Study on the thermal sensitivity of cnidarian-algal symbiosis using Aiptasia model system

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Table of Contents

Chapter 1
General Introduction

Coral belongs to the group Anthozoa of phylum Cnidaria and is generally categorized into major two groups, reef-building, or hermatypic, corals and non-reef-building, or ahermatypic, corals. Reef-building corals have rigid skeleton made by calcium carbonate ($CaCO_3$). Non-reef-building corals have a flexible body with no significant skeleton; they make skeletal elements in the cells of epithelial tissue. A colony of corals consists of massive amount of individual structures called polyps. An individual coral polyp has a stomach and tentacles which is used for defense and to capture food. In colonies, individual polyps are connected to each other via gastrovascular canals and can share nutrition.

Reef-building coral provides a biologically enriched coral reef ecosystem. Coral reef covers only 0.1% of the surface area of ocean, however, it supports at least 25% of total marine species (Hoegh-Guldberg et al., 2007). 4,000 species of fish, 800 species of hard corals and other species are living around reefs (Costanza et al., 1997). Coral reefs are also important in the economic aspect; the value of fisheries from coral reefs in the U.S. is over 100 million dollars (NMFS/NOAA. 2001). The most famous reefs, the Great Barrier Reef, offer fishing and tourism and provide 1.5 billion dollars every year (Queensland museum). Coral reefs in the world generate 375 billion dollars per year with goods and services (NMFS/NOAA. 2001). However, today more than 50% of coral reefs in the world have already declined (Bruno and Selig, 2007), mainly due to coral bleaching caused by increase in the seawater temperature (Lough and Barnes, 2000). The seawater temperature is predicted to increase 1-3 °C over the next 100 years (IPCC 2001), suggesting that severe damage to corals could cause the destruction of coral reef ecosystems in future (Descombes et al., 2015). Some studies predicted that as much as 60 % of coral reefs will have declined by 2030 (Hughes et al., 2007). Despite the predicted loss of coral reefs, whether corals can acquire tolerance to survive in the warmer environment remains unknown. Further understanding about the mechanism of coral bleaching is necessary.

Reef building corals form a symbiotic relationship with dinoflagellates of the family Symbiodiniaceae. Symbiotic algae transport photosynthetic products to the host tissue (Burriesci et al., 2012). The nutrition supplied by algae is essential for growth and building the calcium carbonate skeleton in the coral. In return, algae receive nitrogen, phosphorus and CO_2 from the coral (Ateweberhan et al., 2013), and a stable position to escape from UV-damage. Corals recruit algae by the inheritance of algae from the parent or the acquisition of algae from

the environment. The latter transmission mechanism is expected to give an advantage in possessing suitable algae in the habitat. The first step in initiating a symbiotic relationship is recruiting algae into host cells. The algal cell is expected to enter a host cell through phagocytosis. The algal cell entered into the host cell is then surrounded by symbiosome membrane. The density of algae within corals can reach more than 10^6 cells per cm^2. This relationship is fragile, however, and is severely damaged by climate changes (Goreau, 1964; Hughes et al., 2003). Breakdown of the symbiosis in the form of coral bleaching is induced by a small increase in seawater temperature (0.5 – 1.5 °C) over several weeks or a large increase (3 – 4 °C) over a few days (Jokiel and Coles, 1990).

The thermal sensitivity of corals differs among taxa; for example, *Acropora* significantly declined during the high temperature period of 1998 in Japan and it is known as a heat sensitive coral species (Hongo and Yamano, 2013). On the other hand, *Goniastrea* successfully survived during the high temperature period and it is known as a heat tolerant coral species (Hongo and Yamano, 2013). Generally, boulder-shaped corals have higher tolerance to warm environments than branching corals (Loya et al., 2001; Marshall and Baird, 2000). However, interestingly, the thermal sensitivity sometimes differs among colonies in a same coral species (Fong and Glynn, 2001). Coral translocation experiments showed that the thermal sensitivity of corals can be changed by the environment, as occurs when corals change their symbiotic algae (Rowan, 2004). Moreover, juvenile corals infected by algae from a cooler reef underwent bleaching and tissue death at elevated temperatures while those infected by algae from a warmer reef did not (Berkelmans and van Oppen, 2006). These reports suggest that the thermal sensitivity of a coral can be influenced by their symbiotic algae.

The family Symbiodiniaceae includes seven genera, formerly in nine clades (LaJeunesse et al., 2018). Each genus includes different species or strains. Thus, the family Symbiodiniaceae consists of genetically and physiologically diverse species. Among genera in the family of Symbiodiniaceae, the difference can be seen in genome size, cell size, photosynthetic activity and stress tolerance for such as heat and strong light (Berkelmans and van Oppen, 2006; Karim et al., 2015; LaJeunesse et al., 2010; Sampayo et al., 2008; Takahashi et al., 2008; Takahashi et al., 2009). Such differences can be sometimes seen among strains in a same genus. These differences might affect to the susceptibility of the symbiotic relationship with host cnidarians. This hypothesis is supported by many reports which showed corals with Symbiodiniaceae *Cladocopium* (formerly clade C) tend to get more severe bleaching than corals with Symbiodiniaceae *Durusdinium* (formerly clade D) under increased temperatures

(Baker, 2014; Kemp et al., 2014). Thus, physiological feature of corals including the thermal tolerance is decided by the combination of coral type and symbiotic algal type.

The effect of heat stress is seen in the process of establishing the cnidarian-algal symbiosis. Most reef-building coral species must establish the symbiotic relationship with algae in each generation at the stage of larvae or juveniles (Cumbo et al., 2018). The source of the algae is free-living algae from the environment (Cumbo et al., 2018). The process of symbiosis establishment (This process is referred as uptake of algae or infection of algae in this study) consists of several steps: acquisition of algae into polyps, retention of algal cells in gastrovascular cavity, transportation of algal cells through gastrovascular system, engulfment of algae into gastrodermal tissue through phagocytosis and recognition. However, previous studies have shown that heat stress suppresses the uptake of algae to larvae (Abrego et al., 2012; Cumbo et al., 2018; Schnitzler et al., 2012). Since the photosynthetically produced energy from algae is required for growth and skeleton building of corals, failure of algal uptake crucially decreases the survival rate of coral juveniles (Schnitzler et al., 2012). Similar phenomenon has been observed in the apo-symbiotic polyps of Aiptasia (Gabay et al., 2019). However, its mechanism has remained unknown.

The adult coral polyps can suffer serious damage through the loss of algal density (bleaching) due to the stress of elevated temperature (Fujise et al., 2014; Glynn and D'Croz, 1990; Hughes et al., 2003; Hughes et al., 2018; Jokiel and Coles, 1990). Bleaching occurs through several mechanisms, however, the most major mechanism is the expulsion of algal cells from coral polyps (Bieri et al., 2016). Corals show active expulsion of algae by few days of high temperature period and their color turn to pale due to significant loss of algal density (Bieri et al., 2016; Tolleter et al., 2013). It is expected that the trigger of the expulsion is the production of harmful reactive oxygen species (ROS) in algal photosystem, which has been observed in the similar timing with expulsion (Dykens et al., 1992; Lesser, 1996; Lesser, 1997; Lesser et al., 1990). Higher expression of antioxidant proteins in corals under higher temperature supported this hypothesis (Downs et al., 2000). However, a recent study showed that loss of algal cells from corals occurs under heat stress in complete darkness, suggesting that neither ROS production nor damage on photosystem is necessary for the expulsion (Tolleter et al., 2013). Therefore, the mechanism for bleaching under heat stress is still controversial.

Corals rely on their symbiotic algae for most of the energy they use. Therefore, serious bleaching can cause mortality of corals by starvation. Bleaching is not always lethal because corals can recover their algal density before they completely starve. Recovery of algal density

in corals has been reported in natural condition (Luke Thomas 2017), as well as in experiments (Line K Bay 2016). However, at the global scale, most of corals die after bleaching, implying the recovery is often limited (Hoegh-Guldberg, 1999; Hughes et al., 2017; Hughes et al., 2018). One of the inhibition factors should be the prolonged high temperature periods because the uptake of algae to coral tissue can be suppressed by heat stress (Abrego et al., 2012; Cumbo et al., 2018). Therefore, the recovery may occur effectively once the temperature returns to normal. However, in most cases, corals still do not recover even at lower temperatures.

The mechanisms associated with coral-algal symbiosis breakdown have been intensively studied across the world for the last few decades. However, we still have a big gap to understand it at molecular level. One reason is that most of the coral studies use coral colonies from the field, which can introduce uncontrollable varieties of different environmental and biological background which results in lower reproducibility. Another problem is difficulty in the maintenance of fragile coral samples in the artificial environment, which slow down the study. To solve these problems, the sea anemone Aiptasia model system has been developed recently (Baumgarten et al., 2015; Hambleton et al., 2014; Rädecker et al., 2018; Van Treuren et al., 2019). Both coral and sea anemone Aiptasia belong to Anthozoa of Cnidaria and they establish the symbiotic relationship with same algae of Symbiodiniaceae (Hambleton et al., 2014). Furthermore, increase in the seawater temperature leads to bleaching of Aiptasia as seen in corals (Tolleter et al., 2013). Moreover, it is easy to grow Aiptasia in laboratory conditions with or without symbiotic algae (Hambleton et al., 2014), which makes it possible to artificially infect specific type of algae.

In this study, using the sea anemone Aiptasia model system, I investigated how heat stress harms cnidarian-algal symbiosis. I introduce my results in the three chapters. In the first chapter, I show the effect of heat stress on the establishment of cnidarian-algal symbiosis. In the second chapter, I show the effect of heat stress on the maintenance of cnidarian-algal symbiosis. In the third chapter, I show the effect of heat stress on the re-establishment of the symbiosis, i.e. recovery from bleaching. In the last section, general discussion, I propose how heat stress has damaged coral reefs through destruction of the cnidarian-algal symbiotic relationship.

Chapter 2

The effect of heat stress on the establishment of cnidarian-algal symbiosis

2.1 Introduction

Most reef-building corals establish the symbiotic relationship with symbiotic algae of Symbiodiniaceae by acquiring free-living algal cells from the environment when they are in the stage of larvae or juveniles (Cumbo et al., 2018). However, increase in the seawater temperature suppresses the symbiosis establishment (Abrego et al., 2012; Cumbo et al., 2018). Similar suppression has been observed in adult Aiptasia polyps (Hawkins et al., 2016). The process of establishing symbiosis between a cnidarian host and algae (This process is referred as uptake of algae or infection of algae in this study) consists of several steps including acquisition of algae through mouth from the environment, retention of algae in the gastrovascular cavity, transport of algae through gastrovascular system, engulfment of algae into the endoderm tissue through phagocytosis and recognition. The suppression of algal uptake by heat should occur due to the inhibition of either of these steps, however, the mechanism is not yet clear.

Another question has been which symbiotic partner (host cnidarians or symbiotic algae) is directory affected by heat stress. Previous reports described heat stress responses of the holobiont at molecular level. Heat shock proteins (HSP) were identified in host cnidarians, such as corals and sea anemones following the first report with *Goniopora dijboutensis* (Black et al., 1995; Branton et al., 1999; Sharp et al., 1994). These HSPs were also discussed about their potential role in symbiosis (Richier et al., 2005). In Aiptasia, the expression of several HSPs has been reported to be symbiotic state specific (Ishii et al., 2019). These changes in gene expressions of cnidarian hosts may be related to the breakdown of the symbiosis with algae, however, their direct relationship is not clarified.

Heat stress responses in symbiotic algae also has been described. Some HSP gene families are up-regulated under heat stress especially when they are hosted by Aiptasia (Ishii et al., 2019). These stress reactions can be related to symbiosis, although further analysis is needed.

Thus, heat stress responses have been described in host cnidarians as well as in symbiotic algae. However, it is still unclear whether cnidarians or algae, whose stress response plays the main role in the suppression of symbiosis. In this study, this question was solved using Aiptasia model system. By measuring the symbiosis efficiency after the Aiptasia and/or the algae of Symbiodiniaceae were separately heat-treated, contributions of their thermal

sensitivity on the symbiosis were analyzed. I repeated the experiment with another algal strain to consider the effect from difference of algal strain. Furthermore, in this study, the effect of heat stress on each symbiosis step was investigated to clarify which steps can change the efficiency under heat stress. These findings give new insights to the mechanism of heat-induced suppression of symbiosis and what determines the thermal sensitivity.

2.2 Materials and methods

Materials and culture conditions.

Sea anemone *Exaiptasia pallida* (strain H2, commonly called Aiptasia) polyps were provided by Professor John Pringle (Stanford University). To eliminate their original symbiotic algae, polyps were incubated at 34 °C in darkness for 1 month. The elimination of all algae was confirmed by the inability to detect any fluorescence from algae under a fluorescence microscope (Leica M165FC) under 200 X magnification. The polyps of apo-symbiotic Aiptasia were cultured in filtered artificial seawater (REI-SEA marine 2; IWAKI) at 25 °C in darkness and fed freshly hatched *Artemia* nauplii once a week, until used in the experiments.

Symbiodiniaceae CCMP2459 (ITS2 type B2) was obtained from the National Center for Marine Algae and Microbiota (USA), and Symbiodiniaceae CS-164 (ITS2 type B1) from the Australian National Algae Culture Collection (AUS). To ensure that cultures were monoclonal, obtained cultures were subcultured from a single cell and their genotypes confirmed in the previous study (Biquand et al., 2017). Algal cells were grown in filtered (0.22 µm pore filter; Steritop-GP Filter Unit, Merck Millipore) artificial seawater (sea salt; Sigma-Aldrich) containing Daigo's IMK medium for marine microalgae (Wako). They were cultured in a growth cabinet MLR-350 (SANYO) at 25 °C under a cycle of 12 h of fluorescent light (100 µmol photons m^{-2} s^{-1}) and 12 h of darkness.

