A Dissertation

Entitled

Effects of CO₂ and Nitrogen on Plant Response to Heat Stress

By

Dan Wang

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Advisor: Dr. Scott A. Heckathorn

Dr. Jiquan Chen

Dr. Jonathan M. Frantz

Dr. Stacy M. Philpott

Dr. Asko Noormets

College of Graduate Studies

The University of Toledo

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More intense, more frequent, and longer heat-waves are expected in the future due to global warming, both of which could have dramatic ecological impacts. It is necessary to determine how elevated CO₂ and N affect plant responses to heat stress because atmospheric CO₂ and N deposition will increase in the future. In the first project, we found thermotolerance of Pₙ in elevated (vs. ambient) CO₂ increased in C₃, but decreased in C₄ (especially) and CAM (high growth temperature only), species. In contrast, elevated CO₂ decreased electron transport in 10-of-11 species. High CO₂ decreased gₛₚ (stomatal conductance) in 5 of 9 species, but stomatal limitations to Pₙ increased during heat stress in only 2 cool-season C₃ species. Thus, benefits of elevated CO₂ to photosynthesis at normal temperatures may be partly offset by negative effects during
stress, especially for C₄ species, so effects of elevated CO₂ on acute heat tolerance may contribute to future changes in plant productivity, distribution, and diversity. The second project showed that effects of elevated CO₂ on plant tolerance to heat stress are also dependent on N availability. Negative effects of high CO₂ were associated with decreased CE (carboxylation efficiency) and rubisco activase (except high-N barley) and HSPs (especially HSP70). My meta-analysis results showed that elevated CO₂ affects plant physiology and growth to varying degrees under different temperature regimes. The field study examined the effects of N availability on plant response to heat-stress (HS) treatment in naturally-occurring vegetation. The results indicated that increasing nitrogen (N) availability will likely impact plant responses to heat stress, and thus carbon (C) sequestration in terrestrial ecosystems, which suggests that heat waves, though transient, could have significant effects on plants, communities, and ecosystem N cycling, and N can influence the effect of heat waves.
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1.1 Climate change

Climate change in IPCC (Intergovernmental Panel on Climate Change) usage refers to a change in the state of the climate that can be identified (e.g. using statistical tests) by changes in the mean and/or the variability of its properties, and that persist for an extended period, typically decades or longer. It refers to any change in climate over time, whether due to natural variability or as a result of human activity. Since pre-industrial times, increasing emissions of green house gases (GHGs) due to human activities have led to a marked increase in atmospheric GHG concentrations (IPCC 2007). Between 1970 and 2004, global emissions of CO₂, CH₄, N₂O, HFCs, PFCs and SF₆, weighted by their global warming potential (GWP), have increased by 70%. The emissions of these gases have increased at different rates. CO₂ emissions have grown between 1970 and 2004 by about 80% and represented 77% of total anthropogenic GHG emissions in 2004. Carbon dioxide is the most important anthropogenic green house gas. The global atmospheric concentration of carbon dioxide has increased from a pre-industrial value of about 280 ppm to 379 ppm in 2005, which exceeds by far the natural range over the last 650,000 years (180 to 300 ppm) as determined from ice cores.

The combined radiative forcing due to increases in GHGs and the rate of increase
in radiative forcing during the industrial era is very likely to have been unprecedented in more than 10,000 years (IPCC 2007). The carbon dioxide radiative forcing had increased by 20% from 1995 to 2005, the largest change for any decade in at least the last 200 years. Anthropogenic increases in atmospheric CO₂ are likely largely responsible for recent increases in global mean surface temperatures, which rose by 0.76 °C from 1850-1899 to 2001-2005 and are projected to increase by another 1.4-to-5.8 °C by 2100 (IPCC 2007). Eleven of the last twelve years (1995-2006) rank among the warmest years in the instrumental record of global surface temperature (since 1850). The temperature increase is widespread over the globe and is greater at higher northern latitudes. Widespread changes in extreme temperatures have also been observed over the last 50 years. Cold days, cold nights and frost have become less frequent, while hot days, hot nights and heat waves have become more frequent (IPCC 2007). These weather extremes have changed in their frequency and/or intensity over the last 50 years (Wagner, 1996; Haldimann and Feller, 2004). Thus, in the future, plants will likely experience increases in heat stress, which can impact plant growth and development, decreasing crop and ecosystem productivity (Ciais et al., 2005) and biodiversity (Davis, 1986; Thomas et al., 2004).

1.2 The effect of CO₂ and temperature on net photosynthesis

Heat stress has a negative effect on plants, resulting mainly from negative effects on photosynthesis, which is among the most thermosensitive aspects of plant function (e.g., Berry and Björkman, 1980; Weis and Berry, 1988; Wise et al., 2004; Kim and Portis, 2005). Both the light (electron transport) and dark (Calvin cycle) reactions of photosynthesis have thermolabile components, especially photosystem II (PSII) in the
light reactions (Santarius, 1975; Berry and Björkman, 1980; Weis and Berry, 1988; Heckathorn et al., 1998, 2002) and rubisco (ribulose 1, 5-bisphosphate carboxylase/oxygenase) activase in the Calvin (CO$_2$-fixation) cycle (Eckardt and Portis, 1997; Crafts-Brandner and Salvucci, 2000). However, increases in atmospheric levels of CO$_2$ above current levels can increase photosynthesis by decreasing photorespiration (fixation of O$_2$ rather than CO$_2$ by rubisco, the first step of the Calvin cycle), which increases with temperature and is higher in plants with C$_3$-type photosynthesis (the majority of plants) vs. plants with the other two types of photosynthesis, C$_4$ and CAM plants (Sage and Monson, 1999; Taiz and Zeiger, 2004). It has therefore been predicted that photosynthetic response to increased CO$_2$ in plants with C$_3$ metabolism will be larger at higher temperatures (Bowes et al. 1996; Gifford 1995; Long 1991). In contrast, it has been generally considered that C$_4$ species will show little CO$_2$ stimulation irrespective of temperature because of the CO$_2$ concentration mechanism in C$_4$ species.

Numerous studies have reviewed the evidence for temperature by CO$_2$ interactions in plant carbon balance (Morison and Lawlor 1999), so the basic points need little repetition. In contrast, the effects of elevated CO$_2$, or the interactions between elevated CO$_2$ and higher mean growth temperature, on plant responses to acute heat stress have been examined in only a few studies, and the results have been variable. For example, elevated CO$_2$ has yielded positive (Faria et al., 1996, 1999; Ferris et al., 1998; Huxman et al., 1998; Hamerlynck et al., 2000; Taub et al., 2000), negative (Bassow et al., 1994; Huxman et al., 1998; Roden and Ball, 1996a, b; Taub et al., 2000), and no effects (Coleman et al., 1991) on photosynthetic and plant tolerance to acute heat stress. More studies are needed to investigate how elevated CO$_2$ affects plant tolerance to heat stress at
different growth temperatures and different N levels. Due to the contrasting effect of CO₂ at mild heat stress (ET) and severe heat stress (HS), it is critical to examine the role of CO₂ under different temperature treatments and for different functional groups to better understand plant responses to multiple environmental changes in the future.

In addition to temperatures, human activities are increasing global N availability (IPCC 2007). N availability is likely to affect plant, community, and ecosystem responses to increasing heat stress, which will then impact ecosystem C sequestration. Understanding effects of N on the responses of vegetation to heat stress requires insight into how stress physiology and community structure interact. While the influence of plant N status on response to acute heat stress has been previously examined, past studies have largely focused on laboratory experiments examining physiological responses (Heckathorn et al. 1996a, b; Lu & Zhang 2000). Further, because of the difficulties of imposing heat stress on naturally-occurring vegetation, little experimental work has been conducted on response to acute heat stress in field-grown plants (Morison & Lawlor 1999; Weis & Berry 1987). To date, there have been only a handful of studies in which plant communities were exposed to extreme high temperatures, and these focused on community processes (e.g., recolonization, competition, invasion, and the role of species richness during extreme events) and were conducted on grassland (Van Peer et al. 2004; White et al. 2001) or arctic species (Marchand et al. 2006; Marchand et al. 2005). Also, N availability had significant effects on plant N-relations responses to moderate warming (rather than acute heat stress) in a tallgrass prairie (An et al. 2005). Thus, little is known as to how heat stress in general, and N interactions with heat stress in particular, will affect natural plant communities. Furthermore, whether N availability interacts with heat
stress differently in C3 vs. C4 species remains to be determined, but will have a bearing on the relative impact of global environmental change on C3 and C4 species abundance and distribution.

1.3 Objectives

To amend this lack of knowledge with respect to heat stress and physiological, community, and ecosystem effects, I conducted a series of lab and field experiments and a meta-analysis. The particular objectives of the dissertation were to:

1. To help clarify the influence of elevated CO2 on the tolerance of photosynthesis to acute heat stress and to determine if any observed differences are related to photosynthetic pathway or to organismal heat tolerance.

2. To determine whether the photosynthetic responses of C3 and C4 plants to acute heat stress are dependent on different CO2 and N level.

3. To provide estimates of the magnitude and significance of elevated CO2 effects on plant biomass accumulation and partitioning, gas exchange, PSII, stomatal conductance, Rubisco activity and nitrogen concentration under different global warming scenarios and to test for differences among plant functional groups and growth forms in affecting these responses.

4. To examine the influence of N on plant response to heat stress in naturally-occurring mixed C3-C4 vegetation.
References


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Chapter 2

Effects of elevated CO$_2$ on the tolerance of photosynthesis to acute heat stress

Abstract:

Determining the effect of elevated CO$_2$ on the tolerance of photosynthesis to acute heat-stress (AHS) is necessary for predicting plant responses to global warming, as photosynthesis is heat-sensitive and AHS and atmospheric CO$_2$ will increase in the future. Few studies have examined this, and past results are variable, which may be related to methodological variation among studies. In this study, we grew 11 species (including: cool- & warm-season, and all 3 photosynthetic pathways- C$_3$, C$_4$, CAM) at current or elevated (370 or 700 ppm) CO$_2$ and at species-specific optimal growth temperatures and at 30$^\circ$C (if optimal $\neq$ 30$^\circ$C). We then assessed thermotolerance of net photosynthesis (P$_n$), stomatal conductance (g$_s$), leaf internal [CO$_2$], and photosystem II (PSII) and post-PSII electron transport during AHS. Thermotolerance of P$_n$ in elevated (vs. ambient) CO$_2$ increased in C$_3$, but decreased in C$_4$ (especially) and CAM (high growth temperature only), species. In contrast, elevated CO$_2$ decreased electron transport in 10-of-11 species. High CO$_2$ decreased g$_s$ in 5 of 9 species, but stomatal limitations to P$_n$ increased during AHS in only 2 cool-season C$_3$ species. Thus, benefits of elevated CO$_2$ to photosynthesis at normal temperatures may be partly offset by negative effects during AHS, especially for C$_4$ species, so effects of elevated CO$_2$ on acute heat tolerance may contribute to future changes in plant productivity, distribution, and diversity.
2.1 Introduction

Anthropogenic increases in atmospheric CO$_2$ are likely largely responsible for recent increases in global mean surface temperatures, which rose by 0.6°C from 1990-2000 and are projected to increase by another 1.4-to-over 5°C by 2100 (Houghton et al., 2001; IPCC 2001). In addition to mean increases in annual temperatures, there will also be increases in the frequency, duration, and severity of periods with exceptionally high temperatures (i.e., heat waves) (Wagner, 1996; Haldimann and Feller, 2004). Thus, in the future, plants will likely experience increases in acute heat stress, which can impact plant growth and development, decreasing crop and ecosystem productivity (Ciais et al., 2005) and biodiversity (Davis, 1986; Thomas et al., 2004).

Negative effects of heat stress on plants are caused, to a large extent, by negative effects on photosynthesis, which is among the most thermosensitive aspects of plant function (e.g., Berry and Björkman, 1980; Weis and Berry, 1988; Wise et al., 2004; Kim and Portis, 2005). Both the light (electron transport) and dark (Calvin cycle) reactions of photosynthesis have thermolabile components, especially photosystem II (PSII) in the light reactions (Santarius, 1975; Berry and Björkman, 1980; Weis and Berry, 1988; Heckathorn et al., 1998, 2002) and rubisco (ribulose 1, 5-bisphosphate carboxylase/oxygenase) activase in the Calvin (CO$_2$-fixation) cycle (Eckardt and Portis, 1997; Crafts-Brandner and Salvucci, 2000). However, increases in atmospheric levels of CO$_2$ above current levels can increase photosynthesis by decreasing photorespiration (fixation of O$_2$ rather than CO$_2$ by rubisco, the first step of the Calvin cycle), which increases with temperature and is higher in plants with C$_3$–type photosynthesis (the majority of plants) vs. plants with the other two types of photosynthesis, C$_4$ and CAM
plants (Sage and Monson, 1999; Taiz and Zeiger, 2004); thus, elevated CO2 might benefit C3 plants more than C4 plants during heat stress. Additionally, elevated CO2 can also increase water-use efficiency, in part by decreasing stomatal conductance and transpiration (Ainsworth et al., 2002), which may increase acute-heat tolerance by increasing plant water status. On the other hand, both C3 and C4 plants experience reductions in stomatal conductance (opening) with increasing CO2 (e.g., 20% for C3 and 50% for C4 species with a doubling of CO2) (Sage, 1994; Wand, 1999; Reich et al., 2001; Maherali et al., 2002), so the lower average stomatal conductance of C4 plants at any given CO2 level means lower average transpiration (water loss) and thus higher leaf temperatures in C4 plants, which may increase heat-related damage in C4 plants compared to C3 plants in the same habitat.

