes or significant changes in the zooxanthellae densities were observed in the coral fragments transferred from naturally shaded conditions to experimental conditions with dim light and an unlimited supply of a zooplankton. This study found that the main adaptive reactions in hermatypic corals to lowered light intensity (20% PAR_0) are zooxanthellae accumulation in polyps and an increase in the zooxanthellae's chlorophyll concentration which take place only in the presence of a sufficient supply of zooplankton. It is postulated that zooxanthellae populations in corals are regulated by the coral animal controlling the zooxanthellae's reproduction and death.

Keywords: *Stylophora pistillata*, hermatypic corals, symbiosis, photoacclimation, zooxanthellae division, zooxanthellae degradation, photosynthesis, respiration, chlorophylls

1. Introduction

Under experimental conditions an increase in symbiotic zooxanthellae densities in hermatypic corals can be induced by a decrease in light intensity (Titlyanov et al., 1983), by an increase in inorganic nitrogen concentrations in the surrounding seawater (Cook et al., 1988, 1992; Hoegh-Guldberg and Smith, 1989; Stimson and Kinzie, 1991; Muller-Parker et al., 1994; Marubini and Davies 1996), and by an increase of zooplankton (Muscatine et al., 1989).

Changes to the density of symbionts lead to physiological changes in the host (Clayton and Lasker, 1984). For example, a decrease in zooxanthellae density leads to a loss of the coral's photosynthetic capacity and a decrease in primary production (Titlyanov, 1991). An increase in zooxanthellae density in corals analogously increases the coral's photosynthetic capacity and primary production (Titlyanov, 1991; Marubini and Davies, 1996), reduces the zooxanthellae's mitotic index (MI), increases the chlorophyll concentrations of zooxanthellae, and often reduces the size of the zooxanthellae (Jones and Yellowlees, 1997).

Intensive studies have been undertaken on the influences of external factors reducing the zooxanthellae population density in symbiotic cnidarians (Searle et al., 1982; Steen and Muscatine, 1987; Gates et al., 1992; Van Woesik et al., 1995; Jones, 1997). Under adverse external conditions hosts generally expel zooxanthellae, at times with endodermal tissue or parts of these cells attached (Gates et al., 1992). However, mechanisms regulating zooxanthellae populations are not clear. Hermatypic coral hosts, in all probability, appear to regulate zooxanthellae numbers by influencing their rate of division and

growth, by controlling mitogenetic factors (Muscatine and Pool, 1979; Jacques and Pilson, 1980), or gradually limiting carbon (Weis et al., 1989) or nitrogen (Trench, 1987; McAuley et al., 1994). Any direct evidence is lacking.

Under normal physiological conditions the expulsion of zooxanthellae in corals amounts to about 0.1–1% per day from the zooxanthellae stock (Hoegh-Guldberg et al., 1987; Stimson and Kinzie, 1991; Titlyanov et al., 1996). Corals with such low rates of zooxanthellae expulsion are not likely to regulate their symbiont population in this manner because the release rate of zooxanthellae is almost ten times lower than the replenishment rate (Muscatine et al., 1985; Hoegh-Guldberg and Smith, 1989; Titlyanov et al., 1996).

A "space limitation" theory has been suggested to explain zooxanthellae regulation in corals, where the area available for the zooxanthellae to occupy space within the endoderm directly determines their densities under steady-state conditions (Drew, 1972; Muscatine and Porter, 1977; Jones and Yellowlees, 1997). But, as Jones and Yellowlees (1997) stated: "The second model also slightly overestimates the recovery of the coral compared to the field data, suggesting an additional loss of zooxanthellae which has not been accounted for." Another suggested mechanism of zooxanthellae regulation in corals, under normal physiological conditions, is through the digestion of symbionts by host cells. The possibility of zooxanthellae digestion by host cells was first pointed out by Boschma (1925). Recently we confirmed degradation of zooxanthellae (probably through semi-digestion) in some hermatypic corals, including *Stylophora pistillata* (Titlyanov et al., 1996). It was shown, that this is a phased and constant process with maximum activity at night-time (releasing 0.5–6% of their zooxanthellae standing stock per day), enhanced through starvation.