Aiptasia infected with cultured algae were obtained by culturing apo-symbiotic Aiptasia polyps with the algae of Symbiodiniaceae (40,000 cells ml^{-1}) in a 10 cm diameter dish with 50 ml of artificial seawater. Polyps were cultured at 25 °C under a cycle of 12 h of fluorescent light (100 µmol photons m^{-2} s^{-1}) and 12 h of darkness. Infection of algae into the host was confirmed by the change in coloration (white/brown). They were fed freshly hatched *Artemia* nauplii once a week, until used.

Temperature treatments.

Cultured algae of Symbiodiniaceae were collected by centrifugation (1,500 g for 3 min) and the seawater was replaced with new seawater (REI-SEA marine 2; IWAKI). Apo-symbiotic

Aiptasia polyps were placed in 6-well plates with 5 ml of fresh seawater. For temperature treatments, algal cells and Aiptasia polyps were separately incubated at a growth temperature of 25 °C or an elevated temperature of 32 °C in darkness.

Measurement of algal uptake in Aiptasia.

Aiptasia polyps were individually placed in 6 well-plates with 5 ml of artificial seawater. Algal cells (approximately 9000 cells) were collected by centrifugation (1,500 g for 3 min) and mixed with fresh *Artemia* nauplii (3-5 individuals in 3 µl seawater) for making a pellet. *Artemia* nauplii were used to accelerate the introduction of an algal pellet into the gastrovascular cavity. The pellet was fed to Aiptasia using a glass Pasteur pipet (Supplementary Fig. 1). Aiptasia polyps were then incubated at 25 °C for maximum 5 days under continuous darkness or a cycle of 12 h of light (100 µmol photons m^{-2} s^{-1}) and 12 h of darkness for infection. Algal density (algal number per area) in Aiptasia was measured by counting algal cells in tentacles with a stereo fluorescence microscope (Leica M165FC or M205FA) after the incubation.

Measurement of microbeads uptake by Aiptasia.

Aiptasia polyps were individually placed in 6 well-plates with 5 ml of artificial seawater. Yellow Green microspheres (Fluoresbrite (YG), 2.5% solids-latex, excitation max.=441 nm; emission max.=486 nm) with diameters of 6.3±0.18 µm (Polysciences, Inc., Warrington, PA, USA) were collected (approximately 9,000 particles) by centrifugation (1,500 g for 3 min) and mixed with fresh *Artemia* nauplii (3-5 individuals in 3 µl seawater) for making a pellet. The pellet was directly fed to Aiptasia using a glass Pasteur pipet. Aiptasia polyps were then incubated at 25 °C for 3 days under a cycle of 12 h of light (100 µmol photons m^{-2} s^{-1}) and 12 h of darkness for uptake of microbeads.

Measurement of algal and microbeads distribution in Aiptasia body.

After an inoculation of Aiptasia polyps with algal cells or with microbeads at 25 °C for 3 days, the density of algal cells or microbeads were measured at their tentacles and stomach cavity using a microscope. Ratio of cells or microbeads transported to tentacles was calculated by the number of cells or microbeads on tentacles divided by the number of them remained in stomach cavity. The ratio of cells or microbeads retained in a body was calculated by measurement of the number of cells or microbeads in the Aiptasia body before and after temperature treatments.

Measurement of algal viability, growth and cell morphology.

The viability of algal cells was measured by Evans blue staining following to a previous study (Morera and Villanueva, 2009). Evans blue stains cells with compromised membrane structures. We first examined with a light microscope (Olympus CX43) that CS-164 cells are stained by Evans blue after the expose to heat shock at 80 °C for 1 hour (Supplementary Fig. 2). Less chlorophyll fluorescence was observed using an inverted fluorescence microscope (Olympus IX71) with filters (WIG filter: Excitation 520~550nm band pass, Detection 580nm~) in Evans blue-stained cells, indicating that Evans blue staining is available for the detection of damaged CS-164 cells.

Algal cells (40,000 cell ml^{-1}; 1 ml) were incubated at 25 °C or 32 °C in darkness for 3 days. Then, cells were used for the measurement of viability. Approximately, 50 cells were used for each viability test. Observations were taken under a light microscope (CX43 Olympus). The cell viability was scored by counting live cells (non-stained cells) versus dead cells (blue-stained cells).

For monitoring cell density, algal cells (40,000 cell ml^{-1}; 10 ml) were incubated at 25 °C or 32 °C in the dark for 5 days. Algal cell density was measured by counting cells using a hemocytometer under a microscope.

2.3 Results

The effect of high temperature on the uptake of two algal strains to host Aiptasia.

To examine the effect of high temperature on the uptake of algae to hosts, two Symbiodiniaceae strains, CS-164 and CCMP2459 were used as the symbiotic algae and Aiptasia H2 as the host. I first eliminated the original algae (Symbiodiniaceae *Breviolum*) from Aiptasia polyps by 1 month of continuous heat treatment. I confirmed their complete loss of algae (apo-symbiosis) by the absence of red fluorescence from algal chlorophyll in polyps (Fig. 2-1). Then, I monitored the number of algae in tentacles, following to the introduction of each algal strain at a growth temperature (25 °C) or at an elevated temperature (32 °C) (Supplementary Fig. 1). At 25 °C, both algal strains quickly and efficiently infected to Aiptasia polyps. However, at 32 °C, CS-164 did not increase the density in polyps, on the other hand, CCMP2459 showed significant increase in the density in polyps (Fig. 2-2).

To examine whether the suppression of algal uptake upon the exposure to elevated temperature occurs due to heat stress on algae or on Aiptasia, CS-164, CCMP2459 and apo-symbiotic Aiptasia polyps were separately incubated at 25 °C or 32 °C for 3 days and then

inoculated at 25 °C for 3 days to infect. After the 3 days inoculation, the density of algal cells in tentacles was measured. Under the optimal temperature at 25 °C, both algal strains infected the host (Fig. 2-3). In CS-164, when both algae and Aiptasia were pre-incubated at 32 °C, the infectivity was significantly lower than both pre-incubated at 25 °C (Fig. 2-3). Importantly, when algae and Aiptasia were pre-incubated at 32 °C and 25 °C, respectively, infectivity was similar to that with both pre-incubated at 32 °C. While, when algae and Aiptasia were pre-incubated at 25 °C and 32 °C, respectively, there was no statistical difference with that when both were pre-incubated at 25 °C (Fig. 2-3). In CCMP2459, neither host nor algae showed change in their infectivity due to the high temperature (Fig. 2-3). Our results demonstrated that uptake of algae to hosts decreases by heat stress on algae and the thermal sensitivity varies depending on algal strain.

The effect of heat stress on the process of algal uptake in Aiptasia body.

Uptake of algae to a host cnidarian has at least four processes: (1) acquisition of algal cells into the host body through mouth, (2) retention of algal cells in the gastrovascular cavity, (3) transport of algal cells to the endoderm tissue, (4) endocytotic engulfment of algal cells into host cells (Fig. 2-4a). To test how heat stress suppresses the symbiosis, algal cells which had pre-incubation at control (25 °C) or heat (32 °C) condition were introduced into the gastrovascular cavity of Aiptasia polyps and the distribution change of algal cells in polyps were monitored (Supplementary Fig. 1). When I introduced algae pre-incubated at 25°C, most algal cells were retained in Aiptasia body during the incubation (Fig. 2-4c). Some cells were transported to endoderm tissue at tentacles from gastrovascular cavity and the ratio of the cells in tentacles linearly increased (Fig. 2-4b, c and d). On the other hand, with algal cells pre-incubated at 32°C, only less than 50% of the introduced cells were retained in Aiptasia body at the end of the observation (Fig. 2-4b and c). However, the ratio of algae transported to tentacles was similar between treatments (Fig. 2-4c and d). These results indicate that the retention of algal cells in an Aiptasia body declines by heat on algae, while the efficiency of algal transportation in the Aiptasia body does not change by heat treatment.

Heat stress on algae and its effect on the activity of algal uptake in Aiptasia.

To test whether the activity of algal uptake in Aiptasia, including the process of algal cell retention, transportation and engulfment, changes by heat stress on algal, I compared the ability of algal uptake in Aiptasia when they were inoculated with algal cells pre-incubated at 25 °C or at 32 °C for 3 days. The distribution of algal cells in polyps can be affected both by host

behavior and by algal behavior. To exclude the effect of algal activity and visualize only the host activity of algal uptake, I introduced algae with fluorescent microbeads to the Aiptasia body and monitored the distribution of the microbeads, instead of algal cells. I chose the microbeads which have similar shape with algal cells to model how Aiptasia retain and transport algal cells in their body. When I introduced microbeads with algae which had been pre-incubated at 25 °C, microbeads spread through the Aiptasia body and some of them were observed at tentacles (Fig. 2-5b and d). However, when I introduced microbeads with algae which had been pre-incubated at 32 °C, the density of microbeads observed at tentacles became significantly smaller than with the algae pre-incubated at 25 °C (Fig. 2-5b and d). When I introduced only microbeads, there was no statistical difference with the result with algae from 25 °C, but the variability became larger compared to the samples with algal cells (Fig. 2-5b and d). The number of microbeads at tentacles was consistent with the number of algal cells on tentacles (Fig. 2-5a and c). These results indicate that the activity of algal uptake in Aiptasia changes by heat stress on algae.

To examine how uptake of microbeads to Aiptasia changed (Fig. 2-5), I monitored the distribution change of microbeads in an Aiptasia body (Fig. 2-6a). When I introduced microbeads and algae pre-incubated at 25°C to Aiptasia, most of the microbeads were retained in the body, and some of the microbeads were transported to tentacles (Fig. 2-6b and c). However, the amount of microbeads retained in the body became smaller when heat-treated algae were introduced together (Fig. 2-6b and c). The ratio of the microbeads in tentacles was also smaller with heat-treated algae (Fig. 2-6c). These results indicate that algal cell retention and transportation by Aiptasia are suppressed under the presence of heat-treated algal cells.

The effect of heat stress on algal cell morphology.

To analyze morphological change in algal cells upon heat stress, I measured the viability, cell size and chlorophyll fluorescence before and after the temperature treatment at 25 °C or 32 °C for three days. I measured the viability of CS-164 cells by Evans blue staining. The viability of the cells did not decrease by high temperature stress (Fig. 2-7a and b). The cell growth of CS-164 cells was measured by counting cell number using a hemocytometer and could not find any difference between temperatures (Fig. 2-7c). Cell size and chlorophyll fluorescence of CS-164 and CCMP2459 cells was measured by Attune Flow Cytometry system. The intensity of chlorophyll fluorescence changed from initial in any samples, but no temperature specific difference was detected (Fig. 2-7d). No temperature specific or strain specific change was detected in cell size which were analyzed as forward scatter area.

The potential effect of heat-induced change in algal culture media to the infectivity.

To test a potential effect of bacteria in the seawater media to the algal infection tests, I collected the seawater media after temperature treatment of algae at 25 °C or 32 °C and then Aiptasia polyps were incubated in these seawater media. During the incubation, the activity of algal uptake by Aiptasia were measured using fluorescent microbeads. There was no significant difference in the distribution of microbeads in Aiptasia between these two seawater medias (Fig. 2-8). This result indicates that there was no considerable change in effectiveness of other organisms in the seawater on the infection of algae to Aiptasia.

2.3 Discussion

Heat stress response in algae, but not in host was related to the suppression of the symbiosis establishment.

Symbiosis establishment between cnidarians and symbiotic algae can be suppressed by heat stress (Abrego et al., 2012; Cumbo et al., 2018). Heat stress responses such as the expressions of heat shock proteins has been described both in host cnidarians and in algae at the transcription level as well as at the protein level (Black et al., 1995; Branton et al., 1999; Ishii et al., 2019; Richier et al., 2005; Sharp et al., 1994). However, it has been unclear which responses are related to the efficiency of algal uptake. In this study, I demonstrated that the uptake of algae by host Aiptasia was suppressed by heat stress only on algae, while no significant effect was observed by heat stress on Aiptasia (Fig. 2-3). These results suggest that heat stress responses in algae are related to the suppression of symbiosis establishment. Using algal strain CCMP2459 and CS-164, I demonstrated that the thermal sensitivity of their infectivity varies by algal strain (Fig. 2-3). These two algal strains with different thermal sensitivity might be useful in future studies to approach the mechanisms of heat-induced symbiosis suppression at the molecular level.

The effect of heat stress on the symbiosis processes.

The process of algal uptake (i.e. establishing of the cnidarian-algal symbiosis) has several processes, including acquisition of algal cells from the environment to a host body, algal retention in the gastrovascular cavity, transportation of algae to the endoderm tissue, engulfment of algal cells into host cells through phagocytosis and recognition (Parrin et al., 2016). In the present study, heat-sensitive algae of CS-164 was used to examine the effect of heat stress on these symbiosis processes (Fig. 2-4, 2-5 and 2-6). The efficiency of algal uptake

was always tested after introducing the same number of algal cells into the gastrovascular cavity of Aiptasia. Therefore, the suppression of algal uptake observed in this study is related to the process after the acquisition of algae to the host cavity. The results in Figure 2-4 showed that the ratio of algal cells transported to the endoderm tissue at tentacles did not change by temperature conditions. This result suggests that the process of algal transportation is not affected by temperature. On the other hand, the cell number remained in the body significantly decreased by heat stress (Fig. 2-4). This result suggests that the process of algal retention in Aiptasia body is suppressed by heat stress and results in less uptake of algae. Heat-treated algal cells lost from Aiptasia body might have been either expelled to outside through mouth or digested in body.

The activity of algal uptake in host Aiptasia changes depending on the condition of *in situ* symbiotic algae.

The results in Figure 2-3 showed that heat-induced suppression of symbiosis was related to the heat stress response in algae. Although Aiptasia did not show any significant change by heat stress, their behavior might change depending on whether algal cells are normal or heat-treated. I examined this possibility by measuring the activity of algal uptake by Aiptasia. In this study, I visualized the activity by introducing fluorescent microbeads into Aiptasia body. The result showed that the amount of microbeads transported to tentacles decreased under the existence of heat-treated (32 °C) algae compared to the result with normal (25 °C) algae (Fig. 2-5b and d). This result suggests that the activity of algal uptake in Aiptasia changes depending on whether algae are normal or heat-treated. Therefore, the suppression of symbiosis establishment under heat stress is not only due to the heat stress response of algal cells but also due to the subsequent changes in the host activity. This result implies that the cnidarian hosts have ability to detect algal cell condition before they engulf them to initiate the endosymbiosis. The activity in the host body might be decided by its tissue movement, such as contraction and relaxation movement.

