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Reactive oxygen species (ROS) and dimethylated sulphur compounds in coral explants under acute thermal stress

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Summary statement

We used coral explants to investigate the direct link between intracellular reactive oxygen species and dimethylated sulphur compounds at the microscale under an acute thermal stress

Key words

Coral explant, reactive oxygen species, dimethylsulphoniopropionate (DMSP), *Fungia granulosa*

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Abstract

Coral bleaching is intensifying with global climate change. While the causes for these catastrophic events are well understood, the cellular mechanism that triggers bleaching is not well established. Our understanding of coral bleaching processes is hindered by the lack of robust methods for studying interactions between host and symbiont at the single-cell level. Here we exposed coral explants to acute thermal stress and measured oxidative stress, more specifically, reactive oxygen species (ROS), in individual symbiont cells. Furthermore, we measured concentrations of dimethylsulphoniopropionate (DMSP) and dimethylsulphoxide (DMSO) to elucidate the role of these compounds in coral antioxidant function. This work demonstrates the application of coral explants for investigating coral physiology and biochemistry under thermal stress and delivers a new approach to study host-symbiont interactions at the microscale, allowing us to directly link intracellular ROS with DMSP and DMSO dynamics.

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Introduction

Coral reefs are vulnerable to climate-induced changes, in particular; increasing water temperatures that can lead to mass coral bleaching events. While much is known about the environmental causes of bleaching, the physiological and cellular mechanisms which trigger coral bleaching are not yet well described (Downs et al., 2002). Previous research has identified excess reactive oxygen species (ROS) as a causative agent for coral bleaching (Lesser, 1997); and linking oxidative stress with the expulsion of *Symbiodinium* cells has since been the focus of numerous publications (Tchernov et al., 2011; Downs et al., 2013).

Despite this focus, our understanding of the symbiotic breakdown between the coral host and *Symbiodinium* cells is hampered by a lack of methods for investigating the symbiotic interaction at the micro-scale (Shapiro et al., 2016). Previously, small scale holobiont physiology has been studied using microscopy methods including histology (Downs et al., 2009), electron microscopy (Tchernov et al., 2004) and more recently nanoscale secondary ion mass spectrometry (Pernice et al., 2012). While these methods provide detailed information about cellular structures and functioning, they are limited in their ability to follow dynamic processes *in vivo* at relevant time-scales, because samples need to be fixed (Shapiro et al., 2016). New approaches allowing study of coral tissues *in vivo* in real-time have recently been developed, including micropropagation of tissue in microfluidic devices (Shapiro et al., 2016) and coral explants (Gardner et al., 2015).

In the past decade, there has been strong interest in the role of dimethylsulphoniopropionate (DMSP) in alleviating cellular oxidative stress (Sunda et al., 2002). As DMSP represents a major fraction of organic sulphur within marine systems (Kiene et al., 1999) and corals are among the largest producers of DMSP (Broadbent and Jones, 2004), it follows that the biochemical processes thought to be involved with coral bleaching and antioxidant quenching need to be explored with respect to DMSP and ROS (Jones and King, 2015). DMSP is an effective antioxidant (Sunda et al., 2002) and it could act as an additional defence mechanism in corals under oxidative stress (Deschaseaux et al., 2014a; Gardner et al., 2016). However; to date, most studies have only linked the antioxidant function of DMSP by inferring ROS through antioxidant activity or concentrations, instead of measuring ROS directly, which requires a single-cell approach.

Here we used coral explants to investigate oxidative stress in coral symbionts at the single cell level, while concomitantly measuring DMSP and its oxidised breakdown product DMSO under acute thermal stress. In conducting this study, we demonstrated the suitability of coral explants for detailed

investigation of the coral host and symbiont physiology at the microscale, offering a novel and valuable experimental approach for studying the physiology and biochemistry of corals, increasing our understanding of the importance of dimethylated sulphur compounds in oxidative stress regulation.