Since increases in temperature and CO2 may have interactive effects on photosynthesis, many studies have examined the effects of elevated CO2 and increased growth temperature (typically 3-5°C) on photosynthesis (reviewed by Morison and Lawlor, 1999). In contrast, the effects of elevated CO2, or the interactions between elevated CO2 and higher mean growth temperature, on plant responses to acute heat stress have been examined in only a few studies, and the results have been variable (Table 1). For example, elevated CO2 has yielded positive (Faria et al., 1996, 1999; Ferris et al., 1998; Huxman et al., 1998; Hamerlynck et al., 2000; Taub et al., 2000), negative (Bassow et al., 1994; Huxman et al., 1998; Roden and Ball, 1996a, b; Taub et al., 2000), and no effects (Coleman et al., 1991) on photosynthetic and plant tolerance to acute heat stress. In the previous studies that compared elevated-CO2 effects on tolerance to acute heat stress in relatively heat-sensitive vs. -tolerant species, or in species with different
photosynthetic pathways (Coleman et al., 1991; Bassow et al., 1994; Roden and Ball 1996a, b; Huxman et al., 1998; Taub et al., 2000), all species were grown under identical thermal regimes, which were likely closer to optimal for some of the species examined, but supra- or sub-optimal for others. Given that growth temperature is known to strongly influence the response and tolerance of organisms and photosynthesis to acute heat stress (e.g., Weis and Berry, 1988; Barua and Heckathorn, 2004), comparisons of the heat-stress responses of species not grown at their respective optimal (or sub- or supra-optimal) growth temperatures may obscure response patterns that otherwise may be evident. In addition, heat stress treatments in the previous studies varied widely in both intensity and duration (Table 2.1), making it difficult to directly compare across studies. Further, only one previous study included C₄ species (1 species in Coleman et al., 1991), but photosynthesis was not measured in this study, and only one study included CAM species (1 species in Huxman et al., 1998). In a preliminary study with *Pisum sativum* (pea, cool-season C₃) and *Zea mays* (corn, warm-season C₄), effects of elevated CO₂ on photosynthetic thermotolerance were positive in the C₃, but negative in the C₄, species (Joshi, 2006). However, it remains to be determined if this difference can be generalized and is related to photosynthetic pathway or organismal thermotolerance, since C₄ species are also warm-season species originating from tropical/subtropical climates (Sage and Monson, 1999).

In this study, to help clarify the influence of elevated CO₂ on the tolerance of photosynthesis to acute heat stress, I grew and heat-stressed 11 species at current or elevated CO₂, including C₃, C₄, and CAM species, as well as both cool- and warm-season C₃ species; thereby allowing us to determine if any observed differences were related to
photosynthetic pathway or to organismal heat tolerance. Plants were grown at both species-specific optimal pre-heat-stress growth temperatures and at 30°C (if optimal was different from 30°C), to permit comparisons at both a common temperature regime, as well as at temperatures that reflect the natural conditions that each species will more-typically encounter in the field. I also determined if CO₂-effects on photosynthetic thermotolerance were associated with changes in stomatal or biochemical (especially electron transport) limitations to photosynthesis (e.g., decreases in net photosynthesis during heat stress caused by stomatal closure at high CO₂).
2.2 Materials and methods

2.2.1 Plant material and growing conditions

Eleven species were examined in this study, including 3 cool-season C3 species \([Pisum sativum\) L. (pea), \(Chenopodium album\) L. (lambs quarters), and \(Triticum aestivum\) L. (wheat)], three warm-season C3 species \([Glycine max\) L.(soybean), \(Helianthus annuus\) L. (sunflower), and \(Lycopepsicon esculentum\) L. (tomato)], three C4 species \([Zea mays\) L. (corn or maize), \(Sorghum bicolor\) L. (Moench) (sorghum), and \(Amaranthus retroflexus\) L. (pigweed)], and two CAM species \([Agave Americana\) L. (century plant) and \(Ferocactus wislizenii\) Britt. & Rose (fish-hook cactus)]. All C3 and C4 species used are annuals and were germinated and grown (ca. 6 weeks) in ambient or elevated CO2 until the early vegetative stage (e.g., to the 6th leaf stage in corn, the species with the largest plants in this study). The agave and cactus species used herein are perennials, and young (ca. 3 years old) plants (initially raised under ambient CO2) were raised under controlled ambient or elevated CO2 levels for three months prior to use to ensure acclimation to CO2 treatments (Note: This was sufficient time for the agave species to produce 2 new fully-expanded leaves, from which photosynthetic data were collected, and for the cactus species to increase in diameter by ca. 2 cm). Plants were grown in 5-L pots in a 1:1:1 mixture of top-soil, sand, and perlite, and placed in growth chambers (E-36HO, Percival Scientific, Iowa, USA) equipped with light, temperature, and CO2 control. All the species were grown under 4 temperature regimes (20/12°C, 25/17°C, 30/22°C and 35/27°C for day/night respectively; CAM species were also grown at 38/30°C) to determine species-specific optimal (or near-optimal) temperatures (based on biomass, Table 2). Plants were grown at either ambient (370±15 ppm) or enriched (700±15 ppm)
CO₂, with a day length of 14 hours and a light level of 1000 µmoles/m²/sec PAR (photosynthetically active radiation) at the canopy level of plants. Plants were rotated at least once per week to avoid position effects in the chambers. Prior to treatment, a subset (15-18) of uniformly-sized plants of each species was selected within each chamber for further use. All the pots were watered as needed and fertilized regularly (every other week with a commercial complete nutrient mix), so that plants were not water or nutrient limited.

2.2.2 Heat-shock treatments

Heat-shock treatment was applied during vegetative growth, and within each species, all plants were at similar stages of development (e.g., similar number of leaves). Prior to heat stress, plants in each chamber were randomly assigned to one of two groups (control = C and heat-stress = HS), with 5-6 plants in each group. The controls were not heat stressed and HS plants were heat stressed at 15°C higher than daytime growth temperatures from 9:00 am to 1:00 pm. Chamber temperatures in all treatments were monitored using Hobo Dataloggers and probes (Onset Computer Corp., MA, USA).

2.2.3 Photosynthesis measurements

Steady-state net photosynthesis (Pₙ; net CO₂ exchange) of leaves was measured with a portable photosynthesis-transpiration system with infrared gas analyzer (model 6400, LiCOR, Lincoln, NE, USA), equipped with a 250-mm³ leaf chamber. Measurements were made within 1-2 min of insertion of leaves into the cuvette, and after stabilization of CO₂ and H₂O flux, to ensure that photosynthetic responses reflected those within the growth chambers. Plants were measured before and during heat stress, as described in Heckathorn et al. (1996), at the same CO₂ levels as the plants were growing.
at (either 370 or 700 ppm CO₂), a light level of 1000 µmol m⁻² s⁻¹ PAR (ca. = to that at the tops of the plants), and at species-specific growth temperatures. During heat stress, \( P_n \) was measured twice at 10:00 am and 1:00 pm. All results were collected from recently-expanded fully-lit leaves of intact plants.

For the CAM species, I monitored treatment effects on net CO₂ fixation by determining the pH of chlorenchyma (green) tissue at the end of the heat-treatment-period in both heat-stressed and unstressed control plants. CAM species photosynthetically fix CO₂ at night into malic acid, lowering tissue pH throughout the night in the absence of other large pools of acid, and they decarboxylate this CO₂ during the day and use it and ATP and NADPH from the light reactions to produce sugars, thereby increasing cell pH throughout the day (Taiz and Zeiger, 2004). If heat stress affected net CO₂ fixation in the CAM species, then pH of photosynthetic tissue should be lower in heat-treated plants compared to controls, since utilization of the CO₂ fixed at night would be hindered and the normal daytime increase in pH would be slowed. Plants were harvested at the end of the heat-stress treatment, and immediately frozen and stored at -70°C. Sub-samples of tissue were later ground completely in a mortar and pestle, diluted with 10-mL of deionized water, and then measured to determine pH.

To examine heat effects on PSII and post-PSII electron transport, PSII efficiency (\( F_v' / F_m' \)) and photochemical quenching (\( q_p \)) in light-adapted leaves were monitored by analysis of chlorophyll fluorescence using a pulse-amplitude-modulated (PAM) fluorometer (Model PAM 101/103; Walz, Germany). Chlorophyll fluorescence was measured in unstressed control plants and on HS plants during heat stress at 10:00 am and 1:00 pm. Basal fluorescence (\( F_s \)) under steady-state illumination (900 µmol m⁻² s⁻¹
PAR) was measured initially, followed by maximum fluorescence ($F_{m'}$) after a 1.0 s pulse of saturating white light (>5000 µmol m$^{-2}$ s$^{-1}$ PAR). Minimum fluorescence ($F_{o'}$) was then measured after turning off both actinic and flash light-sources. $F_{v'}/F_{m'}$ and $q_{p}$ were then calculated as in Genty et al. (1989), where $F_{v'}/F_{m'} = (F_{m'}-F_{o'})/F_{m'}$, and $q_{p} = (F_{m'}-F_{s})/(F_{m'}-F_{o'})$.

2.2.4 Statistical analysis

All photosynthesis and fluorescence results are means derived from independent replicate plants (separate sets of plants were used for controls and each heat-stress time point). Analyses were conducted within each species to determine whether the physiological variables differed as a function of different treatments. Unless indicated otherwise, a split-plot two-way ANOVA (JUMP IN 5.1 software) was used to test for significant effects of CO$_2$, heat stress duration (control, 1-h of heat stress, 4-h of heat stress), and their interaction on $P_{n}$, $g_{st}$, $C_{i}$, $F_{v'}/F_{m'}$, and $q_{p}$ through the time course of the high-temperature period. Three-way ANOVAs were also conducted among C$_3$ and C$_4$ species to determine whether CO$_2$ effects differed as a function of different photosynthetic groups, with $P_{n}$, $g_{st}$, $C_{i}$, $F_{v'}/F_{m'}$, and $q_{p}$ as dependent factors and CO$_2$, heat stress duration, and functional types (cool-season C$_3$, warm-season C$_3$ and C$_4$) as independent factors. Post-hoc Tukey HSD test were made on specific contrasts to examine significant treatment effects among groups. Absolute data were used to conduct correlation analysis (SAS 9.1) between $P_{n}$ and $g_{st}$, $C_{i}$, $F_{v'}/F_{m'}$ and $q_{p}$ for C$_3$ and C$_4$ species, respectively; these results were compared to results with log-transformed data and results of partial-correlation analysis, which yielded similar conclusions.
2.3 Results

Based on the seedling biomass of species grown at different growth temperatures (Table 2.2), near-optimal growth temperature for the cool-season C₃ species was ca. 25°C, and was ca. 30°C for the warm-season C₃ and the C₄ species. Therefore, in subsequent experiments, 25°C and 30°C were used as optimal daytime growth temperatures for cool-season C₃ and warm-season C₃ & C₄ species, respectively.

As expected, prior to initiation of acute heat-stress (AHS) treatments, C₃ species (except for wheat at 25°C and chenopodium at 30°C) grown at elevated CO₂ showed significant stimulation of Pₙ compared to ambient CO₂, while C₄ species exhibited little stimulation of Pₙ in elevated CO₂ (Fig 2.1). Within 1-hour of AHS, decreases in Pₙ were observed for most species, with additional decreases in Pₙ with continuing heat stress, such that after 4 hours of AHS, Pₙ decreased significantly in all species (Table 2.3). For the C₄ species, the decreases in Pₙ caused by AHS were more pronounced in elevated-CO₂-grown plants than in ambient-CO₂-grown plants. For the cool-season C₃ species, the positive effects of elevated CO₂ on Pₙ during AHS were evident both for plants grown at optimal pre-stress daytime growth temperatures and for plants grown at 30°C prior to AHS; however, the stimulatory effects of high CO₂ were smaller at the higher growth temperature.

Stomatal conductance increased or remained essentially unchanged by AHS for all species except for small decreases (absolute and relative to Pₙ) in chenopodium (25 & 30°C) and wheat (30°C) (Fig 2.2; Table 2.3), indicating that the decreases in Pₙ during heat stress were not caused by stomatal closure (confirmed below). And for all species (except for sorghum), plants grown at elevated CO₂ had a lower gₛ than plants at ambient
CO$_2$, though the CO$_2$ effects were biologically insignificant for some species (soybean, sunflower, pigweed). Similar to $g_{st}$, $C_i$ was increased by AHS in 7 of 9 species (Fig 2.3, Table 2.3), and biologically significant decreases were observed only in chenopodium and in pea at 25°C in high CO$_2$. And for all species but pigweed, plants grown at elevated CO$_2$ had a higher $C_i$ than plants at ambient CO$_2$. Hence, decreases in $P_n$ during AHS were caused by negative effects on photosynthetic metabolism, rather than increases in stomatal limitations, in all species except for chenopodium, wherein heat stress both increased relative stomatal limitations to photosynthesis and caused damage to photosynthetic metabolism (see below).

In contrast to $P_n$, prior to the initiation of AHS, the quantum efficiency of PSII ($F_{v'}/F_{m'}$) was not significantly different in plants grown at elevated vs. ambient CO$_2$, except in wheat at 25°C and tomato (Fig 2.4). As with $P_n$, heat stress significantly decreased $F_{v'}/F_{m'}$ in all species, except soybean (note that decreases were small and transient in wheat at 30°C and in tomato) (Table 2.3). During heat stress, elevated CO$_2$ had a negative effect on $F_{v'}/F_{m'}$ in the C$_4$ species, but had no consistent effects on C$_3$ species, (i.e., positive effects on tomato and wheat at 25°C, negative effects on chenopodium and pea at 30°C, and no effects on wheat at 30°C, pea at 25°C, soybean, and sunflower). Similar patterns to $F_{v'}/F_{m'}$ were found for $q_p$ (Fig 2.5, Table 2.3). Heat stress significantly decreased $q_p$ in 5-of-9 species, which means that the capacity of these species to keep PSII reaction centers in an open configuration was decreased by heat stress, indicating heat-stress effects on post-PSII electron transport. Elevated CO$_2$ had a negative effect on $q_p$ for nearly all species (except chenopodium at 25°C). Importantly, elevated CO$_2$ had negative effects on either $F_{v'}/F_{m'}$ or $q_p$ during AHS in all species, and
since the quantum yield of PSII electron transport ($\Phi_{et}$) is the product of the efficiency of PSII and the fraction of open PSII (i.e., $\Phi_{et} = \frac{F_{v'}}{F_{m'}} \times q_p$; Genty et al. 1989), elevated CO$_2$ decreased $\Phi_{et}$ during AHS in all species but tomato.