The first objective of the present work was to experimentally induce an increase in the zooxanthellae population density in *Stylophora pistillata* tissue to identify a possible mechanism that may explain, or be responsible for, algal population changes. The second objective was to assess the influence of zooxanthellae population density on the accumulation rate of zooxanthellae in corals. The third objective was to assess the coral's physiology and to determine its dependence on zooxanthellae concentrations in the polyp endoderm.

2. Materials and Methods

Biological material

Colonies of the coral *Stylophora pistillata* (Esper, 1977) were collected in March (for experiment 1) and in May 1995 (for experiments 2, 3) at a depth of 2

m in the East China Sea near the Sesoko Marine Station, Tropical Biosphere Research Centre, University of the Ryukyus, Okinawa, Japan. The colonies were placed in plastic bags with seawater and transported to a 12 m^3 semi-open aquarium supplied with seawater (turnover rate 5% h^{-1}) and left until morning. At 9:00 h the next day branches (4 cm lengths, 2–4 g of fresh weight) were broken off each colony and transferred to experimental plastic jars.

In the first experiment (Exp. 1) the coral branches were taken from 90% of the incident photosynthetic active radiation (PAR_0) and were subjected to osmotic shock and starvation to reduce their zooxanthellae density. After osmotic shock action and subsequent starvation they declined from 17,000 ± 2,300 cells per polyp to 830 ± 350. Under osmotic shock action (salinity 18‰) during 12 h the coral branches of *S. pistillata* lost about 50% of zooxanthellae population (Titlyanov et al., in press). For experiment 2 (Exp. 2), and for the control experiment (Exp. C), coral branches were taken from well-lit habitats where corals contained 13,500 ± 1,300 zooxanthellae per polyp. In experiment 3 (Exp. 3) coral branches from shaded habitats were used. They supported 24,000 ± 2,300 zooxanthellae per polyp. In this and in previous two papers (Titlyanov et al., 1996, 1998) we went over to standardization of physiological characteristics on the number of polyps that more correct, fast and convenient in comparison with the normalized indications per surface area of *Stylophora pistillata*. From necessity to compare our data with literature ones normalized per surface area of the skeleton *S. pistillata* used in experiments equal 48 ± 6. Coral surface area was estimated using the foil technique (Marsh, 1979).

Experimental design

Coral branches from four colonies, close in appearance and colour, were supported vertically in plastic jars (1.5 l). Four branches (one from every colony) were placed in each jar. The jars were supplied with unfiltered seawater, a light intensity of 20% PAR_0 (shaded by grey plastic mesh), and constant aeration (Exps. 1–3). In the control experiment, the coral branches (2–4 g of fresh weight) from four colonies (n=4) were exposed to 80% PAR_0, close to natural light quality. Four aquariums for each experiment (including control) were fixed in an outdoor pond where the temperature of the water varied from 24–26°C.

Daily, at 18:00–19:00 h, the unfiltered seawater was changed with seawater that contained cultured rotifers, *Branchionus plicatilis* Muller, at concentrations of 10–20 rotifers per 1 ml of seawater. The rotifers were cultured at the Hatchery Center of Okinawa Prefecture at Motobu-cho. The average diameter of the rotifers was approximately 160 μm. The rotifers were active

throughout the 12 h exposure periods. *Stylophora pistillata* polyps were observed actively catching rotifers nightly. Every morning, at 8:00–9:00 h, the seawater with rotifer remnants was replaced with fresh seawater. In the control experiment the unfiltered seawater was replaced twice a day at 8:00–9:00 and 18:00–19:00 h. The experiment was maintained for 30 days. Coral samples were taken for analysis on the first day of all experiments, on the 14th and 30th day for Exp. 1, on the 5th, 15th and 28th days for Exp. 2, on the 6th, 16th and 29th for Exp. 3, and on the 10th and 25th day for the control experiment, Exp. C. Three branches from different colonies were taken for each analysis.