Materials and methods

Explant production and physiology

Individual solitary corals of the species *Fungia granulosa* (Heron Island, Great Barrier Reef, Australia) were used to produce coral explants following the method described in Gardner et al. (2015). Briefly, coral tissue was gently peeled from the coral skeleton, broken into smaller pieces and transferred to an autoclaved borosilicate glass dish (Schott-DURAN, GmbH, Germany) containing 50 ml filtered seawater (FSW, 0.22 µm) and antibiotics (Gentamicin and Kanamycin, both 50 µg ml^{-1}, Life Technologies Australia Pty Ltd, Australia). After 24 h, any viable explants were transferred to fresh dishes containing the antibiotic treatment for a further 2 days, after which explants were maintained in antibiotic-free FSW. Cultured explants were maintained under 100 µmol photons m^{-2} s^{-1} of cool-white light (Hydra52 HD; Aqua Illumination, Iowa, USA) on a 10:14-h light/dark cycle for 1 week until use. To ensure repeatability of the response, four independent experiments were run (N=5 per treatment in each experiment), resulting in 20-22 independent replicates per treatment.

Prior to heat stress, explant diameter was measured on an upright epifluorescence microscope (Olympus BX51) equipped with a CCD colour camera (DP70) at 100 × magnification. Photosynthetic health of the symbiont (dark-adapted maximum quantum yield of PSII; F_V/F_M) was determined using a pulse amplitude modulated fluorometer (Microscopy Imaging PAM—Max/K, RGB, Walz GmbH, Effeltrich, Germany), mounted on a compound microscope (Axiostar plus, Zeiss, Germany). Following F_V/F_M measurements, explants were transferred into individual 50 ml beakers in a water bath where the temperature was increased from 27°C to 32°C over 2.5 h and held at 32°C (a common bleaching temperature for GBR corals; see Suggett et al., 2008; Downs et al., 2013) for an additional 1.5 h in the light (Fig. 1A), while independent control explants were maintained at 27°C for 4 h. Explants were then either prepared for gas chromatography (GC) measurements (N= 10) or used immediately to visualise intracellular symbiont ROS.

DMSP and DMSO under acute thermal stress

Total DMSP and DMSO concentrations in coral explants were analysed using GC (Shimadzu Scientific Instruments (Oceania), NSW, Australia) fitted with a flame photometric detector, following the method based on the 1:1 alkali cleavage of DMSP to DMS (Curran et al., 1998). Samples were run in technical triplicates for each explant. Following DMSP measurements, samples were prepared based on previous

methods (Kiene and Gerard, 1994; Deschaseaux et al., 2014b) to convert DMSO to DMS. DMSP/O measurements were normalised to the volume of the explants (sphere), calculated from the diameter. Due to the destructive sampling required for DMSP/O determination, no measurements prior to incubation were obtained. Instead, experimental conditions were repeated a minimum of four times using a total of 20 explants to ensure representative data.

Oxidative stress measurements

Individual explants were incubated for 20 min in the dark using 20 µM final concentration of a general oxidative stress indicator, CM-H_2DCFDA (Molecular Probes, Thermo Fisher Scientific Inc., Australia). Dye concentration was chosen based on preliminary trials and optimisation that ensured sufficient and even penetration of the dye through the tissue. Explants were rinsed three times in filtered sea-water (FSW, 0.2 µm) before being flattened onto a slide to break apart the internal structure. Using an inverted fluorescence microscope (Nikon Eclipse Ti, Nikon Instruments Inc., Melville, USA) with standard filters; TxRed (red, chlorophyll fluorescence) and FITC (green, CM-H_2DCFDA fluorescence), at least five randomly selected locations of each slide were imaged (200 × magnification) to include >30 individual *Symbiodinium* cells per explant. The fluorescence of all *Symbiodinium* cells was measured, excluding those where animal tissue was obstructing the measurement. A total of 600 *Symbiodinium* cells were measured for the controls and 521 for the treatment. Images were processed in Fiji (Image J) using the cell magic wand plugin (Schindelin et al., 2012). For each cell, the area, diameter and integrated fluorescence density was measured and recorded in the FITC channel, with additional measurements for background fluorescence, which were adjusted to cell area and subtracted from the integrated fluorescence of each cell. As with DMSP/O determination, oxidative stress measurements required destructive sampling and as such, to ensure scientific rigour, experiments were conducted repeatedly with replication.

