

## Temperature tolerance of symbiotic and non-symbiotic coral larvae

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**Abstract** Coral larvae represent the ideal model with which to test many aspects of the relationship between symbiotic zooxanthellae and the coral host because this is the only stage in the life history of the organism where it is easy to produce both symbiotic and non-symbiotic individuals for comparison. We used this larval model to investigate the effect of the presence of the symbiont on the thermal tolerance of coral larvae. We exposed symbiotic and non-symbiotic larvae of *Acropora muricata* to 3 temperatures under a mildly stressful level of light ( $125-150 \mu\text{E m}^{-2} \text{s}^{-1}$ ) for a 7-day period. Many larvae survived well at 28 and 32° C. In contrast, all larvae died within 40 h at 36° C. Interestingly, there was no major difference in survivorship between symbiotic and non-symbiotic larvae at any temperature indicating that the symbionts had little influence on larval susceptibility to heat and light. However, the density of symbionts in the larvae when the experiments were initiated was low and therefore, unlikely to affect the physiology of the host.

**Key words:** adaptation, bleaching, climate change, coral reef, symbiosis

### Introduction

Reef corals form obligate symbiotic associations with dinoflagellates commonly known as zooxanthellae. Under stress, such as high sea water temperature, this association can break down with consequent loss of the pigmented algal symbionts. This process is known as coral bleaching. Zooxanthellae provide 30 % of the total nitrogen and 91 % of carbon needs of the coral host (Bythell 1988). Consequently, when the density of zooxanthellae is reduced following bleaching the coral loses an important source of energy (Porter et al. 1989) with a subsequent reduction in colony growth and/or reproduction (Baird and Marshall 2002). If the loss of symbionts is very high, or

prolonged, the colony will die (Glynn and D'Croz 1990).

Many aspects of the bleaching phenomenon remain puzzling from a physiological perspective (Brown and Ogden 1993). In many coral species, the relationship breaks down at as little as 1° C above long term summer average temperatures (Goreau and Hayes 1994). The absolute temperatures that induce the breakdown in symbiosis are not that high, with bleaching typically occurring between 28 and 32° C, depending on the thermal history of the location (Coles and Brown 2003). Few other animals are affected by these thermal anomalies (at least in the initial stages of the event) and nor would they be predicted to be affected because cellular processes, such as the denaturing of proteins, are affected below 37° C. Similarly, few free-living algae bleach during these events. Furthermore, zooxanthellae isolated from coral hosts can be maintained in cultures for long periods at 37° C (Peter Ralph pers comm.), and many of the zooxanthellae recovered from corals following bleaching are healthy (Ralph et al. 2001). Most theories assume, therefore, that it is the communication between the host and the symbiont that is being disrupted. However, it is also possible that the coral host is unusually susceptible to temperature stress.

Coral larvae are the ideal model with which to test many aspects of the relationship between symbiont and host. In many broadcast spawning coral species, gametes do not contain zooxanthellae (Harrison and Wallace 1990), however, larvae can readily be infected with different strains of zooxanthellae under experimental conditions (Schwarz et al. 1999; van Oppen 2001). Consequently, it is possible to compare symbiotic and non-symbiotic individuals of the same species, and furthermore, readily control the identity of both the host and the symbiont. This control over the make up of the association is not possible in adults, because even completely bleached adults still contain residual densities of symbionts (Hoegh-Guldberg and Smith 1989), and it is not known whether they can take up symbionts from the environment (Hoegh-Guldberg et al. 2002). Furthermore, coral larvae are non-feeding (Fadlallah 1983) with an extraordinary capacity to delay

metamorphosis (Harii et al. 2002) and can therefore be maintained for long periods in sterile conditions (Morse et al. 1996). In addition, larvae are small and abundant making them easy to manipulate and allowing for many individuals to be compared for statistical power. The aim of this research was to use this larval model to compare the thermal tolerance of symbiotic and non-symbiotic individuals.

