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High tolerance of symbiotic larvae of *Pocillopora damicornis* to thermal stress

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Abstract

Background: When coral planulae, which use a horizontal mode of symbiont transmission, are inoculated with *Symbiodinium*, they suffer greater oxidative stress under strong light or high-temperature stress than non-symbiotic counterparts. Thus, dinoflagellate symbionts may become a source of reactive oxygen species (ROS) under stress. However, it remains unknown whether vertically transmitted symbionts negatively affect coral larvae under stress. We investigated the thermal tolerance of symbiotic planulae of a vertical transmitter coral, *Pocillopora damicornis*.

Results: *P. damicornis* larvae, which have a large number of symbionts, survived the high-temperature treatment (32 °C) for 2 weeks. Significant reductions in *Symbiodinium* cell density were observed, but these did not lead to increased mortality of planulae during the 2-week experimental period. Although no significant difference was detected in the percentage of apoptotic cells between temperature treatment groups, pre-bleaching larvae exposed to 31 °C tended to exhibit higher percentages of apoptotic (TUNEL-positive) cells in the gastrodermis than 32 °C-treated larvae, which contained reduced numbers of *Symbiodinium* cells.

Conclusions: Symbiotic larvae of *P. damicornis* survived well under high-temperature conditions, although their *Symbiodinium* cell density decreased. This suggests that *P. damicornis* larvae have the capacity to reduce the symbiont cell density without a harmful effect on their survivorship under thermal stress. Further studies on antioxidant systems and possible suppression of apoptotic pathways are necessary to elucidate the mechanism underlying the high thermal tolerance of symbiotic larvae of *P. damicornis*.

Keywords: Apoptosis; Bleaching; Coral; Larvae; Survivorship; Symbiosis

Background

Approximately 80 % of scleractinian coral species are broadcast spawners and release gametes, while more than 10 % coral species are brooders and release larvae that have developed within their polyps (Baird et al. 2009a; Harrison 2011). Reef-building scleractinian corals associate with symbiotic dinoflagellates called *Symbiodinium* spp. Approximately 80 % of spawning corals release *Symbiodinium*-free eggs, and less than 20 % of brooding species release *Symbiodinium*-free larvae (Baird et al. 2009a). The offspring of these corals must acquire symbiotic algae from the environment (horizontal transmission of symbionts) in each generation. On the other hand, more than 80 % of brooders release *Symbiodinium*-containing larvae (Baird et al. 2009a), and some spawners such as *Porites*, *Montipora*, and some *Pocillopora* species release *Symbiodinium*-containing eggs. The offspring of these corals inherit symbiotic algae from their maternal colony (vertical transmission; Harrison and Wallace 1990).

Coral larvae containing *Symbiodinium* might obtain energy from algal photosynthesis (e.g., Harii et al. 2002). It is suggested that not all larval energy requirements are met by the lipids provisioned within the egg, but that some are sourced from the photosynthetic products of symbiotic algae. Under favorable circumstances, the presence of symbiotic algae within larvae has the potential to extend the larval lifespan (Cantin et al. 2009; Harii et al. 2010). However, under stressful conditions, such as high temperatures or excessive light irradiance, symbiotic dinoflagellates might become a source of reactive oxygen species (ROS), which lead to disruption of the symbiosis, or bleaching (Weis 2008). Thus, coral planulae that contain *Symbiodinium* might be more sensitive

