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RESEARCH ARTICLE

# Inhibition of photosynthetic $CO_2$ fixation in the coral *Pocillopora damicornis* and its relationship to thermal bleaching

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## ABSTRACT

Two inhibitors of the Calvin–Benson cycle [glycolaldehyde (GA) and potassium cyanide (KCN)] were used in cultured *Symbiodinium* cells and in nubbins of the coral *Pocillopora damicornis* to test the hypothesis that inhibition of the Calvin–Benson cycle triggers coral bleaching. Inhibitor concentration range-finding trials aimed to determine the appropriate concentration to generate inhibition of the Calvin–Benson cycle, but avoid other metabolic impacts to the symbiont and the animal host. Both 3 mmol $l^{-1}$ GA and 20 µmol $l^{-1}$ KCN caused minimal inhibition of host respiration, but did induce photosynthetic impairment, measured by a loss of photosystem II function and oxygen production. GA did not affect the severity of bleaching, nor induce bleaching in the absence of thermal stress, suggesting inhibition of the Calvin–Benson cycle by GA does not initiate bleaching in *P. damicornis*. In contrast, KCN did activate a bleaching response through symbiont expulsion, which occurred in the presence and absence of thermal stress. While KCN is an inhibitor of the Calvin–Benson cycle, it also promotes reactive oxygen species formation, and it is likely that this was the principal agent in the coral bleaching process. These findings do not support the hypothesis that temperature-induced inhibition of the Calvin–Benson cycle alone induces coral bleaching.


**KEY WORDS: Coral bleaching, Heat stress, *Symbiodinium*, Calvin–Benson cycle, Dark reactions, Respiration, Photoinhibition, Photosynthesis**

## INTRODUCTION

Reef-building corals form an endosymbiosis with dinoflagellate algae from the genus *Symbiodinium* (also known as zooxanthellae), which reside within the endodermal tissue of the cnidarian. This mutualism is key to the high productivity found on coral reefs, often in oligotrophic, tropical waters, but can be highly sensitive to environmental perturbations, with a breakdown in the symbiosis a common stress response (Hoegh-Guldberg et al., 2007). Coral bleaching involves the expulsion of *Symbiodinium* and/or the loss of photosynthetic pigments from within algal cells, resulting in a paling, or bleaching, of the coral. Elevations in sea surface temperature, as small as 1–2°C above the summer average, in combination with high irradiance, are well known to cause mass coral bleaching events on coral reefs around the world (Hoegh-Guldberg, 1999). It is forecast that ocean temperatures will continue to increase with global warming due to anthropogenic emissions of greenhouse gasses (IPCC, 2007), placing corals more at risk of having sea temperatures exceed their upper thermal maximum. Coral bleaching events are therefore predicted to become more frequent, widespread and severe with climate change (Hoegh-Guldberg, 1999).

Breakdown in the coral symbiosis has been linked to an initial photosynthetic impairment in the endosymbiotic algae. A sustained loss of photosynthetic performance in coral symbionts is a well-defined characteristic of a thermal stress response (Jones et al., 2000). A great deal of evidence has been collected, primarily through non-invasive chlorophyll fluorescence techniques, demonstrating the loss of photosystem II (PSII) photochemical efficiency during a bleaching event (Jones et al., 1998; Warner et al., 1999; Hill et al., 2004a; Hill et al., 2004b). While such results are indicative of impacts to this early stage of the light reactions of photosynthesis, it is not clear whether inhibition of PSII activity is the initial site of damage induced by bleaching conditions, or whether it is a secondary or tertiary impact only detected following inhibition or damage at another site (Smith et al., 2005). Indeed, this initial site of impact remains elusive, with many studies aiming to identify the origin of photosynthetic impairment in *Symbiodinium* under simulated bleaching conditions.

Photoinhibition, which is largely an impairment of PSII caused by high irradiance, has been demonstrated in coral symbionts during thermal stress with the rate of photoinactivation of the core D1 protein exceeding the rate of repair. D1 content has been shown to drop under bleaching conditions, in correlation with declines in PSII photochemical efficiency (Warner et al., 1999; Robison and Warner, 2006; Hill et al., 2011), and this is likely due to the action of reactive oxygen species (ROS) (Lesser, 2011). The cause of this loss of D1 could be due to (1) inability of the D1 repair mechanism to match the rate of D1 damage (Hill et al., 2011), or (2) inhibition of the D1 repair mechanism (Takahashi et al., 2009). While evidence exists for both processes, the end result is the same, with PSII damage leading to photosynthetic impairment. The capacity for D1 repair has been suggested to control thermal bleaching vulnerability, with evidence indicating that bleaching-tolerant species have faster rates of repair compared with more susceptible species (Hennige et al., 2011).

While PSII inhibition has received considerable attention, other components of the photosynthetic apparatus have also been investigated, including antenna proteins (Takahashi et al., 2008; Hill et al., 2012), the oxygen evolving complex (Iglesias-Prieto, 1997; Hill and Ralph, 2008), photosystem I (Hoogenboom et al., 2012), thylakoid membrane integrity (Tchernov et al., 2004; Hill et al., 2009; Díaz-Almeyda et al., 2011) and the dark reactions (Jones et al., 1998; Leggat et al., 2004; Lilley et al., 2010; Hill et al., 2011). Thermal sensitivity of carbon fixation at bleaching-relevant temperatures has been inferred through measures of PSII chlorophyll fluorescence, where initial damage to the dark reactions resulted in an electron sink limitation and, consequently, PSII photoinhibition (Jones et al.,

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**List of symbols and abbreviations**

| | |
|---|---|
| APX | ascorbate peroxidase |
| FIC | fast induction curve |
| $F_M$ | maximum fluorescence in dark-adapted cells during saturating pulse of light |
| $F_O$ | minimum fluorescence in dark-adapted cells |
| $F_v/F_m$ | PSII photochemical efficiency |
| GA | glycolaldehyde |
| KCN | potassium cyanide |
| NPQ | non-photochemical quenching |
| PSII | photosystem II |
| $Q_A$ | primary electron acceptor of PSII |
| $Q_A^-$ | reduced primary electron acceptor of PSII |
| $Q_B$ | secondary electron acceptor of PSII |
| ROS | reactive oxygen species |
| $\sigma_{PSII}$ | functional absorption cross-section of PSII |

1998). Carbon-concentrating mechanisms have been found to be unaffected despite inhibition of photosynthesis (Leggat et al., 2004), and Rubisco protein content of *in hospite* symbionts has also been shown to remain constant during thermal bleaching (Hill et al., 2011). However, at higher temperatures, Rubisco activity does drop rapidly, indicating it is heat sensitive; the results of Leggat et al. (Leggat et al., 2004) were somewhat equivocal but those of Lilley et al. (Lilley et al., 2010) were well substantiated. More recently, Bhagooli (Bhagooli, 2013) demonstrated that inhibition of Calvin–Benson cycle activity in the coral *Stylophora pistillata* by the inhibitor glycolaldehyde (GA) causes photoinhibition and coral bleaching even at optimal temperatures. Therefore, in this species at least, there is some evidence that bleaching can be initiated by damage to the dark reactions of photosynthesis in the absence of thermal stress. To date, the literature contains no conclusive, unifying model that can explain the thermal bleaching response in corals, but rather an extensive series of independent experiments that provide detailed responses of specific components of the photosynthetic apparatus (see review by Lesser, 2011). The focus of these experiments has been predominantly on PSII (in part because of the ease of direct PSII measurements using chlorophyll fluorescence), with other photosynthetic components, such as the dark reactions, receiving less attention. The existing studies provide divergent lines of evidence of a primary site of photosynthetic impairment, which highlights the need for further detailed analyses into the mechanism of photosynthetic impairment of coral symbionts during the onset of thermal bleaching events, especially the Calvin–Benson cycle, which has recently been shown to activate bleaching when chemically inhibited (Bhagooli, 2013).

The application of chemical inhibitors can provide a useful means to manipulate the function of metabolic processes. At low concentrations, GA and cyanide have been used to inhibit the operation of the Calvin–Benson cycle (Sicher, 1984; Wishnick and Lane, 1969), essential in the fixation of organic carbon from carbon dioxide. GA is known to inhibit ribulose-1,5-diphosphate synthase in this cycle (see Miller and Canvin, 1989). However, as later reported by Salon et al. (Salon et al., 1998), GA also inhibited carbonic anhydrase in *Synechococcus* UTEX and at 10 mmol $l^{-1}$ GA, only 20% of the total inhibition of photosynthesis could be ascribed to reduced $CO_2$ fixation. Potassium cyanide (KCN) is also an inhibitor of the Calvin–Benson cycle, although its specific mode of action is different to that of GA. KCN inhibits $CO_2$ fixation by binding to Rubisco activase and preventing its release from Rubisco (Wishnick and Lane, 1969; Sicher, 1984), and it also causes inhibition of plastoquinone-oxidoreductase (Buchel and Garab, 1995), ascorbate peroxidase (APX) (Ishida et al., 1998) and the scavenging of hydroxyl radicals (Jakob and Heber, 1996). The exact mechanism of cyanide action is not well described in the literature, and because *Symbiodinium* have Type 2 rather than Type 1 Rubisco (i.e. the Rubisco is composed of mainly two large subunits rather than eight large and eight small subunits), they may not react in the same way to cyanide, i.e. the binding of substrates and the heat sensitivity may be quite different. Furthermore, in a symbiosis such as that between corals and *Symbiodinium*, the effect of these inhibitors on host (animal) tissue has to be taken into account.

GA has a number of effects in animal tissues, where it interacts with several biochemical pathways and is inhibitory at a number of levels (e.g. Al-Enezi et al., 2006). KCN also inhibits mitochondrial respiration, but only at significantly higher concentrations (Barnes, 1985; Jones and Steven, 1997; Jones and Hoegh-Guldberg, 1999). Therefore, at higher concentrations of these inhibitors, multiple metabolic processes can be impacted, with specificity of the compound lost, resulting in ambiguous results that cannot provide convincing evidence of the site of action. The duration of exposure is also an important factor to consider, with a concentration- and time-dependent loss of PSII function and coral bleaching response found for GA and cyanide (Jones and Steven, 1997; Buxton et al., 2012). Therefore, the concentration-dependent action of GA and cyanide on a coral holobiont must be clearly understood in order to investigate the effect of Calvin–Benson cycle inhibition on causing coral bleaching. In this study, we ran a series of concentration- and time-dependent assays to determine the appropriate concentration of the inhibitors. Initially, cultured *Symbiodinium* were used for the large-scale concentration range-finding trails, with subsequent assays on intact corals used to refine and optimise the appropriate treatments for experiments on the intact symbiosis.