Estimating degraded zooxanthellae particles (dzp) and healthy zooxanthellae (hz)

At 8.00–9.00 h the *S. pistillata* branches were taken from the experimental plastic jars and placed into separate 0.5 l glass jars (one branch in each jar) and maintained as described in the experimental design. The released zooxanthellae and their remnants (dzp) were collected and counted twice a day, at 12:00 and 18:00 h, by filtering the water from each jar with a Millipore filter Type JH 0.45 μm and counting the number of individual dzp and hz directly on the filter paper using a microscope. Derived means and standard deviations (here and in other experiments) were based on three coral branches.

Measurement of photosynthetic and dark respiration rates

The rates of net O_2 production and O_2 consumption were measured for the coral branches according to Leletkin et al. (1996). The oxygen flux was measured using a respirometer consisting of a cylindrical glass chamber (400 ml volume) with a Clark oxygen electrode (OYI 53010 Model) coupled to a chart recorder and magnetic stirrer. Each coral sample was set on a plastic grid in seawater at a distance of 3 cm from a magnetic stirring bar. The chamber was blocked off with a stopper that prevented any exchange with the atmosphere. Temperature was maintained at 25 ± 0.5°C with a recirculating water bath. A halogen lamp (150 W) was used for illumination and PAR was selected through a thermal filter (with 2% $CuSO_4$ solution). Light intensity was measured with a Li-Cor radiation sensor (Model Li-192 SB). Quantum flow of PAR in the respirometer, equaled 1,300 $\mu E.sm^{-2}.s^{-1}$, which was enough to saturate the photosynthetic process in *S. pistillata* branches (Titlyanov et al., 1988). The O_2 electrode was calibrated before each measurement according to Green and Carritt (1967). The corals were exposed to light for 30 minutes. Dark exposure

was kept at 40 min. The rates of oxygen (P^{max}_{net}) expulsion in saturated light and oxygen consumption in the dark (R_d) were calculated per cm^2 of the coral surface and were also normalized to 10^6 zooxanthellae in coral tissue. The rate of gross photosynthesis (P^{max}_{gros}) was calculated by summing the (P^{max}_{net}) and R_d values.

Removal of coral tissue and analysis of algal density, proliferating zooxanthellae frequency (pzf), degraded zooxanthellae frequency (dzf) and algal size

The numbers of zooxanthellae were counted after estimating released dzp and hz for each branch, and measuring the rates of photosynthesis and respiration. Living coral tissue was removed with a Water-Pik (Johannes and Wiebe, 1970). Small sub-samples of the tissue homogenate were taken and using a hemocytometer estimations were made of zooxanthellae densities, pzf, dzf and algal diameters. Ten to twelve replicate sub-samples were measured. Counts were made of healthy, dividing and degrading zooxanthellae (Fig. 1). In addition, the pzf and dzf were determined in parallel on a slide glass. Zooxanthellae were classified as dividing if they showed anything from a cell wall across the zooxanthella, to the formation of envelopes in the daughter cells. Degrading zooxanthellae were identified by colour, size and shape. Light and electron microscopy of hz and dzp were undertaken as described in Titlyanov et al. (1996). A total of 500 to 1,000 cells was counted in each sample and the percentage of cells dividing was classified as the pzf, and the percentage of cells degrading was classified as the dzf. These estimates were undertaken at 9:00–10:00 h when the number of dividing cells amounted to 80% of the night maximum (occurring at about 03:00 h) and degraded zooxanthellae numbers are highest (Titlyanov et al., 1996). The diameters of one hundred healthy zooxanthellae were measured from each branch using a calibrated ocular micrometer at 400× magnification.

Chlorophyll

To determine chlorophyll concentrations, tissue homogenates, extracted using a Water-Pik, were filtered under a vacuum (47 mm AP Millipore filters), and stored in 90% aqueous acetone solution in a refrigerator for two days and with daily agitation of the samples. The absorbency of acetone extracts was measured at 630 and 663 nm using a Hitachi U-2000 spectrophotometer. Concentrations of Chlorophyll α and c_2 were determined using the spectrophotometric equations of Jeffrey and Humphrey (1975).