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to environmental stress than *Symbiodinium*-free planulae. Since the larvae of *Acropora* corals acquire symbionts via horizontal transmission, it is possible to investigate the effect of inoculation of *Symbiodinium* cells on the stress sensitivity of the larvae. Yakovleva et al. (2009) showed that larvae of *Acropora intermedia* inoculated with *Symbiodinium* exhibited lower survivorship, higher superoxide dismutase (SOD) activity, and higher contents of malondialdehyde, an indicator of lipid peroxidation, under thermal stress than did *Symbiodinium*-free larvae. It was also reported that if non-symbiotic *Acropora tenuis* larvae are inoculated with *Symbiodinium*, the symbiotic larvae suffer greater DNA damage than *Symbiodinium*-free larvae when exposed to natural sunlight for 3 days (Nesa et al. 2012). These studies suggest that symbiotic larvae suffer more severe oxidative stress compared to non-symbiotic larvae because algal symbionts generate reactive oxygen species (ROS) under stress conditions. Another study using aggregates of dissociated coral cells (tissue-balls) showed that under thermal stress, tissue balls with higher *Symbiodinium* density suffered more severe DNA damage and died more rapidly than those with low *Symbiodinium* density (Nesa and Hidaka 2008; Nesa and Hidaka 2009). This again supports the notion that algal symbionts become a burden for host corals under stressful conditions.

Different *Symbiodinium* types have different physiologies, including different tolerances to heat (Glynn et al. 2001; Baker et al. 2004; van Oppen et al. 2005) and light (Abrego et al. 2008). The physiology of the symbiont helps determine that of the host. For example, the heat-tolerant *Symbiodinium* D increases the tolerance of *A. millepora* colonies by 1–1.5 °C compared to those with *Symbiodinium* C1 (Berkelmans and van Oppen 2006). Similarly, *Pocillopora damicornis* colonies hosting clade D *Symbiodinium* show higher survival rate than those harboring clade C at elevated temperatures without growth disadvantage (Cunning et al. 2015). However, the host also controls some thermal tolerance patterns (Baird et al. 2009b), because *A. tenuis* juveniles associating with clade C1 are more stress-tolerant than those associated with clade D (Abrego et al. 2008).

It is not known how the presence of algal symbionts affects larval survivorship under stress in vertical transmitters and whether they are more sensitive to environmental stress than the *Symbiodinium*-free larvae of horizontal transmitter species. It is also possible that, if algal symbionts become a burden for the larvae during the planktonic stage, the larvae of vertical transmitter corals might have highly efficient defense systems against oxidative stress.

In the present study, we investigated the survivorship of symbiotic larvae of *Pocillopora damicornis*, a vertical transmitter, under thermal stress. We also studied changes in symbiont density and the occurrence and distribution pattern of apoptotic host cells in *P. damicornis* larvae to determine if temperature stress causes decreased symbiont densities and increased mortality of host cells as observed in adult corals. *Symbiodinium* types in some maternal colonies were also identified.

Methods

Collection and maintenance of larvae

Nine and five colonies of *P. damicornis* (ca. 10 cm in diameter) were collected from the reef at Sesoko Island (26°38′53.6″ N, 127°51′13.2″ E) in July 2010 and June 2011, respectively. In June 2012, nine colonies were collected from Bise, 6.7 km north of Sesoko Island. All colonies were collected at depths of less than 1 m during low tide. The sea surface temperature at Sesoko Island ranged from 26.5 to 30.6 °C during the collection months (Record of coastal observation at the Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus). After sampling, colonies were transferred to an outdoor tank supplied with non-filtered seawater at Sesoko Station. Planulae released during the first quarter moon of August 2010, July 2011, and June 2012 were collected using planula collectors (Hidaka et al. 1997). Released planulae were removed from the collectors and transferred into 0.5-L plastic chambers filled with filtered seawater (0.2 μm). Each chamber contained planulae derived from one source colony. The seawater in the chambers was changed daily until the planulae were used for the experiment.

Stress experiment

Planulae derived from three maternal colonies were used in the 2010 and 2011 experiments, while planulae derived from four maternal colonies were used in the 2012 experiment. Thirty planulae derived from the same maternal *P. damicornis* colony were placed in a glass bottle filled with 100 ml of filtered seawater, and three such bottles, each containing planulae derived from different maternal colonies, were prepared for each temperature treatment in the 2010 and 2011 experiments. In 2012, four bottles, each containing 20 planulae derived from the same maternal colony, were prepared for each temperature treatment.