Statistical analysis

Unpaired t tests were performed on F_V/F_M, DMSP, DMSO and DMSO:DMSP ratio (data met the assumptions of normality following a Gaussian distribution) using GraphPad Prism v.6 (GraphPad Software, Inc, USA). Mann-Whitney test for non-equal distributions for the chlorophyll *a* and ROS fluorescence were performed using IBM SPSS Statistics v.21 (IBM Corporation, New York).

Results and discussion

By investigating DMSP and DMSO in conjunction with symbiont ROS under acute thermal stress, we validate the suitability of coral explants for detailed physiological and biochemical studies (Fig. 1A). Coral explants consisted of host tissues and *Symbiodinium* (Fig. 1B), with a comparable internal complexity to the parent *Fungia granulosa* coral (Gardner et al., 2015). Symbiont F_V/F_M significantly

declined over the 4 h temperature stress from 0.59 ± 0.02 to 0.40 ± 0.02 (Unpaired t test; $t(40) = 5.76$, $P<0.001$; Fig. 1C) indicating photoinactivation of photosystem II (PSII). This decline is the result of a temporary down regulation of photochemistry attributed to enhanced thermal dissipation from the PSII reaction centres (Falkowski and Raven, 1997).

Using coral explants allowed us to investigate single-cell processes in endosymbionts *in vivo*. Fluorescence microscopy showed a significant decline of autofluorescence in heat treated explants (67,902 ± 1,250 a.u., Mann-Whitney test, $P<0.001$) compared with the control (137,111 ± 1,498 a.u.; Fig. 2A,C), indicative of chlorophyll *a* degradation in the chloroplasts (Coles and Jokiel, 1977; Warner et al., 1996). Loss in the integrity of the photosynthetic apparatus is often accompanied by increases in the cellular concentration of ROS, as well as increases in enzymatic antioxidant defences in the host and/or in different strains of *Symbiodinium* (Tchernov et al., 2004). Indeed, our results show a concomitant increase in the fluorescence intensity of the general ROS dye (CM-H_2DCFDA) in the heat-treated explants, from 16,943 ± 355 a.u. in the control to 26,196 ± 373 in the treatment (Mann-Whitney test, $P<0.001$; Fig. 2B). The increased fluorescence under heat stress would suggest net accumulation of hydrogen peroxide in *Symbiodinium* in response to acute thermal stress (Fig. 2D,E), which has been reported previously (Suggett et al., 2008). While it is generally believed that the primary trigger for coral bleaching during heat stress is ROS produced in the light (Downs et al., 2002; Lesser, 2011), bleaching can also be triggered in the dark (Tolleter et al., 2013), suggesting that photosynthetically-derived ROS are not a pre-requisite for bleaching (Tolleter et al., 2013). The oxidative stress in our study is a measure of ROS generated during the 20 min dark incubation with the dye and is possibly an indication of mitochondrial produced ROS, driven in part by negative effects of temperature on mitochondrial integrity (Dunn et al., 2012).

Thermal stress caused a significant decline in concentrations of intracellular DMSP (Unpaired t test; $t(19) = 3.67$, $P<0.001$; Fig. 3A) and DMSO (Unpaired t test; $t(19) = 3.47$, $P<0.01$; Fig. 3B), resulting in a significant decrease in the ratio of DMSO:DMSP (Unpaired t test; $t(19) = 3.64$, $P<0.001$; Fig. 3C), a measure often used as an indicator for the conversion of DMSP to DMSO, potentially mediated by ROS. This decrease in DMSO:DMSP ratio under thermal stress indicates greater use (or faster rate of quenching) of DMSO than DMSP, highlighting the enhanced potency of DMSO as an ROS quencher (Sunda et al., 2002). Furthermore, DMSO can be oxidised to methanesulphonic acid (MSNA) by reacting with and scavenging hydroxyl radicals (Lavoie et al., 2016), this additional oxidation step could account for the greater loss in DMSO pool compared with the loss in DMSP.