Elevated CO$_2$ effects on the tolerance of $\frac{F_{v'}}{F_{m'}}$, $q_p$, and chlorenchyma pH for CAM species were assayed at three temperatures because of the lack of biomass data to determine optimal growth temperatures (Fig 2.6-2.7; Table 2.4). At 25/18°C, AHS did not decrease $\frac{F_{v'}}{F_{m'}}$ or $q_p$ for either agave and cactus (Fig 2.6), but pH was significantly decreased for agave and cactus (Fig 2.7). At 30/23°C, $\frac{F_{v'}}{F_{m'}}$, $q_p$, and pH were decreased by AHS for agave after 4 hours, but for cactus, only pH was decreased by AHS. At 35/28°C, AHS decreased $\frac{F_{v'}}{F_{m'}}$, $q_p$, and pH for both agave and cactus, though the effect on $\frac{F_{v'}}{F_{m'}}$ and $q_p$ were transient for cactus. For cactus, CO$_2$ had no significant effect on $\frac{F_{v'}}{F_{m'}}$, $q_p$, and pH during AHS at 25/18°C and 30/23°C, but had a negative effect on $q_p$ at 35°C and a positive effect on pH (in controls). For agave, CO$_2$ had no effect on $\frac{F_{v'}}{F_{m'}}$, $q_p$, and pH at 25/18°C, a positive effect on $\frac{F_{v'}}{F_{m'}}$ and $q_p$ at 30/23°C, but a negative effect on $\frac{F_{v'}}{F_{m'}}$ and $q_p$ for plants grown at 35/28°C. For both agave and cactus, there was a significant interaction between CO$_2$ and heat stress at 35°C, such that high CO$_2$ decreased pH relative to either low CO$_2$ in heat-stressed plants (agave) or relative to high-CO$_2$ controls (cactus).

Correlation analysis between $P_n$ and the other photosynthetic variables ($g_{st}$, $C_i$, $\frac{F_{v'}}{F_{m'}}$, and $q_p$) was conducted (for C$_3$ and C$_4$ data only) to determine which variables were correlated with $P_n$ and whether the relationships changed with heat stress (Fig 2.8). For C$_3$ species, $P_n$ was positively correlated with $g_{st}$, $\frac{F_{v'}}{F_{m'}}$, and $q_p$ before heat stress.
(especially Fv'/Fm'), was not correlated with any of the other variables after 1 hour of heat stress, but was again positively correlated with gsts, Fv'/Fm', and (especially) qp after 4 hours of heat stress (and weakly negatively correlated with Ci). In contrast, for the C₄ species, Pn was positively correlated with Fv'/Fm' before and during heat stress and with qp only during heat stress, but was negatively correlated with Ci before and during heat stress and with gst before heat stress.
2.4 Discussion

Elevated CO₂ increased the tolerance of net photosynthesis (Pn) to acute heat stress in C₃ species, but decreased thermotolerance of Pn in C₄ species. Small, but significant, negative effects of high CO₂ were also observed on chlorenchyma pH in heat-stressed plants of the CAM species (high growth temperature only), suggesting that net photosynthesis was negatively impacted by high CO₂ during heat stress for CAM plants. Increases in Pn thermotolerance with high CO₂ in C₃ species were observed in both cool- and warm-season species; hence, CO₂ effects were related to photosynthetic pathway, and not organismal thermotolerance (confirmed by three-way ANOVA, where \( P < 0.01 \) for the interactive effect of functional types and CO₂, not significant when comparing C₃ warm- and cool- season species). Also, the pattern of CO₂ effects observed here were evident whether comparing plants grown at their respective optimal pre-stress growth temperatures, or when comparing plants raised at a common growth temperature (30⁰C). Notably, the relative benefits of elevated CO₂ on Pn thermotolerance were significantly reduced in the C₃ species grown at supra-optimal pre-stress growth temperatures (i.e., in the cool-season C₃ species grown at optimal + 5⁰C = 30⁰C). Although elevated CO₂ decreased stomatal conductance in most species, stomatal limitations to Pn increased during heat stress in only 2 species examined; hence the effects of elevated CO₂ on Pn thermotolerance were caused by negative effects of high CO₂ on photosynthetic metabolism in the majority of species, rather than by CO₂-induced decreases in stomatal conductance. In agreement with this, elevated CO₂ decreased the thermotolerance of electron transport, either by decreasing the efficiency of PSII (\( F_{v'}/F_{m'} \)) and/or by decreasing the performance of post-PSII electron transport relative to PSII (\( q_{p} \)), such that
PSII quantum yield ($\Phi_{\text{PSII}}$) decreased with high CO$_2$ in all species (including the 2 CAM species) but one (tomato, C$_3$).

The correlation analyses also indicated that, across species, $P_n$ was not limited by $g_s$ or $C_i$ during heat stress in either C$_3$ or C$_4$ species, but was likely limited (or co-limited) by damage to electron transport at some point in the heat stress period. Although $P_n$ was positively correlated with $g_s$ prior to heat stress in C$_3$ species, as might be expected, during heat stress, $P_n$ was mostly not (or even negatively) correlated with $g_s$ and $C_i$ during heat stress in both C$_3$ and C$_4$ species (because $P_n$ decreased in all species during heat stress, but $g_s$ and $C_i$ did not generally decrease). In contrast, $P_n$ was strongly correlated with electron transport before and during heat stress in both C$_3$ and C$_4$ species. Interestingly, in C$_4$ species, $P_n$ was only correlated with PSII efficiency before heat stress, but with both PSII and post-PSII efficiency during heat stress. However, in C$_4$ species, the strength of the correlation between $P_n$ and electron transport decreased with duration of heat stress (i.e., from time 0, to 1 h, and then 4 h, of heat stress), suggesting the possibility that the relative importance of electron transport in limiting $P_n$ during heat stress decreased with the duration of heat stress. In contrast, in C$_3$ species, $P_n$ was strongly correlated with electron transport (both PSII and post-PSII), both before heat stress and after 4-h of heat-stress treatment; yet, no such correlation was observed after 1-h of heat stress, indicating that some aspect of photosynthesis other than electron transport (e.g., rubisco activity via rubisco activase) limited $P_n$ early in the heat stress and electron transport became an important limitation later in the heat-stress treatment.

Together, these results suggest that the benefit of elevated CO$_2$ to $P_n$ thermotolerance in C$_3$ plants is related to decreased photorespiration during heat stress,
and that the negative impact of elevated CO$_2$ on photosynthetic light reactions was offset by decreases in photorespiration (as also indicated in Roden and Ball, 1996a). Given that photorespiration increases with temperature, and that C$_3$ species have high levels of photorespiration compared to C$_4$ and CAM species (Sage and Monson, 1999; Taiz and Zeiger, 2004), then the photorespiratory benefits of elevated CO$_2$ to C$_3$ plants during heat stress should outweigh negative effects until such a point that rubisco is damaged or that damage to electron transport becomes limiting to net photosynthesis. This prediction is supported by the relative decrease in the benefits of elevated CO$_2$ to P$_n$ in the cool-season C$_3$ species grown at supra-optimal pre-stress growth temperatures (30°C), compared to those grown at optimal temperatures (25°C). Studies of the thermal sensitivity of rubisco, or rubisco activase, activity indicates that rubisco function (primarily via damage to rubisco activase) begins to decrease significantly by ca. 35-40°C in representative cool- and warm-season species (Eckardt and Portis, 1997; Feller et al., 1998; Crafts-Brandner and Salvucci, 2000), so negative effects of elevated CO$_2$ on photosynthetic electron transport in C$_3$ species should begin to limit P$_n$ during acute heat stress at temperatures over ca. 35-40°C. Thus, in a future warmer world wherein there will be increases in both mean and extreme temperatures, negative effects of elevated CO$_2$ on electron transport may commonly limit P$_n$ in C$_3$, as well as C$_4$ and CAM species.

The positive effect of elevated CO$_2$ on net photosynthetic (P$_n$) thermotolerance in C$_3$ species have also been observed in other studies (Faria et al., 1996, 1999; Ferris et al., 1998; Huxman et al., 1998, 1-of-2 species only; Hamerlynck et al., 2000; Taub et al., 2000). Previous studies had also observed negative effects of high CO$_2$ on photosynthetic heat tolerance in C$_3$ species (Roden and Ball, 1996a), and negative effects
of high CO₂ during heat stress on biomass were observed for three C₃ tree species (Bassow et al., 1994). No previous studies had examined CO₂ effects on photosynthetic thermotolerance in C₄ species, and only one had done so in a CAM species (neutral effect; Huxman et al., 1998).

As in this study, negative, positive, and neutral effects of elevated CO₂ on heat tolerance of Fᵥ/Fₘ have been observed in C₃ species (no C₄ species examined) (Faria et al., 1996, 1999; Roden and Ball, 1996a, b; Huxman et al., 1998; Hamerlynck et al., 2000; Taub et al., 2000). Previously, only 1 CAM species had been examined, wherein high CO₂ decreased Fᵥ/Fₘ during heat stress (Huxman et al., 1998), as in this study. Also, similar to this study, in the two previous studies to measure quantum yield of electron transport (Φₚₛᵢᵢ), elevated CO₂ decreased thermotolerance of Φₚₛᵢᵢ in all species (4 C₃, 1 CAM) (Roden and Ball, 1996a; Huxman et al., 1998). A notable difference between the current study and Huxman et al. (1998) is the magnitude and duration of the temperature stress imposed upon the plants (I subjected plants to a 4-h treatment at 40-50°C, depending on species, whereas Huxman et al. subjected plants to a maximum daily temperature of 55°C for 9 d), yet the two studies obtained similar results, suggesting that negative effects of elevated CO₂ on heat tolerance of electron transport may be generalized to heat stress of various durations.

In addition to photorespiration, there are other aspects of metabolism that are affected by growth under elevated CO₂, including cellular adaptations conferring plant tolerance to acute heat stress, and CO₂-related changes in these heat-stress adaptations will likely impact photosynthetic thermotolerance. For example, heat-shock proteins (Heckathorn et al., 1998, 2002); lipid saturation level (Larkindale and Huang, 2004), the
carotenoids (especially zeaxanthin) (Havaux, 1998), protective compatible solutes (Williams et al., 1992), and isoprene production (Velikova and Loreto, 2005) all are known to confer photosynthetic thermotolerance. Williams et al. (1998) found that growth at elevated CO$_2$ increased saturation of some classes of thylakoid lipids. Both increases and decreases in isoprene emission have been reported for plants grown under elevated CO$_2$ (Sharky et al., 1991; Togenetti et al., 1998). Growth at elevated CO$_2$ affects profound alterations in cellular and subcellular concentrations of many soluble compounds (Poorter et al., 1997). And, HSP content is decreased at low N and at high CO$_2$ in leaves (Heckathorn et al., 1996; Wang and Heckathorn, in review). Further experiments are needed to supply direct evidence of an association between lipid changes, protective solutes, isoprene, HSPs, etc., and thermotolerance at elevated CO$_2$, to determine if high-CO$_2$-related decreases in heat-stress adaptations are linked to decreased thermotolerance of electron transport observed in this study.

Regardless of underlying mechanisms, results of this study indicate that increases in atmospheric CO$_2$ will alter plant photosynthetic responses to acute heat stress (or heat waves), and that the effect of CO$_2$ will likely vary with photosynthetic pathway (C$_3$, C$_4$, CAM). Given that the frequency, duration, and severity of heat stress will increase for plants in the coming decades (Wagner, 1996; Haldimann & Feller, 2004), and that photosynthesis is relatively heat sensitive (Weis and Berry, 1988), these results indicate that interactions between elevated CO$_2$ and plant thermotolerance may contribute to future changes in plant (including crop) productivity, distribution, and diversity associated with global environmental change. Specifically, my results indicate that increases in atmospheric CO$_2$ and acute heat stress in combination may further tip the
balance towards C₃ species, beyond what high CO₂ alone might do, which may contribute to increases in C₃ vegetation, both globally and in communities containing a mix of C₃ and C₄ species (e.g., mid-continental grasslands in the U.S.). However, the negative effects of elevated CO₂ to C₄ species during heat stress may be alleviated by higher water-use efficiency of C₄ species at both the leaf and whole-plant level, especially in years with water stress (Hamerlynck et al. 1997; Owensby 1993; Owensby et al. 1999), and the benefits of elevated CO₂ to C₃ species at near-optimal growth temperatures may be offset by expected increases in mean growth temperatures, or by likely changes in other environmental factors which influence thermotolerance differentially (e.g., increases or decreases in precipitation which might increase or decrease tolerance, respectively, and increasing ozone which might decrease tolerance).
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Table 2.1 Overview of past studies examining effects of elevated CO2 on tolerance to acute heat stress (GT: day growth temperature; HS: heat-stress temperature).