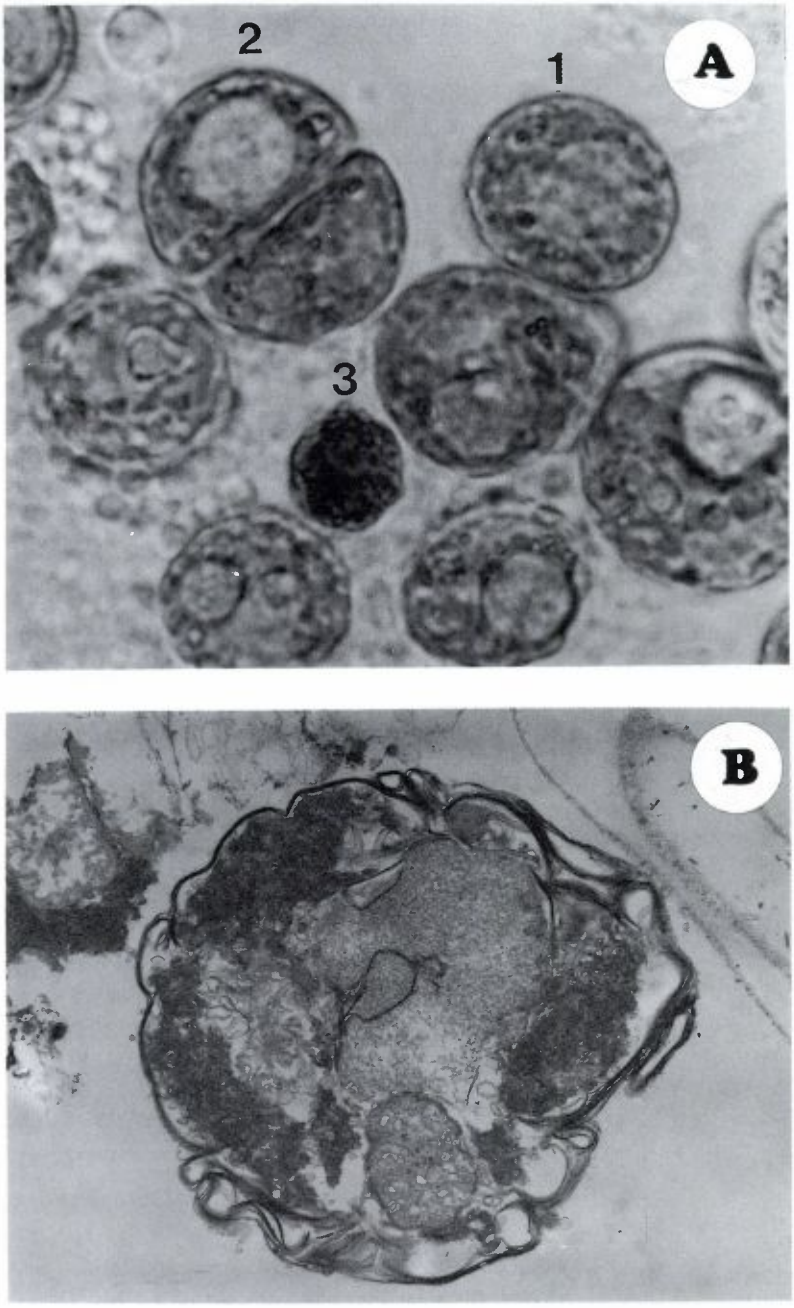

Figure 1. *Stylophora pistillata*. A: healthy (1), dividing (2) and degrading (3) zooxanthellae released from living coral tissue. Light microscopy, magnification 3,200×. B: a degraded zooxanthellae particle (dzp) released by *S. pistillata*. Electron microscopy, magnification 16,000×.

3. Results

Experiment 1 on corals with low zooxanthellae number

Rapid, steady, increases in zooxanthellae, from 1,000 to 12,000 zooxanthellae per polyp, were observed during the 30-day experiment (Fig. 2). Zooxanthellae volume was reduced from an average of 780 to 630 μm^3 (Fig. 3). During the first 14 days the chlorophyll content in zooxanthellae increased approximately 1.3 times (Fig. 4). The P^{max}_{gros} rate (calculated per cm^2 of coral surface) increased by more than 10 times (Fig. 5). The photosynthetic capacity of the zooxanthellae increased by 60% (Fig. 6); coral respiration (R_d) increased three times (Fig. 7); dzf dropped from 9 to 1% while pzf (on the 14th day of the experiment) increased from 0.5 to 5% (Figs. 8 and 9). The ratio pzf/dzf increased from 0.06 to 4. During the experiment the number of dzp released was about 30–100 per polyp per day (Fig. 10) and hz released increased from 5 to 15 zooxanthellae per polyp per day (the data are not included in the figures).