The loss of heat-treated algae and microbeads from Aiptasia body (Fig. 2-5) can occur either by active expulsion from the Aiptasia body or by passive diffusion. The uptake of microbeads to tentacles became significantly smaller when heat-treated algae were introduced together with microbeads, compared to the result when only microbeads were introduced (Fig. 2-5b and d). Introduction of only microbeads should visualize the passive diffusion by inner flow of Aiptasia body without any host-algal communication. Therefore, this result suggests that loss of heat-treated algae occurs by active expulsion by host, rather than only passive

diffusion. This result implies that cnidarian hosts control their expulsion activity depending on the condition of algal cells retained in their body. There is a previous study about the expulsion phenomenon at normal condition in corals (Baghdasarian and Muscatine, 2000). Algal cells expelled from corals under a normal condition had greater ratio of dividing cells compared to the cells retained in the coral body, suggesting dividing algal cells are preferentially expelled (Baghdasarian and Muscatine, 2000). It has been unknown whether this expulsion occurs due to algal mobility or elimination by host. Today, I demonstrated that host Aiptasia changed their expulsion activity depending on the condition of algae. This result implies that algae in specific conditions, not only heat-treatment but also cell division, might activate the expulsion in host cnidarians.

Different thermal sensitivity between the two algal strains.

The results in Figure 2-2 and 2-3 showed that the thermal sensitivity of symbiosis varies between two algal strains, CS-164 and CCMP2459. Further observations using CS-164 in Figure 2-4, 2-5 and 2-6 showed that suppression of algal uptake by heat stress was due to increase in the expulsion of algal cells from the host mouth. Heat-treated CCMP2459 might be able to infect host because they could be less expelled from host body.

The photosynthetic activity of algae is an important factor which can affect the susceptibility of the symbiotic relationship. CS-164 and CCMP2459 shows similar photosynthetic capacity in a normal condition. In higher temperature, the activity of CCMP2459 can be lower due to the higher thermal sensitivity to occur photobleaching (Karim et al., 2015). However, in this study, the heat treatment was conducted in darkness in which algal cells can avoid photobleaching. Therefore, the photosynthetic activity is not directly related to the suppression of symbiosis observed in this study.

Previous study has suggested that algal cell size can limit the process of symbiosis (Biquand et al., 2017). The uptake might be suppressed if algal cell size increases. However, in this study, there was no significant difference in cell size of both strains between conditions (Fig. 2-7).

Recent studies have demonstrated that recognition molecules on host and algal cell surface are important for the establishment of symbiotic relationship. Previously, they were assumed to be important for the phagocytosis. However, since several studies suggested that they are not necessary for the process of phagocytosis (Biquand et al., 2017; Parkinson et al., 2018), they are probably related to other processes such as recognition. Therefore, the suppression of algal uptake under heat stress potentially occurs through changes in composition

or amount of the recognition molecules on algae or host cells. However, further studies are required to prove this hypothesis.

Potential effect of the suppression of algal uptake in coral reef ecosystem.

Algal density in cnidarian tissue is maintained by the balance between release and uptake of algal cells. Therefore, heat-induced suppression in algal uptake may contribute to the loss of algal density in corals (bleaching). I examined this possibility in Chapter 3 with the bleaching experiment using two algal strains which have different thermal sensitivity (CS-164 and CCMP2459).

Increase in algal density in corals by infection of algae is crucial not only for the larvae, but also for adult polyps when they recover from bleaching after a high temperature period. Such recovery from bleaching can be suppressed if free-living algae lose their ability to infect. To assess this possibility, further observations about the recovery potential of the infectivity in algae are needed. I examined this hypothesis in Chapter 4.

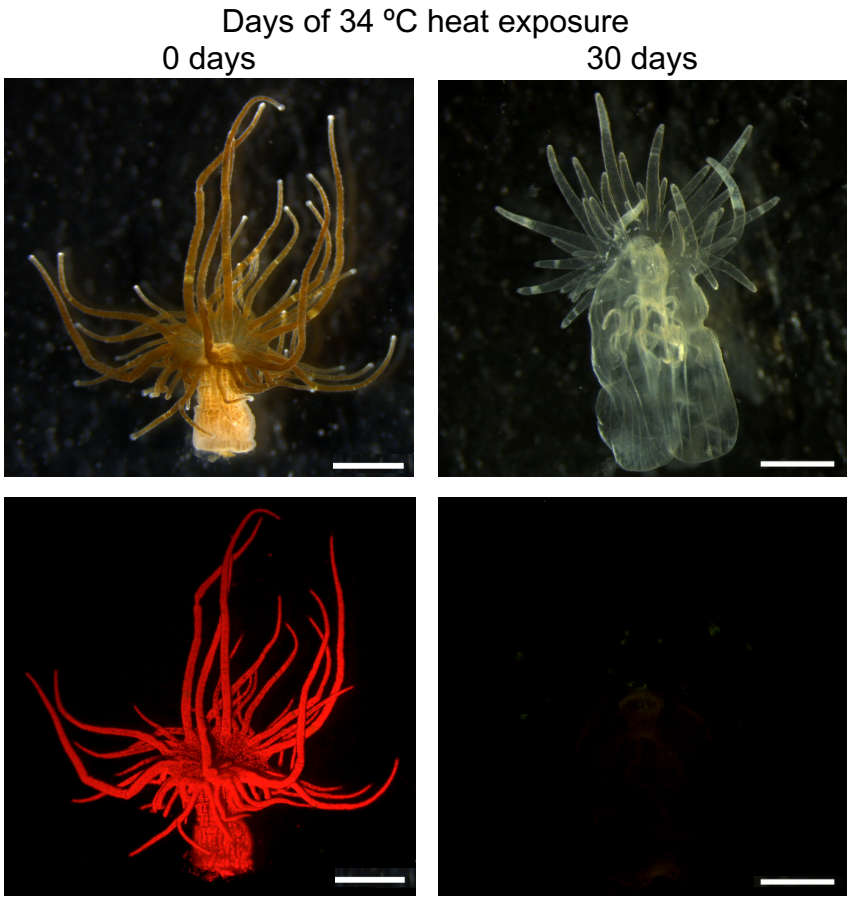


Figure 2-1. Photographs of Aiptasia with and without algae of Symbiodiniaceae.

Aiptasia H2 polyps were incubated at 34 °C for 4 weeks to remove their original algae. Upper and lower photographs are bright-field and fluorescence images, respectively. Red fluorescence represent chlorophyll fluorescence from algae. Scale is 1 mm.

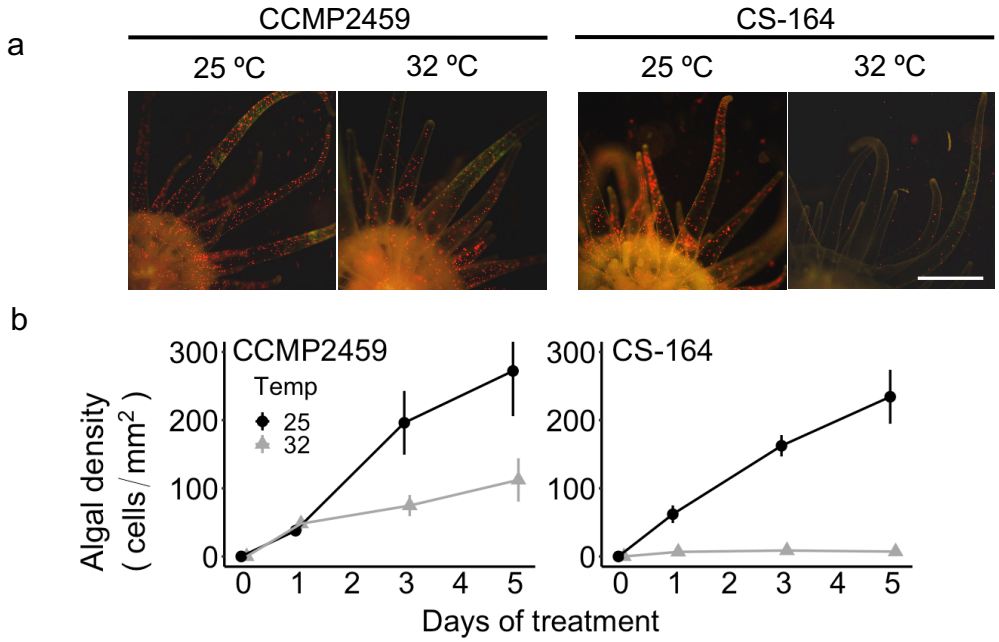


Figure 2-2. Effect of elevated temperature on the infection of the cultured symbiotic algae CCMP2459 and CS-164, in Aiptasia.

(a and b) Apo-symbiotic Aiptasia polyps were inoculated with cultured algae of CCMP2459 or CS-164 at 25 ℃ (black) or 32 ℃ (grey) for 5 days. (a) Aiptasia polyps under fluorescent microscope after 5 days incubation. Red spots indicate chlorophyll fluorescence from the algae. Scale is 1mm. (b) Number of algae in tentacles was counted. Values are mean ± SE of three separate experiments.

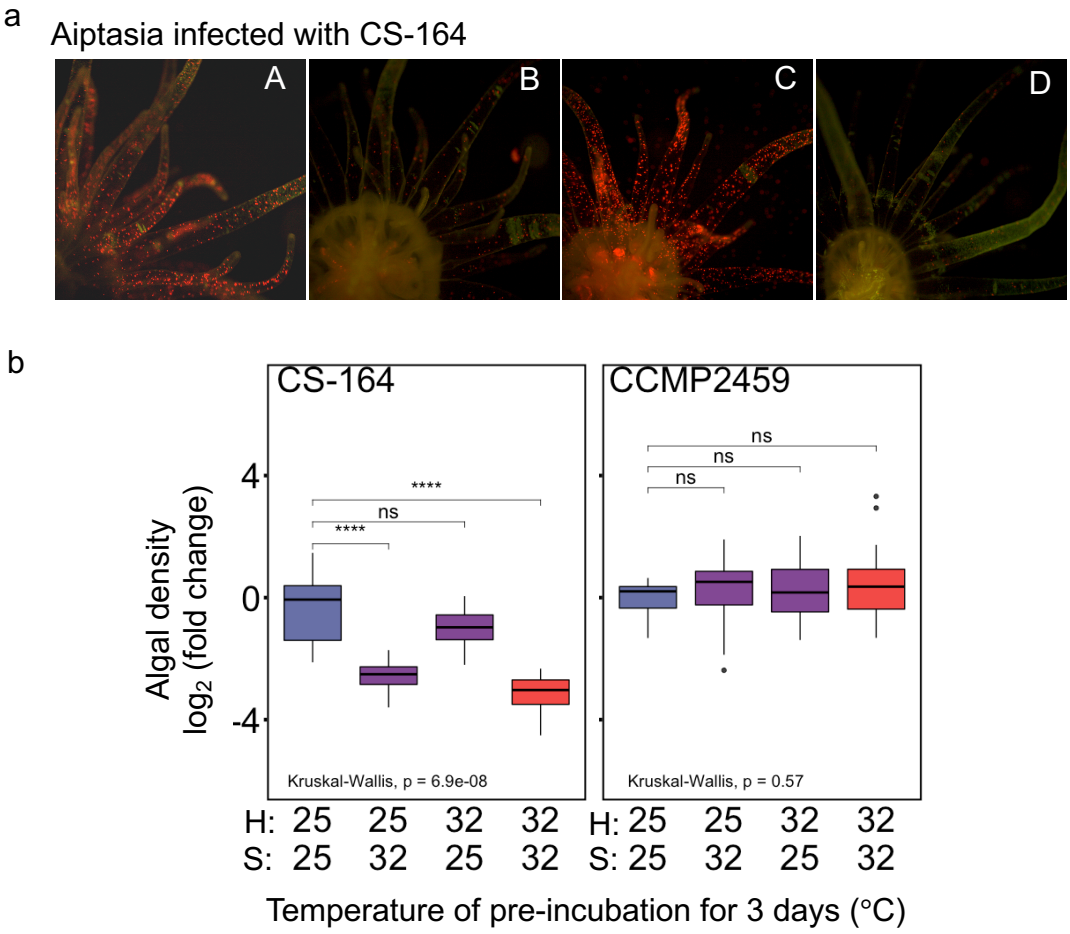


Figure 2-3. Effect of pre-incubation at the elevated temperature on following infection of algae to Aiptasia.

(a) Either cultured algae (B) or apo-symbiotic Aiptasia polyps (C), or both of them (D) were pre-incubated at 32 °C for 3 days and mixed together at 25 °C for 3 days to compare the infectivity with the condition without heat stress (A). Photographs are taken after 3 days from mixing them. (b) Algae in tentacles was counted after the inoculation for 3 days. Values are log2 fold changes to the control 25 °C. Each value is from six separate experiments. The temperatures of pre-incubation for algae (S) and Aiptasia (H) are shown below the plot. Cultured algae of CS-164 and CCMP2459 were used in the test. Symbols indicate p-values (ns: $p > 0.05$, ****: $p \leqq 0.001$).