<table>
<thead>
<tr>
<th>Species and photosynthetic pathway</th>
<th>GT</th>
<th>HS and duration</th>
<th>Response factors**</th>
<th>CO2 effects</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betula populifolia, C3</td>
<td>28°C</td>
<td>45°C, 1d</td>
<td>Biomass, gst, leaf damage,</td>
<td>Negative effects, especially the most shade-tolerant species</td>
<td>Bassow et al. 1994</td>
</tr>
<tr>
<td>Betula alleghaniensis, C3</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Acer pennsylvanicum, C3</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Abutilon theophrasti, C3</td>
<td>20°C</td>
<td>32-40°C, 4h</td>
<td>Biomass, [N]</td>
<td>No effects on biomass, negative on [N]</td>
<td>Coleman et al. 1991</td>
</tr>
<tr>
<td>Sinapis alba, C3</td>
<td></td>
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<td>Amaranthus retroflexus, C4</td>
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<td></td>
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<tr>
<td>Quercus suber, C3</td>
<td>25°C</td>
<td>45°C, 4h</td>
<td>A_{net}, A_{max}, g_{st}, F_v/F_m, rubisco activity</td>
<td>Positive effects on carbon assimilation and F_v/F_m</td>
<td>Faria et al. 1996</td>
</tr>
<tr>
<td>Quercus suber, C3</td>
<td>25°C</td>
<td>45°C, 4h</td>
<td>A_{max}, F_v/F_m, SOD, CAT</td>
<td>Positive effects on A_{max}, F_v/F_m, and SOD activity, negative on CAT</td>
<td>Faria et al. 1999</td>
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<tr>
<td>Glycine max, C3</td>
<td>17°C</td>
<td>30°C, 8d</td>
<td>A_{max}, g_{s}</td>
<td>Positive effects on A_{max} and recovery from water deficit and heat stress</td>
<td>Ferris et al. 1998</td>
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<tr>
<td>Larrea tridentata, C3</td>
<td>45°C</td>
<td>53°C, 9d</td>
<td>A_{net}, A_{max}, g_{st}, F_v'/F_m', carbon fixation efficiency</td>
<td>Positive effects on recovery</td>
<td>Hamerlynck et al. 2000</td>
</tr>
<tr>
<td>Yucca whipplei, C3</td>
<td>45°C</td>
<td>53°C, 9d</td>
<td>A_{net}, g_{st}, F_v'/F_m', Φ_{PSII}</td>
<td>Positive and negative effects, depending on species and variable</td>
<td>Huxman et al. 1998</td>
</tr>
<tr>
<td>Yucca brevifolia, C3</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Yucca schidigera, CAM</td>
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<tr>
<td>Eucalyptus macrorhyncha, C3</td>
<td>20°C</td>
<td>45°C, 3h</td>
<td>A_{net}, g_{st}, F_v/F_m, Φ_{PSII}</td>
<td>Negative effects</td>
<td>Roden and Ball 1996a</td>
</tr>
<tr>
<td>Eucalyptus rossii, C3</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Eucalyptus macrorhyncha, C3</td>
<td>20°C</td>
<td>45°C, 3h</td>
<td>F_v/F_m, q_{Ph}, NPQ</td>
<td>Negative effects on F_v/F_m</td>
<td>Roden and Ball 1996b</td>
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<td>Eucalyptus rossii, C3</td>
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</tr>
<tr>
<td>Cucumis sativus, C3*</td>
<td>28°C</td>
<td>40°C, 4h</td>
<td>A_{max}, F_v/F_m</td>
<td>Positive effects A_{max}, mixed effects on PSII*</td>
<td>Taub et al. 2000</td>
</tr>
</tbody>
</table>

Notes: *Other C3 species were included, but on which only basal PSII fluorescence was measured.

**[N], leaf N concentration; A_{net}, A_{max}, net and maximum CO2 assimilation; g_{st}, stomatal conductance to H2O; F_v/F_m, F_v'/F_m', variable-to-maximum fluorescence of PSII in dark & light-adapted leaves; Φ_{PSII}, quantum yield of PSII; q_{Ph}, NPQ, two different calculations of non-photochemical quenching; SOD, superoxide dismutase activity; CAT, catalase activity.
Table 2.2 Effects of growth temperature on whole-plant fresh mass.

<table>
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<tr>
<th>Crop</th>
<th>20°C</th>
<th>25°C</th>
<th>30°C</th>
<th>35°C</th>
</tr>
</thead>
<tbody>
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<td><strong>C₃, cool-season</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Pea</td>
<td>2.57 (.30)</td>
<td>3.26 (.23)</td>
<td>2.30 (.28)</td>
<td></td>
</tr>
<tr>
<td>Chenopodium</td>
<td>2.61 (.22)</td>
<td>3.14 (.22)</td>
<td>2.53 (.31)</td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td>0.61 (.02)</td>
<td>0.99 (.10)</td>
<td>0.59 (.05)</td>
<td></td>
</tr>
<tr>
<td><strong>C₃, warm-season</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean</td>
<td>1.01 (.15)</td>
<td>2.29 (.22)</td>
<td>1.08 (.06)</td>
<td></td>
</tr>
<tr>
<td>Sunflower</td>
<td>1.80 (.04)</td>
<td>2.24 (.27)</td>
<td>1.85 (.31)</td>
<td></td>
</tr>
<tr>
<td>Tomato</td>
<td>0.83 (.11)</td>
<td>1.06 (.13)</td>
<td>1.02 (.14)</td>
<td></td>
</tr>
<tr>
<td><strong>C₄</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn</td>
<td>2.24 (.33)</td>
<td>3.34 (.27)</td>
<td>3.00 (.20)</td>
<td></td>
</tr>
<tr>
<td>Sorghum</td>
<td>0.49 (.08)</td>
<td>0.67 (.09)</td>
<td>0.45 (.08)</td>
<td></td>
</tr>
<tr>
<td>Pigweed</td>
<td>2.13 (.23)</td>
<td>3.72 (.44)</td>
<td>3.78 (.41)</td>
<td>2.52 (.30)</td>
</tr>
</tbody>
</table>

Notes: Results are means (+ 1 se); n= 3-5. Temperatures shown are daytime growth temperatures; growth CO₂ level was 370 ppm. Plants were 2.5 weeks old post-planting (5 wks for chenopodium and pigweed). Similar temperature effects were observed for shoot dry mass after 6 weeks of growth in separate experiments (not shown).
Table 2.3 Results from analysis-of-variance (F values) for treatment effects in each species at their species-specific optimal growth temperature, with time (duration of heat stress), CO$_2$, and their interactions as independent factors, and P$_n$, g$_s$, C$_i$, F$_v$/F$_m'$, and q$_p$ as dependent variables (* significance at $\alpha=0.05$, **significance at $\alpha=0.01$).

<table>
<thead>
<tr>
<th>Species:</th>
<th>Pea</th>
<th>Chenopodium</th>
<th>Wheat</th>
<th>Soybean</th>
<th>Sunflower</th>
<th>Tomato</th>
<th>Corn</th>
<th>Sorghum</th>
<th>Pigweed</th>
</tr>
</thead>
<tbody>
<tr>
<td>P$_n$:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time-</td>
<td>38.0**</td>
<td>101.2**</td>
<td>24.4**</td>
<td>11.9**</td>
<td>124.4**</td>
<td>2.9</td>
<td>143.6**</td>
<td>118.0**</td>
<td>99.1**</td>
</tr>
<tr>
<td>CO$_2$:</td>
<td>28.8**</td>
<td>16.4**</td>
<td>6.3*</td>
<td>29.6**</td>
<td>28.8**</td>
<td>16.6**</td>
<td>94.3**</td>
<td>38.1**</td>
<td>4.5*</td>
</tr>
<tr>
<td>TimexCO$_2$:</td>
<td>0.1</td>
<td>3.4</td>
<td>0.8</td>
<td>3.4</td>
<td>14.4**</td>
<td>5.6*</td>
<td>38.7**</td>
<td>24.2**</td>
<td>2.7</td>
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<tr>
<td>g$_s$:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time-</td>
<td>6.1**</td>
<td>5.1*</td>
<td>17.8**</td>
<td>6.8**</td>
<td>8.3**</td>
<td>13.9**</td>
<td>31.3**</td>
<td>5.1*</td>
<td>13.9**</td>
</tr>
<tr>
<td>CO$_2$:</td>
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<td>65.7**</td>
<td>12.4**</td>
<td>9.1**</td>
<td>5.7*</td>
<td>4.6*</td>
<td>16.2**</td>
<td>0.2</td>
<td>77.8**</td>
</tr>
<tr>
<td>TimexCO$_2$:</td>
<td>3.4</td>
<td>5.6*</td>
<td>1.3</td>
<td>3.0</td>
<td>0.2</td>
<td>1.3</td>
<td>7.2**</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>C$_i$:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time-</td>
<td>20.1**</td>
<td>15.2**</td>
<td>39.6**</td>
<td>10.8**</td>
<td>48.8**</td>
<td>13.2**</td>
<td>91.5**</td>
<td>252.0**</td>
<td>185.3**</td>
</tr>
<tr>
<td>CO$_2$:</td>
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<td>10.4**</td>
<td>229.8**</td>
<td>160.3**</td>
<td>391.2**</td>
<td>488.2**</td>
<td>1571.3**</td>
<td>906.6**</td>
<td>2.4</td>
</tr>
<tr>
<td>TimexCO$_2$:</td>
<td>7.4**</td>
<td>1.5</td>
<td>5.1*</td>
<td>11.0**</td>
<td>10.1**</td>
<td>0.7</td>
<td>45.9**</td>
<td>92.2**</td>
<td>1.9</td>
</tr>
<tr>
<td>F$_v$/F$_m'$:</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time-</td>
<td>100.9**</td>
<td>93.1**</td>
<td>17.4**</td>
<td>2.6</td>
<td>22.4**</td>
<td>4.5*</td>
<td>95.2**</td>
<td>11.7**</td>
<td>15.9**</td>
</tr>
<tr>
<td>CO$_2$:</td>
<td>0.1</td>
<td>11.5**</td>
<td>2.0</td>
<td>3.1</td>
<td>0.5</td>
<td>13.0**</td>
<td>39.8**</td>
<td>14.1**</td>
<td>7.6*</td>
</tr>
<tr>
<td>TimexCO$_2$:</td>
<td>0.6</td>
<td>2.8</td>
<td>0.6</td>
<td>1.1</td>
<td>0.5</td>
<td>2.2</td>
<td>20.1**</td>
<td>2.9</td>
<td>4.9*</td>
</tr>
<tr>
<td>q$_p$:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time-</td>
<td>1.9</td>
<td>269.9**</td>
<td>32.8**</td>
<td>11.2**</td>
<td>20.3**</td>
<td>0.0</td>
<td>15.5**</td>
<td>2.5</td>
<td>0.2</td>
</tr>
<tr>
<td>CO$_2$:</td>
<td>172.1**</td>
<td>9.7**</td>
<td>5.5*</td>
<td>1.9</td>
<td>0.0</td>
<td>2.1</td>
<td>14.9**</td>
<td>5.6*</td>
<td>0.3</td>
</tr>
<tr>
<td>TimexCO$_2$:</td>
<td>26.8**</td>
<td>8.2**</td>
<td>0.0</td>
<td>0.9</td>
<td>0.7</td>
<td>0.1</td>
<td>4.7**</td>
<td>2.3</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Notes: Similar results were obtained for pea, chenopodium, and wheat at 30 °C (not shown).
Table 2.4 Results from analysis-of-variance (F values) for treatment effects in CAM species at three growth temperatures, with time (for $F_{v}'/F_{m'}$, $q_p$) or heat (for pH), CO₂, and their interactions as independent factors, and $F_{v}'/F_{m'}$, $q_p$ and pH as dependent variables. Time = duration of heat stress (0, 1, or 4 hours of heat stress); heat = heat treatments (control, 4-h heat stress) harvested at the same time at end of the treatment period (* significance at $\alpha$=0.05, **significance at $\alpha$=0.01).

<table>
<thead>
<tr>
<th>Growth temperature</th>
<th>Agave</th>
<th>Cactus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25°C</td>
<td>30°C</td>
</tr>
<tr>
<td>$F_{v}'/F_{m'}$:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time-</td>
<td>0.1</td>
<td>7.8**</td>
</tr>
<tr>
<td>CO₂-</td>
<td>0.0</td>
<td>6.2*</td>
</tr>
<tr>
<td>Time×CO₂-</td>
<td>1.1</td>
<td>4.5*</td>
</tr>
<tr>
<td>$q_p$:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time-</td>
<td>8.4**</td>
<td>11.5**</td>
</tr>
<tr>
<td>CO₂-</td>
<td>0.8</td>
<td>4.3*</td>
</tr>
<tr>
<td>Time×CO₂-</td>
<td>0.7</td>
<td>5.1*</td>
</tr>
<tr>
<td>pH:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat-</td>
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<td>CO₂-</td>
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<td>0.0</td>
</tr>
<tr>
<td>Time×CO₂-</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Fig 2.1 Time course of net photosynthesis ($P_n$) in nine species grown at species-specific optimal daytime pre-stress growth temperatures (solid lines; $= 25^\circ$C in $C_3$ cool-season, $30^\circ$C in all other species), exposed to 370 (dark circles) or 700 ppm CO$_2$ (light circles), prior to and during a 4-hour heat-stress treatment. The dotted lines for the $C_3$ cool-season species are for plants grown at $30^\circ$C growth temperature. Each data point represents the mean ($\pm$SD) of four to six independent replicates.
Fig 2.2 Time course of stomatal conductance to water vapor ($g_{st}$) in nine species grown at species-specific optimal daytime pre-stress growth temperatures (solid lines; $= 25^\circ$C in C$_3$ cool-season, 30$^\circ$C in all other species), exposed to 370 (dark circles) or 700 ppm CO$_2$ (light circles), prior to and during a 4-hour heat-stress treatment. The dotted lines for the C$_3$ cool-season species are for plants grown at 30$^\circ$C growth temperature. Each data point represents the mean ($\pm$SD) of four to six independent replicates.
Fig 2.3 Time course of leaf internal CO$_2$ concentration (C$_i$) in nine species grown at species-specific optimal daytime pre-stress growth temperatures (solid lines; = 25°C in C$_3$ cool-season, 30°C in all other species), exposed to 370 (dark circles) or 700 ppm CO$_2$ (light circles), prior to and during a 4-hour heat-stress treatment. The dotted lines for the C$_3$ cool-season species are for plants grown at 30°C growth temperature. Each data point represents the mean (±SD) of four to six independent replicates.
Fig 2.4 Time course of photosystem II efficiency ($F_{v'}/F_{m'}$) in nine species grown at species-specific optimal daytime pre-stress growth temperatures (solid lines; $= 25^\circ$C in C$_3$ cool-season, $30^\circ$C in all other species), exposed to 370 (dark circles) or 700 ppm CO$_2$ (light circles), prior to and during a 4-hour heat-stress treatment. The dotted lines for the C$_3$ cool-season species are for plants grown at $30^\circ$C growth temperature. Each data point represents the mean ($\pm$SD) of four to six independent replicates.
Fig 2.5 Time course of photochemical quenching ($q_p$) in nine species grown at species-specific optimal daytime pre-stress growth temperatures (solid lines; = 25°C in C$_3$ cool-season, 30°C in all other species), exposed to 370 (dark circles) or 700 ppm CO$_2$ (light circles), prior to and during a 4-hour heat-stress treatment. The dotted lines for the C$_3$ cool-season species are for plants grown at 30°C growth temperature. Each data point represents the mean (±SD) of four to six independent replicates.
Fig 2.6 Heat stress effects on Fv'/Fm' and qP of CAM species (agave and cactus) grown at 25, 30, and 35°C daytime pre-stress growth temperatures, exposed to 370 (dark circles) or 700 ppm CO2 (light circles), prior to and during a 4-hour heat-stress treatment. Each data point represents the mean (±SD) of four to six independent replicates.
Fig 2.7 Heat stress effects on chlorenchyma pH of CAM species (agave and cactus) grown at 25, 30, and 35°C daytime pre-stress growth temperatures following a 4-hour heat-stress treatment (lc: 370 ppm CO₂, without heat stress; lh: 370 ppm CO₂, with heat stress; hc: 700 ppm CO₂, without heat stress; hh: 700 ppm CO₂, with heat stress). Each bar represents the mean (±SD) of 3-4 independent replicates. Different letters above the bars indicate a significant difference among treatments at the same temperature (P<0.05, according to Tukey HSD test).
Fig 2.8 Correlation analysis between $P_n$ and the other photosynthetic variables for $C_3$ and $C_4$ species separately (within each panel, data for all $C_3$ and $C_4$ species combined and not separated by $CO_2$ level). Panel A-D are results between $P_n$ vs. $g_{st}$, $C_i$, $F_v'/F_m'$, and $q_p$, respectively; panel -0 = results from controls, -1 = results after 1 hour of heat stress, and -2 = results after 4 hours of heat stress. $C_3$ species were shown by closed circles and $C_4$ species by open circles.
Abstract:

Determining interactive effects of elevated CO₂ and N on photosynthetic thermotolerance is necessary for predicting plant responses to global warming. I grew *Hordeum vulgare* (barley, C₃) and *Zea mays* (corn, C₄) at current or elevated CO₂ (370, 700 ppm) and limiting or optimal soil N (0.5, 7.5 mM). I then assessed basal and inducible thermotolerance of net photosynthesis (Pₙ), photosystem II efficiency (Fᵥ/Fₘ'), photochemical quenching (qₑ), carboxylation efficiency (CE), and rubisco activase content. I also assayed content of several major heat-shock proteins (HSPs), as HSPs are primary adaptations to heat stress and affected by N. For barley, thermotolerance of Pₙ, Fᵥ/Fₘ', and qₑ was decreased slightly by elevated CO₂ at low N, but increased slightly at high N. However, for corn, Pₙ, Fᵥ/Fₘ', and qₑ were decreased substantially by elevated CO₂ under high and low N. Negative effects of high CO₂ were associated with decreased CE, rubisco activase and HSPs (especially HSP70). These results indicate that stimulatory effects of elevated CO₂ at normal temperatures on photosynthesis and growth may be partly offset by negative effects during heat stress, especially for C₄ species and low-N conditions. Thus, CO₂ and N effects on photosynthetic thermotolerance may
contribute to changes in plant productivity, distribution, and diversity.

Abbreviations

CE, carboxylation efficiency; C_i, intercellular CO₂; F_v/F_m', photosystem II efficiency; g_s, stomatal conductance; HS, heat stress; HSPs, heat-shock proteins; PHS, pre-heat-stress; P_n, Net photosynthesis; q_p, photochemical quenching
3.1 Introduction

Global mean surface temperatures are projected to increase by 1.4-to-5 °C by 2100, which is thought to be caused largely by anthropogenic increases in atmospheric CO₂ (Houghton et al. 2001, IPCC 2007). In addition to mean increases in annual temperatures, there will also be increases in the frequency, duration, and severity of periods with exceptionally high temperatures (acute heat stress) (Wagner 1996, Haldimann and Feller 2004), which could have dramatic ecological, economic, and sociological impacts (Esterling et al. 2000, Diffenbaugh et al. 2005).

Acute heat stress adversely affects plant growth and survival, to a large extent, via negative effects on photosynthesis, which is thought to be among the most thermosensitive aspects of plant function (e.g., Berry and Björkman 1980, Weis and Berry 1988, Wise et al. 2004, Kim and Portis 2005). Both the light (electron transport) and dark (Calvin cycle) reactions of photosynthesis have thermolabile components, especially photosystem II (PSII) in the light reactions (Santarius 1975; Berry and Björkman 1980, Weis and Berry 1988, Heckathorn et al. 1998, 2002) and rubisco (ribulose 1, 5-bisphosphate carboxylase/oxygenase) activase in the Calvin cycle (Eckardt and Portis 1997, Crafts-Brandner and Salvucci 2002). However, increases in atmospheric levels of CO₂ can increase photosynthesis by decreasing photorespiration (fixation of O₂ rather than CO₂ by rubisco), which increases with temperature and is higher in C₃ than C₄ and CAM plants (Sage and Monson 1999, Taiz and Zeiger 2004); thus, elevated CO₂ might benefit C₃ plants more than C₄ plants during heat stress. Additionally, elevated CO₂ can also increase water-use efficiency, in part by decreasing stomatal conductance and transpiration (Ainsworth et al. 2002), which may increase acute-heat tolerance by
increasing plant water status. On the other hand, both C$_3$ and C$_4$ plants experience
reductions in stomatal conductance with increasing CO$_2$ (e.g., 20% for C$_3$ and 50% for C$_4$
species with a doubling of CO$_2$) (Sage 1994, Wand 1999, Reich et al. 2001, Maherali et
al. 2002), the lower average stomatal conductance of C$_4$ plants at any given CO$_2$ level
means lower average transpiration and thus higher leaf temperatures in C$_4$ plants, which
may increase heat-related damage in C$_4$ plants compared to C$_3$ plants in the same habitat.

Since global increases in temperature and CO$_2$ may have interactive effects on
photosynthesis, many studies have examined the effects of elevated CO$_2$ and increased
growth temperature (typically 3-5 °C) on photosynthesis (reviewed by Morison and
Lawlor 1999). In contrast, the effects of elevated CO$_2$, or the interactions between
elevated CO$_2$ and higher mean growth temperature, on plant responses to acute heat stress
have been examined in only a few studies, and the results have been variable and are not
fully understood. For example, elevated CO$_2$ has yielded positive (Faria et al. 1996, 1999,
Ferris et al. 1998, Huxman et al. 1998, Taub et al. 2000), negative (Bassow et al. 1994,
Roden and Ball 1996), and no effects (Coleman et al. 1991) on photosynthetic and plant
tolerance to acute heat stress. In the previous studies that compared elevated-CO$_2$ effects
on tolerance to acute heat stress in relatively heat-sensitive vs. tolerant species, or in
species with different photosynthetic pathways (Coleman et al. 1991, Bassow et al. 1994;
Roden and Ball 1996, Huxman et al. 1998, Taub et al. 2000), all species were grown
under identical thermal regimes, which were likely closer to optimal for some of the
species examined, but supra- or sub-optimal for others. Given that growth temperature is
known to strongly influence the response and tolerance of organisms and photosynthesis
to acute heat stress (e.g., Weis and Berry 1988, Barua and Heckathorn 2004),
comparisons of the heat-stress responses of species not grown at their respective optimal (or sub- or supra-optimal) growth temperatures may obscure response patterns that otherwise may be evident. So, in this study, I grew species in their optimal temperatures to understand how elevated CO₂ will impact thermotolerance to acute heat stress when they were grown at natural temperatures.

Furthermore, while the previous studies above have examined the impacts of elevated CO₂ on the basal tolerance of photosynthesis to acute heat stress, no studies have examined the influence of high CO₂ on inducible thermotolerance. Inducible (or acquired) thermotolerance, an increase in thermotolerance with a recent exposure to a moderate heat stress, is dependent to a large extent on the increased synthesis of heat-shock proteins (HSPs). HSPs are general stress proteins that protect cells from acute heat (and most other) stress, or facilitate recovery from heat-related damage (Vierling 1991, Heckathorn et al. 2002, Barua and Heckathorn 2003, Wang et al. 2004). HSPs play a prominent role in protecting photosynthesis from acute heat stress, and in inducible thermotolerance of photosynthesis (e.g., Heckathorn et al. 1998, 2002, Barua and Heckathorn 2003, and references therein), but synthesis of HSPs is known to be nitrogen costly (Heckathorn et al. 1996). The growth of plants in elevated CO₂ usually decreases tissue N concentrations (e.g., by an average of 14%; Cotrufo et al. 1998), though the effects of elevated CO₂ on N concentration depends on species and N conditions. Also, tissue C: N ratio often increases under elevated CO₂ (e.g., by an average of 15%; Gifford et al. 2000), though in some species this effect is small or absent (Zak et al. 1993). Consequently, I predict that HSPs production will often decrease at high CO₂, due to increased C:N and decreased %N, which will negatively affect cellular and
photosynthetic tolerance to acute heat stress, especially under low-N conditions.

In this study, two important crops, *Hordeum vulgare* (barley, C_3) and *Zea mays* (corn, C_4), were grown at species-specific optimal temperatures, current (370 ppm) or elevated CO_2 (700 ppm), and exposed to 0.5 (limiting) or 7.5 (optimal) mM soil N. The effects of these treatments on photosynthesis and HSPs during acute heat stress was examined to determine (1) whether the photosynthetic responses of C_3 and C_4 plants to acute heat stress are dependent on different CO_2 and N levels; (2) whether the responses are associated with photosynthetic electron transport (especially PSII) or rubisco/rubisco activase; (3) whether effects of CO_2 and N are associated with changes in HSPs production; and (4) whether effects of CO_2 and N are different for C_3 and C_4 species.
3.2 Methods

3.2.1 Plant material and growing conditions

*Hordeum vulgare* L. (barley, *C*₃) and *Zea mays* L. (corn, *C*₄) were germinated and grown in ambient or elevated CO₂ until the adult vegetative stage (8 weeks). Plants were grown in 5-L PVC pipes in a 1:1:1 mixture of top-soil, sand, and perlite, and placed in growth chambers (E-36HO, Percival Scientific, Iowa, USA) equipped with light, temperature, and CO₂ control. All the plants were grown under species-specific optimal (or near-optimal) temperatures (based on biomass and determined in pilot studies wherein each species was grown over a range of temperatures). Barley was grown at 28/22 °C and corn was grown at 32/26 °C for day/night, respectively. Plants were grown at either ambient (370±25 µmol mol⁻¹) or elevated (700±25 µmol mol⁻¹) CO₂, with a day length of 14 hours and a light level of 1000 µmoles m⁻² sec⁻¹ PAR (photosynthetically active radiation) at canopy height. Plants were fertilized twice per day with 50 ml of half-strength Hoagland nutrient solution containing either 7.5 mM or 0.5 mM N (NO₃⁻ : NH₄⁺ =7:1). These N levels were determined to be optimal and limiting respectively from a pilot experiment, where N ranged from 0.05 to 10 mM.

3.2.2 Heat-shock treatments

Heat-shock treatment was applied during vegetative growth, and all plants, within each species, were at similar stages of development (e.g., similar number of leaves). Prior to heat stress, plants in each chamber were randomly assigned to one of three groups (control = C, pre-heat-stress = PHS, and heat-stress = HS), with 9-10 plants in each group. The controls were not heat stressed and were measured on day 1 from 12:00 pm to 2:00 pm. The PHS plants were exposed to a moderate heat stress on day 1 for 4
hours (from 3:00 pm to 7:00 pm) at 8 °C above daytime growth temperatures. Such a pre-treatment is commonly used to induce a subsequent temporary increase in thermotolerance (referred to as inducible or acquired thermotolerance), and elicits the increased synthesis of heat-shock proteins (HSPs) (Kimpel et al. 1990, Vierling 1991). On day 2, all the plants except for the control group were heat stressed at 15 °C higher than daytime growth temperatures for 4 hours from 9:00 am to 1:00 pm. Chamber air temperatures in all treatments were monitored using Hobo Dataloggers and probes (Onset Computer Corporation, MA, USA). During the day, both heat-stressed and control plants were kept well-watered, keeping growth chamber humidity high, to limit occurrence of water stress during heat stress. Following heat stress, all the above and below-ground biomass was harvested and weighted.

3.2.3 Photosynthesis measurements

Steady-state net photosynthesis (Pn; net CO2 exchange) of leaves was measured with a portable photosynthesis system with infrared gas analyzer (model 6400, LiCOR, Lincoln, NE, USA), equipped with a 250-mm³ leaf chamber. Measurements were made within 1-2 min of insertion of leaves into the cuvette, and after stabilization of CO2 and H2O flux, to ensure that photosynthetic responses reflected those within the growth chambers. Photosynthesis was measured in unstressed control plants on day 1, and on PHS and HS plants during heat stress at 10:00 am and 1:00 pm on day 2, as described in Heckathorn et al. (1996), at the same CO2 levels as the plants were growing at (either 370 or 700 ppm CO2), a light level of 1000 µmol m⁻² s⁻¹ PAR (ca. = to that at the tops of the plants), and at species-specific growth temperatures. All results were collected from recently-expanded fully-lit leaves of intact plants.
To monitor heat-stress effects on photosynthetic CO₂ carboxylation, before and during heat stress, A-C_i (net assimilation rate vs. internal CO₂ concentration of leaves, where A=P_n) response curves were measured on three individuals from each treatment, with a light level of 1000 µmol m⁻² s⁻¹ PAR and the cuvette temperature held the same as species-specific optimal growth temperature. The measurements started at 400 ppm CO₂. Once steady-state photosynthesis was reached, the CO₂ concentration was gradually lowered to 50 ppm and then increased stepwise up to 1000 ppm. Net photosynthesis values were plotted against the respective internal leaf CO₂ concentrations (C_i) to produce an A-C_i response curve. The initial linear slope of the A-C_i curve represents carboxylation efficiency (CE) (Farquhar and Sharkey 1982).

