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Acclimatization of *Anemonia viridis* (Forskäl 1775) by thermal preconditioning; potential role of catalase

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Abstract

The phenomenon of cnidarian bleaching has attracted much attention in the literature, as the frequency of coral bleaching events and mass mortality have increased over the last few decades, driving the degradation of coral reefs and loss of coral cover worldwide. This has also led to increased interest in exploring the underlying physiological and biochemical mechanisms of acclimatization of reef building corals and other symbiotic cnidarians to thermal stress. Thermal history and/or previous exposure to thermal stress have been linked with the thermal acclimatization of scleractinian corals in many studies. In this study, it was demonstrated that short term thermal preconditioning by stepwise increase in temperature for a period of 16 days ($19 \pm 1^\circ\text{C}$ for 7 days and $21 \pm 1^\circ\text{C}$ for 9 days) provides thermal tolerance to a temperate symbiotic anemone *Anemonia viridis*. Preconditioned *A. viridis* exposed to the experimentally determined bleaching threshold temperatures for a period of 9 days ($23 \pm 1^\circ\text{C}$ for 7 days and $25 \pm 1^\circ\text{C}$ for 2 days) did not show bleaching whereas non-preconditioned *A. viridis*, exposed to the same bleaching threshold temperatures, for the same duration, showed significant bleaching. Biochemical measurements of samples revealed significant increase in the activity of antioxidant enzyme catalase in preconditioned *A. viridis*, suggesting the potential role of catalase in acclimatization of *A. viridis* to thermal stress and thermal stress-induced increased production of reactive oxygen species.

Introduction

One of the key environmental concerns in this age of rapid climate change is cnidarian bleaching. This phenomenon is one of the principle causes responsible for degradation of ecologically and economically important coral reefs and the loss of coral cover from the world's oceans (Hoegh-Guldberg 1999; Hoegh-Guldberg *et al.* 2007). There has been a dramatic increase in the reports of mass coral bleaching events and related coral mortalities since 1979, and it is estimated that mass bleaching events would further increase in frequency and severity in this century (Donner *et al.* 2005). Bleaching can be triggered in cnidarians and in other animals that live in symbiotic association with the dinoflagellate of the genus *Symbiodinium* (also known as zooxanthellae), by a variety of stressors. Cnidarian bleaching is defined as the loss of symbiotic dinoflagellates (and/or their pigments) from the cnidarian host tissue (Douglas 2003). It can also be explained as stress-induced breakdown of cnidarian-dinoflagellate symbiotic relationship (Leggat *et al.* 2011). Among the variety of stressors that trigger bleaching, thermal stress or higher than normal sea surface temperatures is the major cause of current climate change related-mass bleaching events and associated mortality (Hoegh-Guldberg 1999; Donner *et al.* 2005).

However, marine organisms including cnidarians are capable of acclimatising to thermal stress by modifying various components of their cellular metabolism so that they can perform better under elevated temperature conditions (Hoegh-Guldberg 1999). Maynard *et al.* (2008) reported increased thermal tolerance in three coral genera *Acropora*, *Pocillopora* and *Porites*, from the Great Barrier Reef, Australia during the 2002 bleaching event. This bleaching event of 2002 was even more severe than the notorious 1998 bleaching event, which affected more than 16% of the world's tropical coral reefs, with 50-90% mortality at few locations (Maynard *et al.* 2008). Likewise, Thompson & Woesik (2009) analysed data of many tropical reefs in the Red Sea, Indian and Pacific Oceans and found that the bleaching events of 1998 and 2005-06 were associated with similar elevations in temperature and irradiance; but the bleaching response in 2005-06 was reduced compared to the response during the 1998 bleaching event. One of the proposed mechanisms of thermal tolerance is acclimatisation to thermal stress through long-term physiological memory of past events (Maynard *et al.* 2008). Indeed, thermal history of the site increasingly appears to be important in acclimatization of corals to frequently occurring events of thermal stress. Corals bleach less severely where temperature variability is greater, primarily by acclimatisation to thermal stress. Thermal history and previous exposure to thermal stress determine bleaching response of cnidarians to contemporary thermal stress (Maynard *et al.* 2008; Guest *et al.* 2012).

Thermal tolerance in cnidarians could also occur by the mechanism of symbiont shuffling (Maynard *et al.* 2008). Buddemeier & Fautin (1993) put forward the adaptive bleaching hypothesis (ABH) which proposes that under thermal stress conditions, bleaching drives shuffling and/or switching of symbiont (*Symbiodinium*) types within a coral host. Thus, thermally sensitive symbiont types are switched and/or shuffled to thermally tolerant ones, in order to achieve thermally tolerant symbiont population within the coral host tissue, under elevated temperature conditions. ABH is being heavily debated in the primary literature. For example, Ware *et al.* (1996), Kinzie *et al.* (2001), Ralph *et al.* (2005), Baird *et al.* (2007) and Silverstein *et al.* (2012) found evidence in support of ABH, whereas McClanahan *et*

al. (2004), Thornhill *et al.* (2006), Jones (2008) and Stat *et al.* (2009) challenged ABH.

Thermal tolerance by acclimatization can be brought about by conditioning cnidarians with the previous exposure of thermal stress (Maynard *et al.* 2008). Bellantuono *et al.* (2012a) performed a thermal pre-conditioning study on a tropical scleractinian coral *Acropora millepora*, in which they conditioned the coral to thermal stress by exposing it to moderately high (above ambient but below bleaching threshold) temperature. Thermal stress-conditioned *A. millepora* samples did not show bleaching when stressed with the bleaching threshold temperature (the temperature at or beyond which bleaching occurs, under certain irradiance level) The authors did not find any change in the resident *Symbiodinium* community composition of acclimated *A. millepora*, as suggested by ABH. Such physiological acclimatization could involve cnidarian host acclimatization as well as *Symbiodinium spp.* acclimatization to thermal stress where stress response proteins such as heat shock proteins may play key role in the process of acclimatization (Bellantuono *et al.* 2012a).

