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

Running title: Dark reaction inhibition in *Symbiodinium*

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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 mM GA and 20 μM 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.

Keyword index

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 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 i) inability of the D1 repair mechanism to match the rate of D1 damage (Hill et al., 2011), or ii) 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 bleaching tolerant species have faster rates of repair, compared to 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., 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 rapidly drop indicating it is heat-sensitive; the results of Leggat et al. (2004) were somewhat equivocal but the results of Lilley et al. (2010) were well substantiated. More recently, 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 due to the ease of direct PSII measurements using chlorophyll fluorescence), with other

112 photosynthetic components, such as the dark reactions, receiving less attention. The existing
113 studies provide divergent lines of evidence of a primary site of photosynthetic impairment
114 which highlights the need for further detailed analyses into the mechanism of photosynthetic
115 impairment of coral symbionts during the onset of thermal bleaching events, especially the
116 Calvin-Benson cycle which has recently been shown to activate bleaching when chemically
117 inhibited (Bhagooli, 2013).
118
119 The application of chemical inhibitors can provide a useful means to manipulate the function
120 of metabolic processes. At low concentrations, GA and cyanide have been used to inhibit the
121 operation of the Calvin-Benson cycle (Sicher, 1984; Wishnik and Lane, 1969), essential in
122 the fixation of organic carbon from carbon dioxide. GA is known to inhibit ribulose-1, 5-
123 diphosphate synthase in this cycle (see e.g. Miller and Canvin, 1989). However, as later
124 reported by Salon et al., (1998), GA also inhibited carbonic anhydrase in *Synechococcus*
125 UTEX and at 10 mM GA, only 20% of the total inhibition of photosynthesis could be
126 ascribed to reduced CO_2 fixation. KCN is also an inhibitor of the Calvin-Benson cycle,
127 although its specific mode of action is different to GA. KCN inhibits CO_2 fixation by binding
128 to Rubisco activase and preventing its release from Rubisco (Wishnik and Lane, 1969;
129 Sicher, 1984) and it also causes inhibition of plastoquinone-oxidoreductase (Buchel and
130 Garab, 1995), ascorbate peroxidase (Ishida et al., 1998) and the scavenging of hydroxyl
131 radicals (Jakob and Heber, 1996). The exact mechanism of cyanide action is not well
132 described in the literature, and since *Symbiodinium* have Type 2 rather than Type 1 Rubisco
133 (i.e. the Rubisco is composed of mainly 2 large subunits rather than of 8 large and 8 small
134 subunits), they may not react in the same way to cyanide, i.e. the binding of substrates and
135 the heat sensitivity may be quite different. Furthermore, in a symbiosis like corals the effect
136 of these inhibitors on host (animal) tissue has to be taken into account.
137
138 GA has a number of effects in animal tissues, where it interacts with several biochemical
139 pathways and is inhibitory at a number of levels (e.g. Al-Enezi et al., 2006). KCN also
140 inhibits mitochondrial respiration, but only at significantly higher concentrations (Barnes,
141 1985; Jones and Steven, 1997; Jones and Hoegh-Guldberg, 1999). Therefore, at higher
142 concentrations of these inhibitors, multiple metabolic processes can be impacted, with
143 specificity of the compound lost, resulting in ambiguous results that cannot provide
144 convincing evidence of the site of action. The duration of exposure is also an important factor
145 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 i) the Calvin-Benson cycle, the site of carbon dioxide fixation (Jones et al., 1998; Lilley et al., 2010), and ii) photosystem II (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 with 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 focussed on the bleaching response of the common scleractinian coral, *Pocillopora damicornis* 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 *Pocillopora 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

Glycolaldehyde (GA) and potassium cyanide (KCN) concentration range finding tests were performed on cultured and *in hospite Symbiodinium* of *Pocillopora damicornis*. These 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 F_V/F_M was found for both inhibitors between 0 to 10 mM for GA, and 0 to 100 µM 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 mM. In the case of KCN, inhibition of photosynthetic activity was largely reversible in darkness up to 50 µM. Therefore, long term GA treatment should be restricted to low mM concentrations, while KCN treatment does not seem to induce irreversible side effects up 50 µM after a few hours of exposure time. Fig. 2 shows the fast induction curves which are double normalized to the O and P steps (relative variable fluorescence). In the presence of both GA and KCN the J step increases after incubation of the cells in growth light. These findings demonstrate that the reduction level of the PQ pool increases due to partial inhibition of the Calvin-Benson cycle, which limits electron flow towards CO_2 that 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 *Pocillopora 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 mM GA, as well as 10 and 100 µM KCN. Respiration rate was used as an indicator of coral host condition and only declined in the highest GA and KCN concentrations over the 3 h experiment, suggesting lower concentrations did not have an inhibitory effect on animal respiration over this time period. Following these trials, 3 mM GA and 20 µM 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°C, 30°C or 31°C and a subsequent 14 h of darkness at 26°C. This was done in i) the absence of any inhibitors, ii) 3 mM GA or iii) 20 µM 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 to 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 the presence of GA at 26°C. However, after 24 h, a significant loss of symbionts was found in the 30°C and 31°C treatments compared to 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 to 0 h (one way ANOVA, $F_{1,6} = 7.726$, $P = 0.032$; Fig. 5B). Nubbins exposed to 24 h of 30°C and 31°C in the presence of KCN experienced a similar loss of chlorophyll *a* to 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°C and 31°C compared to 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, which is a measure of PSII photochemical efficiency, declined in all three temperature treatments over time (rmANOVAs, P values < 0.001; Fig. 6). In the absence of inhibitors, F_V/F_M in the 30°C 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 to 30°C and 31°C 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 to 26°C at 10 h (one way 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°C 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 significantly change 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 to 26°C 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, P values < 0.001).

The respiration rate of coral nubbins in all temperatures and at all time points did not significantly change 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 to 26°C and 31°C; one way ANOVA, $F_{2,9}$ = 12.167, P = 0.003) and in the 30°C and 31°C treatment at 24 h (compared to 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°C and 31°C compared to 26°C (one way ANOVA, $F_{2,9}$ = 12.981, P = 0.002; Fig. 7C).

Fast induction curves (FICs)

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 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 (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 (Fig. S1D-I).