Experiments 2 and 3 on corals with moderate and high zooxanthellae numbers

The dynamics of zooxanthellae populations differed considerably between experiments 2 (Exp. 2), supporting moderate numbers of zooxanthellae, and experiment 3 (Exp. 3), supporting high numbers of zooxanthellae. For the first

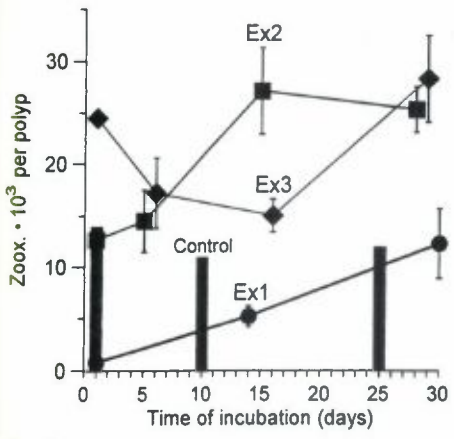

Fig. 2.

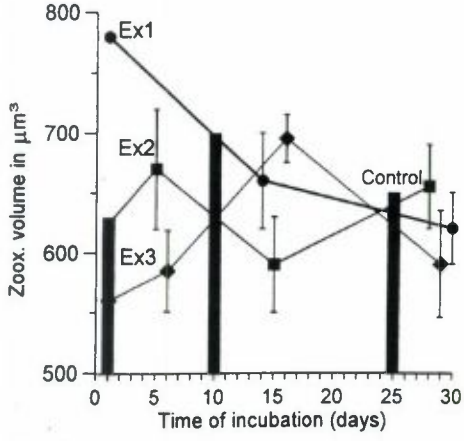

Fig. 3.

Figure 2. *Stylophora pistillata*. Changes in zooxanthellae population density in experiments 1 (Exp. 1), 2 (Exp. 2), 3 (Exp. 3), and control. The means and deviations for Figs. 2–9 were derived from counts on three branches (n=3). Here and for other figures deviations in control are close to deviations in experiments and amounted to about 10–20% of means.

Figure 3. *Stylophora pistillata*. Changes in zooxanthellae volume in experiments 1–3 and control.

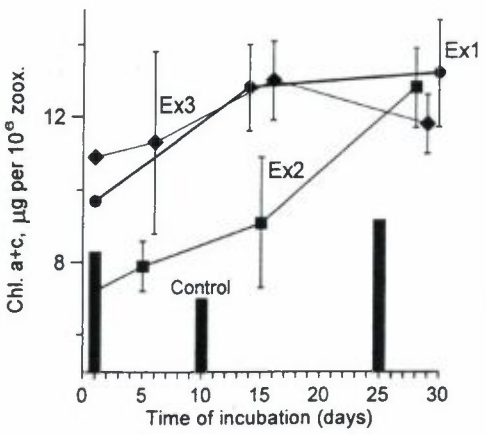

Fig. 4.

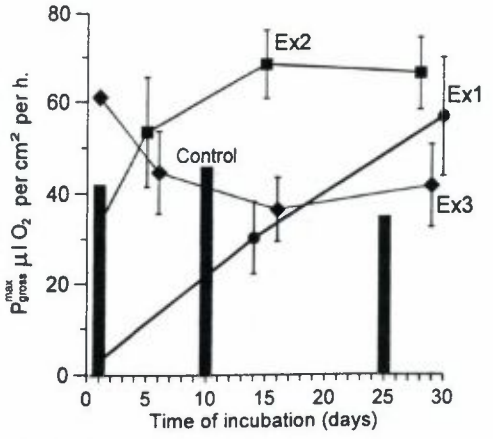

Fig. 5.