Cnidarian bleaching is tightly linked with the phenomenon of oxidative stress and many recent studies have pointed out the significance of oxidative stress in cnidarian bleaching (Lesser 1997; Downs *et al.* 2002). Aerobic metabolism is always prone to production of toxic oxygen intermediates such as the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^{\cdot}), collectively called as reactive oxygen species (ROS) (Fridovich 1998). ROS production is particularly increased during environmental stress (Mittler 2002). ROS are highly reactive and damage various vital bio-molecules such as DNA, proteins and lipids. Antioxidant defences such as superoxide dismutase, catalase and peroxidases protect aerobic organisms from ROS (Fridovich 1998). However, when ROS formation overwhelms their antioxidant-mediated degradation, then this imbalance between pro-oxidant and antioxidant creates the situation of oxidative stress where elevated levels of cellular ROS cause oxidative damage to cellular components such as DNA, mitochondria and cell membranes (Halliwell 2007). ROS produced in the cnidarian-dinoflagellate symbiotic system during thermal stress play a fundamental role in driving breakdown of this symbiosis (bleaching) (Weis 2008). Cnidarian bleaching is closely linked with the antioxidant capacity of the symbiotic cnidarian. The oxidative theory of cnidarian bleaching proposes that loss of zooxanthellae may be the final strategy to protect the cnidarian host from oxidative stress when all the antioxidant defence mechanisms are overwhelmed (Downs *et al.* 2002). Previous studies have suggested a link between regulation of antioxidant enzyme activity and acclimatization to thermal stress, in reef building corals (Gates & Edmunds 1999).

Like reef building corals, the temperate sea anemone *Anemonia viridis* also harbours zooxanthellae (genus: *Symbiodinium*) in the cells of gastrodermal tissue layer (Ganot *et al.* 2011). *A. viridis* is believed to be an ideal model organism for studying cnidarian-dinoflagellate symbiosis and its stress-induced breakdown (Bythell *et al.* 1995; Ganot *et al.* 2011). In this study, thermal preconditioning experiments were conducted on *A. viridis* and effects of preconditioning on the host physiology were studied. It was investigated if antioxidants (catalase and glutathione) play any role in thermal preconditioning induced-acclimatization of *A. viridis*. Additionally, lipid peroxidation of the samples was also measured to further explore the role of oxidative stress in thermal acclimatization mechanism of *A. viridis*. It was found that,

on exposure to bleaching threshold temperatures, anemones from preconditioning treatment were resistant to bleaching while anemones from non-preconditioning treatment significantly bleached. Enzymatic antioxidant catalase may play a role in the thermal preconditioning induced-acclimatisation of *A. viridis*.

Materials & Methods

Sample collection and maintenance

Snakelocks anemones (*Anemonia viridis*) were collected from three different locations within the coastal region of south west England, all near the city of Plymouth, UK. These locations included Mount Batten (50°21'34"N, 4°07'47"W), Wembury beach (50°19'01"N, 4°04'59"W) and Bigbury (50°17'03"N 3°53'41"W). Anemones were carefully removed by hand and collected in small containers from the intertidal region during September and October 2013. All the collected anemones were transported to a laboratory at Plymouth University and maintained in aquaria with aerated seawater at ambient temperature of $16 \pm 1^\circ\text{C}$ and a metal halide lamp giving light ($\sim 10,500$ lx) with a 12 h/12 h cycle of light and dark. Anemones were fed with few small pieces of cockles (*Cerastoderma edule*) once every two weeks and half of the aquarium water was changed every week. In such a way, anemones were acclimatised for 25 days before proceeding with thermal manipulative experiments.

Thermal manipulative experiments

In total, nine healthy-looking anemones of similar sizes (approximately 15 cm wide spread of tentacles) were chosen for the study. They were divided in three different treatment groups, with each treatment group having three replicate anemones. These three treatment groups were control (C), pre-conditioning (P) and non-preconditioning (N). The whole thermal stress experiment lasted for a total of 25 days, beginning from day 0 when chosen anemones (all at $16 \pm 1^\circ\text{C}$ of ambient temperatures) were sampled for the first time and then placed in different treatment groups. After that, sampling took place on day 7, day 16, day 18, day 23 and day 25. Temperature change took place in the afternoon of each of the sampling days, except day 18 (after the sampling). The anemones of control treatment did not receive any kind of manipulation of temperature and were maintained under an ambient temperature of $16 \pm 1^\circ\text{C}$ throughout the experiment. The pre-conditioned anemones were initially exposed to $19 \pm 1^\circ\text{C}$ for one week and to $21 \pm 1^\circ\text{C}$ for nine days and then challenged with the thermal stress of $23 \pm 1^\circ\text{C}$ for one week and of $25 \pm 1^\circ\text{C}$ for two days. Hence, this treatment group received step-wise increase in temperature prior to thermal challenge. In contrast to that, non-preconditioning anemones did not receive such a step-wise increase in temperature prior to thermal challenge. These anemones were challenged with the thermal stress of $23 \pm 1^\circ\text{C}$ for one week and of $25 \pm 1^\circ\text{C}$ for two days, directly from the ambient temperature of $16 \pm 1^\circ\text{C}$. This method of thermal acclimation by pre-conditioning cnidarians to thermal stress was adapted from Bellantuono *et al.* (2012a) with a few differences compared to their methodology (See Figure 1 for schematic representation of the experimental design).

Each treatment consisted of a water-bath maintained at constant set temperature for required period of time. Anemones were placed in separate beakers; each water-bath representing a single treatment contained three replicate beakers, with one anemone in every beaker. Water temperature was measured on each day of the experiment using a digital thermometer. Change of the water in the beakers took

place once a week using artificial seawater maintained at the same temperature as that of the respective beaker to ensure that the temperature of seawater inside beakers did not alter because of the water change.

Sampling was carried out by extracting approximately two tentacles of the anemone. The chopped tentacles were slightly blotted on a tissue paper and then weighed. The tentacles were then added to 1 ml of phosphate-buffered saline (PBS) solution (100 mM potassium phosphate buffer solution containing sodium chloride, pH 7.4), and homogenised using a Potter homogenizer. For each sample, 20 μ l were removed for zooxanthellae count. The remaining part of samples was centrifuged at 7000 g for 10 min so that cellular debris and zooxanthellae cells could be separated from the host tissue extract. The supernatant was retained and stored at -80°C for biochemical measurements. The majority of the reagents used for biochemical measurements were bought from Sigma-Aldrich (Poole, UK).