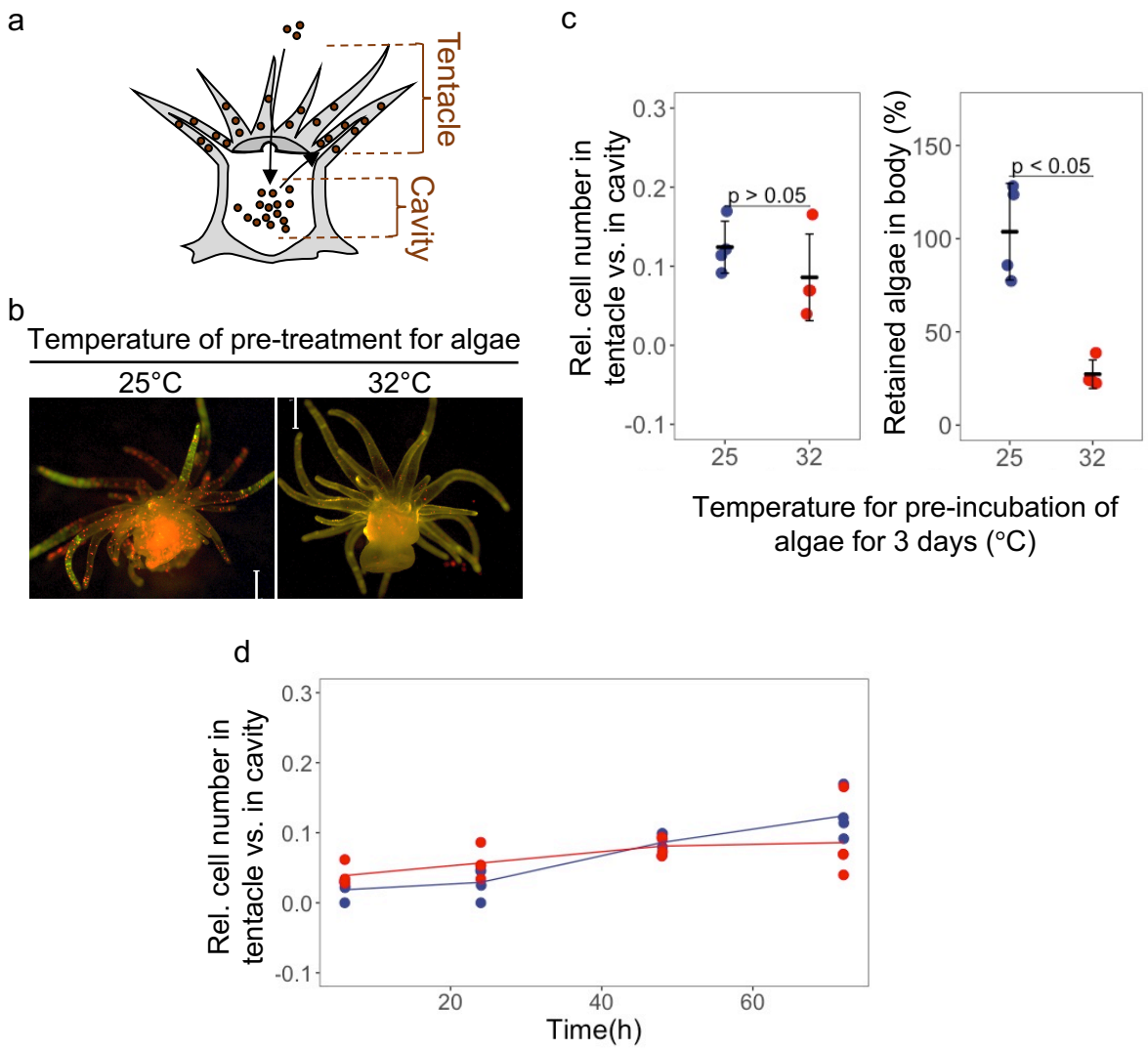


Figure 2-4. Effect of elevated temperature on symbiosis steps.

(a) Description of symbiosis steps including uptake of algal through mouth, transfer of algae to endoderm tissue (tentacles) and engulfment of algae to the tissue. (b-c) Aiptasia polyps were inoculated with algal cells which were pre-incubated at 25 °C or 32 °C for 3 days. (b) Aiptasia after 3 days inoculation with pre-incubated algae. (c) Number of algae in the cavity and in the tentacles were counted after 3 days inoculation. Each dot represents the individual experiment. Mean ±SE of the value is shown in black. P-values are shown in the plot. (d) The ratio of the algal cell number in the tentacles versus in the cavity was monitored every 24 hours. Algal cells pre-incubated at 25 °C (blue) or 32 °C (red) were used in the infection test.

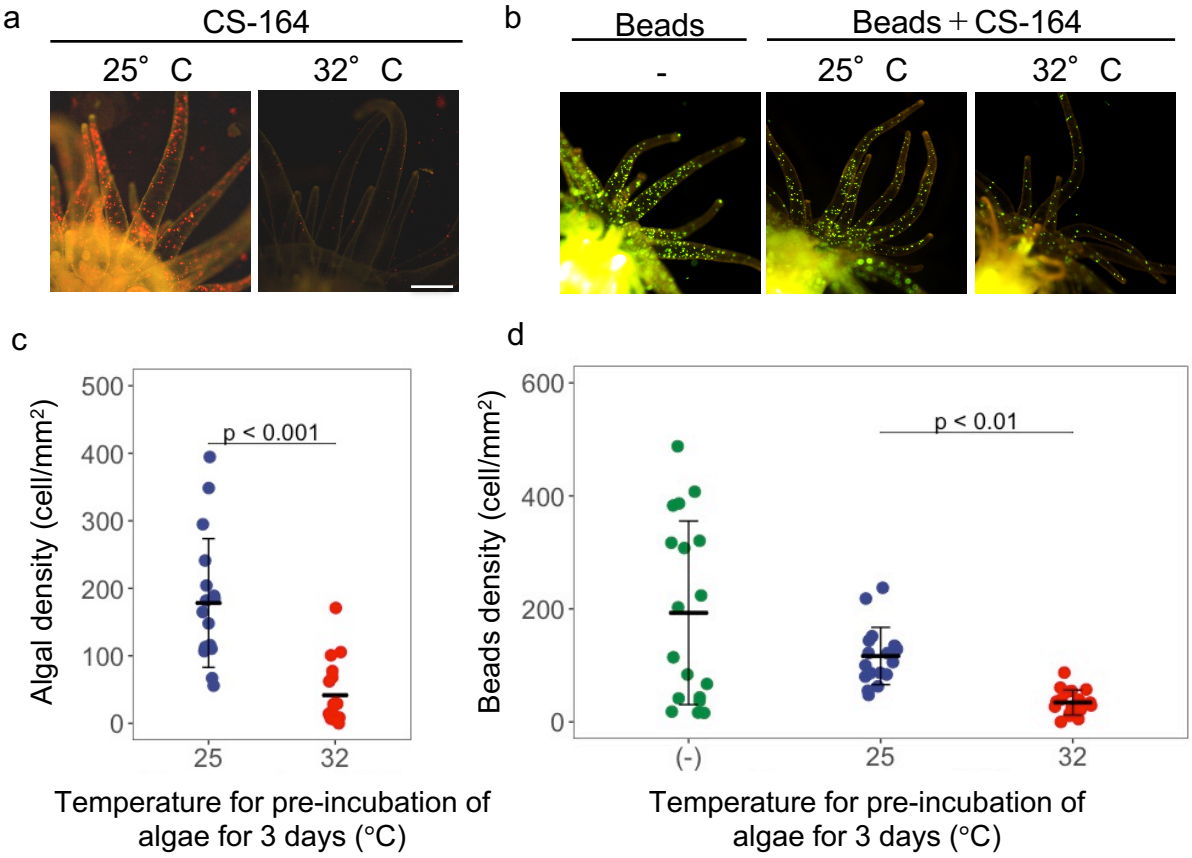


Figure 2-5. Effect of elevated temperature on uptake of algae and microbeads in Aiptasia polyps.

Apo-symbiotic Aiptasia polyps were inoculated with the algae which were pre-incubated at 25 °C or 32 °C for 3 days (a, c) or with the mixture of the algal cells and green-yellow fluorescent microbeads (b, d). (a, b) Aiptasia polyps after 3 days incubation with the algae or with the mixture of the microbeads and algae. Temperatures of the pre-incubation of algae are shown above the photographs. (c, d) Number of algae or microbeads in the tentacles of Aiptasia was counted after the 3 days incubation. The column without temperature (-) indicates the sample with only microbeads without algae.

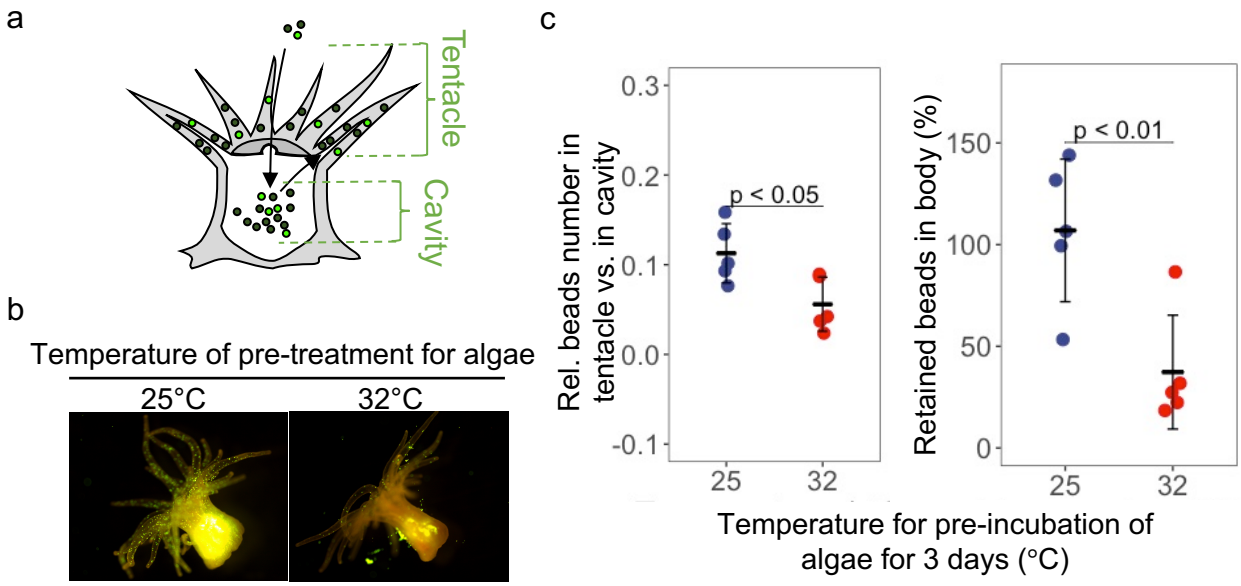


Figure 2-6. Effect of elevated temperature on uptake process of algae and microbeads in Aiptasia polyps.

(a) Description of the uptake of microbeads including acquiring of microbeads through mouth, transfer of microbeads to tentacles and engulfment of microbeads. (b-c) Apo-symbiotic Aiptasia polyps were inoculated with the mixture of the microbeads and algae which were pre-incubated at 25 °C or 32 °C for 3 days. (b) Aiptasia after 3 days inoculation with the microbeads and algae. Temperatures of the pre-incubation of algae are shown above the photographs.(c) Number of microbeads in the cavity and in the tentacles was counted after 3 days of inoculation.

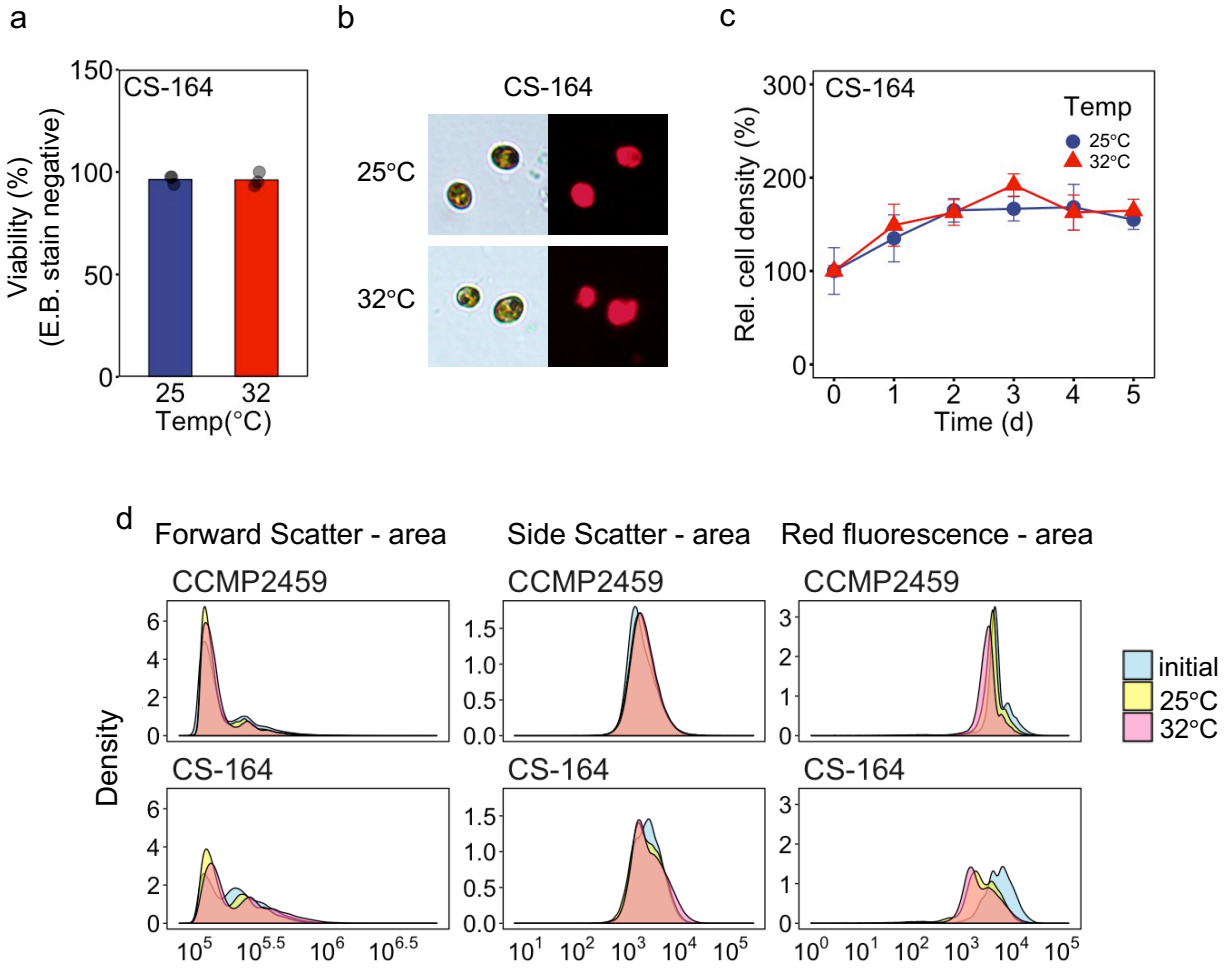


Figure 2-7. Effect of elevated temperature on cultured algae.