It has been proposed that the trigger for coral bleaching lies in the effect of elevated temperature on the photosynthetic process of the symbiotic zooxanthellae (Lesser, 2011). Two specific sites in the photosynthetic apparatus have been identified as likely targets: (1) the Calvin–Benson cycle, the site of carbon dioxide fixation (Jones et al., 1998; Lilley et al., 2010), and (2) PSII (Warner et al., 1999; Hill et al., 2011). Despite the fact that coral bleaching has emerged over the last two decades as a complex interaction of many environmental and genetic factors, operating at several levels within the coral holobiont (Buxton et al., 2012; Tolleter et al., 2013), these two potential triggers of coral bleaching are still worthy of detailed research. There has been considerable focus on impacts to PSII, with fewer studies concentrating on the operation of the dark reactions. Here we focused on the bleaching response of the common scleractinian coral *Pocillopora damicornis* (Linnaeus 1758) under optimal and elevated temperature while manipulating the function of the Calvin–Benson cycle using chemical inhibitors. This is the first study to investigate whether inhibition of the Calvin–Benson cycle by chemical inhibitors promotes bleaching under thermal stress. The outcomes provide a detailed insight into the role of a functional Calvin–Benson cycle in maintaining an intact symbiosis. Specifically, the aim of this work was to characterise impacts to the Calvin–Benson cycle and the consequences of its inhibition in *P. damicornis* under thermal bleaching conditions. We also sought to determine whether inhibition of the dark reactions induced bleaching at optimal growth temperatures in this species and whether Calvin–Benson cycle inhibition by GA or KCN accelerated thermal bleaching.

## RESULTS

### Inhibitor range-finding trials

GA and KCN concentration range-finding tests were performed on cultured and *in hospite Symbiodinium* of *P. damicornis*. These

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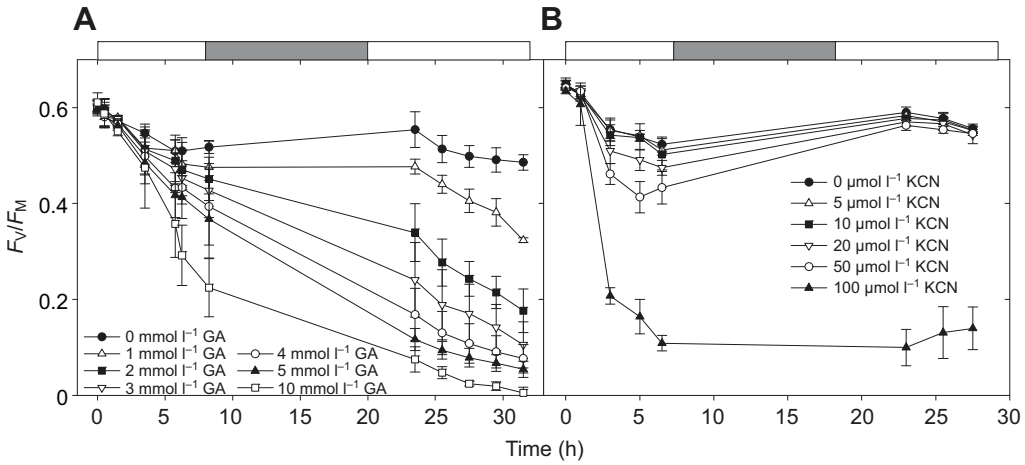


**Fig. 1. Photosystem II photochemical efficiency ($F_V/F_M$) of cultured *Symbiodinium*.** $F_V/F_M$ in the presence of (A) glycolaldehyde (GA; 0–10 mmol l$^{-1}$) and (B) potassium cyanide (KCN; 0–100 µmol l$^{-1}$) during 8 h of exposure to 40 µmol photons m$^{-2}$ s$^{-1}$, followed by 12 h darkness and a further 12 h light. The white bars indicate periods of light and the grey bars indicate darkness. Data are means ± s.d. ($n$=3–6).

trials were essential for determining the appropriate concentrations to use to generate inhibition of the Calvin–Benson cycle, while avoiding other metabolic impacts to the symbiont and the animal host. In the *Symbiodinium* culture, a concentration-dependent decline in the ratio of variable fluorescence to maximal fluorescence ($F_V/F_M$; a measure of PSII photochemical efficiency) was found for both inhibitors between 0 and 10 mmol l$^{-1}$ for GA, and between 0 and 100 µmol l$^{-1}$ for KCN after 8 h light exposure (Fig. 1). Following a 12 h dark period, $F_V/F_M$ continued to decline in all GA-treated samples, while recovery was found in all but the highest KCN concentration. A subsequent light period resulted in continued decline of $F_V/F_M$. These data show that in *Symbiodinium* cells, the application of long-term GA treatment damages the photochemical activity when the concentration is higher than 1 mmol l$^{-1}$. In the case of KCN, inhibition of photosynthetic activity was largely reversible in darkness up to 50 µmol l$^{-1}$. Therefore, long-term GA treatment should be restricted to low (mmol l$^{-1}$) concentrations, while KCN treatment does not seem to induce irreversible side effects up to 50 µmol l$^{-1}$ after a few hours of exposure time. Fig. 2 shows the fast induction curves (FICs), which are double normalized to the O and P steps (relative variable fluorescence). In the presence of both GA and KCN, the J step increased after incubation of the cells in growth light. These findings demonstrate that the reduction level of the plastoquinone pool increases as a result of partial inhibition of the Calvin–Benson cycle, which limits electron flow towards $CO_2$, which acts as the final electron acceptor.

The goal of these inhibitor assays was to determine concentrations high enough to induce Calvin–Benson cycle inhibition in the algal symbionts, detectable through measures of PSII function and oxygen production, while limiting impacts to animal host condition. In the *in hospite Symbiodinium* of *P. damicornis*, $F_V/F_M$ showed a concentration-dependent decline (Fig. 3) over 3 h of exposure, indicating damage to PSII function, while gross photosynthetic rates (Fig. 4) declined during exposure to 5 mmol l$^{-1}$ GA, as well as 10 and 100 µmol l$^{-1}$ KCN. Respiration rate was used as an indicator of

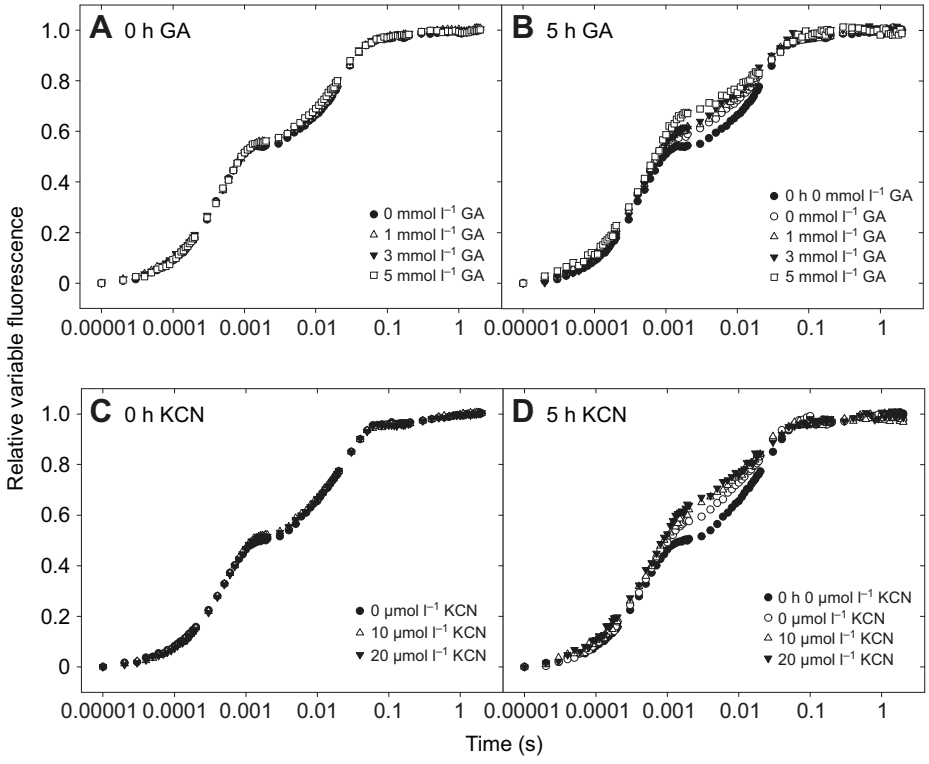


**Fig. 2. Double normalised fast induction curves (FICs) showing relative variable fluorescence [($F_t$–$F_O$)/($F_M$–$F_O$)] of cultured *Symbiodinium*.** Relative variable fluorescence in the presence of (A,B) GA (0, 1, 3 and 5 mmol l$^{-1}$) and (C,D) KCN (0, 10 and 20 µmol l$^{-1}$) after 3 min exposure to inhibitors (A,C) and after a further 5 h exposure to 40 µmol photons m$^{-2}$ s$^{-1}$ (B,D). The control 0 h measurements are also shown for comparison with the FICs from 5 h (B,D). Averages are shown ($n$=5).