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

Functional absorption cross section of PSII (σ_{PSII})

Measures of σ_{PSII} provide an estimate of the fraction of absorbed light that leads to oxygen evolution. No change in σ_{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, σ_{PSII} was significantly lower at 4 and 7 h compared to other time points (Fig. 10B; rmANOVA, $F_{4,12} = 3.896$, $P = 0.030$). In the 30°C and 31°C GA temperature treatments, as well as all KCN temperature treatments (Fig. 10C), σ_{PSII} significantly declined after 0 h, due to a decrease in the cross sectional area of the photosynthetic light-harvesting antenna, after which it remained stable (rmANOVAs, P values < 0.005). In the case of the GA treatment, it was not possible to record reasonable σ_{PSII} values after the 24 h treatment, due to the lack of detectable variable fluorescence (see e.g. Fig 8 D,E,F); therefore, this data point has been omitted. Significant negative correlations were found between σ_{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, signified by a

311 high J:P ratio, has a direct impact on σ_{PSII}, where and over-reduced Q_A^- resulted in a decrease
312 in the cross-sectional area of photosynthetic light-harvesting antenna.
313

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: glycolaldehyde (Buxton et al., 2012; Bhagooli, 2013) and cyanide (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 other microalgae (Whittingham, 1952; Takahashi and Murata, 2005). With both these inhibitors, care was taken to ensure 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* (Fig. 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 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 mM GA and 100 µm KCN over 3 h, but neither process was affected by 1 mM GA or 10 µm KCN. An intermediate concentration of 3 mM GA and 20 µm 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; Fig. 1-3), but have little impact on coral host function (Fig. 4). Nevertheless it should also be borne 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 critical 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 thermal stress was sufficient to induce a bleaching response characterised by the expulsion of symbionts, rather than 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°C and 31°C, while there was no change in the 26°C treatment.

GA at 3 mM 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. 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 mM 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 (see e.g. Hill et al., 2012), and it was clear (Fig. 5 and 6) that at this time there were effects other than inhibition of the Calvin-Benson cycle. GA when applied at a similar concentration (2.5 mM) in the cyanobacterium *Synechocystis* PCC 6803 it induced a 16% loss of PSII activity in 4 hours; 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 as it was in the presence of KCN, and therefore we conclude that GA did not induce coral bleaching conditions at any time.

Potassium cyanide (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 µM KCN, not only strongly inhibited photosynthetic oxygen production (without having an impact on respiration rate), as did 3 mM 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 might suggest that KCN has 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 (MAP) 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 ascorbate peroxidase (Sigeoka 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 inhibits also 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 to 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°C and 31°C in the absence of any inhibitors. This suggests the inhibition caused by KCN at an optimal temperature (26°C) applied the same bleaching pressure as when thermal stress was applied at 30°C and 31°C,

regardless of whether KCN was present or absent. If our hypothesis is correct, that ROS 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 µM) is comparable to that used in other experiments on the inhibition of the Calvin-Benson cycle in algal and coral bleaching studies (Wishnik and Lane, 1969; Sicher, 1984; Jones and Hoegh-Guldberg, 1999). The concentrations of KCN, which have been used to inhibit algal ascorbate peroxidase have traditionally been higher than here, viz 1 mM (Sigeoka et al., 2002). However, a structural study by Hill et al. (1997) showed that ascorbate peroxidase had a binding constant to the enzyme of Kd = 11.6 µM and it is entirely reasonable that ascorbate peroxidase was inhibited in our experiments.

Inhibition of PSII function

The two inhibitors of the Calvin 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 due to 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 due to 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, PSII photochemical efficiency (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 J, I and P steps. The initial decline in F_V/F_M and 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 6 fold increase in irradiance was sufficient to lower 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 to 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 Fig. S1 that 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 due 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 with the greatly inhibited F_V/F_M values in the presence of GA by 24 h in all 3 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 to 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 (PQ) pool reduction (Strasser et al., 1995; Hill et al., 2004a). Together, these impacts to 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 fluorescence induction curves along with the data of functional absorption cross-sectional area of PSII (σ_{PSII}). The negative correlations between the J:P ratio and σ_{PSII} (Table 1) demonstrated how the development of over-reduced Q_A^- lead 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 σ_{PSII} in GA treatments was poor at 26°C but improved towards the 31°C heat treatments, indicating that the correlation between reduction state of Q_A and changes in cross-sectional area was more expressed when the heat stress was more severe during the GA treatment. Nevertheless, a clear tendency of σ_{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 σ_{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 Farrel, 2004; Hennige et al., 2011). Changes in σ_{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 data of σ_{PSII} and fluorescence induction curves, the effect of GA and KCN was similar, but not the same. The lack of correlation between over-reduced Q_A^- and changes in σ_{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 (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 of σ_{PSII} cannot be given (Table 1), in agreement with the finding of 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 directly be linked to declined σ_{PSII}, possibly due to KCN blocking ascorbate peroxidase (APX). A recent study showed that APX deficient mutants of rice (*Oryza sativa*) exhibited enhanced excitation energy pressure on PSII due to 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 ascorbate peroxidase 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 strong correlation between reduction state of plastoquinone pool and σ_{PSII}.

The changes in F_V/F_M for control, GA and KCN 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 PSII photosynthetic efficiency 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). The temperature-dependent reduction in O_2 production in the light by GA and KCN indicates 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 to 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 focussed 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 (2013) found 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 Bhagooli (2013) may be due to, 1) the higher concentration of GA used in Bhagooli (2013; 5 mM GA compared to 3 mM GA used in our study) which may have had additional impacts to the symbiosis or which may induce a more rapid and/or chronic response, or 2) due to the different coral species used; a factor which 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 ascorbate peroxidase, thereby stimulating ROS production, needs further testing.

Materials and methods

Symbiodinium cultures

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

Coral specimens

Four colonies of *Pocillopora damicornis* (Linnaeus) 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 approximately 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), 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 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 which is designed to measure algal in suspension (Photon Systems Instruments, Brno, Czech Republic).