Cultured algae of CS-164 were incubated at 25 °C or 32 °C in darkness for maximum 5 days. (a) Viability was measured by Evans Blue staining after the incubation for 3 days**.** (b) Photographs of CS-164 cells were taken after Evans blue staining in bright field (left) and in fluorescence (right) with a microscope under 32 X magnification. (c) Cell density was monitored during the temperature treatment for 5 days by counting cell number using a hemocytometer. Values are percentages of the initial 40,000 cell ml^{-1} (100%). Values are shown as mean±SE from three independent experiments. (d) The cell size and chlorophyll fluorescence of algal cells (CS-164 and CCMP2459) was analyzed using Attune flow cytometer at day 0 (blue) and day 3 (yellow: 25 °C, pink: 32 °C) of the incubation.

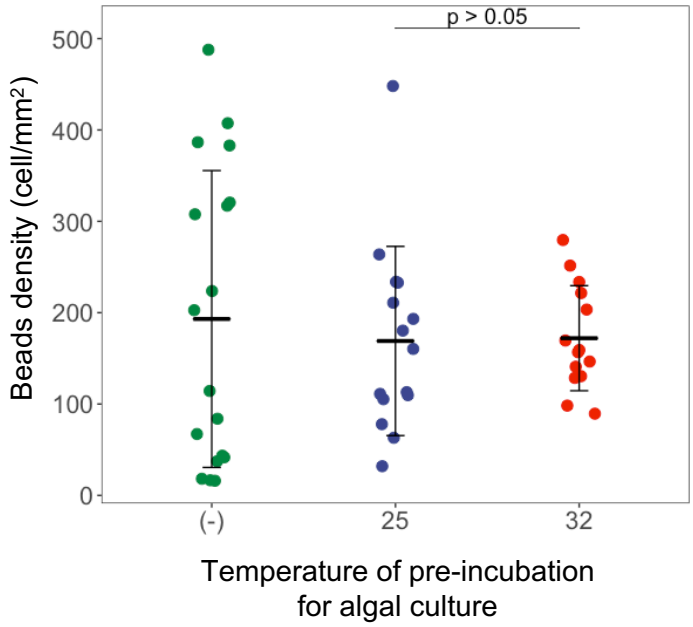
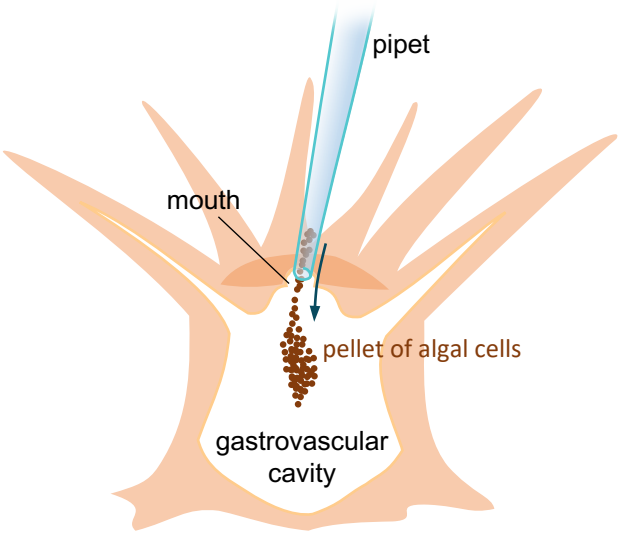
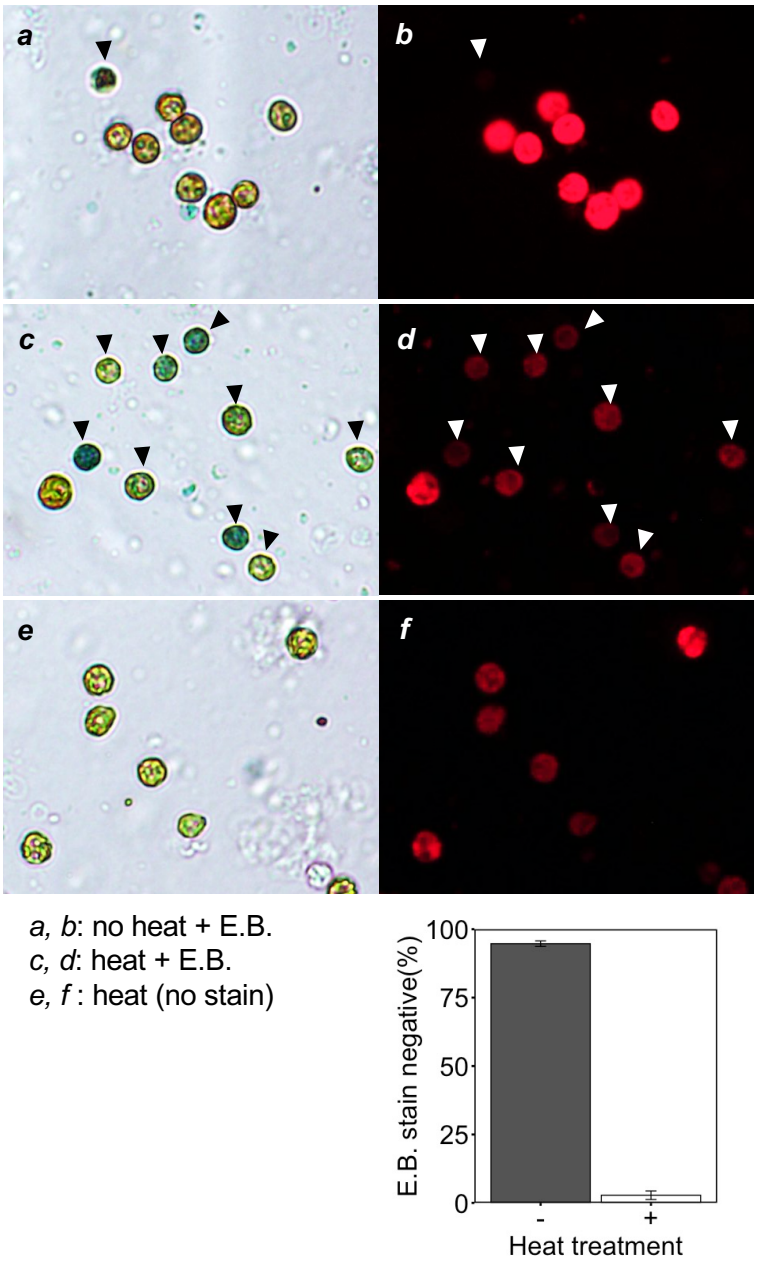


Figure 2-8. Effect of algal culture media to the symbiosis efficiency.

Cultured algae of CS-164 were pre-incubated at 25 °C or at 32 °C for 3 days. Seawater media in the algal culture was collected after the incubation and used in the infection test of microbeads and Aiptasia. Temperature of pre-incubation for algal culture is shown below the plot. The column without temperature (-) indicates the control sample using fresh seawater media instead of the seawater from algal culture. The density of microbeads were counted at tentacles of Aitpasia after 3 days of inoculation with microbeads in the seawater media.



Supplementary Figure 1. Introduction of algal cells into Aiptasia body.
Algal cells (approximately 9000 cells) were collected by centrifugation (1,500 g for 3 min) and mixed with fresh *Artemia* nauplii (3-5 individuals in 3 µl seawater) for making a pellet. The pellet was directly fed to Aiptasia using a glass Pasteur pipet. Aiptasia polyps were then incubated at 25 °C for infection. Microbeads were laso itroduced to Aiptasia polyps with the same thethod.



Supplementary Figure 2. Positive and negative control of Evans blue staining. Cultured algal cells of CS-164 were incubated at 80 °C in darkness for 1 h. Photographs of the cells were taken before (a, b) and after (c-f) the heat treatment, with (a-d) or without (e, f) Evans blue staining under 32 X magnification. Left side and right side are bright field images and fluorescence (WIG filter: Excitation 520~550nm band pass, Detection 580nm~) images, respectively. Evans blue stain-positive cells (dead cells) are indicated by black arrows in bright field images and white arrows in fluorescence images. The ratios of stain-negative cells (living cells) were measured before and after the heat treatment.

Chapter 3

The effect of heat stress on the maintenance of cnidarian-algal symbiosis

3.1 Introduction

In a normal condition, symbiotic algae increase their density by cell division in the symbiosome of the cnidarian cell. Therefore, host cells occasionally release algal cells into the lumen side of the gastrovascular cavity, most probably thorough exocytosis (Davy et al., 2012). Some of released algae are taken into host cells again, but other are expelled from polyps to the environment (Parrin et al., 2016). Thus, expulsion of algae can be seen under optimal growth conditions (Fujise et al., 2014). The algal density is maintained by the dynamic balance between uptake and release of algae, and excess algae in the gastrovascular cavity are expelled into the environments. Currently, heat-induced decline in the algal density, i.e. bleaching, is assumed to occur due to acceleration of algal release from host cells (Hoegh-Guldberg, 1999). Thereby, the thermal sensitivity of corals to lose their algae is proposed to be determined by the efficiency of algal release from host cells under heat stress (Tchernov et al., 2004). However, the loss of algae can potentially occur through suppression of algal uptake into host cells under heat stress. Then, bleaching sensitivity might be determined by the efficiency of algal uptake. However, this hypothesis has not been experimentally tested.

Previous studies have demonstrated that the increase in the seawater temperature suppresses uptake of algae into the host using coral larvae (Cumbo et al., 2018). In this study, I demonstrated that the higher temperature suppresses uptake of algae to adult Aiptasia polyps (Chapter 2). These findings provide a hypothesis that cnidarian bleaching through the loss of algae might occur due to suppression of algal uptake into host cells. To test this hypothesis, I used Aiptasia and two algal strains with different thermal sensitivities in their infectivity. The extent of bleaching of Aiptasia was measured by monitoring the algal density and the amount of algal cells expelled from bodies and was compared between Aiptasia which are infected by heat-sensitive algae and Aiptasia infected by heat-tolerant algae.

3.2 Materials and Methods

Materials and growth conditions.

Sea anemone Aiptasia (strain H2) polyps were provided by Professor John Pringle (Stanford University). To eliminate their original algae, they were incubated at 34 °C in the dark for 1 month. The elimination of all algae was confirmed by the inability to detect any symbiotic algae under a fluorescence microscope (Leica M165FC). The apo-symbiotic Aiptasia polyps

were cultured at 25 °C in darkness and fed freshly hatched *Artemia* nauplii once a week, until needed.

Symbiodiniaceae CCMP2459 was obtained from the National Center for Marine Algae and Microbiota (USA), and Symbiodiniaceae CS-164 from the Australian National Algae Culture Collection (AUS). Both strains were cultured from a single cell and confirmed to be the genus *Breviolum* (previous Clade B) in previous study (Biquand et al., 2017). Both strains have been demonstrated to infect in the cnidarian hosts, sea anemone Aiptasia and coral *Acropora tenuis* (Biquand et al., 2017). Algal cells were grown in filtered (0.22 µm pore filter; Steritop-GP Filter Unit, Merck Millipore) artificial seawater (sea salt; Sigma-Aldrich) containing Daigo's IMK medium for marine microalgae (Wako). They were cultured in a growth cabinet MLR-350 (SANYO) at 25 °C under a cycle of 12 h of light (100 µmol photons m^{-2} s^{-1}) and 12 h of darkness.

Colonies of *Acropora tenuis* were collected in 2015, a few days prior to spawning off the coast of Sesoko Island (26°38'N127°51'E), in Okinawa (Japan) with local government permission (No.27-1). They were maintained in a tank with regular seawater changes at the Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus. During the spawning event, bundles from different colonies were collected and mixed in a container containing natural seawater filtered by 3 µm polypropylene cartridge (TCW-3N-PPS, Advantec). Fertilized eggs developed to larvae over the following days. Larvae were placed in fresh filtered seawater daily. Eight days after fertilization, larvae were mixed with 1 µM Hym-248 (epiphytic hormone isolated from Hydra (Iwao et al., 2002))and placed in non-treated 6-well microplates (AGC Techno Glass). Juvenile coral polyps were cultured at 25 °C under a cycle of 12 h of light (100 µmol photons m^{-2} s^{-1}) and 12 h of darkness. All juvenile polyps were cultured in filtered artificial seawater.

Infection of algae to polyps of apo-symbiotic Aiptasia and *A. tenuis*.

Cultured algal cells were collected by centrifugation at 1,500 g for 3 min and resuspended in fresh filtered seawater. Apo-symbiotic polyps were incubated with algal cells (40,000 cells ml^{-1}) in 6-well microplates with 10 ml (*A. tenuis*) or 5 ml (Aiptasia) of artificial seawater (REI-SEA Marine, IWAKI). Algal cells were counted with an automated cell counter (TC20, Bio-Rad). Polyps were cultured at 25 °C under a cycle of 12 h of light (100 µmol photons m^{-2} s^{-1}) and 12h of darkness. Infection of algae into the polyps was confirmed by the change in coloration (white/brown).

Temperature treatments.