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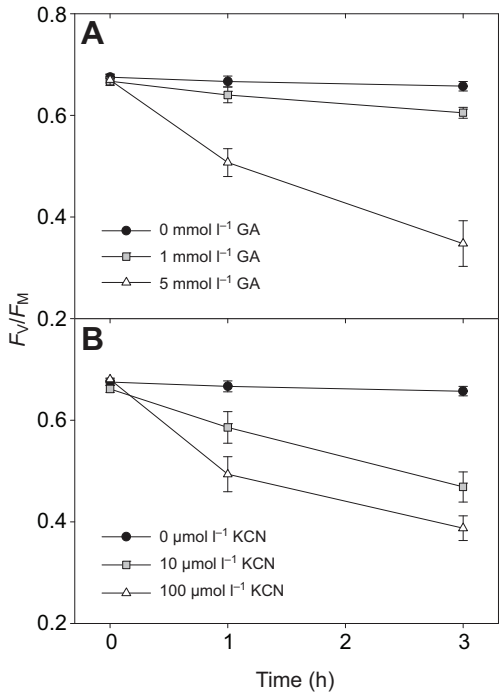


**Fig. 3. $F_V/F_M$ of *in hospite* symbionts of *Pocillopora damicornis*.** Treatments are as follows: (A) 0, 1 and 5 mmol $l^{-1}$ GA and (B) 0, 10 and 100 µmol $l^{-1}$ KCN after 0, 1 and 3 h at 26°C and under 250 µmol photons $m^{-2}$ $s^{-1}$. Data are means ± s.e.m. (*n*=4).

coral host condition and only declined in the highest GA and KCN concentrations over the 3 h experiment, suggesting that lower concentrations did not have an inhibitory effect on animal respiration over this time period. Following these trials, 3 mmol $l^{-1}$ GA and 20 µmol $l^{-1}$ KCN were chosen as appropriate concentrations to apply in the subsequent experiments.

### Quantification of bleaching

Nubbins of *P. damicornis* were exposed to 10 h of 26, 30 or 31°C and a subsequent 14 h of darkness at 26°C. This was done in (1) the absence of any inhibitors, (2) 3 mmol $l^{-1}$ GA or (3) 20 µmol $l^{-1}$ KCN. Bleaching in the coral nubbins was quantified through measures of symbiont density and chlorophyll concentration (Fig. 5). After 24 h in the 26°C treatments, a significant decline in symbiont density per $cm^2$ of coral host was only found in the presence of KCN when compared with initial symbiont densities at 0 h (one-way ANOVA, $F_{1,6}$=13.520, $P$=0.010; Fig. 5A). No loss of symbionts was found in the absence of an inhibitor (control) or in the presence of GA at 26°C. However, after 24 h, a significant loss of symbionts was found in the 30 and 31°C treatments compared with the 26°C treatment in the absence of inhibitors (one-way ANOVA, $F_{2,9}$=6.288, $P$=0.020) and in the presence of GA (one-way ANOVA, $F_{2,9}$=10.232, $P$=0.005). Nubbins in all three temperature treatments in the presence of KCN experienced bleaching to a similar extent by 24 h.

Chlorophyll *a* concentration per $cm^2$ of coral host was significantly lower after 24 h at 26°C in the KCN treatment compared with the concentration at 0 h (one-way ANOVA, $F_{1,6}$=7.726, $P$=0.032; Fig. 5B). Nubbins exposed to 24 h of 30 and 31°C in the presence of KCN experienced a loss of chlorophyll *a* similar to that of the 26°C treatment. In the absence of any inhibitor, chlorophyll *a* concentration was not affected by thermal exposure after 24 h. However, in the presence of GA, nubbins lost more chlorophyll *a* following 24 h at 30 and 31°C compared with the 26°C treatment. In contrast, no significant changes were found in chlorophyll $c_2$ concentration per $cm^2$ of coral host (Fig. 5C) or total chlorophyll per cell (Fig. 5D) over time or between thermal treatments in the presence or absence of inhibitors.

### Changes in PSII photochemical efficiency

In the presence or absence of inhibitors, $F_V/F_M$ declined in all three temperature treatments over time (repeated-measures ANOVAs, all $P$<0.001; Fig. 6). In the absence of inhibitors, $F_V/F_M$ in the 30 and 31°C treatments was significantly lower than the 26°C treatment at 7 h (one-way ANOVA, $F_{2,9}$=27.705, $P$<0.001) and 10 h (one-way ANOVA, $F_{2,9}$=17.504, $P$=0.001; Fig. 6A). Partial recovery of $F_V/F_M$ was seen in all treatments to a similar level after the 14 h recovery period, but values did not recover to 0 h levels in any temperature treatment. In the presence of GA, $F_V/F_M$ was significantly higher in the 26°C treatment compared with the 30 and 31°C treatments at 10 h (one-way ANOVA, $F_{2,9}$=7.050, $P$=0.014; Fig. 6B). At the 24 h mark, an almost complete loss of PSII photochemical efficiency occurred in the 31°C treatment, which was significantly lower than $F_V/F_M$ at 26°C (one-way ANOVA, $F_{2,9}$=5.630, $P$=0.026). In the presence of KCN, the decline in $F_V/F_M$ was intermediate between the controls and the GA treatment (Fig. 6C). The 31°C treatment showed the greatest decline compared with 26°C at 10 h (one-way

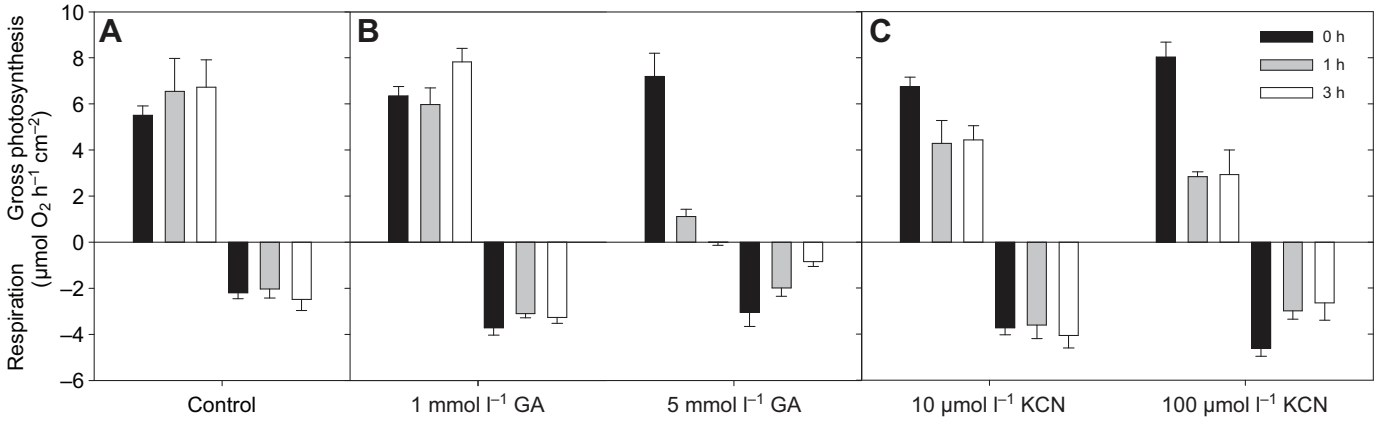


**Fig. 4. Gross photosynthesis and respiration rate (µmol $O_2$ $h^{-1}$ $cm^{-2}$) in *Pocillopora damicornis*.** Treatments are as follows: (A) control (no inhibitors), (B) in the presence of 1 and 5 mmol $l^{-1}$ GA, and (C) in the presence of 10 and 100 µmol $l^{-1}$ KCN, after 0 h (black bars), 1 h (grey bars) and 3 h (white bars) of exposure. Data are means ± s.e.m. (*n*=4).

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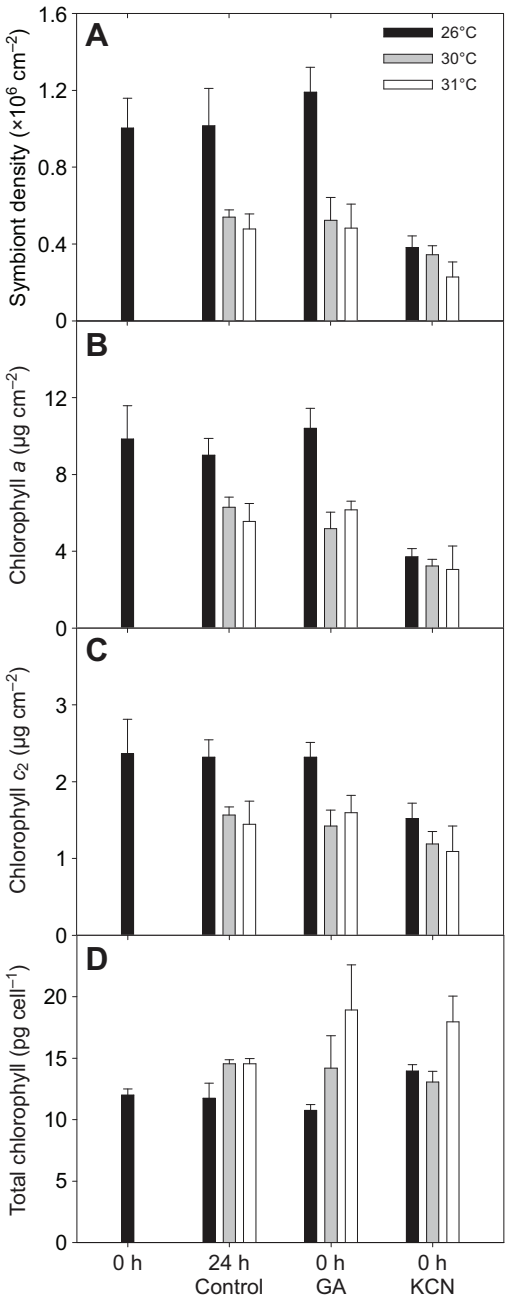


**Fig. 5. Symbiont density and chlorophyll concentration in *P. damicornis*.** (A) Symbiont density (×$10^6$ cm$^{-2}$); (B) chlorophyll *a* concentration (µg cm$^{-2}$); (C) chlorophyll $c_2$ concentration (µg cm$^{-2}$); (D) total chlorophyll concentration (pg cell$^{-1}$). Values were taken at 0 and 24 h in each of the three inhibitor treatments (control, GA and KCN) at 26°C (black bars), 30°C (grey bars) and 31°C (white bars). Data are means ± s.e.m. ($n$=4).

ANOVA, $F_{2,9}$=9.817, $P$=0.005), and although no significant recovery was observed in $F_V/F_M$ by 24 h, the 31°C treatment was lower than both the 26 and 30°C treatments at this time point (one-way ANOVA, $F_{2,9}$=10.306, $P$=0.005).

