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Temperature-induced stress leads to bleaching in larger benthic foraminifera hosting endosymbiotic diatoms

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Abstract

Physiological mechanisms of bleaching were studied on larger benthic foraminifera (LBF) hosting endosymbiotic diatoms. *Amphistegina radiata*, *Heterostegina depressa,* and *Calcarina hispida* were exposed to increasing temperatures in static temperature experiments (23°C to 33°C, 6 d). Photosynthetic activity ($F_v : F_m$, measured with a pulse-amplitude modulated fluorometer), chlorophyll *a* (a proxy for symbiont biomass), and motility (a proxy for overall fitness of the foraminifera) were reduced in specimens at 32°C to 33°C, and cytoplasm color changes associated with bleaching were observed. A 30-d flow-through experiment at three temperatures (26°C to 31°C) and three levels of inorganic nitrate concentration (0.5 to 1.4 μmol L^{-1}) confirmed negative effects of temperature at 31°C for *A. radiata* (including growth) and *H. depressa.* Another *Calcarina* species, *Calcarina mayorii,* was not affected. This suggests that temperature effects are species-specific. However, elevated nutrient concentrations did not affect any of the parameters measured. Temperatures $>$ 30°C stress the foram–diatom endosymbiosis in some LBF species, which may lead to subsequent bleaching of the host. Given that a 2–3°C increase led to rapid bleaching of most species, we propose that, similar to corals, these species are threatened by sea-surface temperature increase predicted for tropical reef waters in the near future.

Benthic foraminifera are unicellular eukaryotes (Rhizaria) with external shells, which are typically produced by active biomineralization of calcite. Together with calcareous green algae, molluscs, and corals, they are important producers of reef sediments (Scoffin and Tudhope 1985). Eighty percent of global foraminiferal reef carbonate originates from larger benthic foraminifera (LBF; Langer et al. 1997). In subtropical to tropical shallow-water areas, especially on coral reefs, representatives of several LBF families join in symbiosis with different algal groups (Lee and Anderson 1991). Until now, LBF have been shown to contain either endosymbiotic diatoms, dinoflagellates, rhodophytes, chlorophytes, or cyanobacteria (Lee 2006). Diatoms are the most prominent of all symbionts and are known to live in association with four LBF families (Lee and Anderson 1991).

Symbiont-bearing foraminifera are able to achieve high calcification rates because the photosynthetic fixation of carbon dioxide raises the pH around the foraminifera and, thus, the saturation state of carbonate (Erez 2003). Besides their positive effect on the calcification ability of the host, the symbionts supply photosynthetic products, such as adenosine triphosphate and carbohydrates, to the host. In return, the endosymbionts living inside the cytoplasm of the foraminifera are protected by the foraminiferal test and are possibly exposed to higher levels of inorganic nutrients compared to free-living algae (Lee and Hallock 1987).

In contrast to the diverse endosymbiosis in foraminifera, dinoflagellates of the genus *Symbiodinium* are the only symbiont type in hermatypic corals (Davies 1984). This symbiotic relationship is sensitive to temperature changes, leading to a stepwise expulsion of zooxanthellae by the host (Strychar et al. 2004). Thermal bleaching thresholds in corals have been identified to lie 1–3°C above local mean summer temperatures and to follow seasonal and spatial patterns; as well, they appear to be species-dependent (Berkelmans and Willis 1999). High water temperatures and high irradiance solely or in combination are known to be among the leading causes of coral bleaching (Fitt et al. 2001). Both elevated photosynthetically active radiation (PAR 400–700 nm) and ultraviolet radiation (UV 280–320 nm) have been shown to harm corals (Gleason and Wellington 1993; Brown et al. 1994).

For the last decades, rapidly increasing sea surface temperatures (SSTs) have been a major concern for the coral reefs around the tropics (Lesser 2007). Mean annual SSTs have increased by 0.3–0.4°C within the past two to three decades across the tropical region suitable for coral reef growth (Kleypas et al. 2008). Annual SSTs in the Great Barrier Reef (GBR) reflect this global trend and have already risen by an average of 0.7°C within the last century (Lough 2001; Lough et al. 2006). Based on Intergovernmental Panel on Climate Change (IPCC) scenarios, the frequency and intensity of coral bleaching events are likely to increase within the next 100 yr (Hoegh-Guldberg 1999). Forecasts taking into account the two ''business-as-usual'' scenarios of the IPCC predict that the GBR waters will be 1–3°C warmer than at present by the end of 2100 (Lough 2007; Berkelmans 2009).

Symptoms of bleaching have been observed in other marine invertebrates such as sea anemones (Perez et al. 2001; Sawyer and Muscatine 2001), sponges (Fromont and Garson 1999; Lopez-Legentil et al. 2008), and giant clams (Addessi 2001; Leggat et al. 2003), as well as in crustose coralline algae (Anthony et al. 2008; Negri et al. 2011). Bleaching has been observed in LBF in the species *Amphistegina gibbosa* during post-bleaching coral surveys

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in the Bahamas in 1988 and in field studies in the Florida Keys (Hallock et al. 1992; Hallock et al. 2006*a,b*). The species *A. lessonii, A. lobifera,* and *A. radiata* have been recorded as bleached in a variety of Indo-Pacific locations in the 1990s (Hallock and Talge 1993; Hallock 2000), as well as the species *Sorites dominicensis* in Florida and Belize in 2003 and 2005, respectively (Richardson 2006, 2009).

Light-stressed *Amphistegina* sp. specimens show a patchy distribution of symbionts within the multichambered shell, with pale chambers alternating with healthy-colored chambers in partly bleached individuals; specimens considered bleached typically exhibited nearly total loss of color except for some brown material in the final chambers (Hallock and Talge 1993; Hallock et al. 2006*b*). Bleaching in *A. gibbosa* has been shown experimentally to be associated with light stress, and it has been cytologically documented to cause damage to symbionts and has led to the digestion of the symbionts by the host (Talge and Hallock 1995; Talge and Hallock 2003). Moreover, Talge and Hallock (2003) found that *A. gibbosa* in a 35-d exposure experiment at 13–15 μmol photons m^{-2} s^{-1} PAR exhibited more than double the incidence of partial bleaching (~ 50%) when incubated at 32°C than at 25°C (~ 20%), indicating that elevated temperature accelerated bleaching in these foraminifers when they were experiencing some light stress. Field-stressed *A. gibbosa* similarly exhibited deteriorated symbionts and severe degradation of cytoplasm (Talge and Hallock 1995) that was cytologically indistinguishable from that observed in laboratory experiments (Talge and Hallock 2003). Microbial infestation (Hallock et al. 1995), increased predation (Hallock and Talge 1994), and increased UV radiation (Williams and Hallock 2004) have also been associated with stress and bleaching in LBF.

In combination with rising SSTs, nutrient and sediment runoff are important concerns for the GBR (Fabricius 2005). The symbiosis in corals may show signs of stress under these conditions (Dubinsky and Berman-Frank 2001). The algal symbiosis in corals and LBF share a common characteristic: both are well adapted to nutrient-poor waters of tropical coral reefs (Hallock 1981). This characteristic makes LBF good indicators of water quality because symbiotic species dominate the foraminiferal assemblages in nutrient-poor water and are less abundant under elevated nutrient levels (Foraminifera in Reef Assessment and Monitoring Index; Hallock et al. 2003; Uthicke and Nobes 2008; Uthicke et al. 2010). In situ experiments found that growth rates of *A. radiata* and *H. depressa* are negatively affected by elevated nutrient levels occurring in the GBR (Uthicke and Altenrath 2010). Therefore, hypotheses about the effects of nutrient stress on corals could apply to LBF as well: nutrient stress releases the symbionts from their nutrient limitation, subsequently leading to extensive population growth and reduced carbon translocation to the host (Dubinsky and Jokiel 1994; Dubinsky and Berman-Frank 2001).

The foraminifera *Amphistegina radiata*, *Heterostegina depressa, Calcarina hispida,* and *Calcarina mayorii* used in this study host several species of endosymbiotic diatoms as their only symbionts. It has been shown that these diatoms live inside the protoplasm without their siliceous frustule (Lee and Anderson 1991). The species *A. radiata* and *H. depressa* are mostly found growing on hard substrate, such as coral rubble, in mid-range depth (8–40 m) and occasionally on sand; calcarinids are abundant on hard substrate near the reef edge and are able to withstand high water energy (Hohenegger et al. 1999).

The aim of the experimental work presented here was to test whether bleaching in LBF is prone to increasing temperatures and to characterize the accompanied physiological stress quantitatively. Thus, repeated runs of static temperature experiments were conducted under a temperature range of 23–33°C. The light physiology of the diatom endosymbionts was investigated by pulse amplitude modulated (PAM) fluorometry. Recent studies have used fluorescence techniques to measure the ratio for variable to maximum fluorescence ($F_v : F_m$), which is an indicator of the photochemical efficiency of the photosystem II (PSII) and is called maximum quantum yield (Schreiber et al. 1986; Jones et al. 1998).