Polyps infected with algae (CCMP2459 or CS-164) were incubated in a growth cabinet (MLR-350) at 25 °C or 32 °C for 5 days in darkness. To measure the number of algal cells expelled from each host polyp, seawater from each sample was carefully collected and replaced with fresh artificial seawater (REI-SEA Marine) every 24 h.

Measurement of the algal density in polyps.

Algal density in Aiptasia and corals was measured by counting algal cells on tentacles with a microscope (Leica M165FC) before and after the temperature treatment for 5 days. Values in figures are mean ±SE from three (Aiptasia) or six (coral) independent experiments.

Measurement of algal cells expelled from host polyps.

Number of algal cells in seawater samples collected after the temperature treatment was measured with a flow cytometer (Attune® flow cytometer). 1 ml of collected seawater was applied to the flow cytometer. Algal cells were discriminated from host cells and debris by their combination of side scatter and red fluorescence from algal chlorophyll. Values in figures are mean ±SE from three (Aiptasia) or six (coral) independent experiments.

3.3 Results

The effect of high temperature on the extent of bleaching in Aiptasia with CS-164 or CCMP2459.

Aiptasia polyps infected by either a heat-sensitive strain CS-164 or a heat-tolerant strain CCMP2459 were examined their bleaching sensitivity under high temperature. In general, heat-induced bleaching occurs through the loss of algal cells or the loss of algal pigments in *in situ* algae. The former shows light-independent manner (Tolleter et al., 2013) and the latter shows light-dependent manner (Takahashi et al., 2008). To focus on bleaching caused by the loss of algal cells, temperature treatments for bleaching were conducted under continuous darkness in this study. When Aiptasia polyps infected by CS-164 were incubated at 25 °C or 32 °C for 5 days, bleaching occurred at 32 °C but not at 25 °C; the algal density declined to 80% of initial at 32 °C (Fig. 3-1a and b). However, in Aiptasia polyps infected by CCMP2459, bleaching was not observed at both 25 °C and 32 °C (Fig. 3-1a and b). These results indicate that Aiptasia polyps harboring CS-164 are more sensitive to heat-induced bleaching through the loss of algal cells than those harboring CCMP2459.

The effect of high temperature on the algal expulsion in Aiptasia with CS-164 or CCMP2459.

To examine that heat-induced bleaching through the loss of algal cells in Aiptasia polyps with CS-164 is due to increase in algal expulsion from polyps, I monitored the number of algal cells expelled from polyps at 25 °C and 32 °C. I determined the background number of cells expelled from each polyp by counting the number of algae expelled to seawater per day for 3 days at 25 °C. Then I examined the effect of incubation at 25 °C and 32 °C on it for 5 days. In polyps with CS-164, the number of cells expelled from polyps was higher at 32 °C than 25 °C, especially on day 3 with 5 times more than the background (Fig. 3-1c). However, in polyps with CCMP2459, there was no significant difference in it between temperatures (Fig. 3-1c). These results demonstrate that heat-induced bleaching through the loss of algal cells in Aiptasia polyps with CS-164 is attributed to the acceleration of algal expulsion from polyps.

Expulsion of algae in coral polyps of *Acropora tenuis* under the increased temperature.

In Aiptasia, the sensitivity to bleaching through the loss of algal cells under high temperature conditions varied between polyps harboring different algal strains. I examined whether it can be also seen in coral polyps. I prepared juvenile apo-symbiotic polyps of *A. tenuis* and separately inoculated with either CS-164 or CCMP2459. In coral polyps with CS-164, incubation at 32 °C, but not at 25 °C, for 5 days caused bleaching due to decrease in algal density (Fig. 3-2a and b). The decrease in algal density corresponded to higher algal expulsion at 32 °C than 25 °C (Fig. 3-2c). In coral polys with CCMP2459, bleaching was not observed at both temperatures (Fig.3-2a and b). Furthermore, there was no significant difference in the rate of algal expulsion between temperatures (Fig. 3-2c). These results demonstrate that results in coral *A. tenuis* are consistent with the results in model sea anemone Aiptasia.

3.4 Discussion

Bleaching sensitivity varies between cnidarian hosts possessing heat sensitive and tolerant symbiotic algae.

Increase in seawater temperature causes coral bleaching through loss of algae, and its thermal sensitivity varies by *in situ* algal types in the host cnidarians, such as sea anemones (Perez et al., 2001) and corals (Berkelmans and van Oppen, 2006; Jones et al., 2008; Rowan, 2004; Silverstein et al., 2015). Previously, heat-induced photoinhibition of symbiotic algae was assumed to be related to the expulsion of algal cells and subsequent loss of algal density, as bleaching often follows severe photoinhibition (Jones et al., 1998; Venn et al., 2008; Weis,

2008). However, a recent study has shown that photoinhibition is not necessary in the process of coral bleaching through algal expulsion (Tolleter et al., 2013), although photoinhibition might be still associated with coral bleaching through the loss of algal pigments (Takahashi et al., 2008). Currently, mechanisms associated with coral bleaching through the expulsion of algal cells and how their thermal sensitivity can be decided have been remained unknown. Today, I used two algal strains of Symbiodiniaceae which showed different thermal sensitivities and I examined whether the difference in their infectivities under high temperature influences on the extent of bleaching. The results demonstrated that the host, both sea anemone (Fig. 3-1) and corals (Fig. 3-2), lost their algal density under heat stress when they possessed heat-sensitive, but not heat tolerant algal strain. Therefore, the ability of algal uptake by hosts under high temperature might be a factor which decides the thermal sensitivity of bleaching.

How does heat-induced loss of infectivity cause bleaching?

The algal density in cnidarians are maintained by the dynamic balance between uptake of algae and release of them (Parrin et al., 2016). Therefore, the high temperature can potentially decrease algal density through acceleration in the release of algal cells from the host tissue and through suppression in the uptake of algae into the host tissue. In the current model, high temperature accelerates the algal release from the host tissue, resulting in decrease of algal density (Hoegh-Guldberg, 1999). It is suggested that the production of reactive oxygen species (ROS) in algae accelerated by heat stress becomes a stress to host and induces the algal release (Jones et al., 1998; Venn et al., 2008; Weis, 2008). However, in this study, I focused on bleaching due to suppression of algal uptake independent from ROS production. Therefore, I used algal strains with different infectivities to host under heat stress (Fig. 2-2) and conducted heat treatments in darkness, which can avoid ROS production from algal photosynthetic components. This study demonstrated that the host, both sea anemone and corals, decreased their algal densities through the expulsion of algal cells from polyps under heat stress when they possessed algal strain which does not possess infectivity under heat stress (Fig. 3-1 and 3-2). This result suggests that in the hosts with heat-sensitive algae, algal cells released from host tissue are unable to re-infect to the host tissue under heat stress, resulting in decrease of algal density and increase in algal expulsion from polyps (Fig. 3-3). While, in the hosts with heat-tolerant algae, algal cells released from the host tissue are still able to re-infect host tissue under heat stress, resulting in less decrease in algal density and less expulsion of algal cells from polyps (Fig. 3-3). These results provide a new hypothesis that bleaching can occur through suppression of algal uptake into the host tissue under heat stress. I need to note that this study

does not exclude the possibility that high temperature accelerates the release of algal cells from host tissue and the thermal sensitivity varies between algal strains. Therefore, difference of bleaching sensitivity seen in this study might be also due to difference of the thermal sensitivity of algal release.

Thermally induced loss of algae might be avoidable in corals through recruiting suitable algae.

Shuffling of symbiotic algal community during high temperature period is expected to bring thermal tolerance if corals recruit heat-tolerant algae (Silverstein et al., 2015). This hypothesis is supported by the report that algal community was changed during a heat exposure and subsequently the coral adapted to higher temperatures (Howells et al., 2012). However, detailed mechanism has been unclear without experimental researches. In the present study, I demonstrated in corals that bleaching sensitivity is related to the algal infectivity under heat stress (Fig. 2-2, 3-1 and 3-2). This is the first experimental evidence suggesting that if corals meet algae which can infect even at high temperatures, they will get higher tolerance on future bleaching threat with effective uptake of algae.

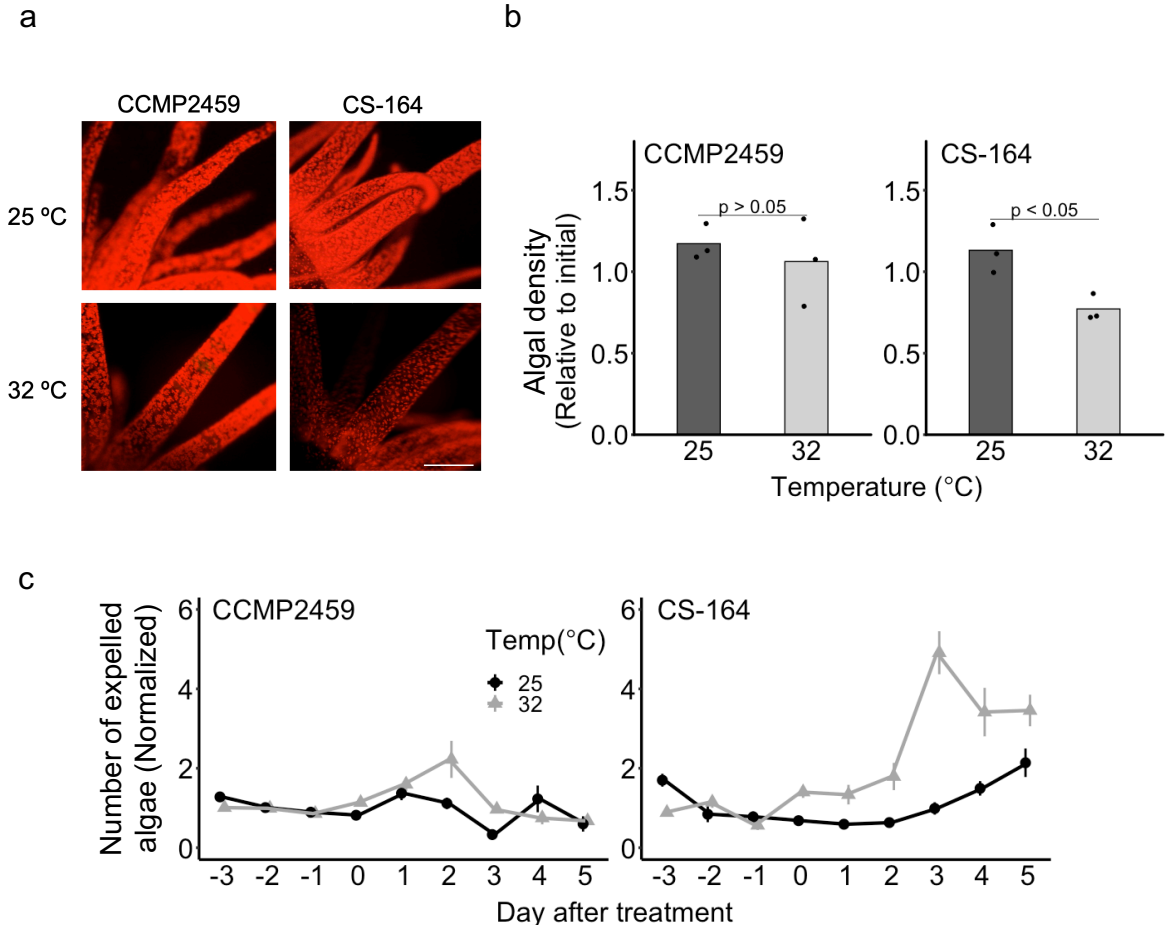


Figure 3-1. Effect of elevated temperature on the density of symbiotic algae CCMP2459 and CS-164, in Aiptasia.

Apo-symbiotic Aiptasia polyps were inoculated with the cultured algae of CCMP2459 or CS-164 for more than 4 weeks. And then polyps were tested their algal density change at 25 °C or 32 °C for 5 days in darkness. (a) Aiptasia polyps observed under a fluorescent microscopy after 5 days incubation. Scale is 200 µm. (b) Algal density after 5 days incubation, normalized to the density before the treatment. Each dot represent an independent experiment. Bars show mean from the experiments. (c) Number of algal cells expelled from host polyps was counted before and during 5 days incubation at 25 °C (black) or 32 °C (grey) using Attune flow cytometer. Mean of the algal cell number expelled in the growth condition for 3 days before the temperature treatment was used to normalize tests. Values are mean ± SE from three independent experiments.