### Oxygen production and consumption

In the absence of an inhibitor, the rate of net oxygen production did not change significantly over time, nor was it different between temperature treatments, except at 24 h, where the rate of $O_2$ production was significantly lower at 31°C compared with 26 and 30°C (one-way ANOVA, $F_{2,9}$=11.628, $P$=0.003; Fig. 7A). The addition of GA (Fig. 7B) and KCN (Fig. 7C) resulted in temperature-dependent declines in net $O_2$ production, with the higher temperature treatments experiencing the most rapid loss over the 24 h period (one-way ANOVAs, all $P$<0.001).

The respiration rate of coral nubbins in all temperatures and at all time points did not change significantly in the absence of an inhibitor (control; Fig. 7A). In comparison, in the presence of GA, respiration rate approached zero over the 24 h experimental period in all temperature treatments, with respiration rate significantly lower in the 30°C treatment at 10 h (compared with 26 and 31°C; one-way ANOVA, $F_{2,9}$=12.167, $P$=0.003) and in the 30 and 31°C treatments at 24 h (compared with 26°C; one-way ANOVA, $F_{2,9}$=67.058, $P$<0.001; Fig. 7B). In the presence of KCN, respiration rate remained constant across temperatures for the first 10 h. After the recovery period at 24 h, respiration rate had significantly declined at 30 and 31°C compared with 26°C (one-way ANOVA, $F_{2,9}$=12.981, $P$=0.002; Fig. 7C).

### Fast induction curves

During the application of a saturating pulse, chlorophyll fluorescence shows a polyphasic rise from minimum ($F_O$ or the O step) to maximum ($F_M$ or the P step) fluorescence, with two intermediate inflection points, the J and I steps. This OJIP curve provides detailed information on the reduction and oxidation of the primary ($Q_A$) and secondary ($Q_B$) electron acceptors of PSII. In the absence of any inhibitor, declines in the J, I and P steps were found along the FICs for each temperature treatment, indicating a loss of variable fluorescence (Fig. 8A–C). The relative decline in the J, I and P steps was the same for each curve, as verified in the double normalisation to the O and P steps, where all FICs showed a very similar pattern at each time point (supplementary material Fig. S1A–C). Application of GA resulted in a rapid decline in variable fluorescence, with the amplitude of the J, I and P steps approaching zero relative to the O step over the length of the experiment (Fig. 8D–F). Exposure to KCN showed an intermediate response with respect to the decline of the J, I and P steps during the exposure period, with the greatest loss of amplitude in the 31°C treatment (Fig. 8G–I). No change in the FICs was found during the 14 h recovery period in the KCN treatment. Calculation of the relative variable fluorescence for GA- and KCN-treated nubbins revealed an elevation of the J step over time for all temperature treatments, indicating a greater reduction of $Q_A$ (supplementary material Fig. S1D–I).

Calculation of the J:P ratio of FICs revealed significant increases over time in all inhibitor and temperature treatments (repeated-measures ANOVAs, all $P$<0.005; Fig. 9A–C). A sharp increase was found from 0 to 10 h in the presence of both GA and KCN at 26, 30 and 31°C. By the end of the recovery period, the J:P ratio was close to 1.0 for all temperature treatments, indicating that J and P amplitudes were similar, confirming the loss of variable fluorescence between the J and P steps.

### Functional absorption cross-section of PSII

Measures of the functional absorption cross-section of PSII ($\sigma_{PSII}$) provide an estimate of the fraction of absorbed light that leads to oxygen evolution. No change in $\sigma_{PSII}$ was found in the absence of inhibitors in any of the temperature treatments, indicating no change to the light harvesting capabilities of PSII (Fig. 10A). In contrast, in the 26°C GA treatment, $\sigma_{PSII}$ was significantly lower at 4 and 7 h compared with other time points (repeated-measures ANOVA, $F_{4,12}$=3.896, $P$=0.030; Fig. 10B). In the 30 and 31°C GA

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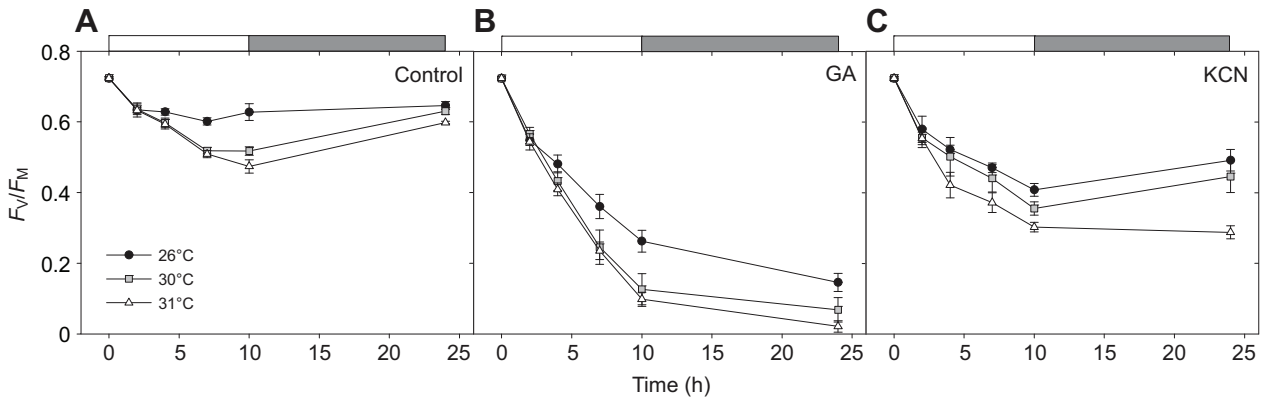


**Fig. 6. $F_V/F_M$ of *P. damicornis*.** Treatments are as follows: (A) in the absence of any inhibitor (control), (B) in the presence of 3 mmol $l^{-1}$ GA and (C) in the presence of 20 µmol $l^{-1}$ KCN at 26°C (black circles), 30°C (grey circles) and 31°C (white circles). Temperatures were ramped from 26°C to treatment temperature over the first 2 h of the experiment and corals were exposed to 600 µmol photons $m^{-2}$ $s^{-1}$ for the initial 10 h (white bars). At the 10 h time point, all corals were given a 14 h recovery period at 26°C in darkness (grey bars). Data are means ± s.e.m. (*n*=4).

temperature treatments, as well as all KCN temperature treatments (Fig. 10C), $\sigma_{PSII}$ significantly declined after 0 h, owing to a decrease in the cross-sectional area of photosynthetic light-harvesting antennae, after which it remained stable (repeated-measures ANOVAs, all $P<0.005$). In the case of the GA treatment, it was not possible to record reasonable $\sigma_{PSII}$ values after the 24 h treatment because of the lack of detectable variable fluorescence (e.g. Fig. 8D–F); therefore, this data point has been omitted. Significant negative correlations were found between $\sigma_{PSII}$ and the J:P ratio in the 31°C GA treatment, plus all three KCN temperature treatments (Table 1). This indicates that the reduced state of $Q_A$ ($Q_A^-$), signified by a high J:P ratio, has a direct impact on $\sigma_{PSII}$, where over-reduced $Q_A^-$ resulted in a decrease in the cross-sectional area of photosynthetic light-harvesting antennae.

## DISCUSSION

### Inhibitors of the Calvin–Benson cycle and considerations for the coral holobiont

Here we tested the hypothesis that inhibition of the Calvin–Benson cycle induces the release of endosymbionts and/or loss of photosynthetic pigments from a bleaching-sensitive coral species under thermal stress. We used two inhibitors of the Calvin–Benson cycle that have been used with corals before: GA (Buxton et al., 2012; Bhagooli, 2013) and KCN (Chalker and Taylor, 1975; Barnes, 1985; Jones and Steven, 1997; Jones and Hoegh-Guldberg, 1999). They have also been widely used for studies on higher plants (Bishop and Spikes, 1955) and microalgae (Whittingham, 1952; Takahashi and Murata, 2005). With both these inhibitors, care was taken to ensure that concentrations affected only (or at least predominantly) the Calvin–Benson cycle. It was therefore necessary to carry out experiments on the effect of GA and KCN at a range of concentrations on algal photosynthesis and animal host respiration. GA and KCN concentrations were carefully chosen following a series of range-finding assays on cultured and *in hospite Symbiodinium* (Figs 1–4). The goal was to apply GA and KCN in concentrations high enough to induce inhibition of photosynthesis in the algal symbionts, but not to interfere with the functioning of host metabolism. Indeed, our quantifications of photosynthetic damage in the preliminary experiments highlighted the concentration-dependent decline in $F_V/F_M$ over time that was due to the inhibitory effect of GA and KCN on mechanisms such as photosynthetic dark reactions (Fig. 1). In addition, we also probed for signs of damage to the animal host through measures of oxygen consumption in the dark. Gross primary production and respiration rate declined at 5 mmol $l^{-1}$ GA and 100 µmol $l^{-1}$ KCN over 3 h, but neither process was affected by 1 mmol $l^{-1}$ GA or 10 µmol $l^{-1}$ KCN. An intermediate concentration of 3 mmol $l^{-1}$ GA and 20 µmol $l^{-1}$ KCN was therefore chosen for subsequent experiments as it was expected to cause inhibition to the Calvin–Benson cycle (as probed via measures of $F_V/F_M$ and oxygen production; Figs 1–3), but have little impact on coral host function (Fig. 4). Nevertheless, it should also be kept in mind that GA and KCN at the chosen concentrations may affect other enzymes or processes.

During subsequent experimentation, GA and KCN exposure lasted for 24 h and measures of respiration rate suggested that GA did have some effect on host metabolism that was accelerated by thermal stress (Fig. 7). KCN also impacted host respiration, but only at 31°C after 24 h of exposure. As the bleaching response was consistent in the presence and absence of GA (Fig. 5), we suggest that although the host experienced damage from the chemical inhibitor, it did not alter the bleaching process. In comparison, while damage to host respiration was only found after 24 h in the highest temperature treatment during KCN exposure, bleaching was accelerated in all treatments by 24 h. Therefore, host condition may not have played a crucial role in the bleaching response.