Similar decreases in physiological health, increases in ROS (Franklin et al., 2004) and decreases in DMSP have been shown for *Symbiodinium* cultures (Deschaseaux et al., 2014c), and previous work has found that changes in DMSO and DMSP often correlate (Deschaseaux et al., 2014a). The loss of

DMSP/O in our study could be explained by an increase in DMSP lyase activity elicited by elevated temperatures, and thus the subsequent oxidation of DMS to DMSO. This has been described previously, where decreases in DMSP and DMS over 48 h in temperature stressed *Acropora intermedia* (Fischer and Jones, 2012). Alternatively, due to the rapid increase in temperature, intracellular DMSP could have been used as an antioxidant (Sunda et al., 2002), with further oxidation of DMSO to MNSA, reducing the available intracellular pools of both DMSP and DMSO well before any *de novo* synthesis could take place. This is plausible, given that DMSP is energetically expensive to produce (Keller et al., 1999), and if coral cellular activity is compromised, as was indicated by the lower F_V/F_M, higher ROS and degradation in chlorophyll *a*, the production of DMSP by the symbiont cells may have slowed or ceased under the acute temperature stress.

Here we use coral explants as model organisms for host-symbiont cellular investigations. Coral explants were used for direct measurements of intracellular ROS *in vivo* during a stress event, allowing for rapid and repeatable measurements of ROS activity in endosymbiotic *Symbiodinium*. While we did not investigate the oxidative stress response of the host, the same methodology could be applied to host cells enabling assessment of both host and symbiont responses in an intact symbiosis. In this study we explored the links between intracellular ROS and DMSP dynamics in corals under acute thermal stress, a procedure previously made difficult due to the complexity of measuring ROS *in vivo*, as the removal of tissue or symbionts from the coral skeleton is invasive and would likely influence the ROS activity being measured. Our results show increased ROS in endosymbionts under acute thermal stress and a decline in both DMSP and DMSO pools, supporting the involvement of sulphur compounds in the quenching of ROS in corals. This study adds to the growing knowledge of the importance of dimethylated sulphur compounds in oxidative stress regulation by linking intracellular ROS with DMSP and DMSO dynamics under acute thermal stress. Investigating these biochemical links in corals at the cellular level will be helpful for future studies, as we uncover the site of DMSP production and its proximity to these short-lived oxygen radicals.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceived and designed the experiments: SG and KP. Performed the experiments: SG and KP. Analysed the data: SG and KP. Wrote the paper: SG and KP, with comments and suggestions from all authors.

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Journal of Experimental Biology • Advance article