The planulae were exposed to temperatures of 27 °C (control), 30 °C or 31 °C (medium), and 32 °C (high) using water baths (E-thermobucket waterbath, TAITEC) under 12:12-h light-dark cycle of moderate light intensity (100 μmol $m^{-2}s^{-1}$). This temperature range encompasses current and future project temperature conditions at Sesoko Island, Okinawa, in summer, as the global temperature is predicted to rise at least 2 °C by the end of this century (Hoegh-Guldberg et al. 2007). Halogen lamps were used as the light source, and light intensity was

measured using a LI-COR light meter (Model LI-250, USA). The stress exposure experiments were conducted for approximately 2 weeks. Seawater in the bottles was changed daily, and the number of live planulae was counted daily for 13–15 days in all experiments.

To enumerate *Symbiodinium* cells per planula, three to four *P. damicornis* planulae were sampled before and after the stress exposure experiments. A planula was homogenized in a 1.5-ml tube with 100-μl of seawater. An aliquot of the homogenate was used to count the number of *Symbiodinium* cells under a light microscope using a hemocytometer.

Symbiodinium *genotypes in maternal colonies*

DNA was extracted from ethanol-preserved samples of adult fragments from three colonies of *P. damicornis* used as source of planulae in the 2011 experiment following the guanidine extraction protocol (Sinniger et al. 2010). We extracted DNA from the adult colony assuming that the parental colony of *P. damicornis* gives a similar type of symbiont to the larvae as described in *Montipora capitata* (Padilla-Gamiño et al. 2012). The internal transcribed spacer 2 (ITS2) region of the rDNA was amplified for each DNA extraction and analyzed by denaturing gradient gel electrophoresis (DGGE). The primer pair ITSintfor and ITS2clamp was used to amplify the ITS2 region under conditions specified by LaJeunesse et al. (2003). Amplified ITS2 fragments were separated on a DGGE DCode system (BIO-RAD) at 90 V for 15 h using 8 % polyacrylamide gels (37.5:1 acrylamide/bis) with an internal gradient of 25–70 % denaturants (formamide and urea). The gels were stained with SYBR Green (Sigma), and the bright bands were cut, eluted in 10 μl H_2O, re-amplified using the ITSintfor and ITS2reverse primers, and sequenced by Macrogen Japan Co. Ltd. Sequences were identified by a local BLAST search using the GeoSymbio database (Franklin et al. 2012).

Detection of apoptotic cells in Pocillopora damicornis *larvae exposed to thermal stress*

After a 14-day stress treatment in 2010, three larvae from each container were fixed in 10 % neutral buffered formalin and stored at 4 °C until processing. They were then dehydrated in a graded series of ethanol and embedded in Paraplast plus (Sigma P3683). Longitudinal sections 5-μm thick were cut, and two consecutive sections were randomly selected from three regions within the specimen. One from each of the consecutive sections was subjected to hematoxylin and eosin staining, and the remaining sections were processed for terminal deoxynucleotidyl transferase dUTP-biotin nick end labeling (TUNEL) assay, which is widely used to detect 3′-ends of DNA fragments in paraffin-embedded tissue sections. The TUNEL assay was performed following the manual provided with the kit (Chemicon S7111) with a slight modification (50 % reduction) of the concentrations of the chemical reagents. The sections were observed under a fluorescence microscope (Nikon Optiphot-2) using a blue excitation filter (wave length; 495 nm) for anti-digoxigenin-fluorescein-labeled (TUNEL-positive) nuclei or an ultraviolet filter (wave length; 365 nm) for nuclei counterstained with DAPI. Photomicrographs of the same areas were taken under blue light and then UV excitation using a digital camera (Nikon Digital Sight DS-L1).