Figure 4. *Stylophora pistillata*. Chlorophyll (a+c) content in zooxanthellae in experiments 1–3 and control.

Figure 5. *Stylophora pistillata*. Maximum gross photosynthetic rates of the coral calculated per cm^2 of coral area in experiments 1–3 and control.

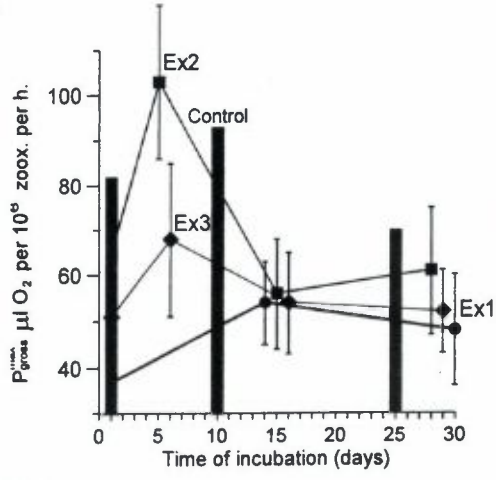

Fig. 6.

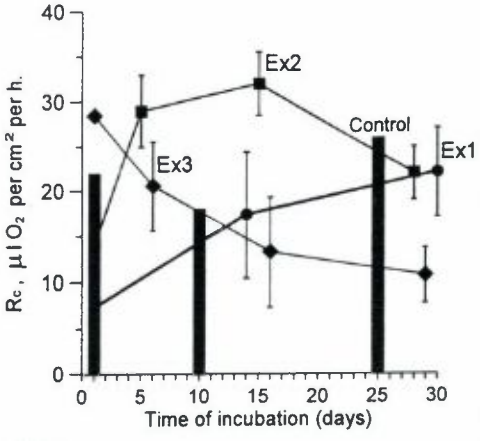

Fig. 7.

Figure 6. *Stylophora pistillata*. Maximum gross photosynthetic rates calculated per 10^6 zooxanthellae in experiments 1–3 and control.

Figure 7. *Stylophora pistillata*. Dark respiration rates (R_c) calculated per cm^2 of coral area in experiments 1–3 and control.

14 d in Exp. 2, the zooxanthellae numbers gradually increased from 10,000–12,000 to 25,000–30,000 zooxanthellae per polyp, then remained constant. In Exp. 3, there were considerable declines in the zooxanthellae population

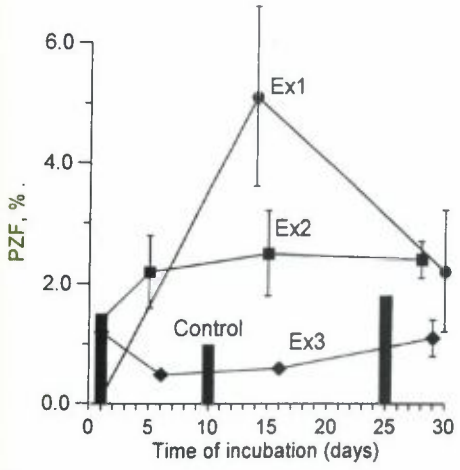

Fig. 8.

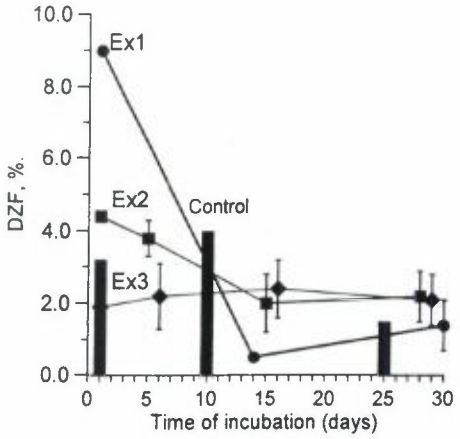

Fig. 9.

Figure 8. *Stylophora pistillata*. Proliferating zooxanthellae frequency (pzf) in tissue homogenate in experiments 1–3 and control.

Figure 9. *Stylophora pistillata*. Degraded zooxanthellae frequency (dzf) in homogenate in experiments 1–3 and control.