In addition, we developed a method to measure chlorophyll *a* (Chl *a*) content from individual foraminifera as a proxy for symbiont biomass and used motility as a proxy for overall fitness of the foraminifera. A further aim was to test if the potential bleaching response in LBF is affected by enhanced nutrients, as was suggested for corals (Dubinsky and Berman-Frank 2001; Wooldridge 2009; Wooldridge and Done 2009). This was experimentally tested in a 30-d orthogonal flow-through experiment in which nitrate was constantly added at three different concentrations in combination with three temperature levels.

Methods

Study area and sample collection—Amphistegina radiata and *Heterostegina depressa* were collected on several field trips in the dryer and colder months (05 June 2009 [24.6°C], 06 August 2009 [22.6°C]) and in the wetter and warmer months (16 January 2010 [28.1°C]) by SCUBA diving in the Whitsundays area, located in the central section of the GBR Marine Park (collection sites: Double Cone Island S 20°06.30′, E 148°43.31′; Border Island S 20°09.10′, E 149°01.45′; Deloraine Island S 20°09.30′, E 149°04.50′; Edward Island S 20°14.80′, E 149°10.22′; 8–10 m Lowest Astronomical Tide [LAT]). Daily average SSTs (at 2.5-m depth) were obtained from Automatic Weather Station (AWS) from the Australian Institute of Marine Science (AIMS) at Hardy Reef, Whitsundays (S 19°45.00′, E 149°10.80′). *Calcarina hispida* specimens were collected from Heron Island (by walking on the reef flat during low tides, S 23°26.45′, E 151°54.57′, 1–2 m LAT, 17 May 2009 [21.8°C]). Daily average SST (at 0.6-m depth) was obtained from AIMS AWS at Heron Island (S 23° 26.40′, E 151° 55.20′). *Calcarina mayorii* from Magnetic Island was collected by SCUBA diving (S 19°09.31′, E 146°52.05′, 8–10 m LAT, 14 August 2009, 23°C ± 0.5°C). SST data were obtained from a reef flat temperature logger at Nelly Bay (S 19°10.31′, E 146°50.88′, 0-m depth).

After collection, all specimens were washed clean of coral rubble with seawater and identified to genus level. In

the lab they were identified to species level under a Leica Stereoscope MX16 A (10–25× magnification). Prior to experiments, specimens were allowed to acclimate for up to 2 weeks in several 500-mL beakers at 24–26°C at low light intensities (10 μmol photons m^{-2} s^{-1}, 12 : 12 light : dark cycle [LD]), with seawater replaced by fresh seawater (unfiltered, sediment removed) every 3–4 d.

In addition to samples for experimental studies, field samples of *A. radiata* and *H. depressa* were collected to establish a natural benchmark for Chl *a* measurements in the experiments. To represent the environmental range under which these foraminifera are found naturally in the study area (Nobes et al. 2008; Uthicke and Altenrath 2010), samples were collected on two field trips on 06 August 2009–10 August 2009 and 16 January 2010–17 January 2010 in shallow water at 6–8 m and at 10–12 m LAT from two reefs close to the coastline (distance: 10–15 km, Daydream Island S 20°15.35′, E 148°48.73′, Double Cone Island) and two outer shore islands (distance: 30–37 km, Deloraine, Edward, and Border Islands). Specimens from the coastal islands are subjected to distinctly lower light levels and higher concentrations of dissolved inorganic nitrogen and suspended sediments compared to specimens from the outer shore islands (Cooper et al. 2007; Uthicke and Altenrath 2010). Foraminifera were sampled and identified as described above. Subsequently, individual samples were cleaned with sable artist brushes, put into preweighed 1.5-mL Eppendorf vials, immediately frozen (−20°C), and later stored at −80°C until further processing.

Ultra performance liquid chromatography (UPLC) was used to validate total Chl *a* contents and test for the presence of pheopigments. For these experiments, *A. radiata* and *H. depressa* were collected on Dent Island (S 20°21′01, E 148°55′37, depth: 6–8 m LAT, 11 August 2009) and from Bait Reef (S 19°49′16, E 149°06′35, depth: 16–18 m LAT, 08 August 2009). Samples were collected and processed as described in the previous paragraph.

Experimental setup—Static experiments: A series of static temperature manipulation experiments with *A. radiata, H. depressa*, and *C. hispida* were carried out in five individually set temperature incubators (23°C, 28°C, 30°C, 32°C, and 33°C) over a 6-d consecutive period. Temperature in the incubators was checked twice daily with a handheld digital thermometer and varied by ± 0.5°C. The chosen temperature range reflects current and future water-temperature predictions for the GBR (Lough et al. 2006; Lough 2007). The experiments were repeated three times (June, August, January) for *A. radiata* and *H. depressa*. The five static temperature incubators have been assigned a different temperature (23–33°C) in each experiment. Effects of potential differences between individual incubators have thus been reduced for these species.

Each of the subsequent experimental runs used light levels of 11–15 μmol photons m^{-2} s^{-1} (12 : 12 LD, 50 : 50 actinic 420 nm : 10 K trichromatic daylight; Catalina Compact). These low light levels yielded optimal growth rates in previous experimental studies (Röttger et al. 1980; Williams and Hallock 2004; Nobes et al. 2008) and are common in microenvironments where LBF are found (Hohenegger et al. 1999). Specimens were kept in six-well plates (size of well: 3 cm, volume of well: 5 mL) standing inside the incubators. One six-well plate generally held 24 specimens (4 specimens × 6 wells). Seawater (unfiltered) was changed daily with fresh inshore seawater that was stored in a settlement tank before it was pumped into the laboratory. Salinity was checked with a handheld refractometer and was consistently between 35–36‰ throughout all experiments. To avoid thermal shock, the specimens were introduced to the five different temperature treatments gradually. All six-well plates were initially kept in the control treatment (23°C), from which four plates were shifted in 1-h intervals toward the nearest higher temperature until the desired temperature was reached.

To document changes in coloration of *A. radiata* and *H. depressa* during the experiment, digital images were taken on representative specimens in the 32°C treatment once per day over a 6-d period until bleaching became clearly visible. Images were taken on a Leica MI65C stereomicroscope with a Canon 30D digital camera and Leica camera attachment. During photography the illumination settings were kept constant on the stereoscope. To warrant comparability between photos taken at different times, digital white balance adjustments were made in the raw file format using the calcite white as a reference point using the software Digital Professional (Canon).

Flow-through experiments: The effects of three temperatures (26°C, 29°C, and 31°C) and three nutrient levels (0.5, 1.0, and 1.4 μmol $L^{-1}NO_3^-$) were studied simultaneously in a flow-through setup constructed at AIMS over a 30-d period. Nine different temperature and nutrient concentrations were replicated three times, so that 27 aquaria were randomly arranged in a climate-controlled room (24–26°C). Incoming seawater was filtered in series (25 μm, 25 μm, 5 μm) into four storage tanks, which were heated using heating rods to the desired temperatures, and pumped through submersible pumps into the treatment aquaria. Computer-controlled automatic temperature sensors were placed into one aquarium per temperature to constantly record the temperature. Once to twice daily, manual temperature measurements were made with a handheld digital thermometer over the 30-d period (range per aquaria: SD ± 0.22–0.82°C).

Each of the aquaria contained a six-well plate which hosted six specimens each of *A. radiata* and *H. depressa.* For sufficient water flow, six circles (3.5 cm) were cut in the lids of the plates. In order to prevent the specimens from escaping the plates, a plankton mesh (1 mm) was placed between the plate and the lid. Nine specimens of *Calcarina mayorii* were contained in polypropylene tubes (50 mL) with four slits (5.5 mm × 1 cm) and wrapped in plankton mesh (1 mm; Nobes et al. 2008).

The light levels of 200 μmol photons m^{-2} s^{-1} in the aquaria (12 : 12 LD, 50 : 50 actinic 420 nm : 10 K trichromatic daylight; Catalina Compact) were achieved using adjustable racks. Light levels were further adjusted to low light regime (6–8 μmol photons m^{-2} s^{-1}) by wrapping double-layer shade cloth around the six-well plates and fixing it with rubber bands.