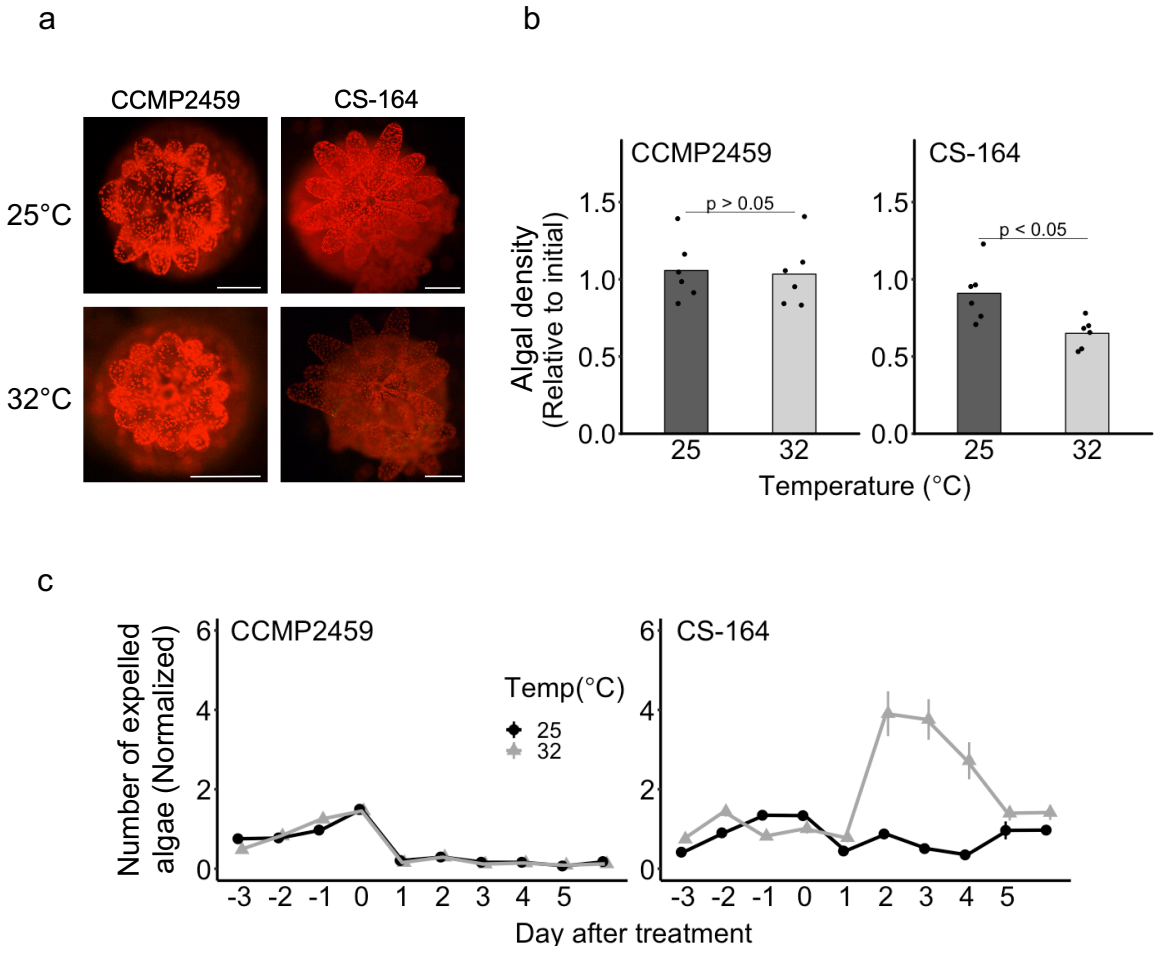


Figure 3-2. Effect of elevated temperature on the density of symbiotic algae CCMP2459 and CS-164, in coral.

Coral polyps were inoculated with the cultured algae of CCMP2459 or CS-164 for more than 4 weeks. And then polyps were tested their algal density change at 25 ℃ or 32 ℃ for 5 days in darkness. (a) Coral polyps observed under a microscopy after 5 days incubation. Scale is 1 mm. (b) Algal density after 5 days incubation, normalized to the density before the treatment. Each dot represents an independent experiment. (c) Number of algal cells expelled from host polyps was counted before and during 5 days incubation at 25 ℃ (black) or 32 ℃ (grey) using Attune flow cytometer. Mean of the algal cell number expelled in the growth condition for 3 days before the temperature treatment was used to normalize tests. Values are mean ± SE from six independent experiments.

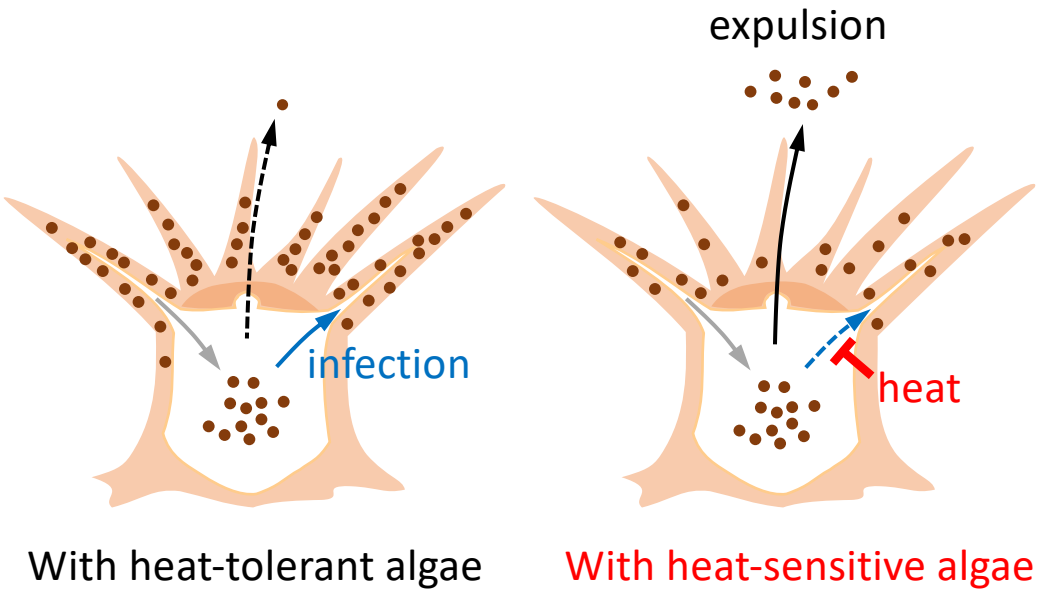


Figure 3-3. Hypothetical model of different algal expulsion by different infectivity under elevated temperatures.

With heat-tolerant algae, a host cnidaria shows low expulsion because it can infect free algae to its tissue. With heat-sensitive algae, expulsion becomes high due to the inhibition of infection of free algae. Algal release from tissue may also affect expulsion, but its efficiency is unknown (grey arrow).

Chapter 4

The effect of heat stress on the recovery potential of cnidarians from bleaching

4.1 Introduction

Cnidarian animals harbor symbiotic algae, from which they derive the majority of their energy. Therefore, the breakdown of the symbiotic relationship (bleaching) can result in the host starving. However, bleaching is not always lethal because the algal density can recover (Fitt et al., 1993; Levas et al., 2018). Recovery from bleaching is driven mainly by the remaining algal cells within bleached corals (the residual population) dividing and spreading throughout the coral colony (Baird and Marshall, 2002), and possibly by recruitment of free-living algal cells from the external environment (Lewis and Coffroth, 2004). In the last few decades, coral reefs have dramatically decreased in many regions due to the frequent mass coral bleaching events (Hughes et al., 2018), implying that recovery from bleaching is often limited by unknown factors. In the present study, I demonstrate that both free-living and residual algal cells lose their capacity to infect cnidarian host cells once they are exposed to high temperature stress. Moreover, the time duration to recover the decreased infectivity becomes longer as the heat period becomes longer. These results suggest that the loss of infectivity in algae as a limiting factor for the host's recovery from bleaching.

4.2 Materials and Methods

Organisms and culture condition.

Sea anemone *Exaiptasia pallida* (strain H2, commonly called Aiptasia) polyps were provided by Professor John Pringle (Stanford University). To eliminate their original algae, they were incubated at 34 °C in darkness for 1 month. The elimination of all algae was confirmed by the inability to detect any fluorescence from symbiotic algae under a fluorescence microscope (Leica M165FC) under 200 X magnification. The apo-symbiotic Aiptasia polyps were cultured in filtered artificial seawater (REI-SEA marine 2; IWAKI) at 25 °C in darkness and fed freshly hatched *Artemia* nauplii once a week, until used in the experiments.

Symbiodiniaceae CCMP2459 (ITS2 type B2) was obtained from the National Center for Marine Algae and Microbiota (USA), and Symbiodiniaceae CS-164 (ITS2 type B1) from the Australian National Algae Culture Collection (AUS). To ensure that cultures were monoclonal, obtained cultures were subcultured from a single cell and their genotypes confirmed in a previous study (Biquand et al., 2017). Algal cells were grown in filtered (0.22

μm pore filter; Steritop-GP Filter Unit, Merck Millipore) artificial seawater (sea salt; Sigma-Aldrich) containing Daigo's IMK medium for marine microalgae (Wako). They were cultured in a growth cabinet MLR-350 (SANYO) at 25 °C under a cycle of 12 h of fluorescent light (100 μmol photons m^{-2} s^{-1}) and 12 h of darkness.

Aiptasia infected with algae were obtained by culturing apo-symbiotic Aiptasia polyps with cultured algae (40,000 cells ml^{-1}) in a 10 cm diameter dish with 50 ml of artificial seawater. Polyps were cultured at 25 °C under a cycle of 12 h of fluorescent light (100 μmol photons m^{-2} s^{-1}) and 12 h of darkness. Infection of algae into the host was confirmed by the change in coloration (white/brown). They were fed freshly hatched *Artemia* nauplii once a week, until used.

Temperature treatment.

Cultured algae of Symbiodiniaceae were collected by centrifugation (1,500 g for 3 min) and seawater was replaced with new seawater (REI-SEA marine 2; IWAKI). For temperature treatments, algal cells were incubated at a growth temperature of 25 °C or an elevated temperature of 32 °C in darkness for maximum 3 days. To shock algal cells within Aiptasia polyps, symbiotic polyps were placed in 6-well plates with 5 ml of fresh seawater and incubated at 25 °C or 32 °C in darkness for 3 days. Algal cells expelled from polyps were collected by centrifugation (1,500 g, 3 min) and re-suspended in fresh seawater for the use in experiments.

Measurement of algal infectivity to Aiptasia polyps.

Aiptasia polyps were individually placed in 6 well-plates with 5 ml of artificial seawater. Algal cells (approximately 9000 cells) were collected by centrifugation (1,500 g for 3 min) and mixed with fresh *Artemia* nauplii (3-5 individuals in 3 μl seawater) for making a pellet. *Artemia* nauplii were used to accelerate the introduction of an algal pellet into the stomach cavity. The pellet was directly fed to Aiptasia using a glass Pasteur pipet. Aiptasia polyps were then incubated at 25 °C for 3 days under a cycle of 12 h of light (100 μmol photons m^{-2} s^{-1}) and 12 h of darkness for infection. Algal density (algal number per area) in Aiptasia was measured by counting algal cells in tentacles with a stereo fluorescence microscope (Leica M165FC or M205FA) after the incubation.

Re-infection of symbiotic algae.

To measure the infectivity of algae in symbiotic condition, algal cells expelled from Aiptasia infected with CS-164 or CCMP2459 were collected after the temperature treatment and infectivity was measured by the method described above. To collect the algal cells expelled from each Aiptasia polyps, seawater around Aiptasia samples was collected and concentrated by centrifugation of 1,500 g for 3 min.

Statistics and reproducibility.

All statistical tests were performed using R 3.5.2 software. Statistical analysis between two samples was performed using Welch's *t*-test. $P < 0.05$ was considered statistically significant.

4.3 Results

The effect of high temperature on the symbiotic algae in host Aiptasia tissue.

I formerly demonstrated that cultured algae of Symbiodiniaceae lose their infectivity upon exposure to elevated temperature (Fig. 2-2 and 2-3). To examine whether symbiotic algal cells in host tissue also lose their infectivity by heat, I collected algal cells expelled from host Aiptasia polyps harboring either CCMP2459 or CS-164 and examined their infectivity using apo-symbiotic host polyps at 25 °C. In CCMP2459, algal cells expelled from Aiptasia succeeded to infect new host polyps with no significant difference between temperatures (Fig. 4-1). In CS-164, algal cells expelled from Aiptasia infected new host polyps at 25 °C, but significantly lower at 32 °C (Fig. 4-1). The difference in thermal sensitivity between two strains is consistent with the result of cultured algae (Fig. 2-3). These results indicate that algal cells lose their infectivity during the exposure to elevated temperature in host tissue as well as outside of host.

Recovery of the algal infectivity after heat exposure.

To investigate the recovering potential of algae after the heat treatment, pre-heat-exposed algae of CS-164 (at 32 °C for 1-3 days) were moved back to 25 °C for maximum 10 days as a recovering period, then infectivity to Aiptasia was measured at 25 °C for 3 days. With 1 day of heat exposure, the infectivity of algae had no difference with control (Fig. 4-2). The infectivity became lower than control with 2 days heat exposure, but it became as high as control after 5 days recovering period (Fig. 4-2). However, with 3 days heat exposure, the infectivity became lower than control and stayed still low even after 10 days of recovering

period (Fig. 4-2). These results indicate that heat exposed algae can recover its infectivity but it is depending on the period of heat stress.

4.4 Discussion

The limiting factor of the recovery of corals from bleaching.

Recovery of algal density in bleached coral occurs in nature, but not often. Inhibition factors have been unknown. One possible mechanism was decrease in algal uptake speed under heat stress. However, it has been still unclear why coral recovery is difficult even after the temperature returns to normal. In this study, using Aiptasia model system, I clarified algal uptake in bleached host can be suppressed by heat driven loss of infectivity in algae, while effect of heat stress on host is minimal (Fig. 2-3). Such function loss was observed in not only cultured algae but also algae in symbiotic form inside host tissue, implying both free-living algae in the environment and algal cells expelled from other host polyps can have less ability to infect corals (Fig. 4-1). Importantly, such heat damage on algal symbiotic function has potential to recover if the stress is a short term, however, recovering rate is time dependent and becomes significantly lower after a long term heat exposure (Fig. 4-2). These results suggest that suppressed algal infectivity can be an inhibition factor of coral recovery from bleaching even after a high temperature period.

Symbiosis establishment between coral larvae and algae can be suppressed by prior high temperature period.

Most stony coral species establish symbiotic relationship with algae in the stage of larvae or juvenile polyps (Cumbo et al., 2018). The spawning of corals occurs at the night of early summer, especially around the days with full moon. The seawater temperature becomes higher during the season of spawning. However, if the temperature becomes extremely high, algal cells lose their function to infect to host (Fig. 2-3). Moreover, when high temperature period becomes a long term, the declined algal infectivity can be prolonged even after the temperature becomes mild (Fig. 4-2). Therefore, if the temperature is already high before the coral spawning night, algae would be inactive for symbiosis during the spawning. If coral larvae cannot catch algae, their surviving rate significantly drops (Abrego et al., 2012; Cumbo et al., 2018). Currently, the average seawater temperature is usually below 30 degrees around the spawning periods, which would not make critical damages. However, if the seawater temperature continues increasing in the future, it might become a critical factor to drop the success rate of the larval symbiosis establishment.