Following these concentration range finding tests on *Symbiodinium* cultures, *in hospite* *Symbiodinium* of *Pocillopora damicornis* were tested at 0, 1 and 5 mM GA and 0, 10 and 100 µM KCN. One nubbin from each of the four colonies was placed in individual 500 mL beakers filled with 250 mL of 0.2 µm filtered seawater (FSW) 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 mins 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, 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 (Rank, Bottisham, England) with a capacity of 3-5 mL 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 two hours of the light treatment, nubbins were ramped from 26°C to their experimental temperature treatments of 26°C (control), 30°C 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 mM GA, or 20 µM 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 FSW and airbrushed to remove all coral tissue from the skeleton. The host and endosymbiont slurry was centrifuged at 1000 *g* for 10 mins and the supernatant 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 mins. The supernatant was discarded and the algal pellet was resuspended in 4 mL 90% acetone. 24 h later, the samples were centrifuged at 1000 *g* for 10 mins 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 by using the equations of 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). The 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 mins of dark adaptation using a Mini-PAM fluorometer (as described above), followed by functional absorption cross section of PSII (σ_{PSII}) on a Fast^{Tracka} II Fast Repetition Rate fluorometer (FRRf; Chelsea Technologies Group Ltd., West Molesey, Surrey). 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 x 1 µs flashlets were given with the interval of 3 µs between each flashlets. These settings resulted in a 240 µs long measuring cycle for saturation. For relaxation, 50 x 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 long measuring cycle for the relaxation phase. To improve the signal-to-noise ratio, 4 sequences were averaged per acquisition, separated by 100 ms. The functional absorption cross-sectional area of photosystem II (σ_{PSII}) was calculated using the FASTPro software according to the biophysical model of Kolber et al. (1998) and is expressed in nm^2*$quanta^{-1}$ using the flash calibration factor for the instrument. The excitation wavelength was at 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_0 levels and the rate of PSII closure during the train of flashlets for the coral nubbins. Upon completion of σ_{PSII} determination, a double-modulation fluorometer (Photon Systems Instruments, FL-3300, Brno, Czech Republic) was used to measured fast induction curves (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 $[(F_t - F_O)/(F_M - F_O)]$ (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 mins of darkness, followed by 5 mins 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 analysis of variance (ANOVA) tests were used to identify differences between inhibitor treatments, temperature treatments or changes over time in independent samples. Repeated-measures analysis of variance (rmANOVA) 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 σ_{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 if assumptions of the parametric analyses were satisfied. The SPSS statistical software (version 21, 2012) was used to perform these analyses.

Author Contributions

R.H., M.S and AWDL 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.

Competing Interests

No competing interests declared.

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935

936 **Tables**

937

938 Table 1: Pearson correlation coefficients (r) between the J:P ratio and σ_{PSII} over time for the
939 control, GA and KCN treatments at 26, 30 and 31°C. Significant negative correlations (where
940 $P < 0.05$, asterisks) indicate that the reduced state of Q_A, signified by a high J:P ratio, resulted
941 in a decrease in the cross-sectional area of photosynthetic light-harvesting antenna.

942

	Control			GA			KCN		
	26°C	30°C	31°C	26°C	30°C	31°C	26°C	30°C	31°C
r	0.365	0.363	-0.096	-0.671	-0.851	-0.944	-0.979	-0.955	-0.957
P-value	0.477	0.480	0.856	0.215	0.967	0.016*	0.001*	0.003*	0.003*

943

944

945 **Figure legends**

946

947 Fig. 1: F_V/F_M of cultured *Symbiodinium* in the presence of A) GA (0-10 mM) and B) KCN
948 (0-100 µM) during 8 h of exposure to 40 µmol photons m^{-2} s^{-1}, followed by 12 h darkness
949 and a further 12 h light. The white bars indicate periods of light and the grey bars indicate
950 darkness. Averages ± s.d.m. (*n* = 3-6).

951

952 Fig. 2: Double normalised fast induction curves (FICs) showing relative variable fluorescence
953 $[(F_t - F_O)/(F_M - F_O)]$ of cultured *Symbiodinium* in the presence of GA (0, 1, 3 and 5 mM; A,B)
954 and KCN (0, 10 and 20 µM; C,D) after 3 min exposure to inhibitors (A,C) and after a further
955 5 h exposure to 40 µmol photons m^{-2} s^{-1} (B,D). The control 0 h measurements are also shown
956 for comparison with the FICs from 5 h (B,D). Averages shown (*n* = 5).

957

958 Fig. 3: F_V/F_M of *in hospite* symbionts of *Pocillopora damicornis* exposed to (A) 0, 1 and 5
959 mM GA and (B) 0, 10 and 100 µM KCN, after 0, 1 and 3 h at 26°C and under 250 µmol
960 photons m^{-2} s^{-1}. Averages ± s.e.m. (*n* = 4).

961

962 Fig. 4: Gross photosynthesis and respiration rate (µmol O_2 h^{-1} cm^{-2}) in *Pocillopora*
963 *damicornis* in the control (A; no inhibitors), in presence of 1 mM and 5 mM GA (B), and in
964 the presence of 10 µM and 100 µM KCN (C), after 0 h (black bars), 1 h (grey bars) and 3 h
965 (white bars) of exposure. Averages ± s.e.m. (*n* = 4).

966

967 Fig. 5: Symbiont density and chlorophyll concentration in *P. damicornis*. Symbiont density
968 ($x10^6$ cm^{-2}) (A), chlorophyll *a* (µg cm^{-2}) (B), chlorophyll c_2 (µg cm^{-2}) (C), and pg of total
969 chlorophyll per cell (D). Values were taken at 0 h and 24 h in each of the 3 inhibitor
970 treatments (control, GA and KCN) at 26°C (black bars), 30°C (grey bars) and 31°C (white
971 bars). Averages ± s.e.m. (*n* = 4).

972

Fig. 6: F_V/F_M of *Pocillopora damicornis* in the absence of any inhibitor (control; A), in the presence of 3 mM GA (B) and in the presence of 20 µM KCN (C) 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 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). Averages ± s.e.m. (n = 4).

Fig. 7: Oxygen production (net primary production; left side of figure) and respiration rate (right side of figure) in µmol O_2 h^{-1} cm^{-2} in *Pocillopora damicornis* in the absence of any inhibitor (control; A), in the presence of 3 mM GA (B) and in the presence of 20 µM KCN (C) 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. Averages ± s.e.m. (n = 4).