## Materials and Methods

### Inoculation of larvae with homologous symbionts

The first priority was to develop techniques to infect a high proportion of the larvae offered symbionts, to ensure the comparisons between the treatments groups were meaningful. Previous research has demonstrated that rates of infection are influenced by a number of variables including the density of the inoculum (Kinzie et al. 2001), the light environment (van Oppen 2001), the strain of zooxanthellae (Weis et al. 2001) and the presence of food (Schwarz et al. 1999). Temperature is also likely to be important. The conditions we settled on for the initial inoculation of larvae were: a temperature of 26-28° C i.e. similar to the sea surface temperature in the adult habitat in June in Okinawa; a low, non-stressful light level (50-80  $\mu\text{E m}^{-2} \text{s}^{-1}$ ); and very high densities of homologous zooxanthellae (approximately  $1.0 \times 10^6$  per ml). The addition of artemia to induce zooxanthellae uptake was considered necessary, because coral larvae are generally considered to be non-feeding and the behaviour previously described as feeding in *Fungia scutaria* larvae (Schwarz et al. 1999) is more likely to be typical settlement behaviour. Larvae of the common scleractinian *Acropora muricata* were cultured following the methods of Babcock et al. (2003). Approximately 72 h after spawning, when larvae were motile with an oral pore and had begun typical settlement behaviour including "testing" the substratum (Harrison and Wallace 1990) they were placed in 1 l glass jars in 0.2  $\mu\text{m}$  filtered seawater (FSW) at initial densities of approximately 1000 larvae per l under cool fluorescent lights (50-80  $\mu\text{E m}^{-2} \text{s}^{-1}$ ) with a 12:12 h light:dark photo-period. Freshly isolated zooxanthellae were used for infection of larvae. *Acropora muricata* nubbins were rinsed gently with 0.2  $\mu\text{m}$  FSW and then coral tissue was removed from the coral skeleton by Water-Pik with 0.45  $\mu\text{m}$  FSW (Johannes and Wiebe 1970). To remove the mucus produced during the isolation procedure, coral blastate was first filtered through a 350- $\mu\text{m}$ -nylon mesh. The blastate was then homogenized with a potter homogenizer. Following centrifugation at 115 g for 5 min, filtration through 40- $\mu\text{m}$  mesh was performed. The algal pellet was washed three times by centrifugation (1500 g for 5 min, 2000 g for 10 min and 3000 g for 15 min). This method of isolating and cleaning zooxanthellae yields intact algal cells. This was checked microscopically under 400 $\times$  magnification using a Nikon OPTIPHOT-2

microscope. The isolated zooxanthellae were then introduced into the larval culture. Under these condition 100 % of larvae became infected within 72 h of the addition of the zooxanthellae. In control culture, where no zooxanthellae were added, none of the larvae became infected. The cultures of infected larvae were followed for another 32 days to estimate the population growth rate of zooxanthellae within the larval hosts. Ten larvae were removed from the cultures, squashed and mounted on a slide, and viewed under blue-violet light using a fluorescent microscope at 7, 12, 15, 18, 24 and 32 days after spawning. The blue-violet light causes the chlorophyll with the zooxanthellae to fluoresce bright red allowing even a single symbiont to be seen in a larva.

### Larval survivorship under different temperatures

Following the successful inoculation of *Acropora muricata* larvae with homologous zooxanthellae, 3 replicate batches of symbiotic and non-symbiotic larvae were introduced into 25 ml glass jars containing 0.2  $\mu\text{m}$  FSW in water baths maintained at 28, 32 and 36° C with RACOMACE, Model HT-10D heaters under a mildly stressful level of light (125-150  $\mu\text{E m}^{-2} \text{s}^{-1}$ ) provided by cool fluorescent globes. Some light stress is required, in addition to temperature, to induce a stress response in zooxanthellae. Water was changed approximately every 72 h. Twenty larvae were placed in each jar, providing a total of 60 larvae per combination of temperature and treatment (i.e. zooxanthellate vs. non-zooxanthellate). The number of larvae alive in each jar was counted directly after 6, 18, 24, 40, 96 and 198 h to estimate survivorship through time. Dead larvae quickly dissolve and any larva visible was considered to be alive.