The labeling index (LI) for TUNEL-positive cells, which indicates the proportion of cells undergoing apoptosis, was calculated by dividing the number of fluorescein-labeled nuclei by the total number of nuclei stained with DAPI. At least three photographs were taken for each of three sections from each specimen. In total, 18 photographs (each photographed area 5.3×10^4 μm^2) were used for each stress treatment. If symbiotic algae become a source of ROS under stress (Weis 2008; Saragosti et al. 2010; McGinty et al. 2012), we expect apoptosis in the neighboring host cells, that is, the gastrodermal cells. Apoptosis of gastrodermal cells was initially observed before the onset of bleaching (Ainsworth et al. 2008). The average LI was calculated for the epidermis and the gastrodermis separately for each specimen. The number of replicates was three for each treatment, as three planulae derived from different colonies were used for LI measurements. Positive control slides were treated with DNase after the process of antigen retrieval (image not shown). No TUNEL-positive signals were detected in the negative control slides that had been incubated with deionized water instead of TdT enzyme (image not shown).

Statistical analyses

Kaplan-Meier tests were used to analyze the survivorships of *P. damicornis* planulae. Significant differences in the numbers of *Symbiodinium* cells per planula were tested among the temperature treatments using a one-way ANOVA followed by Tukey's HSD test. Square root transformation was performed to analyze the 2012 *Symbiodinium* density data so that the data meet the parametric requirements (Shapiro-Wilk test for normality and Levene test for homoscedasticity). When the number of replicates was fewer than three, the data were excluded from the statistical analyses. All tests were performed using STATISTICA (ver. 6.0). Significant differences in the labeling index of TUNEL-positive cells were tested among treatment groups using a one-way ANOVA and between the gastrodermis and epidermis using Welch's *t*-test (R ver. 2.15.1).

Results

Survivorship, Symbiodinium *density of* Pocillopora damicornis *larvae*

Most *P. damicornis* planulae survived the high-temperature treatment (32 °C) for 2 weeks in all of the experiments (Fig. 1). There was no significant difference in the survivorship of *P. damicornis* larvae among different temperature-treated groups (Kaplan-Meier test, df = 3, $p = 0.99$ in 2010, $p = 0.40$ in 2011, and $p = 0.59$ in 2012).

Planulae of *P. damicornis* contained many *Symbiodinium* cells that were vertically transmitted from the maternal colony. The average numbers (±SE) of symbionts at the commencement of the stress exposure experiments were $1.70 \sim 2.78 \times 10^4$ cells planula^{-1} in the 3 years of experiments. After two weeks of exposure, the *Symbiodinium* cell densities were significantly lower at 32 °C compared to the control temperature (27 °C) group (Fig. 2) (Tukey HSD test, $p < 0.01$ in 2010; $p = 0.027$ in 2011; $p < 0.01$ in 2012). *Symbiodinium* densities ranged from $1.27 \sim 2.79 \times 10^4$ cells planula^{-1} at 27 °C, while they ranged from $0.0004 \sim 0.33 \times 10^4$ cells planula^{-1} at 32 °C. The *Symbiodinium* cell density at medium temperature (31 °C in 2010 and 30 °C in 2012) was not significantly different from the control (Fig. 2) (Tukey HSD test, $p = 0.57$ and $p = 0.105$ in 2010 and 2012, respectively). The final symbiont density at the medium temperature (30 °C) in the 2011 experiment was not included in the statistical analysis because the number of replicates was fewer than three.