Fig. 8: Fast induction curves (FICs) of *Pocillopora damicornis* in the absence of any inhibitor (control; A-C), in the presence of 3 mM GA (D-F) and in the presence of 20 µM KCN (G-I) at 26°C (A,D,G), 30°C (B,E,H) and 31°C (C,F,I). Averages shown (n = 4).

Fig. 9: J:P ratio of FICs in *Pocillopora damicornis* in the absence of any inhibitor (control; A), in the presence of 3 mM GA (B) and in the presence of 20 µM KCN (C) 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 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). Averages ± s.e.m. (n = 4). Values close to 1 indicate J=P.

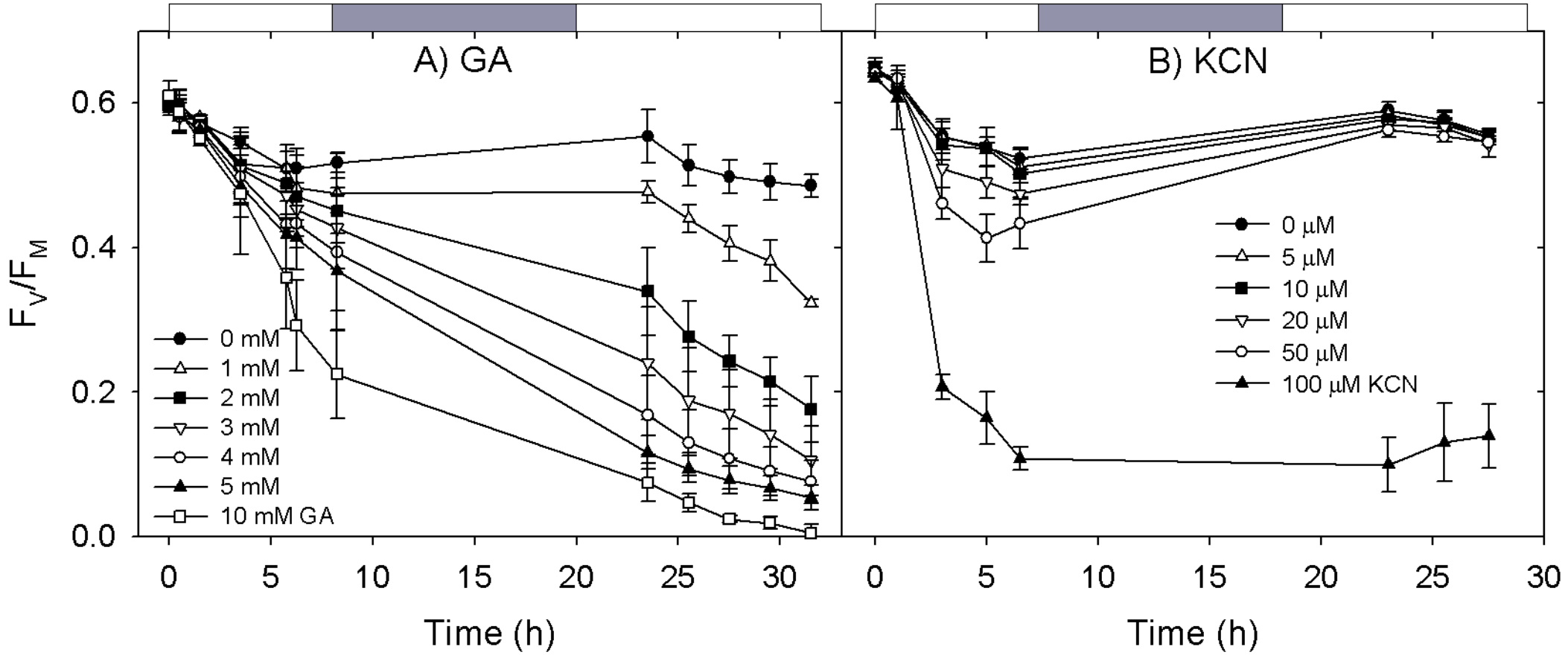
Fig. 10: Functional absorption cross section of PSII (σ_{PSII}) of *Pocillopora damicornis* in the absence of any inhibitor (control; A), in the presence of 3 mM GA (B) and in the presence of 20 µM KCN (C) at 26°C (black circles), 30°C (grey circles) and 31°C (white circles).