The nutrient levels in this study were chosen to represent naturally occurring nutrient concentrations during flood-plume events in the GBR (Devlin and Schaffelke 2009). Nitrate levels (NO_3^-) were chosen to be solely manipulated because these in particular have been increasing over the last decade due to intensive sugar cane cultivation and fertilizer use in the northern GBR catchments (Mitchell et al. 2001). The baseline nutrient level represented the local inshore seawater (on average 0.53 μmol $L^{-1} NO_3^-$), which was pumped from the ocean and stored in a settlement tank prior to its use in the aquaria. Incoming seawater was filtered through three filter systems (25 μm, 25 μm, 5 μm) connected in series in the aquaria room. Two multichannel peristaltic pumps delivered nutrient stock solutions ($K^+NO_3^-$) at a near constant rate (0.8–1 mL min^{-1}) into each aquarium, and water flow rates at 500 mL min^{-1} ensured a steady supply of fresh filtered seawater (5 μm) in every aquarium. Seawater nutrient content was monitored over the entire length of the experiment; duplicate nutrient samples were taken from each aquarium twice weekly, and five randomly selected sets of samples were analyzed by Australian Institute Laboratory Services for dissolved inorganic nutrients (Ryle et al. 1981). Salinity was checked with a handheld refractometer; it remained consistent (between 35–36‰) during the experiment.

PAM fluorometry—The photosynthetic efficiency of the endosymbiotic diatoms was measured as maximum quantum yield ($F_v : F_m$) with two PAM instruments: Mini PAM (used during static experiments) and Imaging PAM (used during flow-through experiment), developed by Walz (Schreiber et al. 1986). The Mini PAM measurements were taken after 15–30 min of dark adaptation in the mornings from below the six-well plate with an 8-mm fiber optic cable. The cable was fixed in a plastic base frame, so that the distance between the plate and the cable stayed constant at all times during the measurements.

The Imaging PAM (Unit IMAG-CM, connected to a MAXI Head with a 300 W LED-array; Walz) was used for measurements during the flow-through experiment. Measurements were taken after 15–30 min of dark adaptation every 3–7 d in the afternoon. The Imaging PAM system allowed homogenous measurements by pulse-modulated excitation and actinic light of the Chl *a* fluorescence of the entire six-well plate resulting in a two-dimensional image (10 × 13 cm).

The initial fluorescence (F_0) was measured by applying a weak pulsed red light, followed by a saturation pulse of actinic light measuring the maximum fluorescence (F_m). The maximum quantum yield ($F_v : F_m$) was calculated as $[(F_m - F_0) : F_m]$.

Chl a *analysis*—To quantify changes in the symbiont population (as a proxy for biomass) the analysis for the total Chl *a* content of the holobiont was developed by modifying existing protocols for freshwater phytoplankton and marine sediment biofilms for LBF (Sartory and Grobbelaar 1984; Uthicke 2006). Foraminiferan samples were kept in low light conditions and on ice during analysis. Wet weight of each specimen was recorded in preweighed Eppendorf vials (1.5 mL) to 0.01 mg accuracy. Individual foraminifera were extracted for 5 min in hot ethanol (95%, 78°C), crushed with a metal rod with 15–20 firm strokes, and vortexed. Subsequently, samples were extracted for 24 h at 4°C in the dark and vials vortexed (3–4 times). After centrifugation (5 min, at 5200 rotations per minute), 320 μL of each extract was placed in a flat-bottomed polystyrene microplate (Sarstedt) and the absorbance at 665 nm and 750 nm measured using a Synergy plate reader (Bio-Tek). The sample size for the individual experiments can be inferred from degrees of freedom (df) given in Table 1.

Initial tests were performed to compare the wet and dry weight for both *A. radiata* and *H. depressa.* Samples were weighed in preweighed Eppendorf vials (1.5 mL) on a balance with 0.01 mg accuracy after drying in a 60°C oven for a minimum of 48 h. Results showed that the wet weight is strongly correlated with the dry weight of these species (linear regression, $R^2 = 0.93$, $F_{1,81} = 1002.23$, $p < 0.001$, function: $y = -1.13 + 0.83x$, $n = 84$). Thus, wet weight (wet wt) measurements were subsequently used to normalize Chl *a* content.

In addition, we conducted a method comparison between the Synergy plate reader and the UPLC, measuring Chl *a* and phaeophytin contents to test whether Chl *a* was partially converted to phaeophytin during processing of the samples. Pigment extracts of the foraminifera (obtained using the method described above) were diluted 1 : 1 with 28 mmol tetrabutyl ammonium acetate pH 6.5 (TBAA), and 35 μL of this diluted extract was injected in a Waters ACQUITY-UPLC® system. Pigments were separated on an ACQUITY UPLC Bridged Ethyl Hybrid C8 column (2.1 × 150 mm; 1.7 μm) using a binary gradient with solvent A (70 : 30, methanol : 28 mmol TBAA, pH 6.5) and solvent B (50 : 50, methanol : acetonitrile). The gradient conditions were as follows (time [min], % B) at a constant flow rate of 0.45 mL min^{-1} and column temperature of 60°C (0, 5; 2, 20; 5, 50; 8, 95; 9, 95; 11, 5; 12, 5). Certified reference pigments were sourced from the Danish Hydraulic Institute, and calibration curves were derived. Pigments in samples were quantified using these calibration curves after identification via retention time and photodiode array spectral confirmation. A regression analysis showed that 90% of the variation of total Chl *a* in the UPLC can be predicted by the spectrophotometric method (regression analysis, $R^2 = 0.91$, $F_{1,10} = 99, 1$, $p < 0.001$, $y = 12.46 + 0.96x$, $n = 13$). The mean phaeophytin content for the species investigated was very low compared to total Chl *a* content. Maximum phaeophytin values measured were < 3 ng (mg wet wt)$^{-1}$ and were assumed negligible for the results.

Motility—The motility of LBF was recorded during static experiments. Foraminifera move by means of their pseudopodial network, which extends out of the shell along flat surfaces (Travis and Bowser 1991). This external network allowed *A. radiata* to orient the shell at an angle (~ 45°) in the water-containing wells, whereas *H. depressa* was observed to position its shell at a very low angle (< 5%). Only in *A. radiata* has this angular behavior been scored as motile because it was very distinct and could have

Table 1. One-way ANOVA for maximum quantum yield ($F_v : F_m$) and Chl *a* content (ng [mg wet wt]$^{-1}$) in *Amphistegina radiata*, *Heterostegina depressa,* and *Calcarina hispida* after 6-d exposure in static studies. $F_v : F_m$ data were arcsine transformed and Chl *a* data was log transformed for statistical analysis. Values in bold are significant at $p < 0.01$. $F_v : F_m \leq 0.1$ have been excluded from the analysis in *A. radiata* (22 : 360 total data points over all runs for 32–33°C) and in *H. depressa* (31 : 320 total over all runs for 32–33°C treatment). df: degrees of freedom; MS: mean square.

	$F_v : F_m$				Chl *a*			
Run (month)	df	MS	*F*	*p*	df	MS	*F*	*p*
A. radiata								
1 (Jun)	4, 99	0.972	81.247	**<0.001**	4, 98	1.300	18.501	**<0.001**
2 (Aug)	4, 111	0.392	38.473	**<0.001**	4, 107	2.137	24.745	**<0.001**
3 (Jan)	4, 105	0.588	31.001	**<0.001**	4, 113	2.990	13.836	**<0.001**
H. depressa								
1 (Jun)	4, 63	0.432	36.255	**<0.001**	4, 65	6.657	22.092	**<0.001**
2 (Aug)	4, 108	1.966	126.721	**<0.001**	4, 106	14.680	55.399	**<0.001**
3 (Jan)	4, 91	0.872	59.342	**<0.001**	4, 112	17.653	37.665	**<0.001**
C. hispida								
1 (Jun)	4, 73		100.782	**<0.001**	4, 71		34.357	**<0.001**

only been achieved by ongoing pseudopodial action. At each observation period, all specimens touching or climbing the wall of the six-well plate after 48 h have been counted as motile. After the measurements, specimens were detached from the walls and moved to the middle of the well to ensure that moving distance stayed constant between recordings.

Growth measurements—Growth in *A. radiata* was determined by cross-sectional shell surface area gain monitored by digital photography in the flow-through experiment. Growth in *H. depressa* and *C. mayorii* were not measured because of their delicate shells and their partial breakage as a result of handling. Pictures of *A. radiata* were taken on a Leica Stereoscope MX16 A using a Canon 30D digital camera with the corresponding attachment before and after the experiment. The software Adobe Photoshop® CS2 (Adobe Systems, 1990–2005) was used to automatically trace the outline of the specimens and compare their surface area gain (derived from pixel area gain) between the initial and final measurement. Most specimens remained in the initial wells over the length of the experiment. In some cases characteristic shell features helped to identify the individuals. Statistical analyses were based on average growth per plate, only including individuals that were alive and healthy-colored at the end of the experiment (Uthicke and Altenrath 2010). In this study, growth (% surface area d^{-1}) was determined by the following equation (daily growth rate = ln [final size (mm^2) : initial size (mm^2)] $\times$ d^{-1} $\times$ 100), modified from Ter Kuile and Erez (1984).