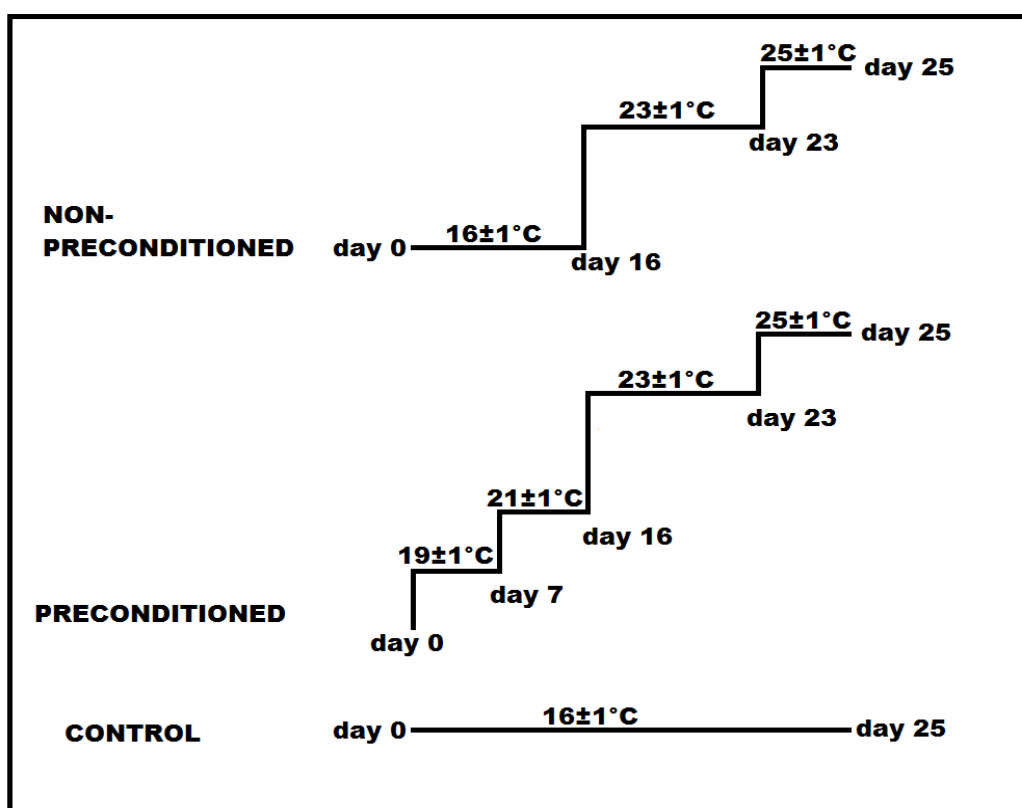


Figure 1: schematic representation of the experimental design, showing the three treatments. All the *A. viridis* replicates from three treatment groups were at $16 \pm 1^{\circ}\text{C}$ before sampling them on day 0. After that sampling, thermal manipulative experiment started on day 0 and finished on day 25. Except those two days, only those days are shown on the line of each treatment, when the temperature change took place.

Zooxanthellae density measurements

The non-centrifuged, 20 μ l part of the tissue extracts was used for measuring zooxanthellae cell densities using a Neubauer haemocytometer. Extracts were placed in the counting chambers of the haemocytometer (depth of chamber, 0.1 mm), and zooxanthellae cells were counted under a compound light microscope

using 40× magnification. Cell count of each sample was calculated to get the zooxanthellae count per gram of the tentacle tissue, for each sample.

Catalase measurements

The spectrophotometric method for measuring decomposition of hydrogen peroxide (H₂O₂) catalysed by catalase of Beers & Sizer (1952) was used for measuring catalase activity of the samples. Sample (100 µl) was added to a quartz cuvette and the reaction with H₂O₂ was started by adding 2.9 ml of 10 mM H₂O₂ solution (0.11 ml of 30% (w/w) H₂O₂ diluted with 100 ml of 100 mM potassium phosphate buffer solution, pH 7.0) at room temperature (approximately 20°C). The rate of decomposition of H₂O₂ was measured at 240 nm using a spectrophotometer (Jenway 7315). The change in absorbance was monitored for 100 s. One catalase unit was defined as the decomposition of 1 µmol of H₂O₂ min⁻¹ and was calculated by using the molar extinction coefficient of H₂O₂ at 240 nm of 39.4 M⁻¹ cm⁻¹ (Merle *et al.* 2007), and the catalase activity of the samples was expressed in µmol min⁻¹ g⁻¹.

Total glutathione measurements

The method, which involves cyclic reactions of reduced glutathione (GSH) and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), developed by Owens & Belcher (1965) with some modifications in the protocol as described by Al-Subiai *et al.* (2009), was used for measuring the total glutathione (GSH and GSSG) content of the samples. Samples (25 µl) were mixed in a 1:1 ratio with 25 µl of buffered DTNB (100 mM potassium phosphate, pH 7.5, containing 5 mM potassium EDTA and 10 mM DTNB). These buffered DTNB treated samples (50 µl) were mixed with 210 µl of glutathione reductase solution (2.56 U/ml, G3664 from *Saccharomyces cerevisiae*). After equilibration for 1 min, the reaction was initiated by adding 60 µl of 1 mM NADPH (Melford, Cambridge) in a 96-well microplate (Sterilin Ltd., UK). Change in absorbance related to colour change in the reaction mixture was monitored at 412 nm over 5 min using an Optimax microplate reader (Molecular Devices, Silicon Valley, CA, USA). During every run, the change in absorbance was monitored for eighteen samples along with a GSH standard (20 µM GSH treated with DTNB) and a blank (no GSH) in triplicate.

Lipid peroxidation measurements

The assay of thiobarbituric acid reactive substances (TBARS), essentially based on Ohkawa *et al.* (1979) and Uchiyama & Mihara (1978), was used to measure lipid peroxidation in the tissue samples, in terms of the malondialdehyde (MDA) concentration in the samples. Samples were added to a 96-well microplate (60 µl per well) and were treated with 210 µl of thiobarbituric acid (TBA) {1.3% TBA w/v} in 0.3% NaOH) and 30 µl of 1 M HCl solution. The 96-well microplate was incubated at 60°C for 40 min. After incubation, absorbance was measured in Optimax microplate reader (Molecular Devices, Silicon Valley, CA, USA) at three different wavelengths (517, 547 and 532 nm). Data from 517 and 547 nm were averaged and then subtracted from the 532 nm data. These absorbance data were converted into MDA concentrations using a standard curve. The standard curve was plotted by measuring absorbance of a range of dilutions of a standard solution (25 µM 1,1,3,3-tetramethoxypropane).

Statistical analyses

The *Symbiodinium* density data were analysed to find the effect of time and the interaction between time and treatments This was done using repeated measures

ANOVA in the SPSS statistical package (IBM Inc Armonk, NY, USA). The data for catalase activity, total glutathione levels and MDA concentration were analyzed to find the effect of different treatment conditions on these biochemical measurements. This was also done using repeated measures ANOVA in the same statistical package. For *Symbiodinium* density data as well as data of biochemical measurements, the significantly different treatment group was compared with the control by conducting a post hoc test – *a priori* contrasts using Tukey's LSD based upon estimated marginal means, in the same statistical package. The level of probability was accepted as significant if $P < 0.05$.