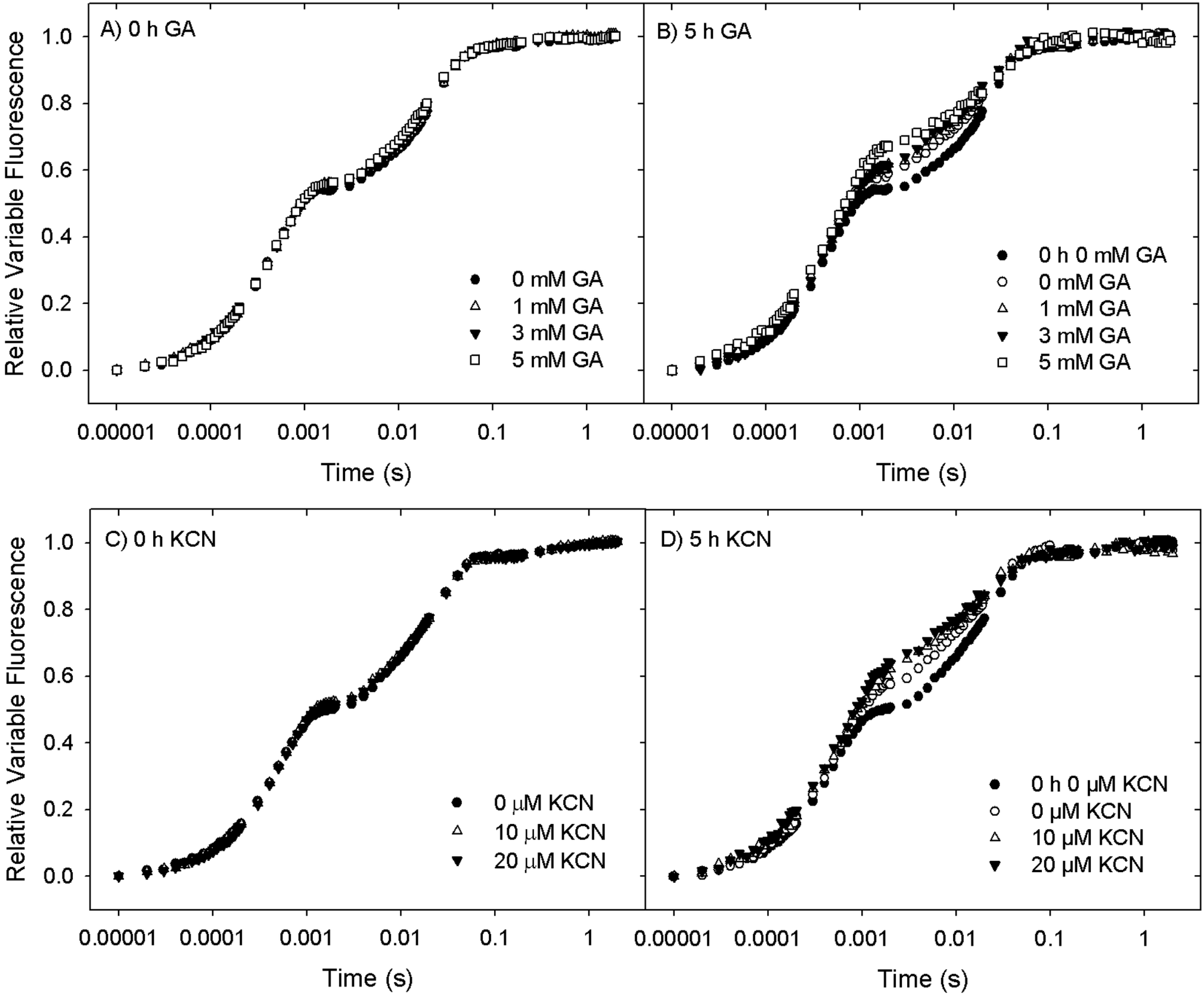
1003 Temperatures were ramped from 26°C to treatment temperature over the first 2 h of the
1004 experiment and corals exposed to 600 µmol photons m^{-2} s^{-1} for the initial 10 h (white bars).
1005 At the 10 h time point, all corals were given a 14 h recovery period at 26°C in darkness (grey
1006 bars). Averages ± s.e.m. (n = 4).