### The effect of Calvin–Benson cycle inhibition on coral bleaching

*Pocillopora damicornis* is a bleaching-sensitive coral species known to live close to its upper thermal threshold (Marshall and Baird, 2000; Loya et al., 2001; Hill et al., 2004a; Hill et al., 2004b). Bleaching is well characterised in this species around the world, and as previously reported, we found that thermal stress was sufficient to induce a bleaching response characterised by the expulsion of symbionts, rather than a loss of intracellular photosynthetic pigments (Fig. 5) (Hill et al., 2011). In this work we were able to confirm that in the absence of any Calvin–Benson cycle inhibitor, both symbiont density and chlorophyll concentration (per $cm^2$ of coral tissue) declined from 0 to 24 h in the 30 and 31°C treatments, while there was no change in the 26°C treatment.

GA at 3 mmol $l^{-1}$ did not affect the severity of bleaching, nor did it induce bleaching in the absence of thermal stress. Clearly, then, this evidence does not support the hypothesis that inhibition of the Calvin–Benson cycle is a key factor in initiating coral bleaching.

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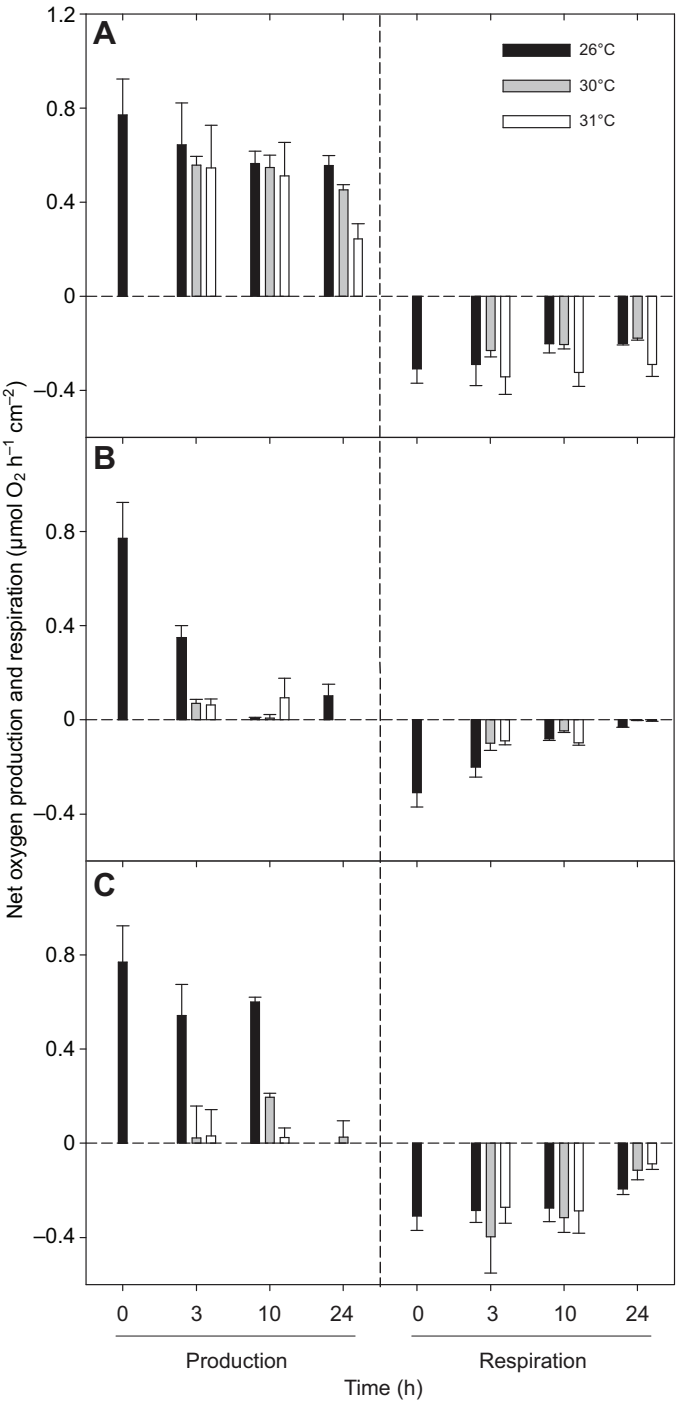


**Fig. 7. Production (net primary production; left side of figure) and respiration rate (right side of figure) in μmol $O_2$ $h^{-1}$ $cm^{-2}$ of *P. damicornis*.** Treatments are as follows: (A) in the absence of any inhibitor (control), (B) in the presence of 3 mmol $l^{-1}$ GA and (C) in the presence of 20 μmol $l^{-1}$ KCN at 26°C (black bars), 30°C (grey bars) and 31°C (white bars). Temperatures were ramped from 26°C to treatment temperature over the first 2 h of the experiment. At the 10 h time point, all corals were given a 14 h recovery period at 26°C. Corals were exposed to 600 μmol photons $m^{-2}$ $s^{-1}$ for the initial 10 h and then 14 h of darkness. Data are means ± s.e.m. (*n*=4).

However, at higher concentrations, GA did have profound effects that were related to an effect on respiration or the integrity of the animal host. While previous research has shown the effects of GA on coral bleaching (Bhagooli, 2013), these experiments were conducted at a higher concentration of GA and only investigated effects on photosynthesis and not respiration, and should therefore be treated with caution. To gain evidence on coral bleaching at 3 mmol $l^{-1}$ GA, we were obliged, for comparison with others' experiments, to carry out our experiments for up to 24 h, a time frame that is a common practice for coral bleaching experiments (e.g. Hill et al., 2012), and it was clear (Figs 5, 6) that at this time point there were effects other than inhibition of the Calvin–Benson cycle. GA, when applied at a similar concentration (2.5 mmol $l^{-1}$) in the cyanobacterium *Synechocystis* PCC 6803, induced a 16% loss of PSII activity in 4 h, an effect assigned to the inhibition of the repair of photodamaged PSII centres (Takahashi and Murata, 2005). However, the consequences of long-term GA treatment in photosynthetic systems have not been investigated previously. Therefore, after 24 h, it is probable that other effects of GA, on respiration and metabolism of the coral host, would have interfered with the coral bleaching mechanism and prevented the expulsion of symbionts. However, it should be noted that at earlier time points, no evidence was found for coral bleaching, unlike in the presence of KCN, and therefore we conclude that GA did not induce coral bleaching conditions at any time.

KCN, a known inhibitor of $CO_2$ fixation in higher plants and microalgae (Whittingham, 1952; Bishop and Spikes, 1955), has been linked with coral bleaching under field conditions (Jones and Steven, 1997) and in laboratory experiments (Jones et al., 1998; Jones and Hoegh-Guldberg, 1999). In our experiments, exposure to 20 μmol $l^{-1}$ KCN not only strongly inhibited photosynthetic oxygen production (without having an impact on respiration rate), as did 3 mmol $l^{-1}$ GA, but, unlike GA, also promoted bleaching, with symbiont density and chlorophyll concentration declining significantly in all three temperature treatments, with no temperature-dependent effect. Thus we have the interesting situation that of these two inhibitors of the Calvin–Benson cycle, only KCN induced bleaching. This suggests that KCN might have other effects besides its effect on the Calvin–Benson cycle.

An early explanation for the coral bleaching effect of KCN was that with the inhibition of the Calvin–Benson cycle, electrons would back up into PSI and PSII and this would generate large amounts of ROS, offset to some extent by the flow of electrons into the Mehler ascorbate peroxidase pathway (Jones et al., 1998; Jones and Hoegh-Guldberg, 1999). However, the fact that GA inhibits the Calvin–Benson cycle but does not induce bleaching seems to require some further explanation for the effect of KCN. We suggest that this is the action of KCN in inhibiting APX (Shigeoka et al., 2002) and stimulating $OH^-$ (hydroxyl radical) production, in a Fenton reaction from $H_2O_2$ (Ishida et al., 1998; Jakob and Heber, 1996). In addition, it has been reported that KCN also inhibits the catalase enzyme in intact chloroplasts of higher plants, which leads to enhanced production of $H_2O_2$ (Forti and Gerola, 1977). Therefore, in contrast with GA, KCN would induce a much greater production of ROS and this could explain the marked difference in bleaching response. Overall, therefore, this evidence supports a strong role for ROS in inducing bleaching (Lesser, 2011). Furthermore, KCN did not significantly enhance bleaching under thermal stress compared with 26°C, as the extent of bleaching was the same in all temperature treatments (Fig. 5), and no different to the extent of bleaching in nubbins exposed to 30 and 31°C in the absence of any inhibitors. This suggests that the inhibition caused by KCN at an optimal temperature (26°C) applied the same bleaching pressure as when thermal stress was applied at 30 and 31°C, regardless of whether KCN was present or absent. If our hypothesis is correct, that ROS