3.2.4 Fluorescence measurements

To examine heat effects on PSII and post-PSII electron transport, PSII efficiency (F_v/F_m') and photochemical quenching (q_p) in light-adapted leaves were monitored by analysis of chlorophyll fluorescence using a pulse-amplitude-modulated (PAM) fluorometer (Model PAM 101/103; Walz, Germany). Chlorophyll fluorescence was measured in unstressed control plants on day 1, and on PHS and HS plants during heat stress at 10:00 am and 1:00 pm on day 2. Basal fluorescence (F_o) under steady-state illumination (900 µmol m⁻² s⁻¹ PAR) was measured initially, followed by maximum fluorescence (F_m') after a 1.0 s pulse of saturating white light (>5000 µmol m⁻² s⁻¹ PAR). Minimum fluorescence (F_o') was then measured after turning off both actinic and flash light-sources. F_v'/F_m' and q_p were then calculated as in Genty et al. (1989), where F_v'/F_m' = (F_m'-F_o')/ F_m', and q_p = (F_m'-F_o)/ (F_m'- F_o').
3.2.5 Immunoblot analysis of rubisco activase and HSPs

After 4h heat-stress treatment, all plants were harvested and tissue was frozen and stored at -70°C. Samples of equal fresh mass were ground with a mortar and pestle in liquid N₂. Then, samples were extracted in buffer containing 200 mM Tris-HCl (pH 8.0), 1% sodium dodecyl sulfate (SDS), 10 mM DTT, and 1 mM phenylmethylsulfonyl fluoride. Samples were boiled for 2 min and then centrifuged at 14,000 x g for 5 min, and the supernatant was collected and stored at -70°C. Aliquots with equal total soluble protein from each treatment were fractionated by SDS-PAGE (11.5% gels) (Heckathorn et al. 2002). For quantification of rubisco activase, HSP60, HSP70, and sHSP, proteins were transferred from SDS-PAGE gels to polyvinylidene fluoride (PVDF) membranes by western blotting (Towbin et al. 1979). Proteins bound to the PVDF membrane were probed with primary antibodies for rubisco activase (generously given by Dr. Archie Portis in University of Illinois at Urbana-Champaign) and HSPs (Preczewski et al. 2000) and then detected using secondary antibodies conjugated to alkaline-phosphatase and nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP). Relative protein content (normalized to a standard protein sample from control corn and barley leaves) on the immunoblots was quantified by Scion Image (Scion Corporation, USA).

3.2.6 Statistical analysis

All photosynthesis and fluorescence results were means from similarly-aged leaves of five different plants; CE was calculated by A-Ci curves generated from three independent plants. Separate sets of plants were used for control (C), heat-stressed (HS), and pre-heat-stressed (PHS) treatments. For gas-exchange and fluorescence results, measurements on HS and PHS plants were made after 1 and 4 h of heat treatment, and
data from both sampling times during heat stress were included in analyses. Analysis-of-
variance (ANOVA) was used to determine whether the physiological responses differed
as a function of treatments (JMP IN 5.1 software, SAS Institute, Cary, NC, USA).
Initially, three-way ANOVA [CO2 x N x heat treatment (HS vs. PHS)] was conducted for
Pn, Fv'/Fm', and qp within each species, and the results indicated significant N effects for
all three response variables in both species (P<0.0024 or less), and significant interactive
effects (P<0.05 or less) with CO2 and heat in all cases but two. Consequently, I then
conducted ANOVAs within each species x N combination, to simplify interpretation of
how N influenced CO2 x heat effects. To test for general heat effects, two-way ANOVA
was conducted wherein effects of heat treatment (C vs. HS + PHS) and CO2 and their
interaction were determined for Pn, gs, Ci, Fv'/Fm', qp, CE, and protein content (only
results for the heat effect, labeled HS, are shown in Table 1). To test for PHS/HS effects
and how heat effects were influenced by CO2, two-way ANOVA was conducted wherein
effects of pre-stress treatment (HS vs. PHS results only, no C data included) and CO2 and
their interaction were determined for each response variable (treatment effects are labeled
PHS, CO2, and CO2*PHS in Table 3.1).
3.3 Results

For both barley and corn, there was a significant positive effect of N on biomass ($P < 0.05$) (Fig 3.1). Elevated CO$_2$ increased shoot biomass only for barley at high N ($P = 0.046$), and increased root mass at low and high N ($P < 0.05$ for each). In contrast, elevated CO$_2$ did not significantly affect corn shoot biomass (root mass not available). Soluble protein concentration (per unit fresh mass) was significantly lower at low N for barley and corn and decreased with elevated CO$_2$ for barley and corn at low, but not high, N (data not shown).

Prior to heat stress, only in high-N barley was there a significant effect of growth in elevated CO$_2$ on $P_n$. Within 60-min of acute heat stress, significant decreases in $P_n$ were observed for both species at both N treatments (Fig 3.2, Table 3.1). Predictably, the proportional decrease in $P_n$ by heat stress varied somewhat among species, and increased with duration of heat stress in corn only. The decreases in $P_n$ during heat stress were not caused by stomatal closure, as indicated by no change, or even increases, in leaf intercellular CO$_2$ concentration ($C_i$) and stomatal conductance ($g_s$) for barley and corn during heat stress (Table 3.2); also, effects on $P_n$ were not related to differences in leaf temperature during heat stress, as leaf temperature was $41.4 \pm 1.3$ SD and $41.7 \pm 1.5$ SD $^\circ$C, respectively for ambient-CO$_2$ and elevated-CO$_2$ grown barley and $45.9 \pm 1.1$ SD and $45.7 \pm 1.3$ SD $^\circ$C for ambient-CO$_2$ and elevated-CO$_2$ grown corn, respectively. Thus, decreases in $P_n$ during heat stress were related to effects on photosynthetic metabolism in mesophyll cells.

For barley with high-N treatment, plants grown at elevated CO$_2$ had slightly but significantly higher $P_n$ than those grown at ambient CO$_2$ during heat stress. For barley
with low-N treatment, plants at elevated CO₂ had slightly but significantly lower Pₙ than those at ambient CO₂ during heat stress. In both low- and high-N corn, Pₙ was lower for elevated-CO₂-grown plants than ambient-CO₂-grown plants during heat stress in both HS and PHS plants. In addition, for barley at both N treatments, there was significant acclimation of Pₙ in PHS plants; in other words, Pₙ was higher in most cases in PHS plants compared to HS plants (evident when comparing left and right panels within each species in Fig 3.2), this was true only for high-CO₂ corn.

In contrast to Pₙ, prior to the initiation of the acute heat stress, Fᵥ/Fₘ′ was not significantly different in plants grown at elevated vs. ambient CO₂ (Fig 3.3). Fᵥ/Fₘ′ declined significantly in both species after the temperature treatment was applied; however, there were species-, CO₂-, and N-treatment-specific patterns to this change in Fᵥ/Fₘ′ (Table 3.1). For barley with high-N treatment, plants grown at elevated CO₂ had a significantly higher Fᵥ/Fₘ′ than those grown at ambient CO₂ during heat stress. But for barley with low N treatment, plants grown at elevated CO₂ had a lower Fᵥ/Fₘ′ than those grown at ambient CO₂ during heat stress. For corn with both low- and high-N treatment, Fᵥ/Fₘ′ was always lower in elevated CO₂-grown plants than in ambient CO₂-grown plants. In addition, in both barley and corn, there was acclimation of Fᵥ/Fₘ′ when plants were pre-heat-stressed (left vs. right panels) (Fig 3.3), and CO₂ effects were similar for HS and PHS plants.

Similar patterns to Fᵥ/Fₘ′ were found for qₚ (Fig 3.4). Heat stress significantly decreased qₚ in both species at both N treatments, which means that the capacity of both species to keep PSII reaction centers in an open configuration was decreased by heat stress, indicating heat-stress effects on post-PSII electron transport. For barley with high-
N treatment, plants grown at elevated CO2 had a slightly but significantly higher qP than those grown at ambient CO2 during heat stress. But for barley with low-N treatment, plants grown at elevated CO2 had a lower qP than those grown at ambient CO2 during heat stress. For corn with both low-and high-N treatment, in comparison with the plants grown at ambient CO2, qP was always lower in plants grown at elevated CO2, and CO2 effects were similar for HS and PHS plants. No significant acclimation of qP was observed for either species at either N treatments.

Carboxylation efficiency (CE) was higher for plants grown at ambient CO2 than those at elevated CO2 for low-N barley and high-N corn (barley, \( P=0.043 \); corn, \( P=0.027 \)) before the initiation of heat stress (Fig 3.5). CE was significantly decreased by heat stress for both species at both N treatments (Table 3.1). During heat stress, CE was slightly higher for plants grown at elevated CO2 than those at ambient CO2 for barley treated with high N. In contrast, for barley treated with low N and corn treated with both high and low N, CE was lower for plants grown at elevated CO2 than those at ambient CO2 (Fig 3.5). Significant acclimation of CE was only found for corn treated with high N (Table 3.1). And CO2 effects overall were similar for HS and PHS plants.

Immunoblot analysis of rubisco activase disclosed the presence of a single band in leaf extracts before heat stress but two activase polypeptides (41 and 43 KDa) after heat stress for both species. Heat stress treatment resulted in a reduction of the total content of soluble rubisco activase for both species at low N and barley at high N (Fig. 3.6, Table 3.1). During heat stress, the content of rubisco activase was always lower for plants grown at elevated CO2 than those at ambient CO2 for both species at both N treatments, though for barley at low N, the difference was not significant (Table 3.1). Thus, since
rubisco activase levels were not affected by CO₂ prior to stress, heat stress decreased soluble rubisco activase level, more under elevated CO₂ (decreases in soluble rubisco activase could result from either decreases in total activase content or increases in the fraction of heat-denatured insoluble activase). There was also a significant acclimation of rubisco activase content for barley treated with high N when plants were pre-heat-stressed, and a decrease in activase for PHS plants of corn at high N.

For barley at high N, only the content of small HSPs was affected by heat stress, CO₂ or pre-heat-stress (Fig 3.7, Table 3.1). But for barley at low N, heat stress significantly increased the content of HSP60, HSP70, and sHSP, and plants grown at elevated CO₂ had a significantly lower content of HSP60 and HSP70 than those grown at ambient CO₂ (Fig 3.7). The content of sHSP in low-N barley was also reduced by elevated CO₂ in HS plants. Pre-heat-stress had a positive effect only on sHSP for barley at both high and low N. For corn at both high and low N, heat stress increased the content of all HSPs, excluding HSP70 at high-N, and HSP60 at low N (Fig 3.8, Table 3.1). For corn at high N, during heat stress, CO₂ negatively affected production of HSP60 and HSP70 in PHS plants, and sHSP only in HS plants. In contrast, for corn at low N, the production of HSP60, HSP70, and sHSP was negatively impacted by growing at elevated CO₂, though for HSP60 and sHSP, the effects were not statistically significant. Pre-heat-stress had a positive effect on the production of HSP60 and sHSP in low-N plants; no pre-heat-stress effect was observed in high-N plants.
3.4 Discussion

For barley (C₃), the tolerance of photosynthesis (i.e., $P_n$, CE, $q_p$, and $F_v/F_m$) to acute heat stress was negatively impacted by elevated CO₂ at low N, but positively affected by high CO₂ at high N; these effects, though sometimes relatively small, were nonetheless statistically significant. In contrast, for corn (C₄), the tolerance of photosynthesis was severely negatively impacted by growth at elevated CO₂ under both high and low N. The same general effects of elevated CO₂ on photosynthesis during heat stress were observed in both pre-heat-stressed plants and plants experiencing their first heat stress. The differential effects of elevated CO₂ on photosynthesis during heat stress between C₃ barley and C₄ corn and between high-N and low-N barley were not the results of differences in photorespiration. Though photorespiration is low in corn and high in barley, and $P_n$ was typically higher within each barley plant when measured at 700 vs. 400 $\mu$mol/mol CO₂, the differential effects of elevated CO₂ during heat stress in low- vs. high-N barley were observed for electron transport ($q_p$ and $F_v/F_m$) and CE, as well as for $P_n$ (measured at a near-ambient CO₂ level of 400 $\mu$mol/mol). As CE is determined from $P_n$ vs. $C_i$ measurements at photosynthetically-limiting CO₂ levels, differential CO₂ effects during heat stress for low- vs. high-N barley on CE, as with electron transport, suggest that these differences in low- and high-N plants were due to effects of N on other aspects of metabolism, rather than on photorespiration (i.e., the rate of photorespiration relative $P_n$ was not different in low-N vs. high-N plants). In support of this, the same N x CO₂ effects during heat stress were observed for $P_n$ in barley when measured at saturating CO₂ (i.e., when photorespiration was negligible) (not shown), and soluble rubisco activase content decreased more during heat stress in high-CO₂ than low-CO₂ plants in both
species. Interestingly, elevated CO$_2$ decreased HSP production in both species and at both N levels (though the specific HSPs affected varied, averaged across HSPs, content always decreased with high CO$_2$). HSPs protect photosynthesis during acute heat stress (e.g., Heckathorn et al. 1998, 2002; Preczewski et al. 2000; Barua et al. 2003), and HSP production is known to be N costly and decreases with plant N status (Heckathorn et al. 1996). Collectively, these results suggest that growth of both C$_3$ and C$_4$ plants in elevated CO$_2$ can decrease general cellular adaptation to high temperatures, likely because these adaptations are often N costly and high CO$_2$ decreases plant N concentration, and this results in greater damage to cells during acute heat stress.