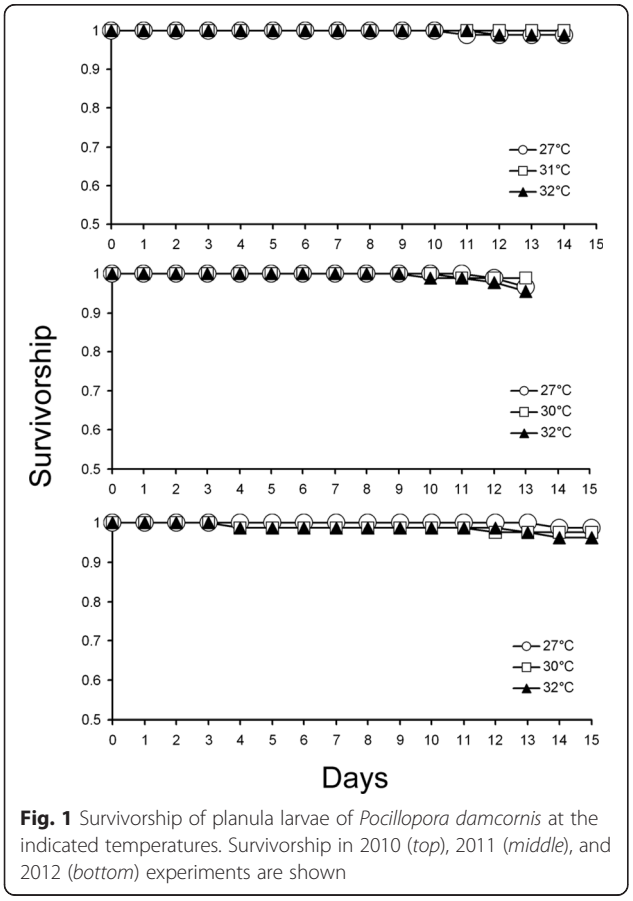

Fig. 1 Survivorship of planula larvae of *Pocillopora damcornis* at the indicated temperatures. Survivorship in 2010 (*top*), 2011 (*middle*), and 2012 (*bottom*) experiments are shown

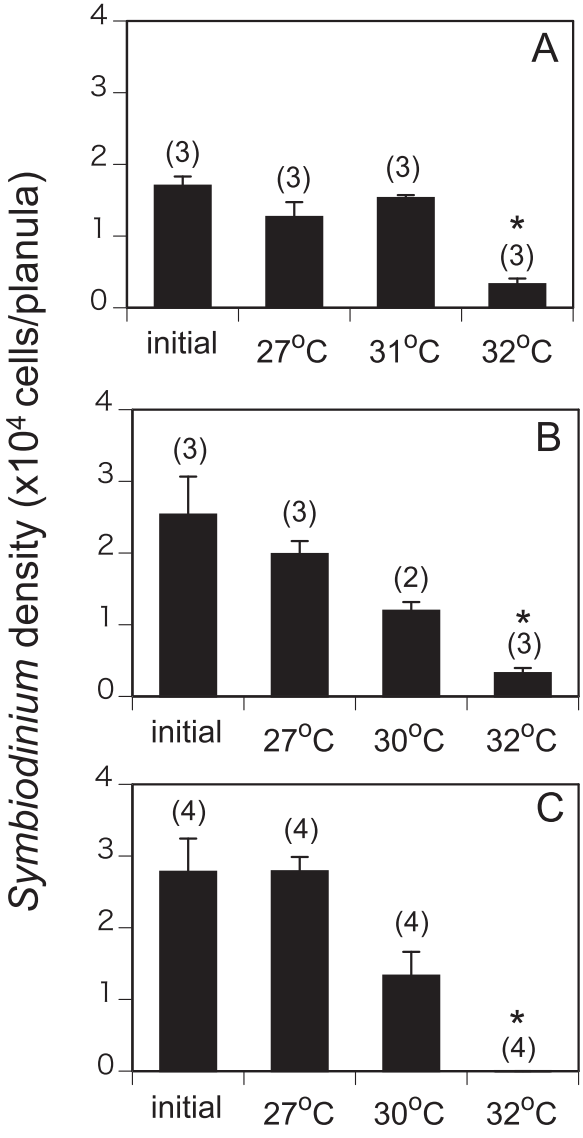

Fig. 2 The number of *Symbiodinium* in *Pocillopora damicornis* planulae before and after exposure to the indicated temperatures. *Symbiodinium* densities 13–15 days after the commencement of stress exposure are shown for the 2010 (**a**), 2011 (**b**), and 2012 (**c**) experiments as means ± S.E. The *numbers* in parentheses indicate the numbers of planulae measured. *Asterisks* indicate significant differences from the control (27 °C; Tukey's HSD test, $p < 0.05$)

Symbiodinium *genotypes of maternal* Pocillopora damicornis *colonies*

Symbiodinium type was analyzed in three colonies that were used as source of planulae in the 2011 experiment.