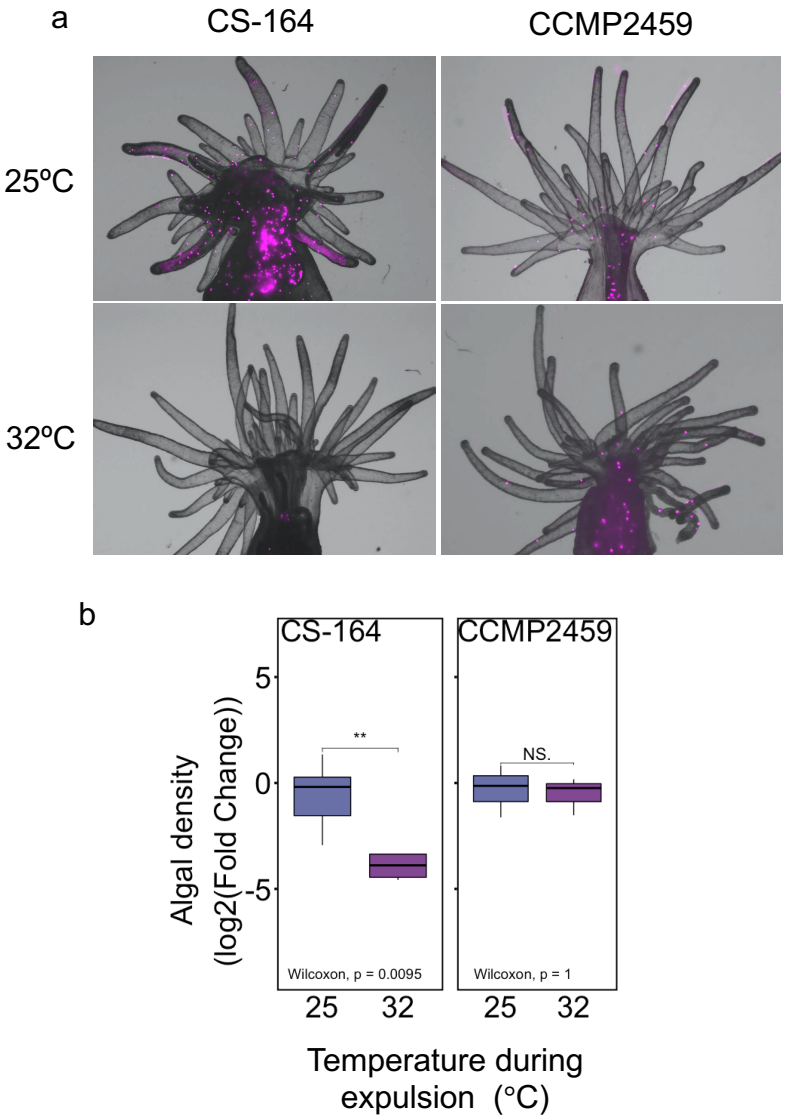


Figure 4-1. Effect of the elevated temperature on re-uptake of expelled algal cells.
Algal cells which were expelled from host Aiptasia during the incubation at 25 or 32 °C for 3 days were tested their re-infectivity into apo-symbiotic Aiptasia. (a) Aiptasia after 3 days infection with CS-164 or CCMP2459. Magenta dots represent chlorophyll fluorescent from algal cells. (b) Algal density in tentacles of Aiptasia was measured after 3 days incubation. Values are log2 fold changes to the control 25 °C. Each value is from six separate experiments.

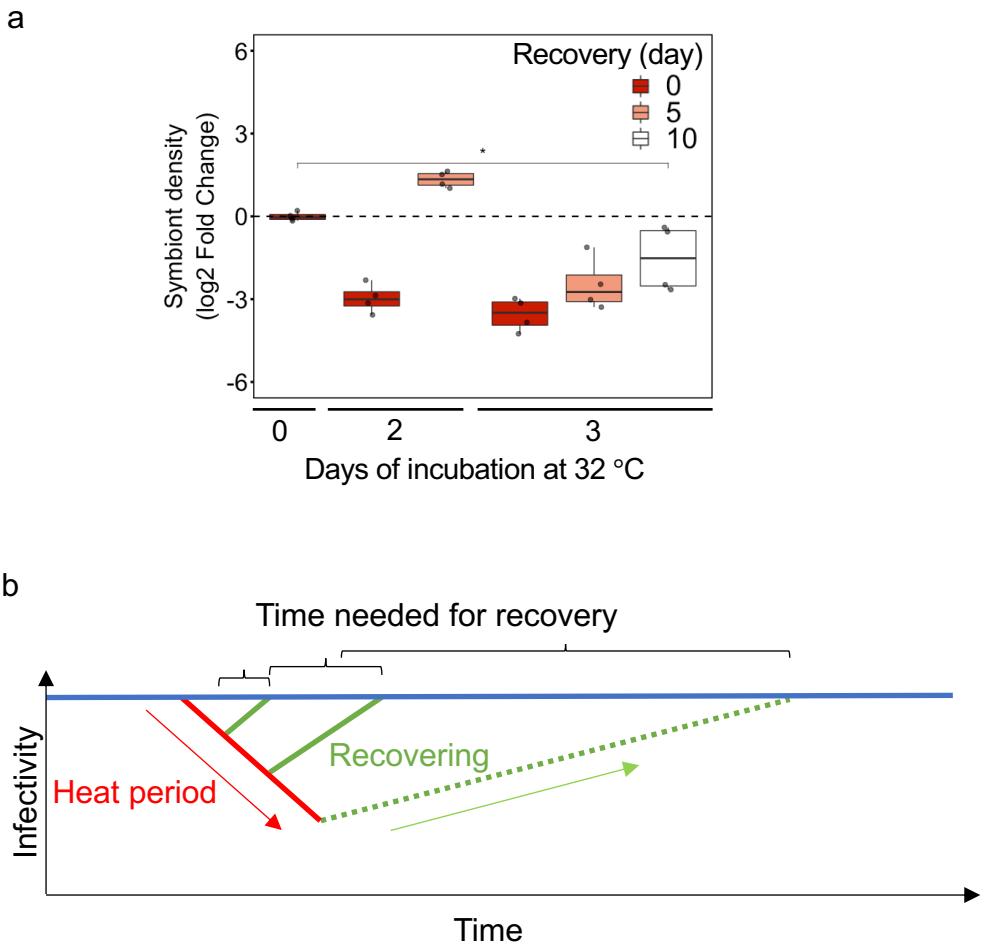


Figure 4-2. Reversibility of the lost infectivity upon the exposure to elevated temperature in algae of CS-164.

(a) The density of algae CS-164 in tentacles of Aiptasia was measured following the treatments as described in the text and shown by the relative amount to control. Values are $\log_2$ fold changes with respect to the control. The box and line represent the quartiles and median, respectively. Each point represents an independent experiment. Symbol indicates p-value (*: ≦ 0.05). (b) Hypothetical model of the recovery of algal infectivity after high temperature stress.

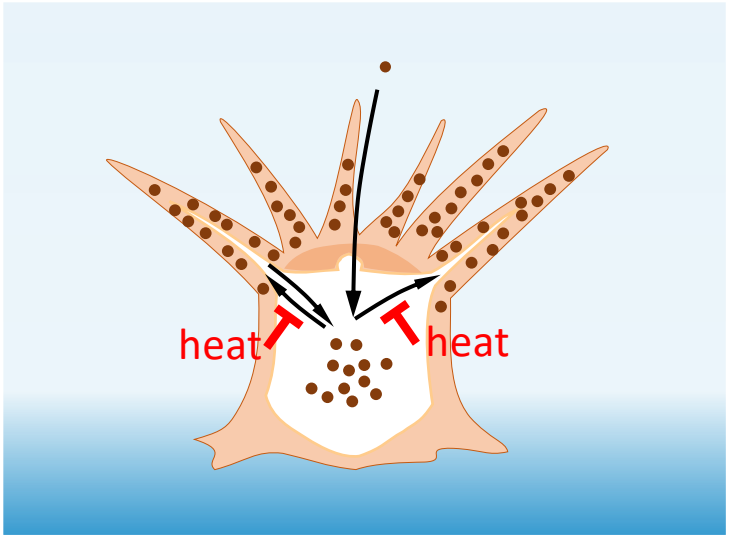


Figure 4-3. Hypothetical model of how recovering from bleaching is limited.

Algal cells both in symbiosis and free-living lose the infectivity under elevated temperature, which suppress re-uptake of expelled algae and acquiring of free-living algae from environment. This suppression continues even after the temperature turns back, might limit the recovering of corals from bleaching.

Chapter 5
General Discussion

Since 1980s, coral coverage is dramatically decreasing worldwide due to ongoing environmental changes and warming. Previous studies have demonstrated that coral-algal symbiosis is sensitive to heat stress, resulting in loss of symbiotic algae, i.e. bleaching and subsequent mortality (Glynn and D'Croz, 1990). However, the mechanism of heat stress-induced destruction of symbiotic relationship remains unknown, although it has been intensively studied in last few decades. In this study, using sea anemone Aiptasia as a model system, the effect of heat stress on the establishment of symbiosis, the maintenance of symbiosis and the re-establishment of symbiosis after bleaching event was examined. Results demonstrated that symbiotic algae lose infectivity upon heat stress, resulting in suppression of the establishment of cnidarian-algal symbiosis, bleaching (decrease in symbiont density) and suppression of the recovery from bleaching (suppression of re-establishment of symbiosis). Furthermore, the effect of heat stress on algal infectivity differed by algal genotypes. These findings give a new insight into how heat stress harms the coral-algae symbiotic relationship in nature and what influences its thermal sensitivity. More details have been discussed below.

In Chapter 2, the effect of heat stress on the establishment of symbiosis was investigated. The process of algal infection to cnidarians has several steps including acquiring of free-living algae from the environment, transportation of algal cells to the endoderm tissue and engulfment of algal cells to the host cells (Parrin et al., 2016). It has been observed in corals and sea anemones that the efficiency of algal infection can be suppressed by heat stress, but the mechanism behind this suppression has remained unknown (Abrego et al., 2012; Cumbo et al., 2018). In this study, it was shown that the infection of algae to host Aiptasia was suppressed by heat stress on algae, resulting in frailer to establish symbiosis under increased temperatures (Fig. 2-3). When algal cells from normal condition were introduced into Aiptasia polyps, almost all cells were kept in the polyps and gradually spread through the polyps within few days. On the other hand, when heat-treated algae were introduced, algal cells were expelled from the polyps before they spread through the body (Fig. 2-4 and 2-6). These results suggest that heat stress on algae induces active expulsion by host Aiptasia, resulting in the suppression of symbiosis establishment. Algal cell morphology was monitored during the temperature treatment by flow cytometry, however, no correlative change with the infectivity was detected (Fig. 2-7). Previous studies suggested that recognition process between algae and cnidarians is the key process for the establishment of symbiosis (Wood-Charlson et al., 2007). It is possible

that the recognition molecules on algae might be impaired by heat stress. However, further analysis in molecular level to clarify the relationship between the recognition system and heat stress is needed.

In Chapter 3, the effect of heat stress on the maintenance of symbiotic relationship was investigated by monitoring the algal density in polyps and by counting number of algae expelled from polyps. The algal density in cnidarian hosts is maintained by the dynamic balance between uptake and release of algae in host cells. In the current model, heat stress causes decrease in the algal density by accelerating the algal release from host cells, resulting in acceleration of algal expulsion from host polyps (Hoegh-Guldberg, 1999). However, suppression of algal uptake into host cells can also decrease the algal density and accelerate the algal release from host polyps. However, this possibility has not been tested. In this study, I used two algal strains which show different thermal sensitivity; one possesses infectivity to cnidarians under heat stress and the other does not. When Aiptasia polyps had the heat-sensitive algae, the algal density declined by increase of temperature with more algal expulsion from host polyps (Fig. 2-2 and 2-3). However, the algal density and number of algae expelled from the host polyps did not change by increase of temperature when Aiptasia had heat-tolerant algae (Fig. 3-1 and 3-2). These results suggest that algal infectivity can be a key factor to maintain the symbiotic relationship under heat stress (Fig. 3-3). Furthermore, these results suggest that the heat-tolerant algae bring higher tolerance of bleaching under elevated temperatures. However, algal density is on the balance of the infection and release. To estimate the direct contribution of infectivity to bleaching, establishing the method to measure the net algal release is necessary.

In Chapter 4, the effect of heat stress on the potential of corals to recover from bleaching was investigated. Coral bleaching is not always lethal because the algal density can recover by re-uptake of algae (Fitt et al., 1993; Levas et al., 2018). However, in the last few decades, bleaching has been spreading in many regions, implying such recovery is often limited in the field (Ateweberhan et al., 2013; Hughes et al., 2018). In this study, it was clarified that heat-treated algae lose infectivity and that it is reversible (Fig. 4-2). However, long term exposure to heat stress showed the lower rate of recovery in the infectivity (Fig. 4-2). This result suggests that during and even after a higher temperature period, algae can be inactive for symbiosis. Therefore, recovery of corals from bleaching can be limited by the loss of symbiont infectivity following exposure to heat stress.

Recent increase in the seawater temperature has threatened the coral reef ecosystems. In the past few decades, field researches of coral reefs have demonstrated that heat stress harms

the symbiotic relationship between corals and algae through inhibition of the symbiosis establishment, decrease of algal density (bleaching) and limitation of the recovery from bleaching. However, the mechanisms behind these phenomena have been unclear. In this study, I found that heat stress declines the infectivity of algae to cnidarian hosts. When the infectivity of algae was declined, there was significant suppression in the process of symbiosis establishment, maintenance of the symbiosis and recovery from bleaching. Overall, my study demonstrates that the suppression of infectivity in algae under heat stress can be an major problem for current coral reefs through suppression of symbiosis establishment in coral larvae, bleaching and limitation of recovery from bleaching. It is expected that future studies about the heat-induced suppression of algal infectivity will reveal the mechanisms for all these phenomena. These findings bring new insights into how increased temperatures damaged the coral-algal symbiotic relationships in last few decades in coral reefs and how corals can adapt to ongoing global warming.

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