As noted above, the negative effects of elevated CO$_2$ on thermotolerance of photosynthesis were evident for both light and dark reactions. The efficiency of PSII electron transport by oxidized (open) PSII reaction centers in the light ($F'_v/F'_m$), and the fraction of open PSII reaction centers ($q_p$), determined by post-PSII electron transport, were reduced to a greater extent in plants (low- and high-N corn, low-N barley) grown at elevated CO$_2$ as compared to plants grown in ambient CO$_2$ conditions, which means that elevated CO$_2$ may enhance photoinhibition at high temperatures. The dark reactions of photosynthesis were also negatively affected by elevated CO$_2$ during heat stress, as carboxylation efficiency (CE) was always lower during heat stress for plants grown at elevated CO$_2$, except for barley grown at high N. Decreased CE during heat stress most likely would be caused by decreased activity of rubisco, rubisco activase, or PEP carboxylase (in C$_4$ plants), or by decreased levels of these enzymes, at elevated CO$_2$, or other Calvin cycle enzymes that are known to be heat sensitive (Berry and Björkman 1980). Recent studies have shown that rubisco activase is especially heat sensitive.
Rubisco activase promotes the activation of rubisco by facilitating the disassociation of sugar phosphates from either decarbamylated sites containing RuBP or analogs of RuBP, or cabamylated sites containing analogs of RuBP or the reaction intermediate (Crafts-Brandner and Salvucci 2000). Rubisco activase is more heat susceptible than rubisco (Eckardt and Portis 1997; Crafts-Brandner and Salvucci 2002) and can be significantly impaired by elevated CO₂, because elevated CO₂ can decrease the ratio of ATP:ADP (Streusand & Portis 1987). In this study, rubisco activase content was reduced more by heat stress in elevated CO₂ than in ambient CO₂ for both species at both N treatments, which may have contributed to reduced CE during heat stress at elevated CO₂ in low- and high-N corn and low-N barley, but not in high-N barley, wherein CE increased with high CO₂. I do not know whether this decrease in soluble rubisco activase content occurred because of decreases in total activase or increases in heat-damaged insoluble activase, but either possibility result in a decrease in functional activase content.

Given that photorespiration increases with temperature, and that C₃ species have higher levels of photorespiration compared to C₄ species (Sage and Monson 1999, Taiz and Zeiger 2004), then the benefits of elevated CO₂ to C₃ plants during heat stress should outweigh any other negative effects until such a point that rubisco activity is decreased or that damage to electron transport becomes limiting to net photosynthesis. This prediction is supported by experiments with heat-stressed plants grown at low or high CO₂ and sub-optimal, optimal, or supra-optimal temperatures, wherein the relative benefit of elevated CO₂ to Pₙ during acute heat stress decreased (or even disappeared) in cool-season C₃ species (e.g., Pisum sativum, pea) (Wang et al. 2008; Hamilton et al. 2008). In CAM species, elevated CO₂ increased photosynthesis during heat stress in plants grown at sub-
or near-optimal pre-heat-stress temperatures, but high CO₂ decreased photosynthesis in plants grown at supra-optimal temperatures, and high CO₂ generally decreased photosynthesis in C₄ species during heat stress regardless of growth temperatures.

It is likely that due to different experimental regimes of growing temperature or heat-treatment temperature in past studies, the effects of elevated CO₂ on plant responses to acute heat stress have revealed no patterns, including with respect to C₃ vs. C₄ plants. For example, elevated CO₂ enhanced photosynthesis in Yucca whipplei at high temperature (up to 53°C), which was accompanied by increases in the quantum yield of photosystem II at high temperatures in elevated CO₂ (Huxman et al. 1998). A notable difference between the current study and Huxman et al. (1998) is the magnitude and duration of the temperature stress imposed upon the plants (I subjected plants to a 4-h treatment at 40-50°C, depending on species, whereas Huxman et al. subjected plants to a maximum daily temperature of 55°C for 9 d). However, growth in elevated CO₂ was found to protect cork-oak leaves from high temperature, resulting in lower decreases in photosynthetic rates, as well as in the efficiency of excitation energy captured by open photosystem II reaction centers (F₀/Fₘ), than in plants grown at ambient CO₂ (Faria et al. 1996). In contrast, well-watered Eucalyptus seedlings grown in elevated CO₂ had lower quantum efficiencies (F₀/Fₘ) than seedlings grown in ambient CO₂ during high-temperature stress (Roden and Ball 1996). None of the above studies had different N treatments. The previous study with both CO₂ and N treatments reported little relationship between plant nitrogen status and the ability of plants to tolerate acute heat stress (Coleman et al. 1991), and only two past studies included C₄ or CAM species (Coleman et al. 1991, Huxman et al. 1998), with no pattern among photosynthetic types
There have been several past studies that have examined heat, CO₂, N or heat x N effects on photosynthesis or growth in barley and corn, and results from these studies are comparable to the current study, indicating that CO₂ x N x heat effects in the current study are not anomalous. For example, effects of low and high N on Pn rates in corn are comparable in this study and those of Lu and Zhang (2000), Makino et al. (2003), and Heckathorn et al. (1996), and heat x N effects on Pn or electron transport here were similar to those in Lu and Zhang (2000; only effects on electron transport presented) and Heckathorn et al. (1996; both Pn and electron transport). Krall and Edwards examined heat effects on photosynthesis in corn, and though their heat-treatment methods were very different than this study, Pn and electron transport values for controls and heat-treated leaves were similar between this past study and the current one. In the case of barley, Sicher and Bunce (2008) obtained similar effects of elevated CO₂ on growth and photosynthesis, and similar values for Pn (given their higher light levels during measurement), compared to this study, and Sicher (1999), also observed decreases in Photosystem II activity in plants grown under elevated CO₂.

Though photosynthesis is very sensitive to acute heat stress, plants are able to increase photosynthetic thermotolerance following recent exposure to severe acute heat stress (termed acquired or induced thermotolerance) or longer exposures to moderate chronic higher temperatures (acclimation) (Weis and Berry 1988, Vierling 1991). Tolerance to acute heat stress and acquired thermotolerance both result, to a large extent, from increased levels of heat-shock proteins (HSPs) (Wang et al. 2004), including for photosynthesis (e.g., Heckathorn et al. 1998, 2002, and references therein), as HSPs help
protect cellular components from damage during heat stress, and facilitate their repair afterwards (Wang et al. 2004). Importantly, HSP production is N costly and increases with plant N status (Heckathorn et al. 1996). Consequently, I had predicted apriori that elevated CO₂ would decrease HSP levels during heat stress and that this would have negative effects on photosynthetic thermotolerance. However, effects of CO₂ on HSPs and correlations between CO₂-related changes in HSPs and photosynthesis during heat stress were dependent on species and CO₂ and N treatments. For example, at high N, elevated CO₂ affected HSP levels in corn but not barley, yet effects of elevated CO₂ on the heat tolerance of photosynthesis for high-N barley and corn (positive and negative, respectively) were not consistently related to HSP levels during heat stress. In contrast, at low N, decreased photosynthetic thermotolerance with elevated CO₂ was accompanied by decreases in HSPs in both corn and barley. Further, these HSP patterns were similar for plants receiving their first heat stress and pre-heat-stressed plants experiencing their second heat stress.

Of course, HSPs are but one of many cellular adaptations to acute heat stress in plants that contribute to the protection of photosynthesis during high temperatures, and these other adaptations may also be affected by elevated CO₂, which might offset, obscure, or add-to CO₂-related changes in HSPs. To illustrate, in high-N plants in this study, effects of CO₂ on photosynthetic thermotolerance were unrelated to changes in HSP levels during heat stress; thus, the CO₂ effects were necessarily caused by other CO₂ responses, which remain to be elucidated. For example, increases in lipid saturation level (Larkindale and Huang 2004), carotenoid pigment zeaxanthin (Havaux 1998), protective compatible solutes (Williams et al. 1992), and isoprene production (Velikova and Loreto
were found to be related to increased thermotolerance. Williams et al. (1998) found that growth at elevated CO₂ increased saturation of some classes of thylakoid lipids. Both increases and decreases in isoprene emission have been reported for plants grown under elevated CO₂ (Sharky et al. 1991, Togenetti et al. 1998). Growth at elevated CO₂ affects profound alterations in cellular and subcellular concentrations of many soluble compounds (Poorter et al. 1997). These experiments were conducted either under ambient CO₂ or under elevated CO₂ but without heat stress. Prior to this study, there were no reports on the effects of elevated CO₂ on HSP production. Further experiments are needed to supply direct evidence of an association between lipid changes, protective solutes, isoprene, HSPs and thermotolerance at elevated CO₂.

To conclude, I have shown that increases in atmospheric CO₂ will alter plant photosynthetic responses to acute heat stress, and that the effect of CO₂ on thermotolerance will likely vary with photosynthetic pathway and N treatment. Effects of CO₂ on photosynthetic thermotolerance were evident for both light (Fᵥ/Fm' and qₚ) and dark reactions (carboxylation efficiency and rubisco activase), each of which may contain thermolabile steps that may limit net photosynthesis during acute heat stress. Additionally, CO₂ effects on HSP levels tended to mirror effects on photosynthesis at low, but not high, N, indicating that CO₂ effects on photosynthetic thermotolerance may be mediated only partly by effects on HSPs, and effects of CO₂ are likely due to changes in other cellular adaptations to acute heat stress too. Given that the frequency, duration, and severity of heat stress will increase for plants in the coming decades (Wagner 1996, Haldimann and Feller 2004), and that photosynthesis is relatively heat sensitive (Weis and Berry 1988), these results indicate that interactions between elevated CO₂ and plant
thermotolerance may contribute to future changes in plant productivity, distribution, and diversity associated with global environmental change. Specifically, my results indicate that increases in atmospheric CO₂ and acute heat stress in combination may further tip the balance towards C₃ species, beyond what high CO₂ alone might do, which may contribute to increases in C₃ vegetation, both globally and in communities containing a mix of C₃ and C₄ species. However, the benefits of elevated CO₂ to C₃ species during acute heat stress at near-optimal growth temperatures may be offset by expected increases in mean growth temperatures, or by changes in other environmental factors which influence thermotolerance (e.g., water or ozone)-possibilities which I am investigating at present.
References


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Phytologist 150: 435-448.


Table 3.1 Summarized results from statistical analyses (analysis-of-variance, ANOVA). Overall heat-stress effects (HS) are shown from an ANOVA comparing unstressed controls vs. PHS + HS plants (heat-treated plants that did or did not receive a pre-heat treatment the previous day, respectively). Effects of CO₂ during heat stress are shown from an ANOVA comparing PHS vs. HS heat treatments (factor labeled as PHS), ambient vs. elevated CO₂ (factor labeled as CO₂), and their interactions as independent factors. Significant F-statistics are shown (*significance at α=0.1, **significance at α=0.05, ***significance at α=0.01); non-significant values were omitted.
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Table 3.2 Stomatal conductance ($g_s$) and internal [CO$_2$] ($C_i$) in barley and corn grown at
low or high N levels and 370 or 700 ppm CO$_2$, prior to (control) and during a 4-h heat-
stress treatment (1 h and 4 h); heat-treated plants did or did not receive a pre-heat-
treatment the previous day (PHS or HS, respectively). Each data point represents the
mean ($\pm 1$ SD) of five independent replicates.

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<td>4 h</td>
<td>0.46 (0.07)</td>
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81
Fig 3.1 Aboveground biomass of barley and corn and belowground biomass for barley, with either low or high N treatment, grown at 370 (□) or 700 ppm CO2 (■). Results are from control plants. Each data point represents the mean (±SD) of five independent replicates.
Fig 3.2 Time course of net photosynthesis ($P_n$) in barley and corn, with either low or high N treatment, grown at 370 (○) or 700 ppm CO₂ (●), prior to and during a 4-hour heat-stress treatment. The left panels contain results from plants being heat-stressed directly, while results in the right panels are from plants having been given a pre-heat-stress. Each data point represents the mean (±SD) of five independent replicates.
Fig 3.3 Time course of photosystem II efficiency (Fv'/Fm') in barley and corn, with either low or high N treatment, grown at 370 (○) or 700 ppm CO₂ (●), prior to and during a 4-hour heat-stress treatment. The left panels contain results from plants being heat-stressed directly, while results in the right panels are from plants having been given a pre-heat-stress. Each data point represents the mean (±SD) of five independent replicates.
Fig 3.4 Time course of photochemical quenching ($q_p$) in barley and corn, with either low or high N treatment, grown at 370 (○) or 700 ppm CO$_2$ (●), prior to and during a 4-hour heat-stress treatment. The left panels contain results from plants being heat-stressed directly, while results in the right panels are from plants having been given a pre-heat-stress. Each data point represents the mean (±SD) of five independent replicates.
Fig 3.5 Carboxylation efficiency (CE) in barley and corn, with either low or high N treatment, grown at 370 (□) or 700 ppm CO₂ (■). Results are from plants without heat stress (CON), with 1-h heat stress (HS), and with 1-h heat stress treatment following 4-h pre-heat-stress treatment (PHS). The value of each column represents the mean (±SD) of three independent replicates.
Fig 3.6 The content of rubisco activase in barley and corn, with either low or high N treatment, grown at 370 (□) or 700 ppm CO₂ (■). Rubisco activase was measured on plants without (CON), with 1-h heat stress (HS), and with 1-h heat stress treatment following 4-h pre-heat-stress treatment (PHS). The value of each column represents the mean (±SD) of five independent replicates.
Fig 3.7 The content of HSP60, HSP70 and sHSP in barley, with either low or high N treatment, grown at 370 (□) or 700 ppm CO₂ (■). Protein contents were measured on plants without (CON), with 1-h heat stress (HS), and with 1-h heat stress treatment following 4-h pre-heat-stress treatment (PHS). The value of each column represents the mean (±SD) of five independent replicates.
Fig 3.8 The content of HSP60, HSP70 and sHSP in corn, with either low or high N treatment, grown at 370 (□) or 700 ppm CO₂ (■). Protein contents were measured on plants without (CON), with 1-h heat stress (HS), and with 1-h heat stress treatment following 4-h pre-heat-stress treatment (PHS). The value of each column represents the mean (±SD) of five independent replicates.
Chapter 4

A meta-analysis of plant physiological and growth responses to elevated CO₂ under different global warming scenarios

Abstract

1) Atmospheric carbon dioxide (CO₂) and global mean temperature are expected to be significantly higher by the end of the 21st century. Elevated CO₂ and higher temperature have been shown to affect plant physiology and growth, but their interactive effects have not been reviewed statistically with consideration for higher mean temperatures and acute heat stress.