Multiple types of *Symbiodinium* were found to be associated with *P. damicornis* colonies, including subclade A1, C1, C3 and C71 in colony 1, A1 in colony 2, and A1, C1, and C3 in colony 3.

Apoptotic cells in Pocillopora damicornis *larvae exposed to thermal stress*

The TUNEL assay of longitudinal sections of *P. damicornis* larvae exposed to 31 °C for 2 weeks showed a high number of TUNEL-positive (apoptotic) cells, especially in the gastrodermis (Fig. 3). The proportion of apoptotic cells was significantly higher in the gastrodermis than in the epidermis in 27 and 31 °C treatment groups (Fig. 4, Welch's *t*-test, df = 2.006, $p < 0.05$; df = 2.259, $p < 0.05$), while no difference was detected in the 32 °C-treated groups (Welch's *t*-test, df = 2.497, $p = 0.2032$). Only 4.5 ± 2.9 % of apoptotic cells were in the epidermis at 27 °C, with twice that amount at 12.2 ± 0.1 % in the gastodermis. Furthermore, only 3.1 ± 1.6 % apoptotic cells were in the epidermis at 31 °C, while the highest numbers recorded (18.1 ± 6.3 %) were in the gastrodermis. However, differences among the number of apopotoic cells in the gastrodermis of larvae exposed to different temperatures were not significant (one-way ANOVA, df = 2, $p = 0.1173$). Many apoptotic signals were observed in the gastrodermis of 31 °C-treated planulae, while diffuse TUNEL-positive signals were occasionally observed in the epidermis of 32 °C-treated planulae. Diffuse TUNEL-positive signals that extended more than a third of epidermis thickness were observed only in one out of three planulae exposed to 32 °C. The number of such diffuse signals was low (1–3 per section of the whole planula) and was not included in the labeling index, as counting the number of TUNEL-positive nuclei was difficult in such cases.

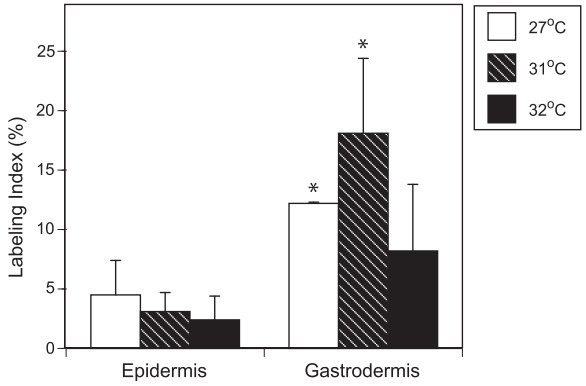

Fig. 4 Labeling index of TUNEL-positive cells in *Pocillopora damicornis* planulae. Planulae exposed to 27, 31, or 32 °C for 2 weeks were subjected to TUNEL assay. The labeling indices of the gastrodermis and epidermis were analyzed separately. Mean ± SD ($n = 3$). The *asterisks* show significant difference between the epidermis and the gastrodermis at each temperature