Results

Response of *Anemonia viridis* to thermal stress (*Symbiodinium* density)

There were significant differences in *Symbiodinium* densities between days and also a significant interaction between days and treatment ($P = 0.008$ and 0.007 , respectively, repeated measures ANOVA; Figure 2). Despite having low replication ($n = 3$ per treatment) the observed power exceeded reasonable expectations (power = ± 0.9 for both hypotheses). On day 16, when both preconditioning and non-preconditioning treatment groups were challenged with the experimentally determined bleaching threshold temperature of the, *Symbiodinium* densities began to show a treatment-specific pattern.

Anemones from the non-preconditioning treatment began to lose their zooxanthellae after day 16 and, by day 25, they had lost considerably higher numbers of zooxanthellae, as reflected in the decline in zooxanthellae density. There was a significant difference in *Symbiodinium* densities in anemones from the control and non-preconditioning treatment groups ($P = 0.005$, repeated measures ANOVA with Tukey LSD; Figure 2).

In contrast, anemones from the pre-conditioning treatment group retained most of their zooxanthellae, even after challenge with the bleaching threshold temperature on day 16. There was no significant difference in *Symbiodinium* densities in anemones from the control and preconditioning treatment groups ($P = 0.147$, repeated measures ANOVA with Tukey LSD; Figure 2).

Catalase activity in tentacle tissue from anemones

Significant difference was found in the catalase activity among treatment groups ($P = 0.029$, repeated measures ANOVA; Figure 3). Although anemones from the non-preconditioning and preconditioning treatment groups were challenged with the same bleaching threshold temperatures (on day 16 and on day 25), they showed different catalase activity.

Catalase activity of anemones that underwent the non-preconditioning treatment did not show any significant changes, compared to catalase activity of controls ($P = 0.851$, repeated measures ANOVA with Tukey LSD; Figure 3).

In contrast, catalase activity of anemones that underwent the preconditioning treatment showed significant increase, compared to the catalase activity of controls ($P = 0.019$, repeated measures ANOVA with Tukey LSD; Figure 3).

Total glutathione level in tentacle tissue from anemones

No significant difference was found in total glutathione levels between the anemones of three treatment groups ($P = 0.270$, repeated measures ANOVA; Figure 4). Anemones from both preconditioning and non-preconditioning treatment groups showed some response (slight increase in total glutathione levels) after thermal challenge on day 16, but it was not statistically significant. For unknown reasons, anemones of control treatment also showed increased levels of glutathione (see Discussion) (Figure 4).

MDA concentration of anemones (tentacle tissue)

Like glutathione, no significant difference was detected in MDA concentration between the treatment groups ($P = 0.715$, repeated measures ANOVA, Figure 5).

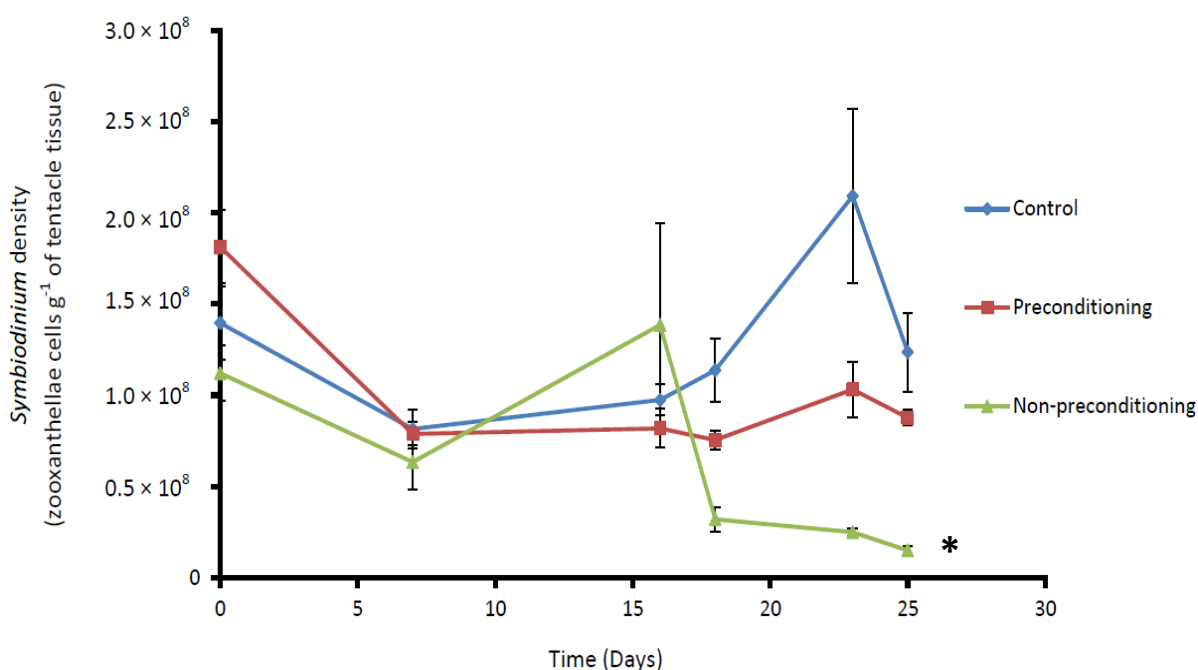


Figure 2 *Symbiodinium* densities of *A. viridis* (tentacle tissue) under three different treatments, over a course of 25 days. Asterisk indicates the treatment which is significantly different from control ($P = 0.005$, repeated measures ANOVA with Tukey LSD, $n = 3$). The blue line represents the control treatment, maintained at ambient temperature ($16 \pm 1^\circ\text{C}$), throughout the experiment. The red line represents the preconditioning treatment which was at $19 \pm 1^\circ\text{C}$ for a week and at $21 \pm 1^\circ\text{C}$ for 9 days prior to receiving thermal challenge of $23 \pm 1^\circ\text{C}$ on day 16 and, of $25 \pm 1^\circ\text{C}$ on day 23. The green line represents the non-preconditioning treatment which was at ambient temperature ($16 \pm 1^\circ\text{C}$) until the day 16, when it received thermal challenge of $23 \pm 1^\circ\text{C}$ and, of $25 \pm 1^\circ\text{C}$ on day 23. Each data point is the mean *Symbiodinium* density \pm S.E.