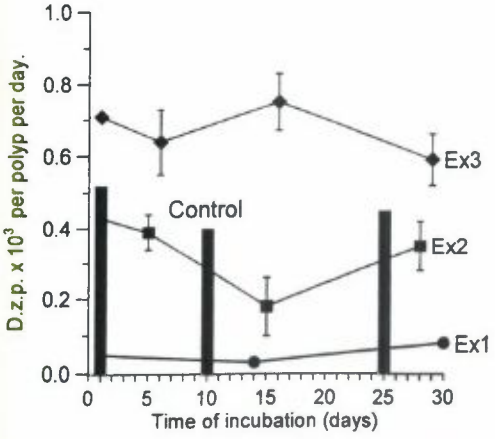

Figure 10. *Stylophora pistillata*. Release rates of degraded zooxanthellae particles (dzp) in experiments 1–3 and control.

density for the first 15 d, followed by an increase (Fig. 2). For both experiments the zooxanthellae volume fluctuated greatly but returned to initial conditions by the end of the experimental period (Fig. 3). For the first 15 days the chlorophyll content in zooxanthellae increased in both experiments, then increased only in Exp. 2 (Fig. 4). In both experiments 2 and 3, changes in the P^{max}_{gros} rate were directly proportional to the changes in zooxanthellae

densities in the polyps (Figs. 2 and 5). Changes in the potential photosynthetic capacity of zooxanthellae were similar in both experiments, increasing for the first few days, dropping over the next 10 days and remaining steady over the last 15 days of the experiment (Fig. 6). The changes in coral respiration rates in both experiments were directly proportional to the changes in zooxanthellae densities (Figs. 2 and 7).

Over the first 15 days, the pzf level increased (Fig. 8) and the dzf level decreased in Exp. 2 (Fig. 9). In concordance, the pzf/dzf ratio increased from 0.3 to 1.3, and the number of degraded zooxanthellae particles (dzp) decreased (Fig. 10). In contrast, the pzf level decreased and the dzf level slightly increased, to give a pzf/dzf ratio change of 1.2 to 0.5 in Exp. 3 (Figs. 8 and 9). During the next 15 days the pzf/dzf ratio was virtually the same in Exp. 2, but increased two-fold in Exp. 3.

4. Discussion

The *S. pistillata* coral branches, with low induced numbers of zooxanthellae, acclimated to well-lit habitats and maintained under dim light (20% of surface PAR) and with a surplus supply of zooplankton, showed recovery of zooxanthellae population density. The recovery characteristics were a decrease in zooxanthellae degradation and an increase in zooxanthellae division. An increase in the symbiont population density under low light intensities has been shown in many instances (Drew, 1972; Zvalinsky et al., 1978; Dustan, 1979; Titlyanov et al., 1980; Titlyanov, 1991). But the opposite has also been reported (Falkowski and Dubinsky, 1981; Dubinsky and Jokiel, 1994). How these processes are regulated is uncertain. Rees (1991) considered that cell division may be regulated by the host restricting nutrient(s) supply.

In this study, reduced light intensity with *ad lib* food led corals to not only increase their zooxanthellae density per polyp but also to increase their overall photosynthetic capacities. However, the photosynthetic capacities of the coral did not stem from the zooxanthellae increasing their individual photosynthetic capacities but was merely due to an increase in zooxanthellae numbers. This is shown by the lack of a direct relationship between the coral's photosynthetic capacity per area of coral tissue and the photosynthetic capacity per number of zooxanthellae. Notably, over the first five days of experiment 2 and 3, the photosynthetic capacities of the zooxanthellae did increase but returned to initial conditions after two weeks. Similarly, Clayton and Lasker (1984), studying the sea anemone *Aiptasia pallida*, reported that although the maximum gross photosynthesis of the anemone, calculated on a mg of protein, increased 1.5 times in fed animals, because of an increase in the zooxanthellae population density, there were no differences in the potential

photosynthetic capacities of zooxanthellae and in the quantity of the photosynthetic units (PSU) for fed and starved animals.