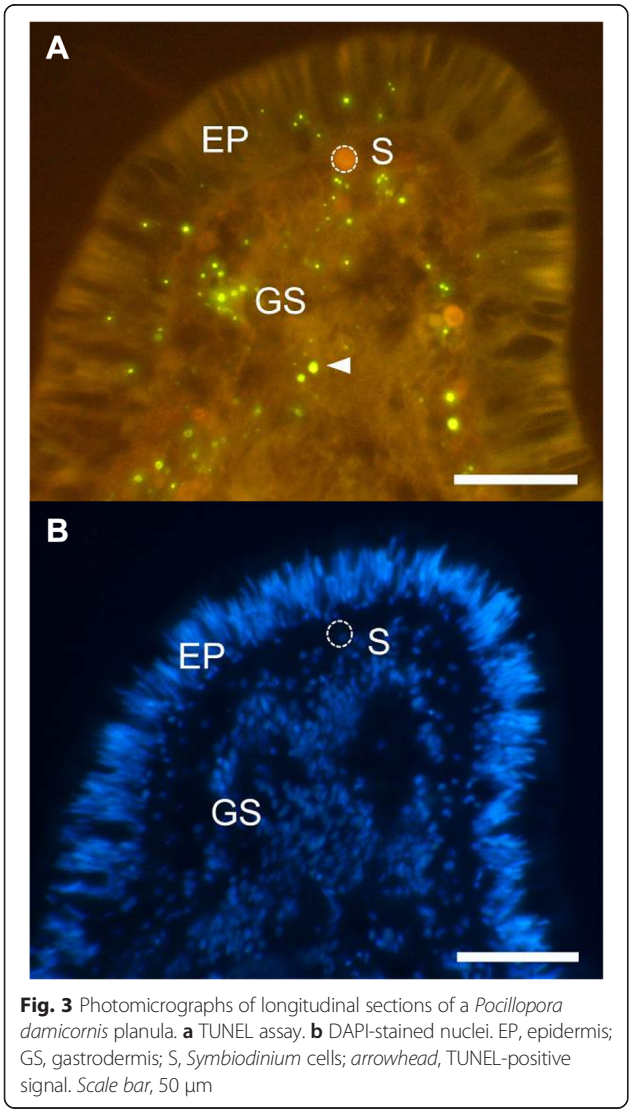

Fig. 3 Photomicrographs of longitudinal sections of a *Pocillopora damicornis* planula. **a** TUNEL assay. **b** DAPI-stained nuclei. EP, epidermis; GS, gastrodermis; S, *Symbiodinium* cells; *arrowhead*, TUNEL-positive signal. *Scale bar*, 50 μm

Discussion

Previous studies on *Acropora* larvae inoculated with *Symbiodinium* isolated from adult colonies have shown that symbiotic larvae are more sensitive to thermal or strong light stress than are *Symbiodinium*-free larvae (Yakovleva et al. 2009; Nesa et al. 2012). Larvae containing *Symbiodinium* show lower survivorship, higher levels of antioxidant enzyme activity and lipid peroxidation, and more severe DNA damage under thermal or strong light stress (Yakovleva et al. 2009; Nesa et al. 2012). However, few studies have investigated the survivorship or stress susceptibility of symbiotic larvae of vertical transmitter corals (Cumbo et al. 2013). We postulated that the symbiotic larvae of *P. damicornis* would be more stress sensitive than the *Symbiodinium*-free larvae of horizontal transmitter corals. Contrary to our expectations, *P. damicornis* planulae with high numbers of

symbionts ($2–3 \times 10^4$ cells/planula) exhibited high survivorship at a high temperature (32 °C) under medium light intensity (100 μmol $m^{-2}s^{-1}$). There was no significant difference in survivorship among larvae exposed to 27, 30 or 31, and 32 °C for at least 13–15 days. Symbiotic planulae of *P. damicornis* showed higher survivorship than *Symbiodinium*-free larvae of *Acropora* corals as well as larvae inoculated with homologous symbionts at high temperatures. For example, Baird et al. (2006) reported that the survivorship of both symbiotic and non-symbiotic larvae of *A. muricata* decreased to ~30 % after a 7-day exposure to 28 or 32 °C. Yakovleva et al. (2009) reported that the survivorship of *A. intermedia* larvae inoculated with *Symbiodinium* from their parents decreased to 50 % after 4 days of exposure to 32 °C.

Despite having high survivorship, the *Symbiodinium* cell density of *P. damicornis* larvae significantly decreased at 32 °C. *P. damicornis* larvae may potentially have the capacity to reduce their symbiont numbers under thermal stress, thereby reducing the oxidative stress within their tissue. This could lead to higher survival rates of larvae under stressful conditions, like what is seen in our study. This agrees with recent research that showed that adult corals with high symbiont-to-host cell ratios were more susceptible to bleaching (Cunning and Baker 2013). Therefore, lower *Symbiodinium* cell densities are advantageous to corals under thermal stress.