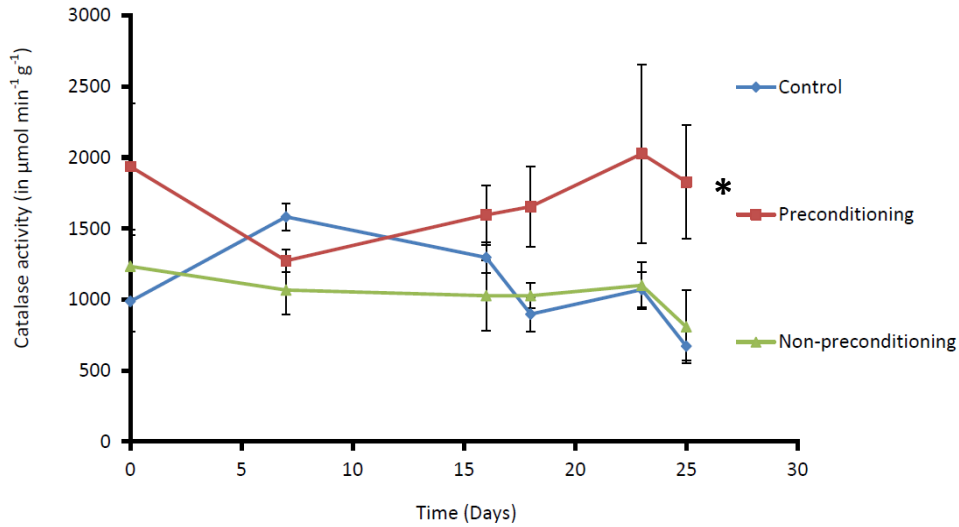


Figure 3 Catalase activity of *A. viridis* (tentacle tissue) under three different treatments, over a course of 25 days. The asterisk indicates the treatment which is significantly different from the control ($P = 0.019$, repeated measures ANOVA with Tukey LSD, $n = 3$). The blue line represents the control treatment, maintained at ambient temperature ($16 \pm 1^\circ\text{C}$), throughout the experiment. The red line represents the preconditioning treatment which was at $19 \pm 1^\circ\text{C}$ for a week and at $21 \pm 1^\circ\text{C}$ for 9 days prior to receiving thermal challenge of $23 \pm 1^\circ\text{C}$ on day 16 and of $25 \pm 1^\circ\text{C}$ on day 23. The green line represents the non-preconditioning treatment which was at ambient temperature ($16 \pm 1^\circ\text{C}$) until the day 16, when it received thermal challenge of $23 \pm 1^\circ\text{C}$ and, of $25 \pm 1^\circ\text{C}$ on day 23. Each data point is the mean catalase activity \pm S.E.

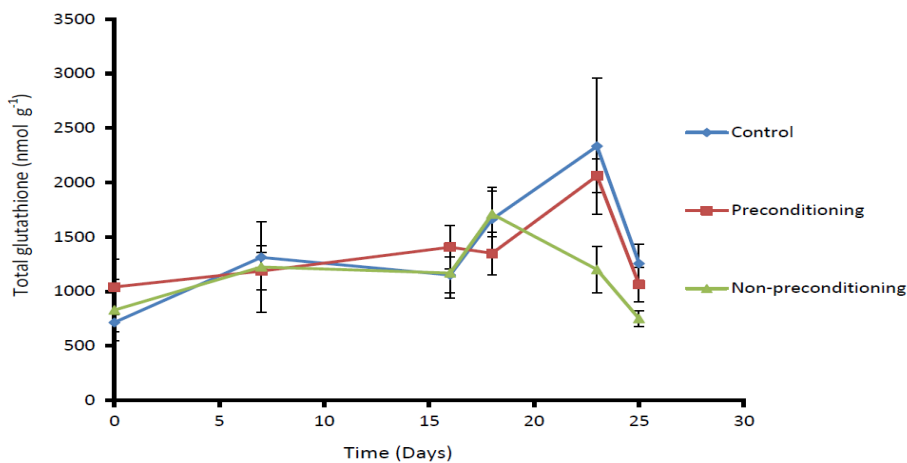


Figure 4 Total glutathione levels of *A. viridis* (tentacle tissue) under three different treatments over a course of 25 days. No significant difference was found in total glutathione levels among treatments ($P = 0.270$, repeated measures ANOVA, $n = 3$). The blue line represents the control treatment, maintained at ambient temperature ($16 \pm 1^\circ\text{C}$), throughout the experiment. The red line represents the preconditioning treatment which was at $19 \pm 1^\circ\text{C}$ for a week and at $21 \pm 1^\circ\text{C}$ for 9 days prior to receiving thermal challenge of $23 \pm 1^\circ\text{C}$ on day 16 and, of $25 \pm 1^\circ\text{C}$ on day 23. The green line represents the non-preconditioning treatment which was at ambient temperature ($16 \pm 1^\circ\text{C}$) until the day 16, when it received thermal challenge of $23 \pm 1^\circ\text{C}$ and, of

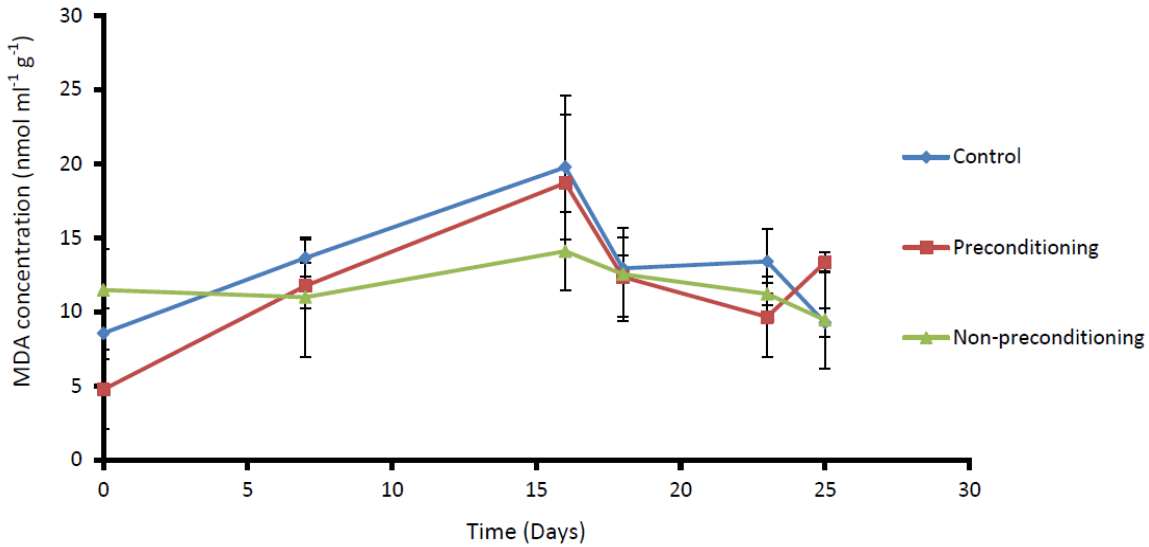


Figure 5: MDA concentrations of *A. viridis* (tentacle tissue) under three different treatments over a course of 25 days. No significant difference was found in total glutathione levels among treatments ($P = 0.715$, repeated measures ANOVA, $n = 3$). The blue line represents the control treatment, maintained at ambient temperature ($16 \pm 1^\circ\text{C}$), throughout the experiment. The red line represents the preconditioning treatment which was at $19 \pm 1^\circ\text{C}$ for a week and at $21 \pm 1^\circ\text{C}$ for 9 days prior to receiving thermal challenge of $23 \pm 1^\circ\text{C}$ on day 16 and, of $25 \pm 1^\circ\text{C}$ on day 23. The green line represents the non-preconditioning treatment which was at ambient temperature ($16 \pm 1^\circ\text{C}$) until the day 16, when it received thermal challenge of $23 \pm 1^\circ\text{C}$ and, of $25 \pm 1^\circ\text{C}$ on day 23. Each data point is the mean concentration of MDA \pm S.E.