It has often been shown that zooxanthellae in corals from deep-water, or from shaded habitats, have reduced photosynthetic capacities in comparison to zooxanthellae in corals from well-lit habitats (Leletkin et al., 1980; Zvalinsky et al., 1980; Falkowski and Dubinsky, 1981; Titlyanov et al., 1988). In this study, zooxanthellae in corals adapting to low light intensity did not lose their photosynthetic capacities. This may be because of the relatively short exposure period to dim light. Increases in the zooxanthellae's chlorophyll were apparent for *S. pistillata* corals fed with zooplankton, but the average cell volume did not change (as in Exp. 2, 3), or even decreased (as in Exp. 1). Muscatine et al. (1989) also found slight increases in chlorophyll content in the zooxanthellae of *S. pistillata* fed with *Artemia salina* nauplii. However, in our experiments we tested colonies from well-lit (Exp. 1 and 2) and shaded habitats (Exp. 3), and since only the zooxanthellae's pigmentation from the well-lit habitats increased we suggest that the zooxanthellae from well-lit habitats were adapting to the low light intensity. Increasing the zooxanthellae's pigment concentration is one of the coral's main adaptive responses to low light (Titlyanov et al., 1980; Falkowski and Dubinsky; 1981; Dustan, 1982; Dubinsky et al., 1984).

In experiments 1 and 2, an increase in zooxanthellae density showed a corresponding increase in the rate of dark respiration, that, in all probability, occurred because of an increase in zooxanthellae respiration. Indeed, it was recently shown that zooxanthellae respiration can amount to 30% of the coral's respiration in *S. pistillata* (Leletkin et al., 1996). Similarly, it has been shown that a reduction in a polyp's respiration rate is affected by reducing the light intensity (as in experiment 3, and see Muscatine et al., 1984; Porter et al., 1984; Titlyanov et al., 1988; Leletkin et al., 1996).

The daily number of dzp released was ten times higher than the number of hz released, which corroborate the supposition by Titlyanov et al. (1996) that the release of hz under normal conditions has little significance in the regulation of zooxanthellae density in hermatypic corals. Jones and Yellowlees (1997) attempted to model recovery and stabilization of zooxanthellae population density in the staghorn coral *Acropora formosa* based only upon mitotic indices and hz expulsion rates during a post-bleaching period. However, the authors point out that "expulsion of zooxanthellae are not sufficient to account for the observed repopulation given a duration of cytokinesis of 11 h without the inclusion of an additional loss of zooxanthellae in the form of degenerate zooxanthellae" (p. 463, Jones and Yellowlees, 1997). In our opinion it is most useful to consider the dzp extrusion, or dzf index, when calculating the changes in algal population densities.

Corals with high numbers of zooxanthellae did not increase their

zooxanthellae population density, either because they were incapable of further accumulation, due to space limitation (Drew, 1972; Jones and Yellowlees, 1997), or they were not receiving a requisite stimulant. We are not inclined to explain the lack of zooxanthellae accumulation due to space limitation because 25×10^3 zooxanthellae per polyp (as in Exp. 3) is far from our maximum symbiont population density recorded for *S. pistillata*. *S. pistillata* adapted to 6% PAR_0 showed 42,400 ± 2,300 zooxanthellae per polyp (unpublished data). We suggest that the S. pistillata coral colonies taken from shaded habitats, on Sesoko Island, do not respond to 20% PAR_0 and high concentrations of rotifers as the signals to algal accumulation. In contrast, corals taken from well-lit habitats and placed to 20% PAR_0 respond by accumulating zooxanthellae.

In conclusion this study found that: 1) rapid increases in zooxanthellae population densities in corals are caused by an increase in the mitotic index and a decrease in zooxanthellae degradation; 2) Zooxanthellae accumulation increased the photosynthetic capacities and respiration level of the coral; 3) The regulation of symbiont population density in the coral *S. pistillata*, under photoacclimation to lowered light intensity (and *ad lib* zooplankton), occurred by changing the rates of zooxanthellae degradation and division. Healthy zooxanthellae are released from the coral but do not play an essential role in the regulation of the algal population density; 4) Corals with high zooxanthellae numbers are not capable to accumulating additional symbionts, even if supplied with an excess food.

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