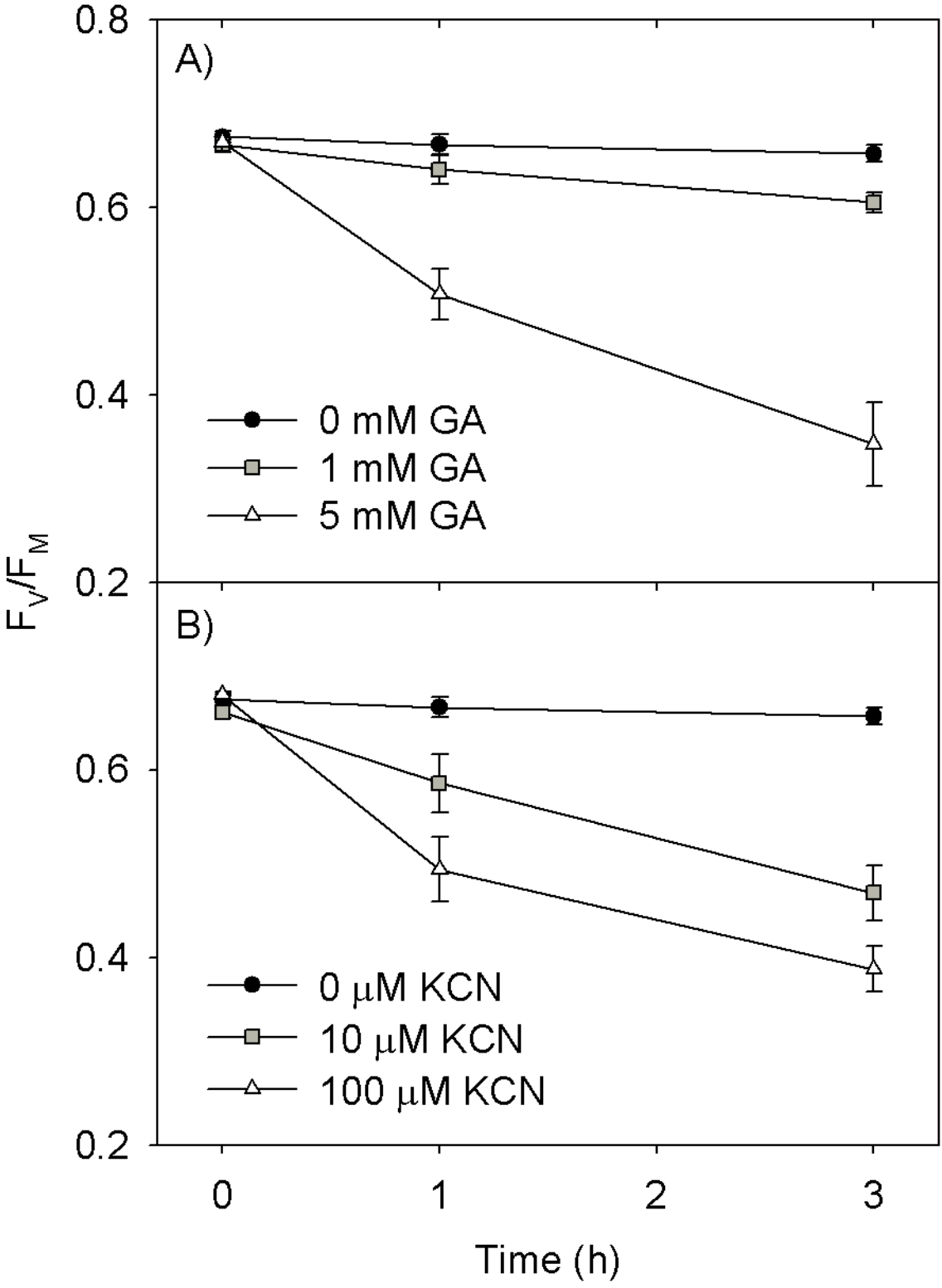
1007

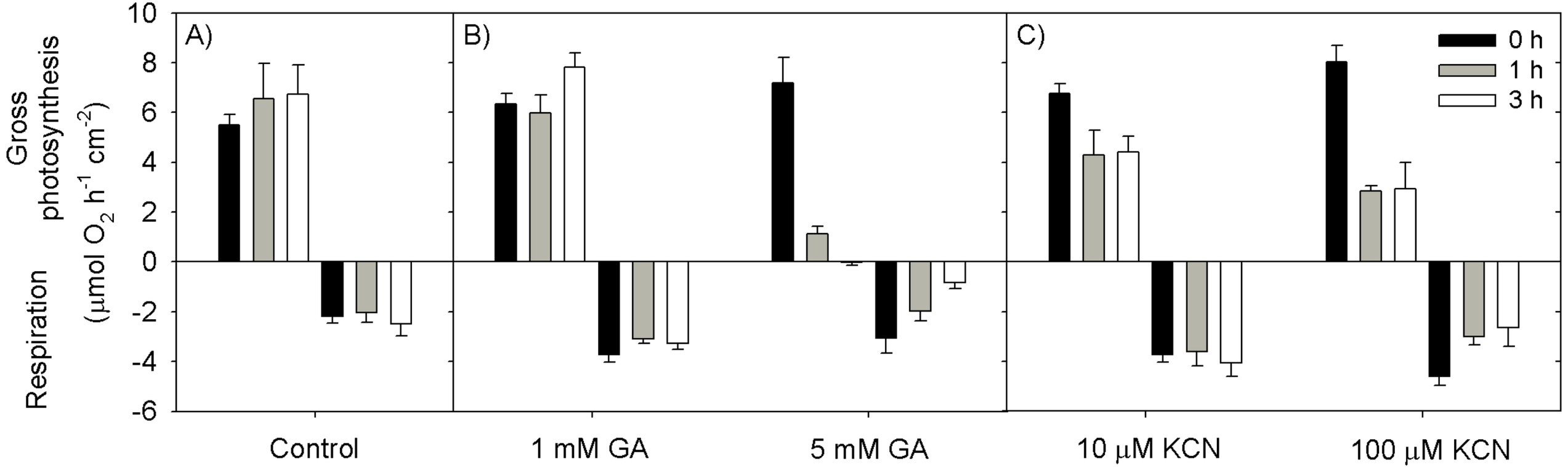
Supplementary material

Fig. S1: Double normalised fast induction curves (FICs) showing relative variable fluorescence [$(F_t - F_O)/(F_M - F_O)$] of *Pocillopora damicornis* in the absence of any inhibitor (control; A-C), in the presence of 3 mM GA (D-F) and in the presence of 20 µM KCN (G-I) at 26°C (A,D,G), 30°C (B,E,H) and 31°C (C,F,I). Only the 0-10 h time points are shown. The 24 h GA time point had many values close to zero resulting in excessive noise in the relative variable fluorescence calculation. Averages shown (n = 4).

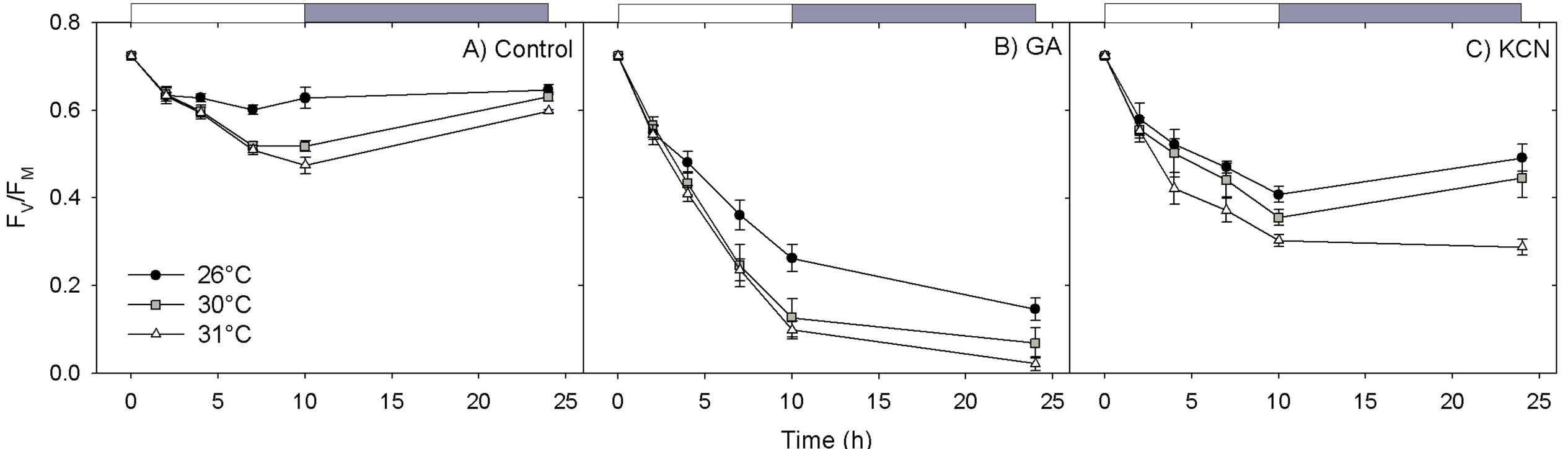

A) GA
B) KCN
F_V/F_M
0.0
0.2
0.4
0.6
0
5
10
15
20
25
30
Time (h)
0 mM
1 mM
2 mM
3 mM
4 mM
5 mM
10 mM GA
0 µM
5 µM
10 µM
20 µM
50 µM
100 µM KCN