Discussion

Anemones (*Anemonia viridis*) from the preconditioning treatment, which received short term exposure to moderately elevated temperatures (higher than ambient but below the bleaching threshold) did not show bleaching after they were stressed with the elevated temperature above the normal bleaching threshold. They maintained *Symbiodinium* densities within their tissue, which were comparable with the *Symbiodinium* densities of controls even after thermal challenge. In contrast, anemones from the non-preconditioning treatment, which did not receive prior exposure of moderately elevated temperatures, suffered from significant losses of *Symbiodinium* densities (bleaching) after the thermal challenge of the same intensity. Such acquired thermal tolerance following pre-exposure to moderately elevated temperatures has been demonstrated previously, in other cnidarians, particularly scleractinian corals. Middlebrook *et al.* (2008) pre-stressed the coral *Acropora aspera* for 2 weeks and 1 week prior to a simulated bleaching event and found that pre-stressed corals showed reduced bleaching in comparison to the corals that were not pre-stressed. In two separate studies, Bellantuono *et al.* (2012a, 2012b) preconditioned the coral *Acropora millepora* by short term exposure to a temperature, 3°C below the bleaching threshold temperature and found no bleaching after stressing it with the bleaching threshold temperature.

Tolerance to thermal stress, acquired by pre-exposure of stress, has also been observed in the field. Griffin *et al.* (2006) reported that the thermally sensitive coral

Pocillopora meandrina from a thermal outfall site in Kahe, Hawaii USA, which was frequently exposed to heated seawater (5°C higher than the surrounding ambient sea surface temperature) did not show bleaching under elevated temperatures (32°C) and had a similar physiological response as that of thermally tolerant coral *Pocillopora damicornis*, suggesting that the thermally sensitive *P. meandrina* from thermal outfall site was acclimated to elevated temperatures. This, acclimated population of *P. meandrina* showed a different physiological response, compared to the conspecifics in the nearby region, which were living in comparatively more stable environmental conditions, including temperature. Such acquired resistance to thermal stress is not restricted to pre-exposure of thermal stress but can also be acquired by pre-exposure to light stress or higher irradiance. On fringing reefs in Thailand, colonies of the coral *Goniastrea aspera* that had more exposure of solar radiation showed bleaching resistance under elevated temperatures compared to colonies in shaded areas, which had less exposure of solar radiation. Coral colonies with more exposure of solar radiation showed resistance to bleaching, primarily because of this pre-exposure and/or prior experience of light stress that also made them tolerant to thermal stress (Brown *et al.* 2002a, 2002b). Our current study and these other previous studies emphasize the significance of stress history and/or pre-exposure of stress, in acclimatization of cnidarians to thermal and other stresses, which is increasingly becoming important in this age of rapid climate change and global warming. Persistence of reef building corals and other cnidarians in this changing environment depends on their physiological capabilities of acclimatization (Gates & Edmunds 1999).

To further explore the physiological mechanisms of this thermal preconditioning-induced acclimatization of *A. viridis*, antioxidant defences of the anemones were investigated. Catalase activity showed an increase in preconditioned anemones, which was significantly different from that of the controls, whereas catalase activity of non-preconditioned anemones did not show any significant changes. This suggests that the enzymatic antioxidant catalase has a potential role in the acclimatization of *A. viridis* to thermal stress. This finding is in agreement with the previous related studies. Merle *et al.* (2009) performed catalase characterization studies in *A. viridis*. They investigated effects of a catalase inhibitor on *A. viridis* symbiotic system and found bleaching of *A. viridis* in response to introduction of catalase inhibitor, even under normal temperature and light conditions. Lesser (1997) exposed the coral *Agaricia tenuifolia* to elevated temperatures and exogenous antioxidants, including catalase and did not find bleaching of stressed *A. tenuifolia*; whereas *A. tenuifolia* exposed to only elevated temperature (without exogenous antioxidants) showed significant bleaching (Lesser 1997). Leutenegger *et al.* (2007) studied a natural bleaching event of temperate anemones *Anemonia rustica* and *Anemonia sulcata* (= *A. viridis*) var. *smaragdina*, occurred in a small bay near Collioure, France. They found reduced catalase activity in significantly bleached anemones. All of these studies highlight the importance of catalase in preventing the process of cnidarian bleaching and the findings of present study are in accordance with them.

Under thermal stress conditions, reactive oxygen species (ROS) production mainly by the chloroplasts of the zooxanthellae, and also by the mitochondria of the cnidarian host increases (Downs *et al.* 2002, Saragosti *et al.* 2010). Photoinhibition of photosynthesis in the symbiont zooxanthellae causes an increase in ROS production in the zooxanthellae (Smith *et al.* 2005). Hydrogen peroxide (H₂O₂) from stressed zooxanthellae diffuses through the membranes, into the host cell (Lesser

1996). At the same time, cnidarian host cells also suffer from stress-induced increased production of ROS (Saragosti *et al.* 2010; Higuchi *et al.* 2010). In such a way, this symbiotic system faces heavy oxidative stress that ultimately leads to the breakdown of this symbiotic relationship (bleaching) (Lesser 1997, Smith *et al.* 2005). The antioxidant enzyme catalase catalyzes the decomposition of toxic H₂O₂ to water and molecular oxygen (Fridovich 1998). It showed significantly increased activity in preconditioned anemones that did not bleach under elevated temperature, suggesting that the preconditioning treatment of *A. viridis* may prepare the anemones for coping with the thermal stress-related increased levels of ROS, probably by stimulating the production of catalase that effectively scavenges H₂O₂, a potent ROS and protects cells from ROS, and thus, from ROS-induced breakdown of this cnidarian-dinoflagellate symbiosis (Weis 2008). Indeed, the regulation of enzyme activity is the characteristic of physiological acclimatization of organisms to environmental stress. Cnidarians have the capacity to rapidly change protein expression in response to altered environmental conditions. Changes in protein turnover during the initial exposure to elevated temperature provide cnidarians with the biological flexibility to acclimatize to elevated temperatures (Gates & Edmunds 1999). In this context, it would be interesting to investigate the role of ubiquitin in preconditioning-related thermal acclimatization of anemones, as ubiquitin plays critical roles in protein turnover (Granados-Cifuentes *et al.* 2013) and previous studies have reported increased protein levels of ubiquitin (Downs *et al.* 2002) in stressed corals and also up-regulation of ubiquitin-related genes in thermally stressed coral *Acropora palmata* (DeSalvo *et al.* 2010).