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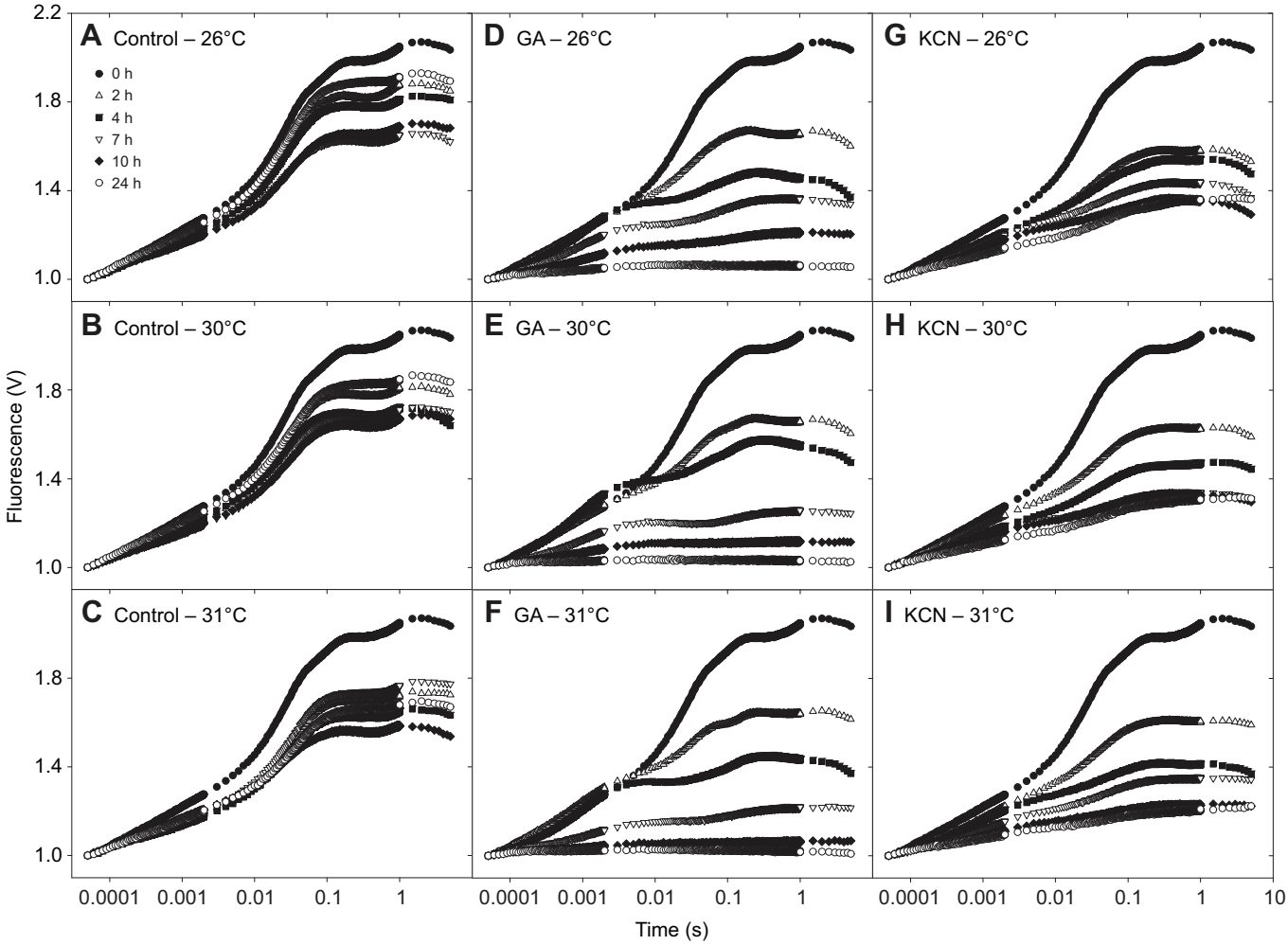


**Fig. 8. FICs of *P. damicornis*.** Treatments are as follows: (A–C) in the absence of any inhibitor (control), (D–F) in the presence of 3 mmol $l^{-1}$ GA and (G–I) in the presence of 20 µmol $l^{-1}$ KCN at 26°C (A,D,G), 30°C (B,E,H) and 31°C (C,F,I). Averages are shown (*n*=4).

is driving the bleaching process, then KCN at 26°C must produce the same degree of ROS as 31°C in the absence of KCN, i.e. ROS production in the presence of KCN is saturating at 26°C.

The concentration of KCN that has been used in our studies (20 µmol $l^{-1}$) is comparable to that used in other experiments on the inhibition of the Calvin–Benson cycle in algal and coral bleaching studies (Wishnick and Lane, 1969; Sicher, 1984; Jones and Hoegh-Guldberg, 1999). The concentrations of KCN, which have been used to inhibit algal APX, have traditionally been higher than here, viz. 1 mmol $l^{-1}$ (Shigeoka et al., 2002). However, a structural study by Hill et al. (Hill et al., 1997) showed that APX had a binding (dissociation) constant to the enzyme of $K_d$=11.6 µmol $l^{-1}$ and it is entirely reasonable that APX was inhibited in our experiments.

### Inhibition of PSII function

The two inhibitors of the Calvin–Benson cycle, GA and KCN, would be expected to have indirect effects on PSII through a limitation on electron transport and ROS production. GA has been shown to inhibit the PSII repair cycle (Takahashi and Murata, 2005), which could be induced by the suppression of D1 protein synthesis at the translation elongation step because of the accumulation of $^1O_2$ in the absence of electron transfer towards $CO_2$ as the final electron acceptor (Nishiyama et al., 2001; Nishiyama et al., 2004). In addition, the effect of superoxide could also contribute to the inhibition of D1 repair in the presence of GA (Takahashi and Murata, 2005). KCN is also expected to enhance $^1O_2$ production as a result of over-reduction of electron transport components at the acceptor side of PSII in the absence of electron flow towards the Calvin–Benson cycle, while the inhibitory effect of KCN on catalase (Forti and Gerola, 1977) would enhance production of $H_2O_2$, which is also an inhibitor of D1 protein synthesis, and therefore of the PSII repair cycle (Nishiyama et al., 2001). Our current findings obtained with the coral symbiosis provide further evidence for the differential effect of inhibiting the process of $CO_2$ uptake by different inhibitors of the Calvin–Benson cycle reported earlier in other microalgae and higher plants.

In contrast to GA and KCN, enhanced temperatures may have a direct effect on one or more functions of PSII (Warner et al., 1999; Hill et al., 2011). In this study, $F_V/F_M$ (Fig. 6) and the amplitude of FIC steps (Fig. 8) showed a temperature-dependent decline even in the absence of inhibitors, with the 31°C treatment experiencing the greatest decrease in $F_V/F_M$ and the J, I and P steps. The initial decline in $F_V/F_M$ and the loss of variable fluorescence in FICs in the 26°C treatment in the absence of inhibitors indicates that the light intensity applied (600 µmol photons $m^{-2}$ $s^{-1}$) caused some photoinactivation of PSII. Prior to experimentation, corals were maintained in aquaria at 100 µmol photons $m^{-2}$ $s^{-1}$ and we conclude that the sixfold increase in irradiance was sufficient to lower

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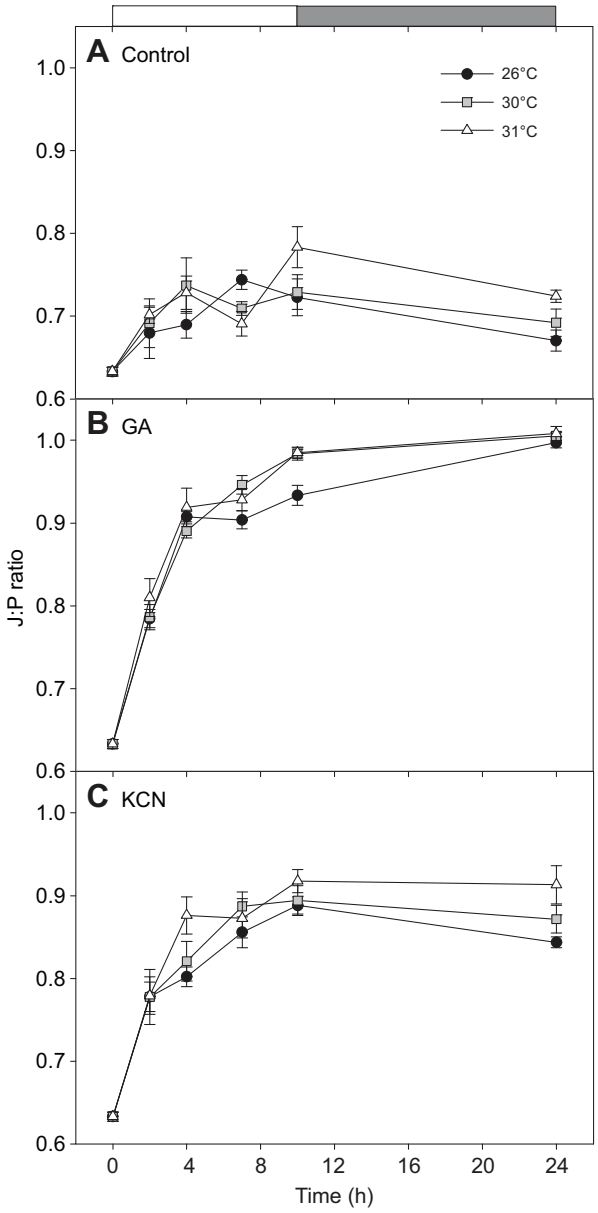


**Fig. 9. J:P ratio of FICs in *P. damicornis*.** Treatments are as follows: (A) in the absence of any inhibitor (control), (B) in the presence of 3 mmol $l^{-1}$ GA and (C) in the presence of 20 µmol $l^{-1}$ KCN at 26°C (black circles), 30°C (grey squares) and 31°C (white triangles). Temperatures were ramped from 26°C to treatment temperature over the first 2 h of the experiment and corals were exposed to 600 µmol photons $m^{-2}$ $s^{-1}$ for the initial 10 h (white bar). At the 10 h time point, all corals were given a 14 h recovery period at 26°C in darkness (grey bar). Data are means ± s.e.m. (*n*=4). Values close to 1 indicate J=P.

photosynthetic efficiency in the absence of thermal stress. With the application of thermal stress, a greater loss of $F_V/F_M$ and amplitude of J, I and P steps was found in all inhibitor treatments compared with the 26°C treatment, with photoinactivation only partially reversible in the absence of GA and KCN after 14 h of recovery in darkness. The decline in FIC amplitude was primarily the result of changes to the J step. This was visualised in supplementary material Fig. S1, which shows the relative variable fluorescence. In the presence of GA or KCN, the non-reversible rise in the J step over time indicates a greater reduction of $Q_A$ owing to closure of PSII reaction centres (Strasser et al., 1995; Hill et al., 2004a; Ulstrup et al., 2005). The loss of variable fluorescence between the J and P steps (Fig. 9) matches the greatly inhibited $F_V/F_M$ values in the presence of GA by 24 h in all three temperature treatments. The low $F_V/F_M$ readings at 24 h indicate an almost complete loss of variable fluorescence, and by comparing the J:P ratio and $F_V/F_M$, we can conclude that the small variable fluorescence that remained was due to increases from the O phase to the J phase. After the 3 ms time point in the FICs, no further change in fluorescence occurred. This indicates that there was still some minor capacity for reduction of $Q_A$ to $Q_A^-$ at this time point, but no potential for $Q_B$ or plastoquinone pool reduction (Strasser et al., 1995; Hill et al., 2004a). Together, these impacts on PSII function suggest that net PSII repair was able to partially counter photoinactivation when the Calvin–Benson cycle was still functional. However, exposure to GA and KCN prevented any net repair to PSII, with no recovery of $F_V/F_M$ or FIC steps found following 14 h of darkness.