## Results and Discussion

Many larvae survived well at 28 and 32° C with approximately 30 % of the cohort remaining alive after 198 h (Fig. 1). While there was no major difference in survivorship between zooxanthellate and non-zooxanthellate larvae at these temperatures, at 28° C survivorship of zooxanthellate larvae was consistently higher than the non-zooxanthellate larvae, and it is possible this trend could have resulted in significant difference in survivorship if the experiments had been continued (Fig. 1). Higher survivorship of zooxanthellate larvae at non-stressful temperatures is to be expected because the acquisition of symbionts give the larvae the potential to be autotrophic and supplement the initial maternal energy reserves with nutrition from the zooxanthellae (Richmond 1981).

In contrast, all zooxanthellate larvae died within 18 h, and all non-zooxanthellate larvae within 40 h, at 36° C (Fig. 1). While this small difference in survivorship between the groups is unlikely to be ecological meaningful, this is the result we would have predicted. Current theories to explain coral bleaching suggest that heat makes light toxic. At high temperatures even normal light levels overload photosystem II of the zooxanthellae (Jones et al. 1998; Takahashi et al. 2004) resulting in the production of reactive oxygen species (ROS) by photosynthetic electron

transport. At least some of these ROS are permeable through the cell wall (Nakamura and van Woesik 2001) and may damage tissue beyond the zooxanthellae. Consequently, we would have predicted that at high temperature larvae containing zooxanthellae would be affected by these ROS and mortality rates would be higher than larvae that lacked zooxanthellae. However, it is also clear that the upper thermal limit of *Acropora muricata* larvae lies somewhere between 32 and 36° C suggesting that the coral host, or at least these early life history stages, are susceptible to temperatures that do not typically challenge many other marine animals.

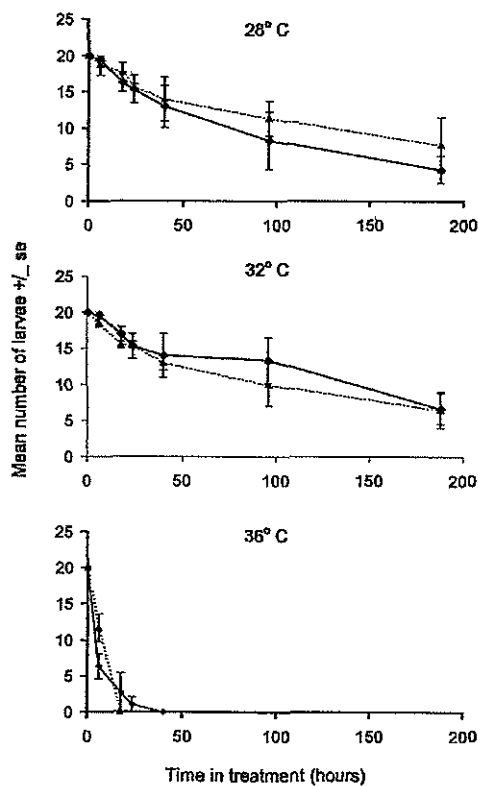


Fig. 1. Survivorship of *Acropora muricata* larvae through time at 3 different temperatures. Dotted lines represent symbiotic larvae and the solid lines are non-symbiotic larvae.

The failure to detect any major difference between zooxanthellate and non-zooxanthellate larvae may have been due to the low the density of symbionts when the experiments were initiated. The mean density of zooxanthellae in larvae at start of the experiments was  $7.1 \pm 2.45$  per larva and possibly too low to affect the physiology of the host. By way of comparison, values for the mean number of zooxanthellae per egg in broadcast spawning species with vertical transmission

include 3000 in *Porites cylindrica* and 1300 in *Montipora digitata* (Hirose et al. 2001). In the original larvae cultures maintained at 26° C under low light the mean symbiont density continued to grow, reaching a mean of  $360 \pm 60$  per larva after 30 d, with no sign of stabilizing (Fig. 2). Clearly, these larvae can accommodate much higher densities of zooxanthellae than those in the larvae at the time the temperature manipulations commenced. Future studies, using the larval model, should be conducted after these densities have stabilized.

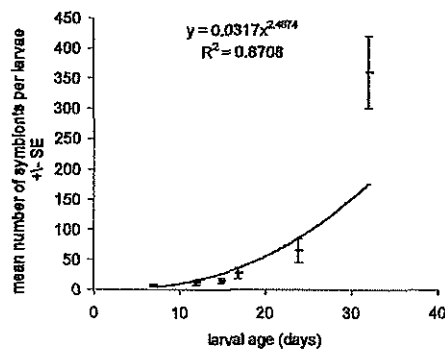


Fig. 2. Mean density of zooxanthellae ( $\pm 1$  SE) in larvae of *Acropora muricata* through time.