Richier *et al.* (2006) noted that the thermal stress induced antioxidant defences in the zooxanthellae-containing host gastrodermal tissue of *A. viridis*, to counteract the elevated levels of ROS. Similarly, the scleractinian coral *A. millepora* undergoing bleaching on the Great Barrier Reef, Australia showed significant up-regulation of the gene for catalase (Seneca *et al.* 2010). Thus, under thermal stress conditions, expression of antioxidant enzymes is induced (Desalvo *et al.* 2008); however, elevated levels of antioxidant enzymes are insufficient to deal with the increased cellular flux of ROS (Lesser & Shick 1989, Lesser 1996). Although, antioxidant defences were induced in the gastrodermal tissue of thermally stressed *A. viridis*, that event was followed by the activation of caspase and initiation of apoptosis (programmed cell death) in thermally stressed *A. viridis*. Increased levels of ROS and consequent oxidative stress caused the induction of apoptosis through the caspase cascade in the gastrodermal tissue of thermally stressed *A. viridis* (Richier *et al.* 2006). Apoptosis is one of the key molecular mechanisms through which cnidarian bleaching occurs (discussed in detail in a review by Weis, 2008). In the present study, catalase activity was significantly increased in preconditioned anemones, which did not show bleaching, whereas non-preconditioned anemones showed bleaching with no substantial increase in the catalase activity. This raises an interesting hypothesis that increased activity of catalase may effectively control elevated levels of ROS and in turn, may inhibit activation of caspase cascade, resultant apoptosis and thus bleaching in preconditioned anemones. This hypothesis is supported by a cDNA microarray study of Bellantuono *et al.* (2012b) who thermally preconditioned scleractinian coral *A. millepora* in a similar way to our current study and explored transcriptomic responses of *A. millepora* specimens under preconditioned, non-preconditioned and control treatments. They found lower expression of NF- κ B inhibitor in non-preconditioned corals than that of

preconditioned corals during and prior to bleaching in non-preconditioned corals. NF- κ B is a transcription factor. As per one model of cnidarian bleaching, thermal-stress related increased levels of ROS activate NF- κ B, which induces apoptotic process directly or through up-regulation of nitric oxide synthase. Thus, in preconditioned *A. millepora*, induction of NF- κ B inhibitor might arrest NF- κ B mediated apoptosis and hence bleaching (Bellantuono *et al.* 2012b). Further studies are required to investigate the effects of thermal preconditioning on signal transduction pathways triggered by elevated cellular ROS, that lead to cellular processes such as apoptosis and autophagy, through which cnidarian bleaching occurs (Dunn *et al.* 2007).

Another antioxidant measured in the present study was a low molecular weight tripeptide thiol, glutathione. Total glutathione content of the samples was measured, which represents both reduced (GSH) and oxidised (GSSG) glutathione. H₂O₂ is reduced to water in the glutathione peroxidase catalysed reaction, in which reduced glutathione (GSH) is used as a substrate and then it gets oxidized (glutathione disulfide, GSSG). Glutathione disulfide (GSSG) is converted to reduced glutathione (GSH) via the enzyme glutathione reductase, depending on the availability of NADPH. A high intracellular ratio of GSH to GSSG is maintained and decrease in this ratio is indicative of oxidative stress (Dickinson & Forman 2002, Sunagawa *et al.* 2008). No significant difference was detected in the total glutathione levels of anemones from three treatments. However, increase in total glutathione levels was found in anemones over the course of experiment, particularly after challenging them with the thermal stress of 23 \pm 1°C on day 16, but this increase was levelled off by the end of the experiment, on day 25 (Figure 4). This finding (although, not statistically significant) is in support with the previous study investigating the effects of thermal stress on total glutathione levels and expression of glutamate-cysteine ligase (GCLC) gene which catalyses rate limiting step in the glutathione biosynthesis, in another symbiotic anemone *Aiptasia pallida*. That study found significant increase in both total glutathione levels and GCLC gene, in response to thermal stress (Sunagawa *et al.* 2008). In the current study, for few unforeseen reasons, total glutathione levels of anemones of control treatment, were also found to be elevated. A possible explanation for the increased glutathione levels of anemones of control treatment could be handling stress while sampling of anemones, as well as their maintenance under artificial conditions, masking the result of total glutathione levels in response to thermal stress alone. Such an elevated response in samples of control treatment has been previously reported for heat shock proteins in thermal stress (heat shock) experiments on *A. viridis* by Sharp *et al.* (1994) and also in a cDNA microarray study of the thermally stressed scleractinian coral *Montastraea faveolata* by Desalvo *et al.* (2008).

In addition to catalase and glutathione, the concentration of malondialdehyde (MDA) which is a product of lipid peroxidation, was also measured. Increases in the products of lipid peroxidation (for example MDA) can be used as a biomarker for ROS-induced cellular membrane damage (Flores-Ramírez & Liñán-Cabello 2007). Elevated cellular ROS can initiate chain reactions and polyunsaturated lipids of cellular membranes are particularly prone to such oxidative damage (Fridovich 1998). No significant difference in MDA concentration was found between the treatments groups. A relevant previous study investigating the effects of thermal stress (gradual temperature increase from 22°C to 32°C) on normal, partially bleached and control samples from different branches of the same coral colony of

the scleractinian coral *Pocillopora capitata*, also found no significant difference in MDA concentration between their treatment groups, despite the finding that partially bleached samples were more tolerant to short term thermal stress (up to 28°C), than normal and control samples (Flores-Ramírez & Liñán-Cabello 2007). Similarly, in a cross-transplantation experiment conducted in the field, on Grande Glorieuse Island (Indian Ocean), on the scleractinian coral *Pocillopora verrucosa*; bleaching and increased antioxidant defence (total oxyradical scavenging capacity and SOD) were found in upward transplanted samples (transplanted from 20 m depth to 5 m depth) in comparison to control and downward transplanted samples, as a result of depth-related increase in temperature and mainly irradiance for upward transplanted samples. However, no significant difference was detected in lipid peroxidation among controls and any of the transplanted samples (Richier *et al.* 2008). A different target of ROS (other than membrane lipids) (Richier *et al.* 2008) could be one explanation for this negative result, but it appears to be unlikely. In the lipid peroxidation assay of thiobarbituric acid (TBA) reactive substances (TBARS), reaction of TBA with MDA produces red colouration (Ohkawa *et al.* 1979). However, in the present study, no significant colour change was detected in any of the samples in the TBARS assay. Hence, the likely explanation for this negative result could be that the TBARS test used in this study was not sufficiently sensitive to detect the subtle changes in MDA concentration of anemones under three different treatments. Similar future studies investigating the TBARS assay need to consider its sensitivity and, how TBARS test could be made sufficiently sensitive, in order to detect the subtle differences.