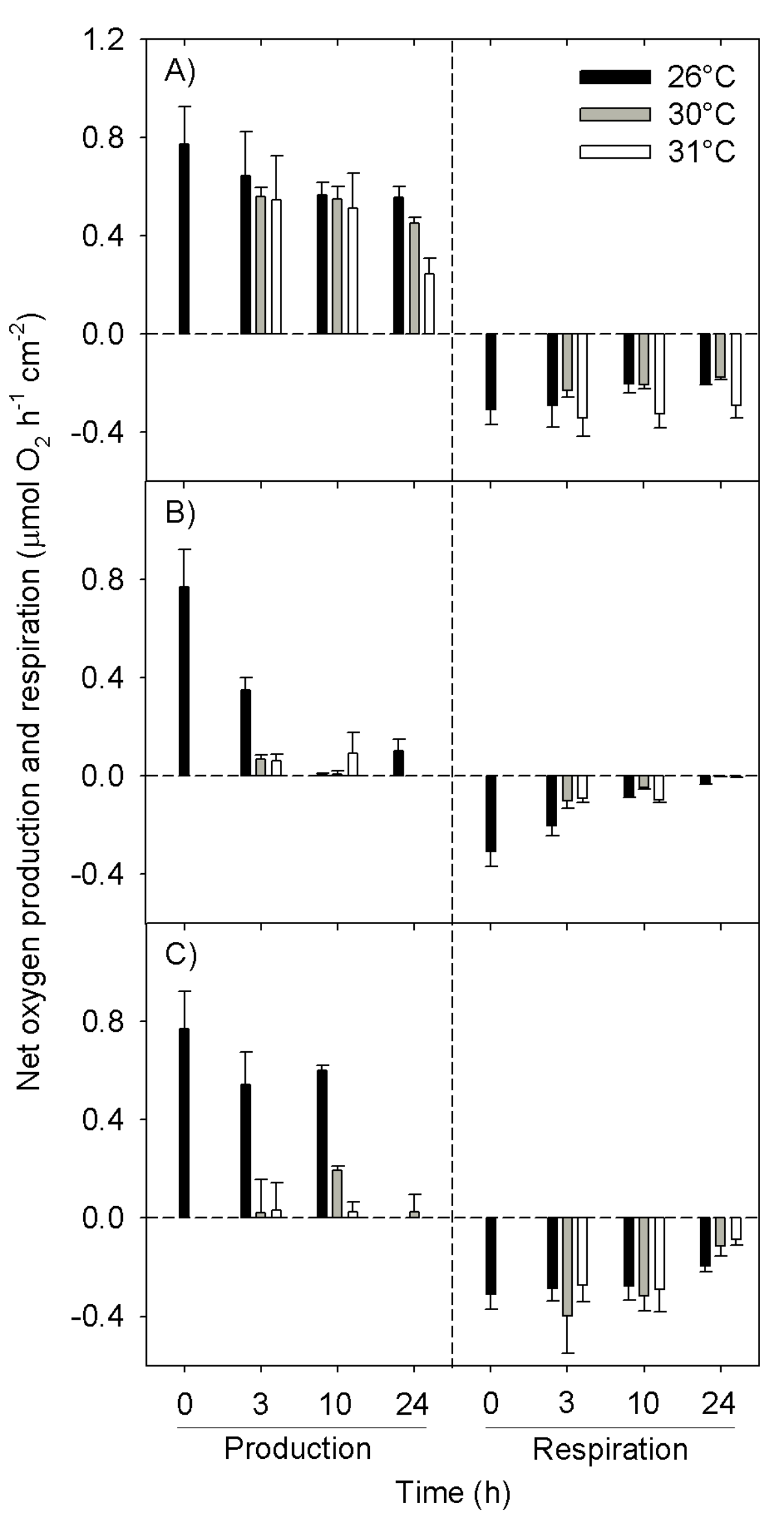

A) 0 h GA
B) 5 h GA
C) 0 h KCN
D) 5 h KCN
Relative Variable Fluorescence
Time (s)
0.00001
0.0001
0.001
0.01
0.1
1
0.0
0.2
0.4
0.6
0.8
1.0
0 mM GA
1 mM GA
3 mM GA
5 mM GA
0 h 0 mM GA
0 μM KCN
10 μM KCN
20 μM KCN
0 h 0 μM KCN


A)
B)
0 mM GA
1 mM GA
5 mM GA
0 μM KCN
10 μM KCN
100 μM KCN
F_V/F_M
Time (h)
0.8
0.6
0.4
0.2
0
1
2
3