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Figures

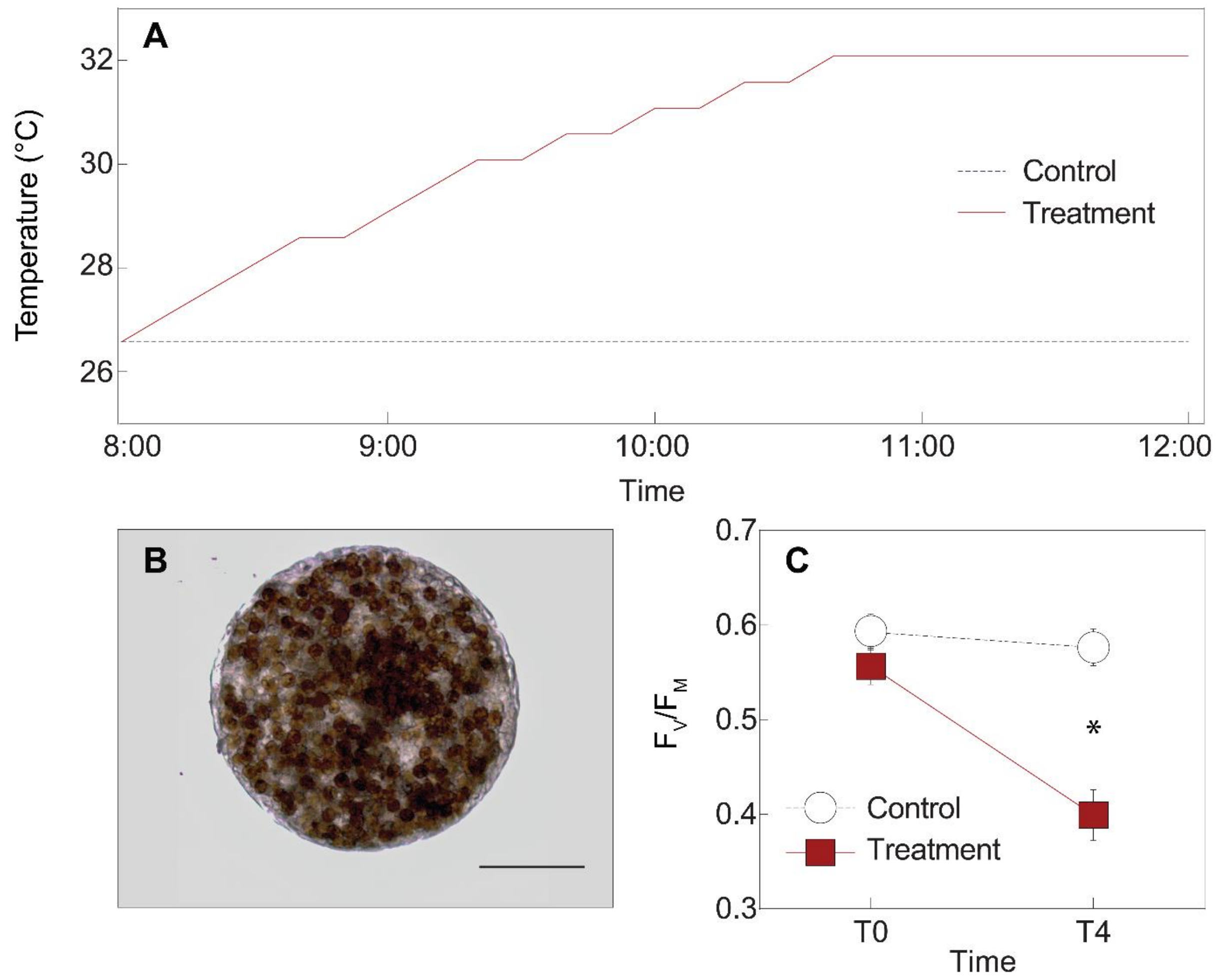

Fig. 1. Temperature treatment and physiological health of coral explants. (A) Temperature treatment **(B)** microscopy image of a coral explant, and **(C)** maximum quantum yield of photosystem II (F_V/F_M) for control (white) and treatment (red). Significant difference shown by asterisk (Unpaired t test; $P<0.001$). Data represent mean ± s.e.m (N=20 for control and N=22 for treatment). Scale bar = 0.2 mm.