Statistical analysis—One-way analysis of variance (ANOVA) was used to test whether temperature had an effect on the photosynthetic efficiency and Chl *a* pigment content of the species in the static temperature experiments. A mixed-model ANOVA was performed for the flow-through experiment to test for interactions of temperature and nutrients. Temperature and nutrients were used as fixed factors. The factor aquaria (n = 3) was regarded as a random factor and nested in temperature and nutrients. Maximum quantum yield and growth data were arcsine transformed, and Chl *a* data were log transformed to meet the assumptions of an ANOVA. Homogeneity of variances was tested using Levene's Test. Tukey-Kramer Honestly Significant Difference (HSD) test was used as a post hoc test to investigate the differences between individual means after significant overall ANOVA results. One-way ANOVAs and associated Tukey-Kramer HSD post hoc tests were performed using the statistical software JMP® (version 7, SAS Institute, 1989–2007), and mixed model ANOVA and associated Tukey-Kramer Multiple Comparison (MC) post hoc tests were done using the program NCSS (Hintze 2007). Severe bleaching was defined as $F_v : F_m \leq 0.1$; such specimens have been excluded from the ANOVA, because values indicate a completely damaged photosystem and do not reflect a gradual reduction in maximum quantum yield.

Results

Static experiments—Three experiments were carried out in constant temperature incubators to determine the effect of increased temperatures on maximum quantum yields ($F_v : F_m$) of PSII of the diatom symbionts and the Chl *a* pigment content of the holobiont. Furthermore, the motility of the specimens was recorded to compare behavioral and physiological changes with the bleaching status of the species determined by digital imaging.

Photophysiology and Chl *a*: The average maximum quantum yield of the endosymbionts remained constant in all species in the 23°C treatment. Initial average maximum quantum yield in the 23°C treatment were 0.734, SE ± 0.005 for *A. radiata*; 0.752, SE ± 0.004 for *H. depressa*; and 0.712, SE ± 0.004 for *C. hispida*.

Increased temperatures above 30°C had a negative effect on the maximum quantum yields of the symbionts. One-way ANOVA revealed a significant decrease in $F_v : F_m$ with higher temperatures obtained after a 6-d exposure (Table 1). After 48 h, the overall trend of significantly reduced

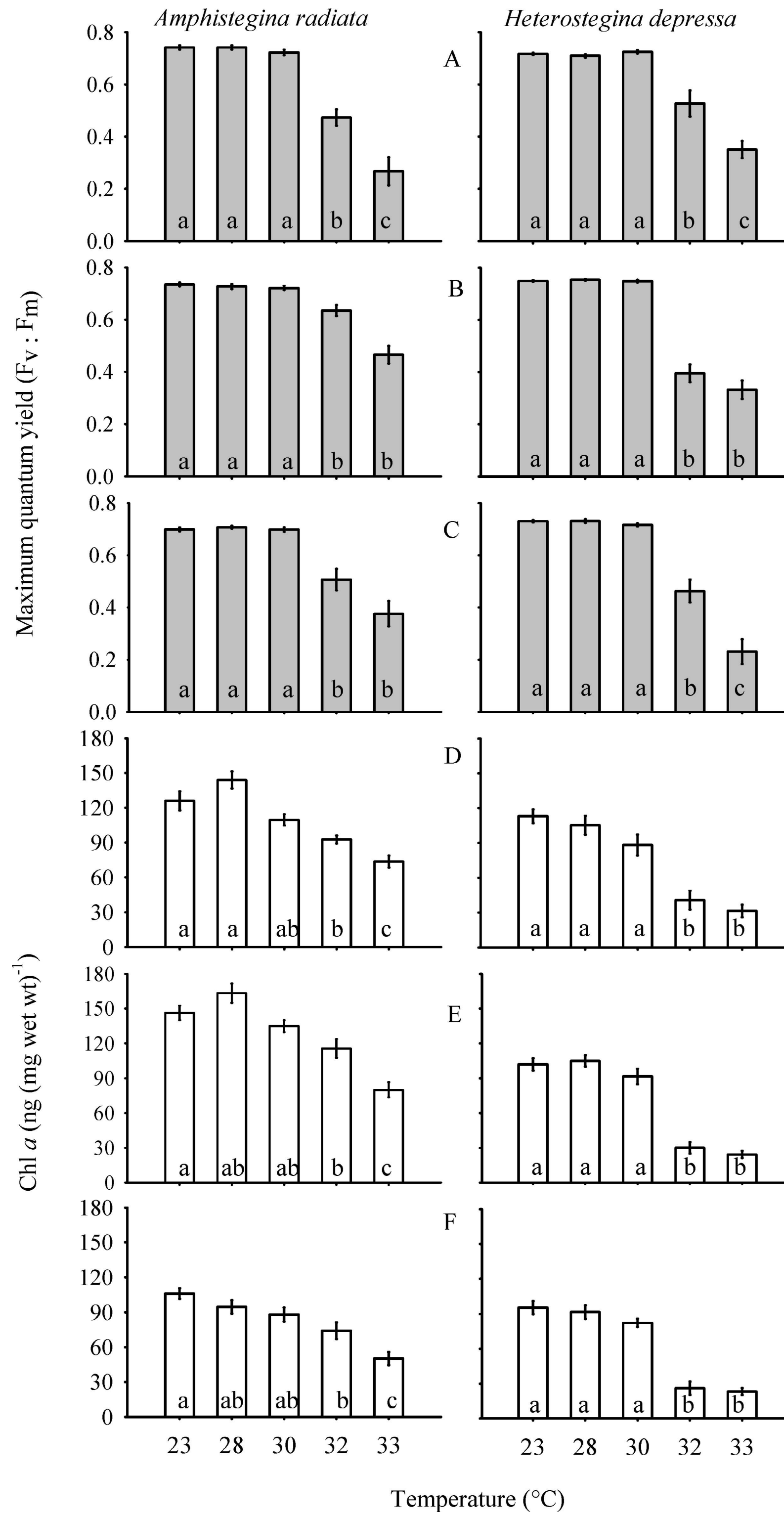

Fig. 1. Mean maximum quantum yield ($F_v : F_m$; gray bars) and Chl *a* content (ng [mg wet wt]$^{-1}$; white bars) in *Amphistegina radiata* and *Heterostegina depressa* in the static studies, three experimental runs: (A, D) June, (B, E) August, (C, F) January after 6-d exposure. $F_v : F_m$ data were arcsine transformed and Chl *a* data log transformed for statistical analysis, but graphs show untransformed data. Error bars = 1 SE; averages sharing the same letter (a–c) were not significantly different ($p > 0.01$) in the Tukey-Kramer HSD post hoc comparisons. Degrees of freedom (df) for each experimental run, *see* Table 1.

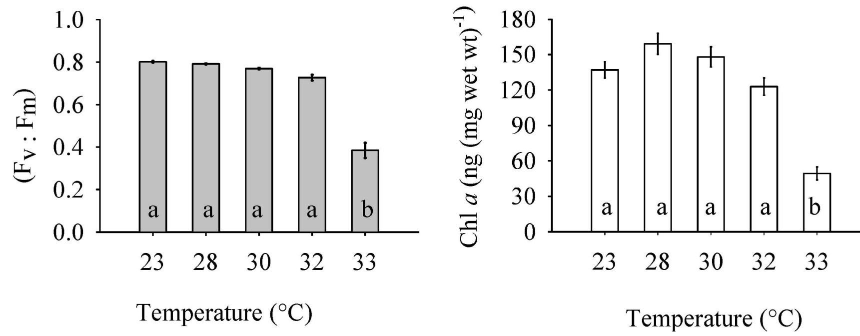

Fig. 2. Mean maximum quantum yield (MQY, $F_v:F_m$, gray 1 bars) and Chl *a* content (ng [mg wet wt]$^{-1}$; white bars) in *Calcarina hispida* in the static studies after 6-d exposure. Error bars = 1 SE; averages sharing the same letter (a–b) were not significantly different ($p > 0.01$) in the Tukey-Kramer HSD post hoc comparisons. Degrees of freedom (df) for each experimental run, *see* Table 1.

maximum quantum yield has been recorded (data not shown). Tukey-Kramer HSD post hoc tests indicate that average $F_v:F_m$ in the 23–30°C treatments in *A. radiata* and *H. depressa* were significantly higher than those in the 32–33°C treatments. The reduction in $F_v:F_m$ to values ≤ 0.01 occurred in 28% of *A. radiata* specimens and 42% of *H. depressa* specimens at the 33°C treatment, summarized over all runs. There was no indication of a seasonal effect (experimental runs in [A, B] summer and [C] winter) in the experiments conducted (Fig. 1). For *C. hispida,* Tukey-Kramer post hoc tests indicate that 23–32°C treatments were significantly higher than those in the 33°C treatment ($p = 0.001$, A = 23–32; B = 33; Fig. 2.). In *C. hispida,* $F_v:F_m$ did not drop below ≤ 0.01 at any point in the experiment.