The elevated excitation pressure on PSII as a consequence of over-reduced $Q_A$ can be judged from the OJIP FICs along with the $\sigma_{PSII}$ data. The negative correlations between the J:P ratio and $\sigma_{PSII}$ (Table 1) demonstrated how the development of over-reduced $Q_A^-$ led to a smaller PSII cross-sectional area, which is a potential photoprotective mechanism to reduce excitation pressure on PSII reaction centres (Falkowski and Owens, 1980; Gorbunov et al., 2001; Suggett et al., 2004). A similar response was observed in the green alga *Chlamydomonas reinhardtii*, where inhibition of Calvin–Benson cycle by GA triggered enhanced non-photochemical quenching (NPQ) as a photoprotective mechanism (Takahashi and Murata, 2005). Interestingly, the correlation between J:P and changes in $\sigma_{PSII}$ in GA treatments was poor at 26°C but improved in the 31°C heat treatments, indicating that the correlation between the reduction state of $Q_A$ and changes in cross-sectional area was stronger when the heat stress was more severe during the GA treatment. Nevertheless, a clear tendency of $\sigma_{PSII}$ and J:P as a function of temperature could not be given. The fact that in the absence of inhibitors J:P was much smaller and no significant change in $\sigma_{PSII}$ was observed indicates that rapid changes in functional cross-sectional area of PSII is not the major photoprotective mechanism of *in hospite Symbiodinium*, which is in agreement with earlier findings (Lesser and Farrell, 2004; Hennige et al., 2011). Changes in $\sigma_{PSII}$ becomes relevant only during the inhibitor treatments, when excitation pressure on PSII increases as a consequence of an over-reduced state of $Q_A$ (elevated J:P ratio) that is triggered by impaired $CO_2$ fixation via the Calvin–Benson cycle. Based on the $\sigma_{PSII}$ and FIC data, the effect of GA and KCN was similar, but not the same. The lack of correlation between over-reduced $Q_A^-$ and changes in $\sigma_{PSII}$ at 26 and 30°C in the presence of GA, but the significant correlations at these temperatures in the presence of KCN, suggests that mechanisms specific to each inhibitor might be responsible for the downregulation of photochemical efficiency and are unrelated to Calvin–Benson cycle activity. In the case of GA, loss of photochemical efficiency may be due to increased NPQ (Takahashi and Murata, 2005), from changes in the light harvesting antenna complexes (Gorbunov et al., 2001; Hill et al., 2012) or reaction centre inactivation (Hennige et al., 2011). Takahashi and Murata (Takahashi and Murata, 2005) suggest that although NPQ increases in *Chlamydomonas* sp. in the presence of GA, the photochemical quenching was unaltered under this treatment, indicating that the reduction state of $Q_A^-$ does result in engagement of the photoprotective processes in all cases. Although our observation in *Symbiodinium* sp. indicates that $Q_A$ becomes reduced in the presence of GA based on the elevated J:P ratio (Fig. 9) a clear correlation between the amount of $Q_A^-$ and the value

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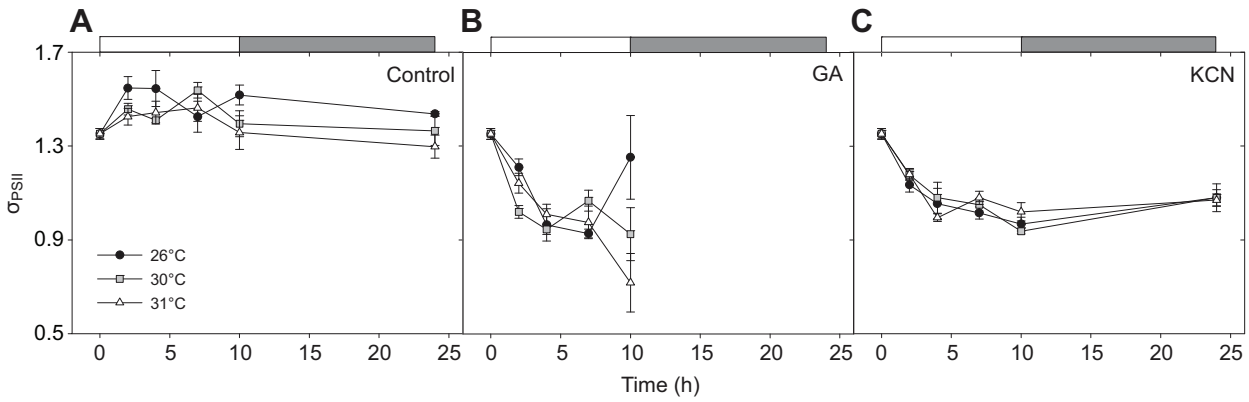


**Fig. 10. Functional absorption cross-section of photosystem II ($\sigma_{PSII}$) of *P. damicornis*.** Treatments are as follows: (A) in the absence of any inhibitor (control), (B) in the presence of 3 mmol $l^{-1}$ GA and (C) in the presence of 20 µmol $l^{-1}$ KCN at 26°C (black circles), 30°C (grey circles) and 31°C (white circles). Temperatures were ramped from 26°C to treatment temperature over the first 2 h of the experiment and corals were exposed to 600 µmol photons $m^{-2}$ $s^{-1}$ for the initial 10 h (white bars). At the 10 h time point, all corals were given a 14 h recovery period at 26°C in darkness (grey bars). Data are means ± s.e.m. (*n*=4).

of $\sigma_{PSII}$ cannot be given (Table 1), in agreement with the finding of Takahashi and Murata (Takahashi and Murata, 2005). The effect of KCN appeared to be somewhat different, with a strong correlation found. In the presence of KCN, the reduction state of $Q_A$ can be linked directly to decreased $\sigma_{PSII}$, possibly as a result of KCN blocking APX. A recent study showed that APX-deficient mutants of rice (*Oryza sativa*) exhibited enhanced excitation energy pressure on PSII because of the accumulation of hydrogen peroxide and hydroxyl radicals under light stress conditions (Caverzan et al., 2014). Although there is no similar detailed study available of the physiological impact of the absence of APX activity in *Symbiodinium*, we propose that the differential response of KCN and GA is related to the additional inhibitory effect KCN has on APX activity and thereby the strong correlation between the reduction state of the plastoquinone pool and $\sigma_{PSII}$.

The changes in $F_V/F_M$ for control, GA and KCN treatments were mirrored in the rates of net oxygen production (Fig. 7), except that instead of recovery at the 24 h time point in the absence of GA and KCN, oxygen production continued to decline, with the greatest loss in the 31°C treatment. While in this treatment $F_V/F_M$ showed potential for recovery, photosynthetic oxygen production showed a significant decline in the thermally treated corals (31°C), as considerable bleaching had occurred and the symbiont density and chlorophyll concentration per $cm^2$ of coral skeleton declined (Fig. 5).

**Table 1. Pearson correlation coefficients between the J:P ratio and the functional absorption cross-section of PSII ($\sigma_{PSII}$) over time for the control, glycolaldehyde (GA) and potassium cyanide (KCN) treatments at 26, 30 and 31°C**

| | Treatment | *r* | *P* |
|---|---|---|---|
| Control | 26°C | 0.365 | 0.477 |
| | 30°C | 0.363 | 0.48 |
| | 31°C | −0.096 | 0.856 |
| GA | 26°C | −0.671 | 0.215 |
| | 30°C | −0.851 | 0.967 |
| | 31°C | −0.944 | 0.016* |
| KCN | 26°C | −0.979 | 0.001* |
| | 30°C | −0.955 | 0.003* |
| | 31°C | −0.957 | 0.003* |

Significant negative correlations (*$P$<0.05) indicate that the reduced state of $Q_A$ (the primary electron acceptor of PSII), signified by a high J:P ratio, resulted in a decrease in the cross-sectional area of photosynthetic light-harvesting antenna.

The temperature-dependent reduction in $O_2$ production in the light by GA and KCN indicates a loss of photosynthetic electron transport, which is confirmed by the $F_V/F_M$ data. Interestingly, however, there was often a large mismatch between $F_V/F_M$ and the rate of oxygen production, particularly in the KCN treatments. Photochemical efficiency was retained in the presence of KCN in the 30°C and 31°C after 3 h of exposure, as well as at 26°C after 24 h. A similar result was seen in the absence of inhibitors after 24 h at 31°C. This indicates that although PSII is still functional when probed with a saturating pulse following dark adaptation, the electron transport chain and dark reactions are inhibited during illumination, resulting in a greatly depressed rate of oxygen production compared with $F_V/F_M$. This suggests that while PSII experiences some level of damage, more severe inhibition is likely occurring at the site of the Calvin–Benson cycle under thermal stress. Alternatively, this mismatch could be related to the bleaching of nubbins, which would affect oxygen production rates, but not $F_V/F_M$, or an inhibition to the oxygen evolving complex that is more severe than damage to the PSII core complex [although this latter possibility is unlikely (see Hill and Ralph, 2008)].