Fig. 3 outlines a generalized stress response in coral larvae. The term "stress" was first proposed by Hans Selye (Selye 1936). Stress was originally defined a nonspecific response independent of the type of stress (Selye 1946). For example, corals bleach in response to high temperature, high light or high salinity stress, and therefore bleaching can be considered a stress response. It is important to note that this stress theory is relevant for all organisms; invertebrates, humans, plants, fungus, and bacteria. In most stress responses, the production of ROS in cells is a common early event. Superoxide ( $O_2^-$ ) is the most commonly produced ROS. Hydrogen peroxide ( $H_2O_2$ ), which is the only diffusive ROS molecule, is produced secondarily. Highly toxic hydroxyl radical ( $\cdot OH$ ) or singlet oxygen ( $^1O_2$ ) can also form under severe stress conditions.

Potentially toxic ROS formed in the cells are removed by antioxidant systems. As long as these scavenging mechanisms are functional, ROS will not accumulate. Under severe stress, however, these antioxidant systems may not be able to destroy all ROS produced, in which case oxidative damage will occur. Oxidative destruction or modification of biomolecules (protein, lipid, DNA) causes enzyme inactivation, lipid peroxidation and DNA damage that leads to metabolic dysfunction, cell destruction, mutation or sterilization. In addition to antioxidant systems, there are repair systems to remove damaged molecules and to replace them with new ones. The photosynthetic reaction center protein D1 requires a protein repair system to maintain photosynthetic activity under light (Takahashi et al. 2004). In this context, antioxidant

systems function as the primary line of defense and the repair systems act as a secondary line of defense against oxidative stress (Fig. 3). If these mechanisms cannot limit or suppress stress damage, living organisms will eventually die.

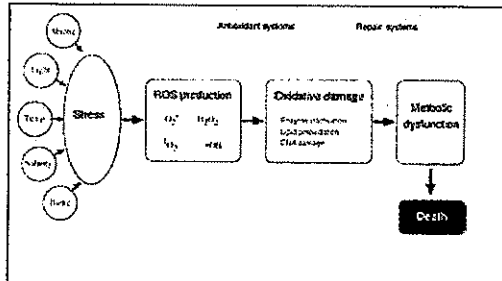


Fig. 3 A sequential diagram for stress-induced events

Except in the eyes, animals are not susceptible to visible light stress because there are few pigments which absorb visible light energy. Thus, animals normally are tolerant of visible light stress. This is not the case for plants and algae because of the presence of photosynthetic pigments such as chlorophylls; even visible light is a potential cause of stress for zooxanthellae. This may account for the difference in survivorship between zooxanthellate and non-zooxanthellate larvae at 36° C. Thus, harboring symbiotic zooxanthellae involves a risk for coral larvae in that the zooxanthellae are an additional source of ROS that may cause oxidative damage. However, this risk may be outweighed by advantages in terms of the acquisition of stress tolerance. One of the obvious benefits of harbouring symbionts is a continuous supply of energy via photosynthetic activity of zooxanthellate.

Marine invertebrates are known to be susceptible to ammonium (NH<sub>4</sub><sup>+</sup>) or nitrite ion (NO<sub>2</sub><sup>-</sup>), i.e. nitrogen toxicity. Recent biochemical investigations have suggested that such inorganic nitrogen can be converted to toxic molecules whose characteristics are very similar to those of ROS; these are referred to as reactive nitrogen species (RNS). Photosynthetic organisms, including zooxanthellae, can remove potential source of RNS (NH<sub>4</sub><sup>+</sup> or NO<sub>2</sub><sup>-</sup>) by driving nitrogen assimilation metabolism. It is important to note that nitrogen assimilation requires the reducing power provided by photosynthesis. The involvement of RNS in stress damage has been proposed in land plants (Yamasaki 2000). For evaluating the contribution of photosynthetic symbionts in stress tolerance, we need to pay much attention to nitrogen assimilation metabolism as well as carbon assimilation process.

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