Like catalase, superoxide dismutase (SOD) is also one of the key antioxidant enzymes. It catalyzes decomposition of $O_2^{\cdot-}$ radicals. Previous studies noted higher expression and isoform diversity of SOD in symbiotic anemone *A. viridis* compared to aposymbiotic anemone *Actinia schmidtii* (Richier *et al.* 2005). Our current study did not measure SOD activity. Further study is needed to investigate the role of SOD in acclimatization of preconditioned *A. viridis*. Similarly, heat shock proteins (HSPs) could have played a role in the acclimatization of preconditioned *A. viridis*. HSPs are molecular chaperones that are associated with the refolding of damaged proteins. They also prevent aggregation of non-native proteins and contribute to the removal of aggregated proteins (Fang *et al.* 1997, Rosic *et al.* 2011, Feder & Hofmann 1999). Choresh *et al.* (2001) found acclimatization of *A. viridis* to fluctuations in seawater temperatures, in their natural habitats by differential expression of HSP60. Choresh *et al.* (2007) reported induced expression of mitochondrial 70-kDa HSP in *A. viridis*, when exposed to heat shock or fluctuating temperature conditions. These and many other studies in other animals on HSPs provide strong background to hypothesize that preconditioned anemones might have differential expression of certain HSPs, compared to controls and non-preconditioned anemones. Further study is needed to experimentally show the role of HSPs in acclimatization of thermally preconditioned anemones.

Epigenetics is concerned with the heritable modifications to genetic material (DNA) that alters gene expression but not the DNA sequence itself (mutations). These modifications include DNA methylation and histone protein modifications which are heritable. Although heritable, these modifications are not permanent and can be reversed (Watson *et al.* 2007) and are believed to be influenced by the environment (Granados-Cifuentes *et al.* 2013). Previous thermal preconditioning studies on the scleractinian coral *A. millepora* have pointed out the potential role of epigenetic

mechanisms in acquired thermal tolerance of cnidarians (Bellantuono *et al.* 2012a, Bellantuono *et al.* 2012b). However, currently there are no studies investigating epigenetic mechanisms in cnidarians and their bleaching sensitivity in response to changes in the environmental conditions (Granados-Cifuentes *et al.* 2013), and thus, further study is needed in this direction.

In this study, sampling of tentacle tissue of anemones was conducted by blotting the tentacle tissue, and then weighing it, as mentioned in the Materials and Methods section. Although, the tentacle tissue was blotted before weighing a moderate amount of water was still associated with it during weighing. Using this wet weight of the tentacle tissue could be a limitation and could be partly responsible for masking the response of total glutathione and MDA concentration. In a future study this limitation could be resolved by measuring the protein content of the samples, as a measure of the amount of tissue present in the sample. Although, anemones from the short term preconditioning treatment showed tolerance to thermal stress for 9 days (to 23°C for 7 days and to 25°C for 2 days), the question of for how long the preconditioned anemones would show this acquired thermal tolerance, remains unanswered, as the experiment was terminated on day 25. In a future study, this could be identified by running the experiment for longer period of time. It would be interesting to see for how long the anemones show acquired thermal tolerance, and after a period of normal environmental conditions including temperatures, if the same anemones are stressed again with the elevated temperatures, then do they retain previously acquired thermal tolerance? One another thing which is important to consider is that this was a laboratory study where all other factors (such as light) were controlled and only the effects of single stressor *i.e.* temperature were investigated. This is highly unlikely to occur in nature where animals are exposed to a combination of different stresses, acting in differing intensities.

Conclusions

In summary, this study showed that prior exposure of sub-lethal thermal stress by stepwise increase in temperature (preconditioning) provides thermal tolerance to *A. viridis*, adding experimental evidence from a temperate symbiotic anemone to the list of studies that have shown thermal history and/or prior stress exposure giving thermal tolerance to scleractinian corals. Additionally, this study showed the potential role of antioxidant enzyme catalase in this physiological acclimatization of *A. viridis*. To our knowledge, this is the first study which has demonstrated thermal acclimatization in *A. viridis* by pre-exposure of moderately elevated temperatures and also the first study that has shown increased catalase activity in thermally acclimatized *A. viridis*. Increased catalase activity in thermally tolerant preconditioned *A. viridis* also corroborates the already established idea that increased production of ROS during thermal stress event plays a fundamental role in leading to breakdown of cnidarian-dinoflagellate symbiosis (Weis 2008). As the catalase of host tentacle tissue showed increased activity, this study also emphasizes the role of the cnidarian host in acclimatization of cnidarian-dinoflagellate symbiosis to thermal stress. Similarly, in the scleractinian coral *Goniastrea aspera*, pre-exposure of light stress-induced tolerance to thermal and light stress was found to have increased production of CuZnSOD, HSP60 and HSP70 in the host tissue (Brown *et al.* 2002b). Cnidarian host is as important as the symbiont zooxanthellae, in influencing the response of their symbiosis to thermal and other stresses. The host contains fluorescent pigments, mycosporine-like amino acids, antioxidant defence systems (as shown in this paper) and HSPs, all of which can aid in alleviating the damaging effects of

thermal and light stress (Baird *et al.* 2008). Equally, physiology of the symbiont zooxanthellae would also contribute in acquiring the thermal tolerance. For example Middlebrook *et al.* (2008) who found reduced bleaching in pre-stressed *A. aspera*, also noted more effective photo-protective mechanisms in the symbiont zooxanthellae of pre-stressed coral, including changes in non-photochemical quenching and xanthophyll cycling. Further studies are needed in order to understand the elaborate cellular, biochemical and physiological mechanisms of both partners of this symbiosis with which preconditioned symbiotic cnidarians manage to acclimatize to thermal and other stresses. This understanding is becoming important in this age of global warming, in order to understand the long term persistence of ecologically and economically important coral reefs and also in their conservation and management strategies (Bellantuono *et al.* 2012a, 2012b).

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