Contour plots summarizing all three experiments illustrate that the reduction in $F_v:F_m$ in *A. radiata* and *H. depressa* was a function of exposure time and temperature (Fig. 3). An exposure of *A. radiata* at 33°C for 2 d had a similar effect on $F_v:F_m$ as did the exposure at 32°C for 4 d (in both cases reduced $F_v:F_m < 0.6$). In *H. depressa,* $F_v:F_m < 0.6$ occurred after 2 d in the 32°C and 33°C treatments (after 4 d, $F_v:F_m < 0.5$ in the 32°C treatment and $F_v:F_m < 0.4$ in the 33°C treatment).

Chl *a* content was measured to record changes of total biomass in the symbionts under temperature stress. Chl *a* content (as a proxy for symbiont biomass) dropped significantly with increasing temperatures (Fig. 1, white bars, one-way ANOVA shown in Table 1). Tukey-Kramer HSD post hoc tests revealed in *H. depressa* that Chl *a* content at the 23–30°C treatments were significantly higher than those measured at 32–33°C treatments. Small variations in the total Chl *a* content of *A. radiata* averaged over all temperatures can be seen between seasons (decline: 128 ng [mg wet wt]$^{-1}$ in run B [August] to 83 ng [mg wet wt]$^{-1}$ in run C [January]). In *C. hispida,* average Chl *a* values in the 33°C treatment were significantly different from the lower temperature treatments ($p = 0.001$, Tukey-Kramer post hoc test).

Motility: In addition to PAM and Chl *a*, motility of the specimens was monitored as a proxy for the overall fitness

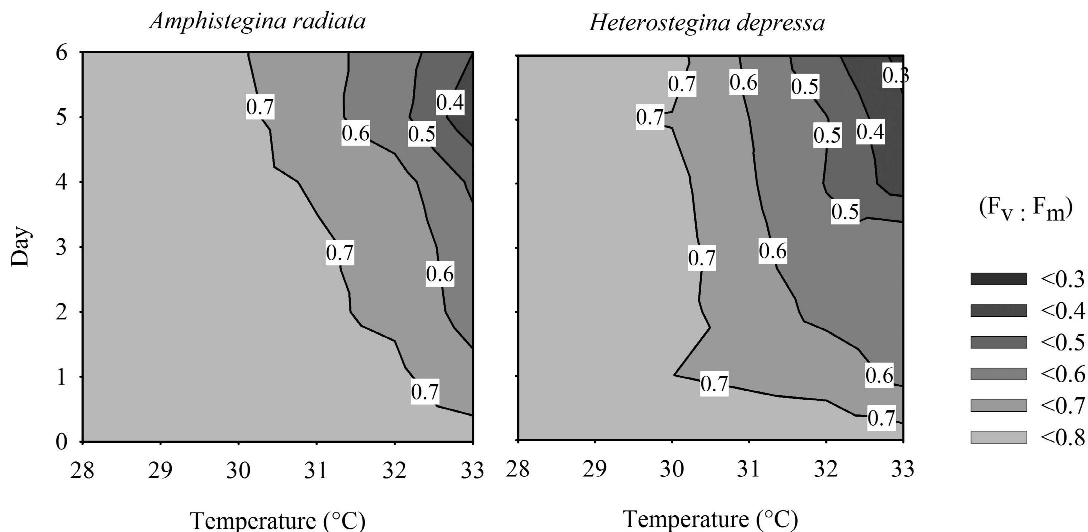

Fig. 3. Contour plots of the photosynthetic response of *A. radiata* and *H. depressa* as maximum quantum yields ($F_v:F_m$) in the static temperature studies. $F_v:F_m$ have been averaged over the three experimental runs.

Table 2. Motility (% active) between initial (2-d), intermediate (4-d), and final (6-d) readings in static studies, data averaged by temperature (T, °C) over three experimental runs (January, June, August). Values in brackets represent 1 SE.

	Motility (% active)					
	A. radiata			*H. depressa*		
T (°C)	2 d	4 d	6 d	2 d	4 d	6 d
23	75(9)	72(12)	70(7)	52(13)	51(10)	48(10)
28	78(6)	74(7)	82(11)	51(13)	45(3)	27(6)
30	85(6)	76(10)	72(5)	40(2)	33(10)	24(7)
32	57(3)	25(6)	28(10)	27(9)	29(2)	29(4)
33	44(10)	35(21)	11(7)	30(3)	21(10)	16(6)

of the holobiont. In all species, the motility (= % active, specimens attached to walls or in angular position) decreased with increasing temperatures (Table 2; Fig. 4). In *A. radiata*, the mean motility stayed at around 70–85% in the 23–30°C treatments over the length of the experiment but decreased from the 30°C to the 32°C treatment by 42%. Overall, the motility in *H. depressa* decreased from 48% in the 23°C treatment to 29% in the 32°C treatment and was lower than in *A. radiata*.

Digital photography: Digital images of representative specimens of *A. radiata* and *H. depressa* were taken from the 32°C treatment daily to illustrate the change in symbiont color (bleaching) over time. After 6 d, exposure pictures indicate less coloration inside the shell and, thus, reflect the results of Chl *a* measurements. Dark material, which could be either the diatom symbionts or debris from their digestion, concentrated at the aperture in *A. radiata* after 4 d (Fig. 5) in the static experiments at 32°C. In *H. depressa*, the symbionts were concentrated along the terminal openings of canals in the marginal cord (indicated by arrow, Fig. 5) after 3 d of exposure to 32°C. No such concentrations or discoloration were observed in temperatures up to 30°C.

Flow-through experiment—A flow-through experiment to test the interactive effects of temperature and nutrient (NO_3^-) was conducted over a 30-d period. Nutrient concentrations in the aquaria averaged by treatment were as follows: baseline: 0.53 (SD = 0.26), middle: 1.00 (SD = 0.44), and high: 1.42 (SD = 0.76) μmol $L^{-1} NO_3^-$. Although there was some variation, nutrient addition has clearly resulted in differences in nutrient concentrations between the treatments. Phosphate concentrations, which were not manipulated, remained the same among the treatments 0.11 (SD 0.04) μmol L^{-1} PO_4^{3-} over the course of the experiment.

Photophysiology and Chl *a*: In the controls, initial $F_v:F_m$ in *A. radiata* (0.682, SE ± 0.010), *H. depressa* (0.716, SE ± 0.003), and *C. mayorii* (0.709, SE ± 0.005) remained constant compared to final $F_v:F_m$ (Fig. 6, gray bars). In *A. radiata* and *H. depressa*, $F_v:F_m$ decreased with increasing temperatures. The mixed-model ANOVA revealed that temperature had a marginally significant (p = 0.073) effect on $F_v:F_m$ in *A. radiata*; in *H. depressa,* $F_v:F_m$ was significantly ($p < 0.001$) reduced with increasing temperatures (Fig. 6). Maximum quantum yields were significantly reduced (by 10%) in the 31°C treatment compared to the controls at 26°C (p = 0.001, Tukey-Kramer MC test). In *C. mayorii*, no significant differences have been found among temperature treatments. Severe bleaching ($F_v:F_m \leq 0.1$) was rare (5–6%) at the end of the experiment in *A. radiata* and *H. depressa* and did not occur in *C. mayorii*. There were neither significant differences between the nutrient treatments, nor significant interactions between temperature and nutrients for $F_v:F_m$ (Table 3).