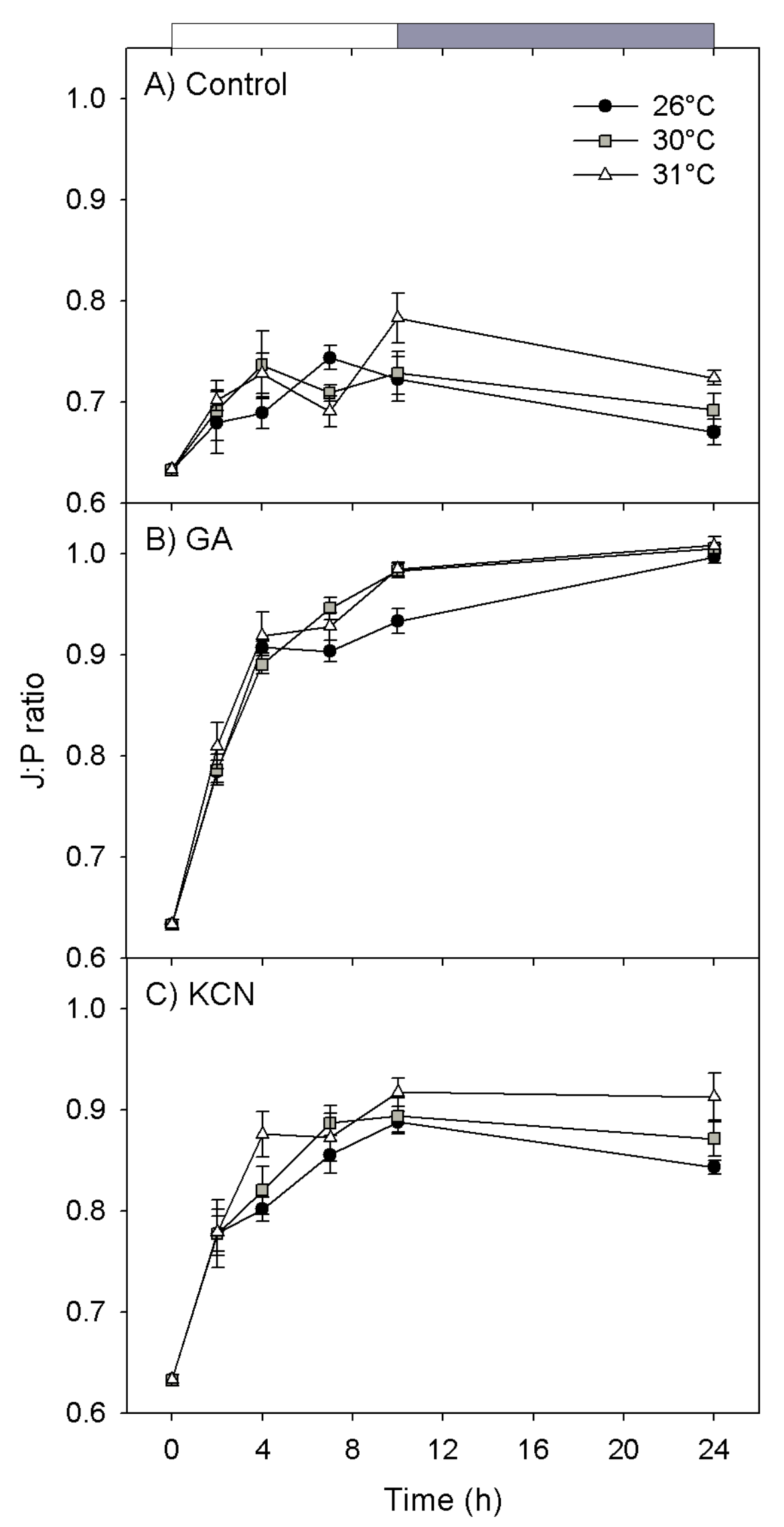

A)
B)
C)
0 h
1 h
3 h
Gross photosynthesis
Respiration
(μmol O_2 h^{-1} cm^{-2})
10
8
6
4
2
0
-2
-4
-6
Control
1 mM GA
5 mM GA
10 μM KCN
100 μM KCN

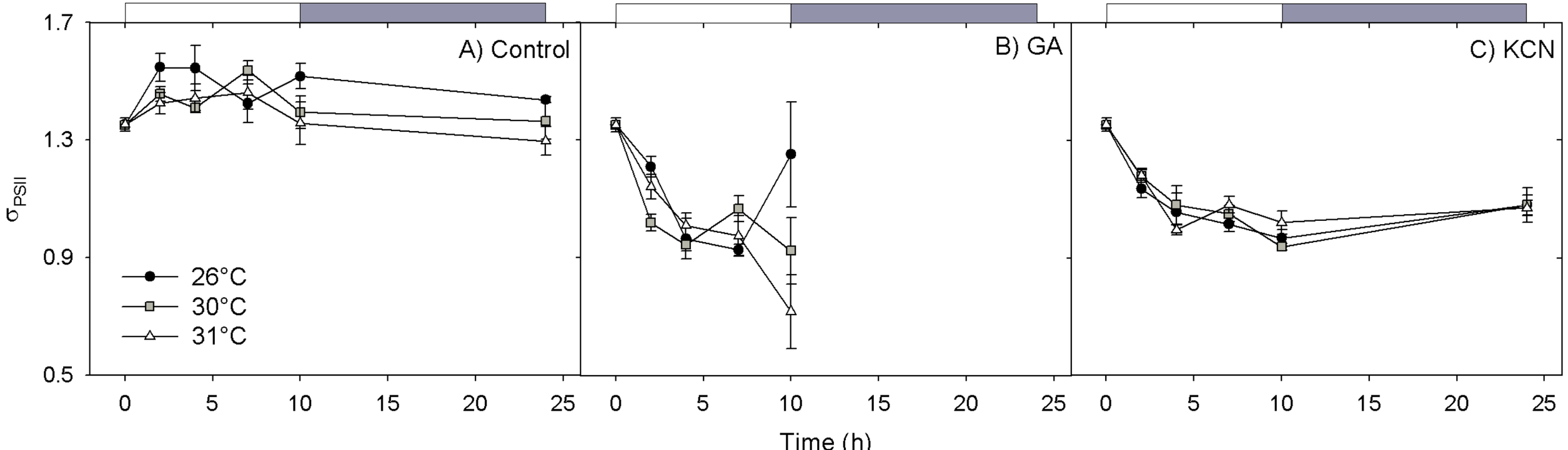
A)
B)
C)
D)
26°C
30°C
31°C
Symbiont Density (x10^6 cm^{-2})
Chlorophyll *a* (µg cm^{-2})
Chlorophyll c_2 (µg cm^{-2})
Total Chlorophyll (pg cell^{-1})
1.6
1.2
0.8
0.4
0.0
12
8
4
0
3
2
1
0
20
15
10
5
0
0 h
24 h Control
24 h GA
24 h KCN


A) Control
B) GA
C) KCN
26°C
30°C
31°C
F_V/F_M
0.0
0.2
0.4
0.6
0.8
0
5
10
15
20
25
Time (h)


A)
B)
C)
26°C
30°C
31°C
1.2
0.8
0.4
0.0
-0.4
Net oxygen production and respiration (µmol O2 h-1 cm-2)
0
3
10
24
Production
Respiration
Time (h)

A) Control - 26°C
0 h
2 h
4 h
7 h
10 h
24 h
D) GA - 26°C
G) KCN - 26°C
B) Control - 30°C
E) GA - 30°C
H) KCN - 30°C
C) Control - 31°C
F) GA - 31°C
I) KCN - 31°C
Fluorescence (V)
2.2
1.8
1.4
1.0
0.0001
0.001
0.01
0.1
1
10
Time (s)


A) Control
26°C
30°C
31°C
B) GA
C) KCN
J:P ratio
Time (h)
1.0
0.9
0.8
0.7
0.6
0
4
8
12
16
20
24


A) Control
B) GA
C) KCN
26°C
30°C
31°C
σPSII
1.7
1.3
0.9
0.5
0
5
10
15
20
25
Time (h)