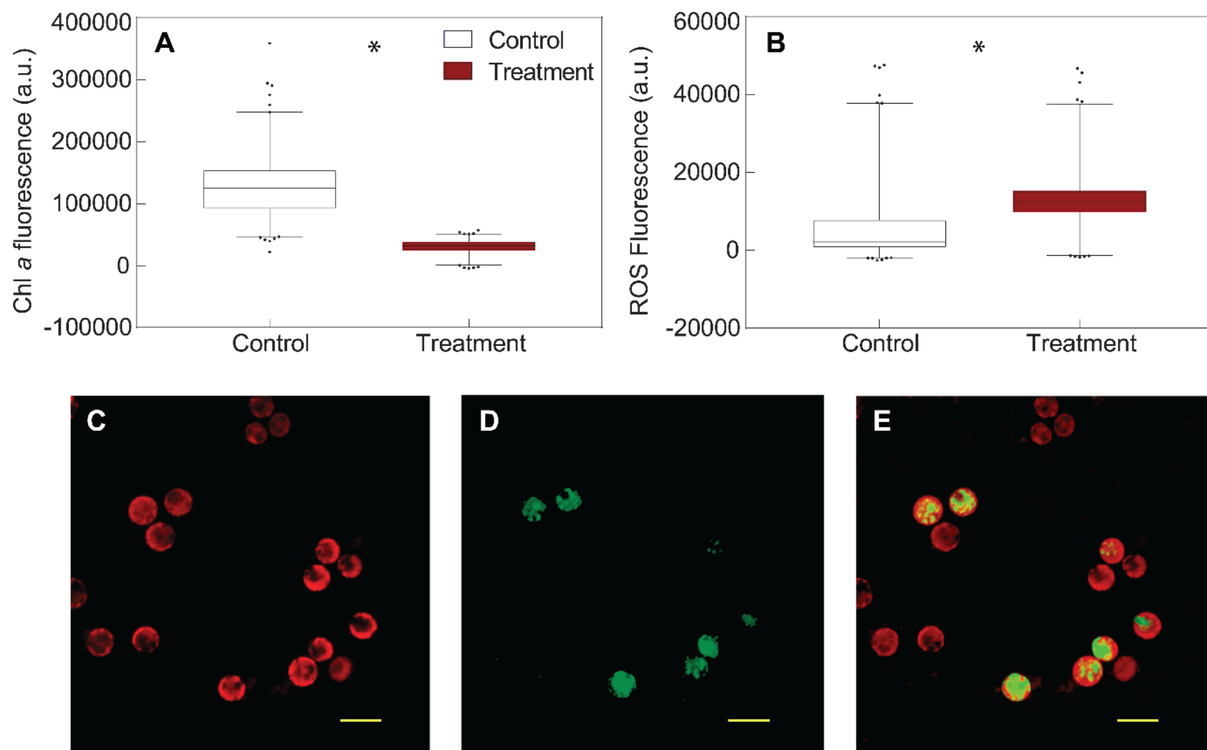

Fig. 2. Fluorescence intensity measurements for chlorophyll *a* and ROS-stained cells. (A) autofluorescence of chlorophyll *a*, **(B)** CM-H_2DCFDA dye (ROS) within the *Symbiodinium* cells, for control (white) and treatment (red), **(C)** autofluorescence (red) of *Symbiodinium* within a coral explant, **(D)** fluorescence of *Symbiodinium* stained with CM-H_2DCFDA dye (green) after acute thermal stress and **(E)** overlay of chlorophyll *a* and CM-H_2DCFDA dye, where ROS is evident within *Symbiodinium* cells. Significant differences shown by asterisks (Mann-Whitney; $P<0.001$). Data represent mean ± s.e.m (N=600 for control and N=521 for treatment). Scale bar = 10 µm.

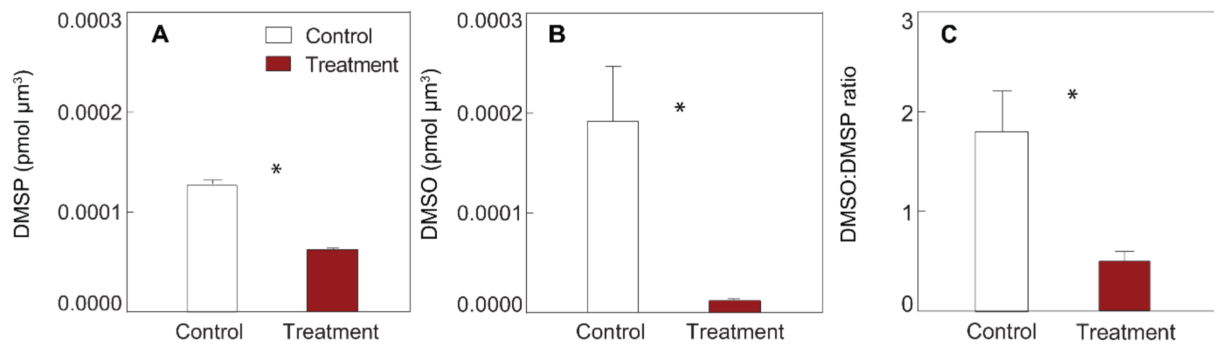

Fig. 3. Intracellular concentrations sulphur compounds measured in coral explants. (A) dimethylsulphoniopropionate (DMSP), **(B)** dimethylsulphoxide (DMSO) and **(C)** DMSO:DMSP ratio in control (white) and heat treated (red) explants. Significant differences shown by asterisks (Unpaired t test; $P<0.001$). Data represent mean ± s.e.m (N=10 for each group).