Chl *a* content (ng [mg wet wt]$^{-1}$) decreased with increasing temperatures in *A. radiata* and *H. depressa* (Fig. 6, white bars; Table 3). In *A. radiata,* Tukey-Kramer MC post hoc tests suggested that mean Chl *a* content in the 31°C treatment was significantly different from that in the 26°C and 29°C treatments. Chl *a* content in *A. radiata*

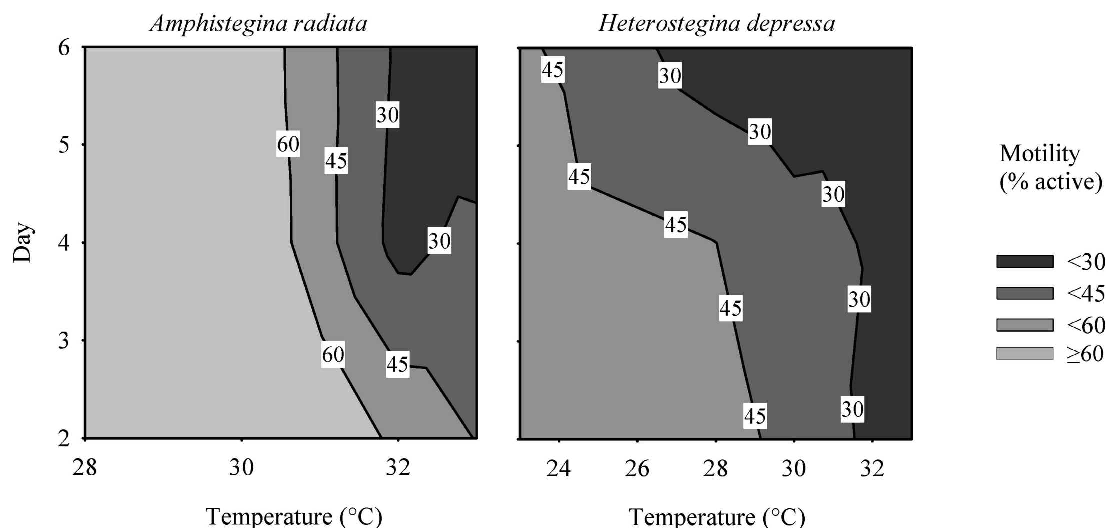

Fig. 4. Contour plots of average motility (% active) of *A. radiata* and *H. depressa* in the static studies. Motility data has been averaged over the three experimental runs.

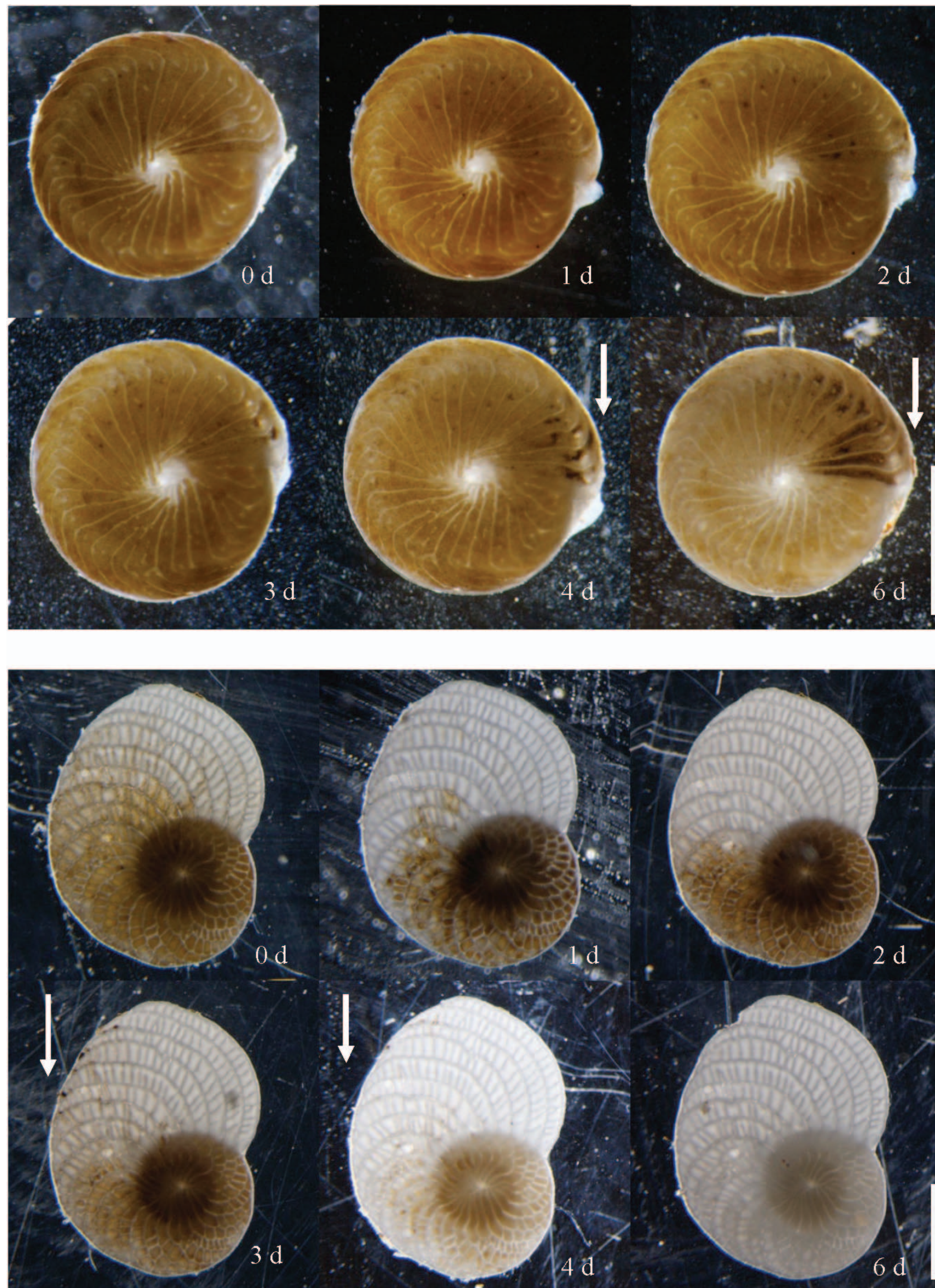

Fig. 5. Bleaching series of *A. radiata* and *H. depressa* from Border Island (6-d exposure at 32°C). White arrows indicate apertures where endosymbiotic diatoms concentrate. Scale bar: 2 mm.

decreased by 35% from the 26°C to 31°C treatments (Fig. 6, white bars). In *H. depressa,* the average Chl *a* content was significantly different among the 26°C, 29°C, and 31°C treatments. Chl *a* content decreased in *H. depressa* by 69% from 26°C to 31°C. *Calcarina mayorii* did not show a significantly reduced average Chl *a* content with increasing temperatures. Nevertheless, Chl *a* was reduced by 16% from the controls to the 31°C treatment. Neither nutrient addition, nor the interaction between nutrients and temperature, had a significant effect on the Chl *a* content of the species (Table 3).

Growth of holobiont: Growth (% surface area d^{-1}) was determined for 162 specimens of *A. radiata* by calculating

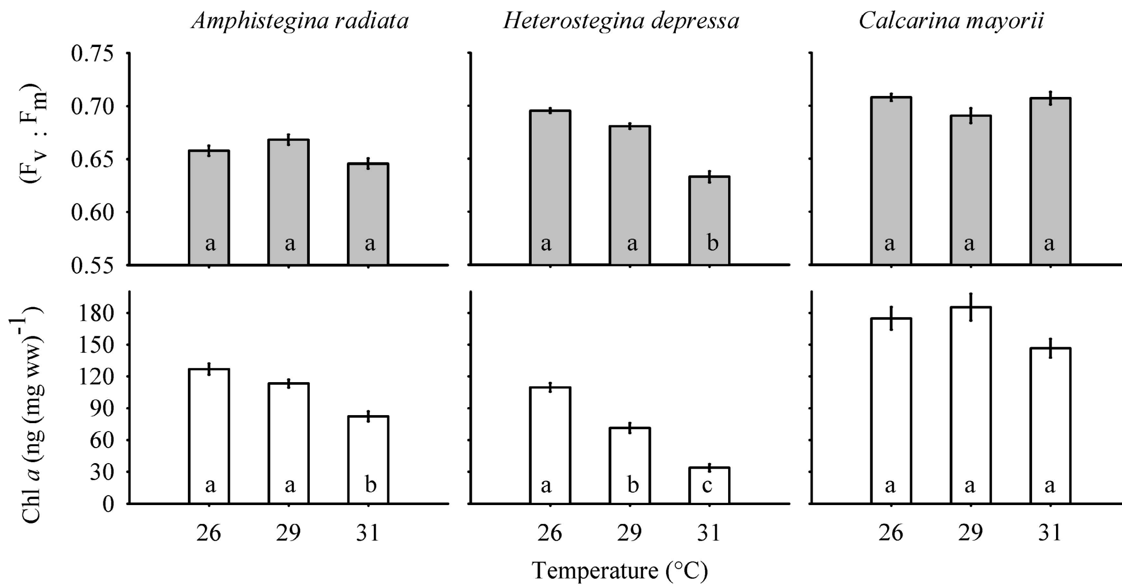

Fig. 6. Mean maximum quantum yield ($F_v : F_m$) and mean Chl *a* content (ng [mg wet wt]$^{-1}$) in *A. radiata* and *H. depressa* after 30-d exposure in flow-through experiment. Maximum quantum yield data ($F_v : F_m$) were arcsine transformed and Chl *a* data log transformed prior to analysis, but graphs show untransformed data. Error bars = 1 SE; averages with different letters (a–c) were significantly different ($p > 0.05$) in the Tukey-Kramer MD post hoc comparisons. Degrees of freedom (df) for each experimental run, *see* Table 3.

percentage surface area gain over the 30-d period. A two-way ANOVA suggested significant effects of temperature on growth in *A. radiata* (Table 4; Fig. 7). The Tukey-Kramer MC post hoc tests showed that growth at 31°C was significantly reduced (approximately by 43%) compared to 26°C. There were no significant differences between the 26°C and 29°C treatments, but a trend toward higher growth at 29°C (~ by 35%) was visible. Nutrient addition did not significantly affect growth of this species (Table 4).