### Inhibition of $CO_2$ fixation as a trigger of coral bleaching

Considerable research effort has focused on understanding the photosynthetic trigger that results in coral bleaching (Lesser, 2011). Here, we used chemical compounds (GA and KCN) known to inhibit the Calvin–Benson cycle to probe their effect on bleaching in coral nubbins exposed to three different temperature regimes. We found that while bleaching was not detected at 26°C in the presence of GA, an almost complete loss of photosynthetic function was found. This supports the conclusion that inhibition of photosynthetic $CO_2$ fixation was not an immediate cause of bleaching in *P. damicornis*. The results from the KCN experiments are in stark contrast to this, with bleaching occurring in all treatment temperatures. We hypothesise that KCN-driven ROS production was the driver of the bleaching response. Bhagooli (Bhagooli, 2013) found that bleaching did occur at the growth temperature of 26°C in *Stylophora pistillata* when $CO_2$ fixation was inhibited by GA. This difference between our study and that of Bhagooli (Bhagooli, 2013) may be due to: (1) the higher concentration of GA used in Bhagooli's study (5 mmol $l^{-1}$ GA compared with 3 mmol $l^{-1}$ GA used in our study), which may have had additional impacts on the symbiosis or which may induce a more rapid and/or chronic response, or (2) the different coral species used, a factor that is believed to play an important role in determining the

site of primary photosynthetic impairment during thermal bleaching (Buxton et al., 2012). Thus we suggest that in *P. damicornis*, elevated ROS production from KCN exposure causes dysfunction to the symbiosis and results in bleaching. The suggestion that in coral bleaching KCN acts by inhibiting APX, thereby stimulating ROS production, needs further testing.

## MATERIALS AND METHODS

### *Symbiodinium* cultures

The CS-156 *Symbiodinium* culture was obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Australian National Algae Culture Collection, and grown in f/2 medium at 25°C and 40 µmol photons $m^{-2}$ $s^{-1}$, with a light:dark ratio of 12 h:12 h. Measurements were performed on cultures with ~7 µg chlorophyll ($a$ + $c_2$) $ml^{-1}$ at 25°C.

### Coral specimens

Four colonies of *Pocillopora damicornis* were collected from Heron Island lagoon, located on the southern Great Barrier Reef of Australia (151°55′E, 23°27′S), and transported to the University of Technology, Sydney, where they were placed in recirculating artificial seawater at 26°C and 100 µmol photons $m^{-2}$ $s^{-1}$. Nubbins ~1 cm in length were broken off from each colony for subsequent use in experimental treatments 1–3 days later.

### Inhibitor range-finding trials

Gross photosynthesis, dark respiration, maximum quantum yield ($F_V/F_M$), chlorophyll fluorescence fast induction curves (FICs), and chlorophyll and zooxanthellae density were measured as detailed below.

Initial experiments were performed on *Symbiodinium* cultures where temperature was kept constant during experimentation and irradiance was applied for 8 h, followed by 12 h darkness and a further 12 h of light (40 µmol photons $m^{-2}$ $s^{-1}$). Photosynthetic activity was assessed by measuring the so-called OJIP transient of variable chlorophyll fluorescence during application of a 2 s saturating pulse (Strasser et al., 1995; Hill et al., 2004a). $F_V/F_M$ was obtained by calculating $(F_M–F_O)/F_M$, where $F_O$ and $F_M$ represent the minimum fluorescence in dark-adapted cells and the maximal fluorescence yield under continuous saturating light, respectively. These measurements were performed using the FL-3000 Fluorometer (Photon Systems Instruments, Brno, Czech Republic), which is designed to measure algal cells in suspension.

Following these concentration range-finding tests on *Symbiodinium* cultures, *in hospite Symbiodinium* of *P. damicornis* were tested at 0, 1 and 5 mmol $l^{-1}$ GA and 0, 10 and 100 µmol $l^{-1}$ KCN. One nubbin from each of the four colonies was placed in an individual 500 ml beaker filled with 250 ml of 0.2 µm filtered seawater and gently mixed during exposure to 600 µmol photons $m^{-2}$ $s^{-1}$ (white LED panels; IronHorse WLED80, Arlec, Blackburn, Australia). $F_V/F_M$ was measured on a Mini-PAM fluorometer (Walz Effeltrich, Germany) after 10 min of dark adaptation (0.8 s saturating pulse of >4500 µmol photons $m^{-2}$ $s^{-1}$, gain=8) at 0, 1 and 3 h. A 6 mm diameter glass fibre optic probe, connected to the Mini-PAM, was used to measure the photosynthetic surface of the coral nubbins. Gross photosynthesis rates were measured using a temperature-controlled oxygen electrode cuvette (3–5 ml capacity; Rank, Bottisham, UK) and a Firesting optical oxygen sensor (PyroScience, Aachen, Germany).

### Experimental protocol

Nubbins of *P. damicornis* were placed in individual beakers as described above for 24 h. For the first 10 h, nubbins were exposed to 600 µmol photons $m^{-2}$ $s^{-1}$, and then 14 h of darkness. During the first 2 h of the light treatment, nubbins were ramped from 26°C to their experimental temperature treatments of 26 (control), 30 and 31°C. At the end of the light period (10 h mark), temperatures were gradually reduced to 26°C over 2 h and maintained at this temperature until 24 h. In each temperature treatment, four nubbins were exposed to seawater only (control), 3 mmol $l^{-1}$ GA or 20 µmol $l^{-1}$ KCN ($n$=4). At the 10 h mark, a 100% water change (containing GA or KCN in the inhibitor treatments) was given to each nubbin.

Four nubbins were harvested at 0 h and another four at 24 h from each of the three temperature treatments and three inhibitor treatments for determination of symbiont density and chlorophyll $a$ and $c_2$ concentration. Nubbins were placed in 15 ml of filtered seawater and airbrushed to remove all coral tissue from the skeleton. The host and endosymbiont slurry was centrifuged at 1000 ***g*** for 10 min and the supernatant was discarded. The algal pellet was resuspended in 4 ml FSW and 100 µl removed for symbiont counting on haemocytometer slides. Eight replicate counts were performed per sample to determine symbiont density. The remaining 3.9 ml was centrifuged again at 1000 ***g*** for 10 min. The supernatant was discarded and the algal pellet was resuspended in 4 ml 90% acetone. Twenty-four hours later, the samples were centrifuged at 1000 ***g*** for 10 min and the absorbance of the supernatant was measured at 630 and 664 nm on a UV–Vis spectrophotometer (Shimadzu, USA). Chlorophyll $a$ and $c_2$ concentrations were then determined using the equations of Jeffrey and Humphrey (Jeffrey and Humphrey, 1975). Symbiont density and chlorophyll $a$ and $c_2$ concentration were normalised to nubbin skeleton surface area, which was determined using the paraffin wax technique (Stimson and Kinzie, 1991). Total chlorophyll ($a$ and $c_2$) per symbiont cell was also calculated.

At 0, 2, 4, 7, 10 and 24 h, $F_V/F_M$ of the *in hospite* symbionts was measured after 10 min of dark adaptation using a Mini-PAM fluorometer (as described above), followed by the functional absorption cross-section of PSII ($\sigma_{PSII}$) on a Fast$^{Tracka}$ II Fast Repetition Rate fluorometer (FRRf; Chelsea Technologies Group Ltd, West Molesey, Surrey, UK). A pre-programmed single turnover acquisition was used for all measurements controlled by FASTPro software (version 2.0). One measuring sequence included both saturation and relaxation phases. For saturation, 60×1 µs flashlets were given with an interval of 3 µs between each flashlet. These settings resulted in a 240 µs measuring cycle for saturation. For relaxation, 50×1 µs flashlets were given, initially with an interval of 100 µs between each flashlet, which increased by 10% in length for each consecutive flashlet interval. This resulted in a 116 ms measuring cycle for the relaxation phase. To improve the signal-to-noise ratio, four sequences were averaged per acquisition, separated by 100 ms. $\sigma_{PSII}$ was calculated using FASTPro software according to the biophysical model of Kolber et al. (Kolber et al., 1998) and is expressed in $nm^2$ $quanta^{-1}$ using the flash calibration factor for the instrument. The excitation wavelength was 470±20 nm and the fluorescence emission was detected at 685±10 nm. The LED intensity and the photomultiplier voltage were adjusted according to the manufacturer's recommendations (see Oxborough et al., 2012) to optimize the $F_O$ levels and the rate of PSII closure during the train of flashlets for the coral nubbins. Upon completion of $\sigma_{PSII}$ determination, a double-modulation fluorometer (Photon Systems Instruments, FL-3300) was used to measured FICs during a 3700 µmol photons $m^{-2}$ $s^{-1}$ 5 s flash from red (640 nm) and blue (455 nm) LEDs (see Hill and Ralph, 2006). Chlorophyll fluorescence measurements were recorded every 10 µs for the first 2 ms, every 1 ms up until 1 s, and then every 500 ms up to 5 s. Curves were normalised to the O step ($F_O$) at 0.05 ms (see Hill et al., 2004a). Relative variable fluorescence of the FICs was calculated using the formula $(F_t–F_O)/(F_M–F_O)$, where $F_t$ is chlorophyll fluorescence at time $t$ (Strasser et al., 1995; Hill et al., 2004a; Ulstrup et al., 2005). The ratio of the amplitude of the J step (inflection at 3 ms) to the P step ($F_M$) was also calculated.

At 0, 3, 10 and 24 h, the rate of gross primary production and respiration was determined using the Rank oxygen cuvette setup described earlier. Nubbins were placed in 5 ml of treatment seawater and held at the treatment temperature. They were exposed to 5 min of darkness, followed by 5 min of 400 µmol photons $m^{-2}$ $s^{-1}$ from a quartz iodine light source. The rate of oxygen consumption or production was expressed per $cm^2$ of nubbin surface area (µmol $O_2$ $h^{-1}$ $cm^{-2}$), determined as described above.

### Statistical analyses

One-way ANOVA tests were used to identify differences between inhibitor treatments, temperature treatments or changes over time in independent samples. Repeated-measures ANOVA was applied in the analysis of samples that were measured continuously throughout the experiment. In cases where significant differences were detected, Tukey's *post hoc* comparisons identified the significantly different treatments. Pearson's correlation tests were used to detect significant correlations between $\sigma_{PSII}$ and the J:P ratio. A significance level of 0.05 was applied throughout these analyses and the

Kolmogorov–Smirnov normality test and Levene's homogeneity of variance test were used to determine whether assumptions of the parametric analyses were satisfied. SPSS statistical software (version 21, 2012) was used to perform these analyses.

### Competing interests
The authors declare no competing financial interests.

### Author contributions
R.H., M.S. and A.W.D.L. wrote the paper and R.H., M.S., I.M., P.J.R. and A.W.D.L. designed the study. Experiments were performed by R.H., M.S., A.R., I.M. and A.W.D.L. All authors read and commented on the manuscript.

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