While larvae exposed to 31 °C retained most of the symbionts in the 2010 experiment, high percentages of apoptotic cells were observed in the gastrodermis of the larvae. The percentage of apoptotic cells in the gastrodermis was significantly higher than that in the epidermis. When adult branches of the coral *Acropora aspera* were exposed to thermal stress, apoptosis of gastrodermal cells was initially observed before the onset of bleaching (Ainsworth et al. 2008). These observations suggest that ROS generated by symbiotic algae induce apoptosis in host gastrodermal cells at early stages of stress response. The frequency of apoptotic cells in the gastrodermis appeared to be lower in the 32 °C-treated, bleached larvae than in the 31 °C-treated, pre-bleaching larvae, although the difference was not significant. This is also consistent with the idea that *P. damicornis* larvae tolerate the thermal stress via reduction of their symbiont densities. It is, however, not clear why the gastrodermis exhibited higher proportion of apoptotic cells than the epidermis even in the control larvae kept at 27 °C in the present study.

In addition to the hypothesis described above, there are several other possible mechanisms by which *P. damicornis* larvae acquire high thermal tolerance. Tchernov et al. (2011) suggested that if the caspase cascade, a process that is activated by apoptotic stimulation and leads to apoptotic cell death was arrested at an early stage, the apoptotic response would not occur and the host would survive the thermal stress, even though it suffered bleaching. It is possible that *P. damicornis* larvae possess a mechanism to suppress the apoptotic pathway to a relatively low level under thermal stress. If so, this may enhance survival of *P. damicornis* larvae under thermal stress. This possibility warrants further investigation. Recently, Padilla-Gamiño et al. (2013) reported that eggs of *Montipora capitata*, a vertical transmitter coral, contain higher concentrations of manganese super oxide dismutase (MnSOD) and higher levels of ubiquitin conjugate than adult colonies. *M. capitata* is a spawner, and its larvae has the potential to stay at the ocean surface, where light levels are high, for long periods of time. The high concentrations of antioxidants in the eggs likely pass into the larvae thereby increasing larval survival under light stress at the ocean surface. Although it is not clear how long *P. damicornis* larvae stay at the ocean surface, it is likely that *P. damicornis* larvae also have a more efficient antioxidant system than adult colonies. This should be confirmed in future studies.

It is also possible that the symbionts associated with *P. damicornis* larvae are highly stress resistant. In this study, *Symbiodinium* type A1 was present in all three maternal colonies, while C1, C3, and C71 were found to be associated with only some of the colonies. A previous study also showed that *P. damicornis* colonies from Okinawa are associated with multiple *Symbiodinium* types A1 and C1 (Magalon et al. 2007). McGinty et al. (2012) reported that type A1 was tolerant and exhibited no increase in ROS production at high temperature (31 °C). If A1 is the dominant symbiont in the larvae used in this study, this might account for the high stress tolerance of *P. damicornis* larvae. It remains to be determined whether the dominant symbiont in the larvae was A1, though it is likely that larvae contained a symbiont composition similar to that of their maternal colonies.

Conclusions

Generally, symbiotic larvae are expected to be more susceptible to environmental stress than non-symbiotic larvae as symbiotic algae become a source of reactive oxygen species under stressful conditions. However, *P. damicornis* larvae, despite having large numbers of *Symbiodinium*, exhibited high tolerance to thermal stress. While symbiont numbers were significantly reduced in larvae under high temperatures, larval survival remained high. This shows that *P. damicornis* larvae have the capacity to reduce the symbiont cell density without a harmful effect on their survivorship under thermal stress. Further studies on antioxidant systems, association with tolerant symbiont types, and possible

suppression of apoptotic pathways might provide insight into the high stress tolerance of symbiotic larvae of *P. damicornis*.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
DH and MH conceived and designed the experiments. DH carried out the survival experiments. NY carried out TUNEL assay and apoptosis data analysis. SH carried out the *Symbiodinium* identification. DH and MH drafted the manuscript, while NY and SH also wrote their parts of the manuscript. All authors read and approved the final manuscript.

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