Chl a *field comparison*—In *A. radiata*, the field Chl *a* (ng [mg wet wt]$^{-1}$) was similar in both seasons (winter mean 80.3, SD = 31.6; summer mean 84.5, SD = 26.2). The Chl *a* content in *A. radiata* was higher in the laboratory than in the field samples on average by 45% in winter and by 11% in summer. The field Chl *a* content was similar in *H. depressa* in both seasons (winter mean 109.2, SD = 32.1; summer mean 131.2, SD = 34.8). In summer, field Chl *a* in *H. depressa* was similar to Chl *a* content measured in the laboratory (field mean 109.2, SD = 32.2; lab mean 101.9, SD = 26.4).

Discussion

The main aim of the conducted experiments was to test whether temperature-induced bleaching occurs in LBF, similar to corals, using four different foraminiferal species as representatives for three families. Diatom symbionts of *A. radiata* and *H. depressa* showed a significant sensitivity toward increased temperatures under laboratory conditions, which has been demonstrated using a variety of response parameters: photosynthetic efficiency, symbiont biomass, and growth and motility of the specimens. In ultrastructural studies, Talge and Hallock (2003) observed partial symbiont loss in 50% of *Amphistegina gibbosa* specimens in the 32°C treatment and 10–20% in the 20–25°C treatment after exposure for 28 d. Our results are consistent because Chl *a*, as a proxy for symbiont biomass, decreased significantly with increasing temperatures. This study shows that bleaching can be triggered by increasing temperatures in the two LBF species *A. radiata* and *H. depressa*.

Reduced maximum quantum yields, as an indicator for photosynthetic activity, significantly decreased at temperatures > 30°C in static and flow-through experiments. This was the first parameter (measured by PAM fluorometry) showing heat stress in the investigated organisms at exposure to 32°C after 48 h. *H. depressa* seemed to be more sensitive to increased temperatures because $F_v : F_m$ decreased sooner and more distinctly than in *A. radiata*. Nutrient enhancement did not have a significant effect on $F_v : F_m$ or symbiont biomass. Results of the static and flow-through experiments show the same trends, but reduction in $F_v : F_m$ occurred sooner and was more distinct in the static setup.

Light levels in the static experiments (11–15 μmol photons m^{-2} s^{-1}) were in the range at which Talge and Hallock (2003) recorded incidences of partial bleaching in *A. gibbosa* at all experimental temperatures (20°C, 26°C, and 32°C). These levels were nearly double as compared with the flow-through experiment (6–8 μmol photons m^{-2} s^{-1}), which held specimens at light intensities comparable to those at which Williams and Hallock (2004) recorded no partial bleaching in *A. gibbosa*. Because $F_v : F_m$ remained constant in the 23–26°C treatments over the course of both experiments, we assume that these light levels alone did not stress the specimens. The decision to use low light intensities was based on previous work that showed that high light can slow growth and reduce maximum quantum yield (Nobes et al. 2008). Incidences

Table 3. Mixed-model ANOVA for the maximum quantum yield ($F_v : F_m$) and Chl *a* content (ng [mg wet wt]$^{-1}$) for *A. radiata*, *H. depressa,* and *C. mayorii* after 30-d exposure in the flow-through study. $F_v : F_m$ were arcsine transformed and Chl *a* data log transformed prior to analysis. Values in bold are significant at $p > 0.05$.

	$F_v : F_m$				Chl *a*			
	df	MS	*F*	*p*	df	MS	*F*	*p*
A. radiata								
Temperature (T)	2	0.001	3.03	0.073	2	3.256	10.44	**<0.001**
Nutrients (N)	2	0.002	0.64	0.537	2	0.065	0.21	0.865
T×N	4	0.003	0.79	0.545	4	0.437	1.40	0.273
Aquaria (T, N)	18	0.003	2.06	**0.012**	18	0.312	3.46	**<0.001**
Error	112	0.002			120	0.090		
H. depressa								
Temperature	2	0.086	36.76	**<0.001**	2	15.525	39.87	**<0.001**
Nutrients	2	0.002	0.72	0.499	2	0.162	0.42	0.666
T×N	4	0.001	0.55	0.698	4	0.367	0.95	0.458
Aquaria (T, N)	18	0.002	2.89	**<0.001**	19	0.389	2.05	**0.013**
Error	120	0.001			99	0.190		
C. mayorii								
Temperature	2	0.005	1.41	0.270	2	0.223	0.72	0.501
Nutrients	2	0.001	0.21	0.811	2	0.201	0.65	0.535
T×N	4	0.003	0.85	0.511	4	0.499	1.61	0.216
Aquaria (T, N)	18	0.003	3.74	**<0.001**	18	0.311	2.97	**0.001**
Error	50	0.001			52	0.105		

of severe bleaching (defined as maximum quantum yields $F_v : F_m \leq 0.1$) were low in the 32°C (3–6%) treatment and high in the 33°C treatment (28–42%) in the static experiment. During the flow-through experiment, there were few incidences of severe bleaching rates, occurring in only 5–6% of total specimens, with 3–4% of the total found in the 31°C treatment.

In several photosynthetic organisms, the molecular basis for temperature sensitivity has been resolved. Temperature and light are important stimuli to trigger the bleaching response in corals: too much of either one in the presence of the other factor or the combination of both can lead to bleaching (reviewed in Fitt et al. 2001). Increased temperature mainly damages the photosynthetic apparatus, especially PSII, as shown in dinoflagellates (Warner et al. 1996). But in the presence of light, nonphotochemical quenching and damage through free oxygen radicals cause photooxidative stress (Jones et al. 1998). We were able to show by PAM fluorometry that the photosynthetic apparatus of the endosymbiotic diatoms was malfunctioning under increasing temperatures in both experimental setups. The exact molecular damage cannot be resolved at present. It has been suggested that in cnidarians reactive oxygen species oxidize membrane lipids of symbiotic algae, leading to death of the latter and their expulsion (Tchernov et al. 2004). Similarly, Talge and Hallock (1995) reported extensive membrane damage in partly bleached *A. gibbosa*; bleached specimens were too structurally compromised to produce meaningful sections.

Table 4. Two-way ANOVA for growth rates (% surface area d^{-1}) in *A. radiata* over 30-d exposure. Data was arcsine transformed prior to analysis, and values in bold are significant at $p > 0.05$.

	Growth			
	df	MS	F	*p*
Temperature (T)	2	0.012	5.226	**0.017**
Nutrients (N)	2	0.001	0.417	0.665
T×N	4	0.006	1.368	0.287
Error	18	0.020		

Chl *a* content (as an indicator for symbiont biomass) was significantly reduced with increasing temperatures (> 31°C). The new method for Chl *a* determination in LBF provides a more objective determination of gradual symbiont loss than visual analysis. The accuracy of the method was confirmed with UPLC (correlation: $R^2 = 0.90$). Phaeophytin content in the UPLC was very low for *A. radiata* and *H. depressa* compared to total Chl *a* values. Previous studies yielded comparable Chl *a* content per LBF (50–250 ng individual^{-1}) but did not normalize Chl *a* content to weight (Hallock and Talge 1993). In corals, symbiont density (per unit surface area or tissue protein biomass) is usually related to the total Chl *a* content, to eliminate the possibility that bleaching is only caused by pigment loss, a rare phenomenon (Hoegh-Guldberg and Smith 1989).

Because a method for symbiont counts is currently not available for LBF without cytological examination (Talge and Hallock 2003), it was difficult to judge whether symbiont numbers decreased or Chl *a* content per cell was reduced. However, given that photographs of temperature-stressed specimens showed apparent clustering of dark material near the aperture (and expulsion), we suggest that most of the decreased Chl *a* actually reflected reduced symbiont numbers, as documented previously by Talge and Hallock (1995, 2003). The Chl *a* contents of field and experimental specimens were generally similar. In *A. radiata*, mean Chl *a* contents in the experiments were even

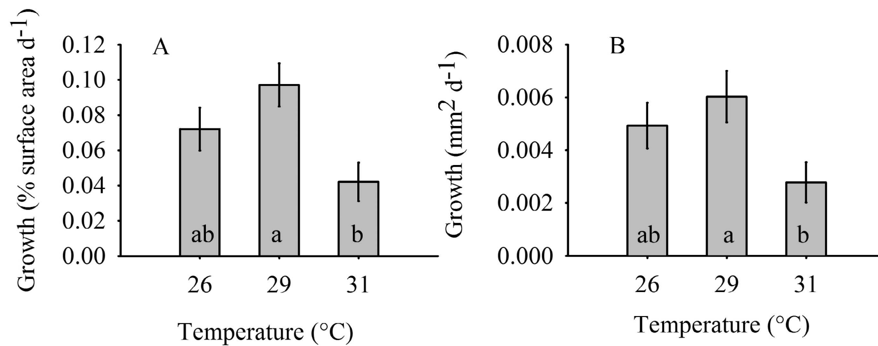

Fig. 7. Growth in *A. radiata* during 30-d exposure in flow-through study (A) expressed as % relative to initial size increase per day (% surface area d^{-1}) and (B) expressed as daily increase in surface area (mm^2 d^{-1}). Averages sharing the same letter (a–b) were not significantly different ($p > 0.05$) in the Tukey-Kramer HSD post hoc comparisons.

slightly elevated compared to Chl *a* contents in the field. This, together with constant $F_v:F_m$, is an indication that experimental conditions under low temperatures (23–28°C) did not stress the symbiont population.

One species, *C. mayorii* from Magnetic Island, was more resistant to increased temperatures in the flow-through study than was *C. hispida* from Heron Island in the static studies. This suggests that the response to thermal stress is highly species-specific. The sensitivity of the symbiosis is an important element in the determination of thermal tolerance limits in the species. To determine whether these two *Calcarina* species have different tolerance limits because of different temperature-tolerant symbionts or because they originate from two different locations (reef flat–dwelling species, intermediate-depth species) requires further investigation. Spatial trends of bleaching exist in corals; the same species from geographically distinct locations show different bleaching thresholds (Berkelmans 2002; Ulstrup et al. 2006). In the GBR, reefs from Magnetic Island have the highest upper thermal bleaching limits from inshore GBR reefs of 31.5°C for exposure > 3–5 d compared to reefs at Daydream Island, Whitsundays, with thresholds of 29.5°C (Berkelmans 2002; Berkelmans 2009; Wooldridge 2009).

Bleaching (symbiont or pigment loss) was additionally documented by digital photography. It was not observed as color loss at temperatures of 23–28°C and was clearly visible only at temperatures > 30°C after 3–4 d of exposure. Mottling (partial symbiont loss) was observed in *A. radiata* similar to observations by Hallock et al. (1992) and Hallock and Talge (1993) in the Caribbean species *A. gibbosa,* and in the Indo-Pacific species *A. lessonii*, *A. lobifera*, and *A. radiata*. Previous studies on *A. gibbosa* by Talge and Hallock (2003) suggested that symbionts are digested because lysosomes were observed surrounding the symbionts and nearly five times more deteriorating symbionts were observed in partly bleached field specimens than in normal-appearing specimens. Thus, the pigment-filled membrane-surrounded bodies at the outside of the aperture in *A. radiata* likely contained digested symbionts. In *H. depressa*, membrane-surrounded bodies have been observed at the outside of the shell along the many terminal openings of canals in the marginal cord that substitute for the terminal aperture (Röttger et al. 1984).

Motility on LBF has been the focus of only a few experiments (reviewed in Travis and Bowser 1991) and has not been systematically investigated in either field or laboratory. Röttger (1973) concluded from microscopic observations that movements are kept to a minimum in *H. depressa* because most of the organism's energy requirements are gained through endosymbiosis and not through active feeding. Motility in LBF used in this study might reflect a search of the species for their optimal microenvironment in situ, but in stressed specimens this ability becomes impaired. Motility decreased with increasing temperature in *A. radiata* (42%) and in *H. depressa* (29%). Motility stayed near constant in the 23°C treatments over the length of the experiment, indicating that results are comparable between the treatments. Repeated observations of pseudopodial activity in heavily bleached specimens have been recorded during the static study and confirm observations by Talge and Hallock (2003). The reduced ability to move under temperature stress puts the species at a disadvantage to cope with additional stressors, such as light or wave energy and possibly others.

Growth (= % growth [surface area gain] d^{-1}) measured in *A. radiata* in the flow-through experiment was reduced by 45% in the 31°C treatment when compared to the 29°C treatment. Generally, growth rates for *A. radiata* were in the range of 0.04–0.10% growth d^{-1}. Experiments conducted by Nobes et al. (2008) yielded slightly higher growth rates of 0.1–0.3% growth d^{-1} for *A. radiata* under similar average light intensities. These experiments were conducted under natural sunlight, and, thus, midday average light peaks (375 μmol photons m^{-2} s^{-1}) might have triggered growth compared to this study using near constant light levels. Calcification in LBF, as in other calcifying systems, is thought to be stimulated by light (Röttger et al. 1980; Ter Kuile et al. 1989). Only a few experiments measured growth of foraminifera in the field. Slightly higher field growth rates (0.2–0.5% growth d^{-1}) were observed for *A. radiata* from the study area (averaged over locations and seasons; Uthicke and Altenrath 2010). Growth from inshore reefs alone in the

latter study (lower light levels) was comparable with results in the present study. In situ growth rates of *A. lobifera* in the Red Sea were higher (1–6% growth d^{-1}) over a 3-week period (Ter Kuile and Erez 1984). Possible reasons for this are that *A. lobifera* is smaller and grows faster than *A. radiata* and typically lives in shallower, much higher light environments (Hallock 1984; Hohenegger et al. 1999; Hohenegger 2004).

Under temperature stress, the effects of reduced maximum quantum yields and reduced symbiont biomass in combination are likely to yield less algal productivity. Thus, reduced organic carbon translocation between symbiont and host seems to correlate with reduced growth rates in *A. radiata.* However, it is not clear whether the breakdown of the algae–symbiont partnership is the reason for decreased growth rates or whether calcification is in general altered with increasing temperatures.

Combined temperature and nutrient effects were investigated in the flow-through experiment. It was hypothesized that excess nitrogen would release symbionts from their nutrient limitation, resulting in increased symbiont biomass (Dubinsky and Jokiel 1994; Dubinsky and Berman-Frank 2001). This has been demonstrated to put stress on the host–algae symbiosis in corals but under much higher nutrient concentrations than used here (20 μmol L^{-1} ammonium and 2 μmol L^{-1} phosphate; Muscatine et al. 1989). The nutrient concentrations used in this study are based on background water quality data (5-yr averages) for the Whitsundays, which range between 0.2–1.2 μmol L^{-1} during the wet season in inshore areas (Uthicke and Altenrath 2010) and are well elevated compared to non–flood-plume scenarios (Furnas et al. 2004). Inorganic nitrogen was applied in the form of dissolved ions (K^+ and NO_3^-) up to 1.4 μmol L^{-1}. Since there was no detectable reaction in the photosynthetic efficiency of any of the species analyzed to elevated nutrients in our experiments, the nutrient levels tested have not been high enough to elevate symbiont biomass or reduce growth of the holobiont. It is interesting to note that one species bearing dinoflagellate symbionts does show clear negative effects from nutrient elevation (Reymond et al. in press). It may be tempting to hypothesize that diatom-bearing species are less vulnerable to nutrient enhancement than dinoflagellate-bearing species. However, given that only one of the latter species has been investigated, further research is required to test the general applicability of this hypothesis.

An in situ study in the Whitsundays demonstrated that growth rates in *A. radiata* and *H. depressa* (Uthicke and Altenrath 2010) are influenced by water-column nutrients. Growth rates were generally significantly lower in inshore compared to offshore reefs, the former being exposed to enhanced nutrient runoff (Cooper et al. 2007). The species also had reduced growth rates at both sites during the wet season, when nutrient levels were highest (Uthicke and Altenrath 2010). The growth rates in *A. radiata* in our experimental controls are similar to inshore growth rates under elevated nutrient levels (0.006–0.010 mm^2 d^{-1}) measured by Uthicke and Altenrath (2010). This indicates that in situ growth, even under offshore nutrient conditions, is generally lower than ex situ.

In summary, the results of these experiments showed that the symbiotic relationship with diatoms and physiological performance of the LBF *A. radiata*, *H. depressa,* and *C. hispida* is negatively affected by increasing SSTs. It is known that corals have suffered from temperature stress within the last decades, leading to large-scale bleaching events (Berkelmans and Oliver 1999; Berkelmans et al. 2004; Berkelmans 2009) but the connection between temperature stress and bleaching in LBF has only now been established. Thus, similar to corals, the future of these species may depend on their ability to acclimatize or adapt to changing conditions. How individual populations of foraminifera respond to temperature stress remains open, because these species can achieve higher evolutionary rates compared to the coral–dinoflagellate symbiosis due to their smaller size and (presumably) shorter generation time. Whether they are better able to adapt appears to be an interesting subject for further experiments.

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