2) In this meta-analysis, I examined the effects of CO₂ on a number of physiological and growth variables in plants subjected to different temperature treatments. Carbon dioxide treatments were categorized into ambient (<400 μmol mol⁻¹) or elevated (>560 μmol mol⁻¹) levels, while temperature treatments were categorized into ambient (AT), elevated (ET; AT + 1.4-6°C), or heat stress (HS; AT + >8°C). Plant species were grouped according to photosynthetic pathways (C₃ and C₄ species) and functional types (legumes and non-legumes).

3) My results show that elevated CO₂ had no effect on net photosynthesis (A) under HS, but enhanced A at AT and ET. C₃ and C₄ plants responded differently to
increases in CO₂ and temperature. In C₄ plants, elevated CO₂ increased A by 8.2% at AT and 23.3% at ET, but reduced A by 27.4% at HS. In C₃ plants, elevated CO₂ enhanced A by 11.6, 19.9, and 31.5% at AT, ET and HS, respectively. It was also found that elevated CO₂ increased A by a greater magnitude in legumes than in non-legumes at HS, suggesting that N status might be important in affecting plant tolerance for HS under elevated CO₂. Total plant biomass (Wₚ) increased by 42.5% at AT, 36.7% at ET and 25.9% at HS for C₃ species, but no CO₂ effect was observed for C₄ species, regardless of temperature treatments.

4) Overall, my results demonstrate that elevated CO₂ affects plant physiology and growth to varying degrees under different temperature regimes. These findings have important implications for biomass accumulation and ecosystem functioning in the future when CO₂ is higher and climate extremes, e.g., heat waves, become more frequent.

Keywords: global environmental change; elevated CO₂; temperature; heat stress; meta-analysis; biomass; photosynthesis.
Abbreviations

A, net CO₂ assimilation rate per unit area; AT, ambient temperature; ET, elevated temperature; Fv/Fm, photosystem II (PSII) efficiency; gₛ, stomatal conductance; HS, heat stress; Nₐₐ, shoot N concentration; Nₕₕ, root N concentration; RA: Rubisco activity; Wₜₜ, total plant biomass; Wₐₜₜ, above-ground biomass; Wₕₕ, below-ground biomass.
4.1 Introduction

As a consequence of human activities, global atmospheric carbon dioxide (CO$_2$) and temperature, key variables affecting plant growth, development, and function, have changed in the recent past and are predicted to change in the future. Increases in atmospheric CO$_2$ and other greenhouse gases are largely responsible for recent increases in global mean surface temperatures, which rose by 0.6$^\circ$C from 1990 to 2000 and are projected to increase by another 1.4-5.8$^\circ$C by year 2100 (Houghton et al. 2001; IPCC 2007). In addition to rising mean annual temperatures, there will also be increases in the frequency, duration, and severity of periods with exceptionally high temperatures (Haldimann & Feller 2004; Wagner 1996). Thus, plants in the future will not only be exposed to higher CO$_2$, but will also likely experience more acute heat stress, which can greatly impact ecosystem productivity (Ciais et al. 2005b) and biodiversity (Davis 1986; Thomas et al. 2004).

The interactive effects of elevated CO$_2$ and temperature on the physiology and growth in a large number of plant species have been investigated. Since net CO$_2$ assimilation rate per unit area (A) is affected by the ratio of CO$_2$ and O$_2$ as they compete for carbon fixation and photorespiration at the active site of the enzyme ribulose bisphosphate carboxylase-oxygenase (Rubisco), increased CO$_2$ reduces C loss through photorespiration. However, elevated temperature increases photorespiration due to reduced solubility of CO$_2$ compared with O$_2$ and reduced specificity of Rubisco for CO$_2$ at higher temperatures. It has therefore been predicted that photosynthetic response to increased CO$_2$ in plants with C$_3$ metabolism will be larger at higher temperatures (Bowes et al. 1996; Gifford 1995; Long 1991). In contrast, it has been generally considered that
C₄ species will show little CO₂ stimulation irrespective of temperature because of the CO₂ concentration mechanism in C₄ species. Recent studies, however, have shown substantial stimulation of both net photosynthesis and biomass in C₄ species (Ghannoum et al. 2000; Ziska 2001; Ziska & Bunce 1997). Therefore, the response of C₄ species to the interactive effect of temperature and CO₂ requires further examination.

Although a large number of empirical studies have examined the interactive effects of elevated CO₂ and elevated growth temperature (ET) (typically 3-5°C) on photosynthesis (reviewed by Morison and Lawlor 1999), the interactions between elevated CO₂ and heat stress (HS; typically over 8°C above normal growth temperature) have been investigated in only a limited number of studies. The results have been variable and have not been reviewed statistically. One of the negative effects of HS on plants is the damage to photosynthesis, often by impairing the photosystem II (PSII) in light (electron transport) (Berry & Bjorkman 1980; Heckathorn et al. 1998; Heckathorn et al. 2002) and rubisco activase in the Calvin-cycle dark reactions (Crafts-Brandner & Law 2000; Crafts-Brandner & Salvucci 2000; Eckardt & Portis 1997). Unlike at mild temperature increase in which CO₂ has consistently positive effect on plants, elevated CO₂ could have positive (Faria et al. 1999; Faria et al. 1996; Ferris et al. 1998; Hamerlynck et al. 2000; Huxman et al. 1998; Taub et al. 2000), negative (Bassow et al. 1994; Huxman et al. 1998; Roden & Ball 1996b; Taub et al. 2000), or no effects (Coleman et al. 1991) on plant photosynthesis and growth at HS. Due to the contrasting effect of CO₂ at ET and HS, it is critical to examine the role of CO₂ under different temperature treatments and for different functional groups to better understand plant responses to multiple environmental changes in the future.
In addition to temperature treatments and species groups, treatment-duration and growth facility also affect CO₂ responses. In the short term, the CO₂ fixation may be stimulated by elevated CO₂; however, in the long term, this effect may be partly lost as a result of down-regulation in photosynthesis (Gunderson & Wullschleger 1994). This down-regulation of photosynthesis might be associated with an over-production of assimilates relative to sink demand and may, therefore, be associated with an accumulation of soluble sugars in the leaves (VanOosten & Besford 1996) or decreased Rubisco activity and production caused by decreased N concentration (Luo et al. 2004). Therefore, in this meta-analysis, reports were categorized into groups based on treatment-duration and growth facility. Key physiological variables, such as Rubisco activity, and N concentration, were analyzed based on different temperature treatments and species groups.

In order to assess the effects of elevated CO₂ and temperature on plant biomass and physiological performance, I conducted a comprehensive meta-analysis of synthesized CO₂ studies published before the end of 2008 in which plant photosynthesis and growth was reported at different temperature and CO₂ treatments. In this meta-analysis, I summarized and interpreted 413 observations of CO₂ effects on plant physiology and growth under different temperature treatments extracted from 73 separate publications (Appendix).

There are two major objectives in this meta-analysis: (1) to provide estimates of the magnitude and significance of elevated CO₂ effects on plant biomass accumulation and partitioning, gas exchange, PSII, stomatal conductance, Rubisco activity and nitrogen concentration under different global warming scenarios; (2) to test for differences among
plant functional groups and growth forms in affecting these responses. I hypothesized that the enhancement of biomass and net photosynthesis by elevated CO₂ would be less pronounced at HS than at AT or ET, which might be associated with similar effects on PSII, gs, and Rubisco activity. I also hypothesized that C₄ species would have a smaller enhancement than C₃ species in biomass and net photosynthesis in response to elevated CO₂ at all temperature treatments due to CO₂-concentrating mechanisms in C₄ plants. I further hypothesized that legume species would have greater enhancement than non-legumes, especially at HS.
4.2 Methods

4.2.1 Data collection

Peer-reviewed journal articles used in building the database for this meta-analysis were obtained by searching the Science Citation Index (SCI) of the Institute of Scientific Information. The list of articles obtained were subsequently cross-checked with references cited in a large number of CO2 review articles and books to ensure inclusion of all articles that have relevant data for this meta-analysis. Any article published in English before the end of 2006 that met all of the following criteria was included: (1) ambient CO2 treatment < 400µmol mol\(^{-1}\) and elevated CO2 treatment between 500 and 1000 µmol mol\(^{-1}\); (2) plants were treated with ambient temperature (AT), either elevated temperature (ET) or heat stress (HS) at both ambient and elevated CO2; and (3) entire plants exposed to CO2 and temperature treatments for the majority of their life cycles. In these studies from which I collected data, the CO2 treatment typically included ambient (360 µmol mol\(^{-1}\)) and twice ambient (700 µmol mol\(^{-1}\)) CO2 concentrations, with additional levels occasionally included (Appendix). On average, the CO2 levels in µmol mol\(^{-1}\) were 364 and 701 at AT, 361 and 690 at ET, 364 and 710 at HS, for ambient and elevated CO2 treatments, respectively. Response variables extracted from these articles include net photosynthesis (A), Photosystem II efficiency (F\(_{v}/F_{m}\)), stomatal conductance (g\(_{s}\)), Rubisco activity (RA), above-ground (W\(_{AG}\)), below-ground (W\(_{BG}\)) and total (W\(_{T}\)) biomass, and nitrogen concentration (%N). For multi-year studies on annual species, results from different seasons were considered independent and all observations were included in this analysis. For multi-year studies on perennial species, only the studies with the longest CO2 exposure were included. If a study included more than one species,
all the observations were considered independent and included in the database. If a study examined the interactive effects of CO₂ and non-temperature stress factors, only the measurements from the non-stressed experiments, e.g., low ozone or well-watered plants, were included. In all, 72 publications that reported plant physiological and growth responses to elevated CO₂ and temperature were included in my database (see Appendix). From these publications, 413 observations were extracted for this meta-analysis.

4.2.2 Categorization of studies

For this analysis, temperature treatments were categorized into three levels: ambient temperature (AT), elevated temperature (ET; 1.4-6°C above ambient), and heat stress (HS; > 8°C above ambient). Originally, the temperature categories used in this meta-analysis were intended to be categorized into AT, ET (AT+1.4-to-5.8°C), as predicted by IPCC (2001, 2007) and then into ET (AT+1.4-6°C) and HS (AT+>8°C) since <6°C and >8°C plus AT were used frequently in the reference papers. Thus, the temperature categories were essentially arbitrary and should not be construed as defining fixed boundaries to the CO₂ exposure. Plant species were categorized based on photosynthetic pathway (C₃ or C₄), and N-fixing ability (legumes or non-legumes). Research facilities used to raise CO₂ concentration were divided into two broad classes: (1) semi-open systems, which include open top chambers (OTC) and screen-aided CO₂; and (2) closed systems, which include greenhouses and growth chambers. Pot size was grouped into < 10 L, > 10 L, or in-ground. These size classes have been previously used in similar meta-analytic review (Curtis 1996; Wand et al. 1999).

4.2.3 Meta-analytical methods

This meta-analysis followed the techniques described in the work of Curtis and
Wang (1998). I used the natural logarithm-transformed ratio (\(\ln r\)) of plant responses at elevated (\(X_{e}\)) to ambient (\(X_{a}\)) CO\(_2\) to estimate effect size of CO\(_2\) treatment (Hedges et al. 1999). In order to include the large percentage of studies that did not adequately report sample sizes and variances, I performed unweighted analysis using the statistical software MetaWin 2.0 (Rosenberg et al. 2000). A mixed-effects model was used in my analysis with the assumption that there were random variations in effect sizes among the diverse studies included in this synthesis. Consequently, the confidence intervals generated are larger than those of a fixed-effect model, and can represent more conservative interpretations. Confidence intervals (CI) for effect-size estimates were generated by bootstrapping the unweighted data using MetaWin 2.0 with a resampling of 9,999 iterations. Elevated CO\(_2\) was considered to have a significant effect on a variable if the bootstrap CIs of its percentage changes did not overlap zero. Response to elevated CO\(_2\) was considered significantly different between temperature treatments if their CIs did not overlap. Significance was established at \(P < 0.05\) unless otherwise noted.
4.3 Results

Across all plant taxa and environmental conditions synthesized in this analysis, elevated CO₂ impacted A, Fᵥ/Fₘ, gₛ, and RA by different magnitudes at different temperature treatments (Fig 4.1). Elevated CO₂ increased A at AT (11.8%) and ET (18.0%), but not at HS (-2.0%) (Fig 4.1a). In contrast, Fᵥ/Fₘ was unaffected by elevated CO₂ at AT or ET, but was decreased at HS (-4.3%) ($P<0.05$; Fig 4.1b). Elevated CO₂ trended towards decreasing gₛ and RA at all temperature treatments, though there were no significant differences (Fig 4.1c, d).

Responses of $A$, $Fᵥ/Fₘ$, and $gₛ$ to elevated CO₂ varied between C₃ and C₄ species at AT, ET, and HS (Fig 4.2). For C₃ species, elevated CO₂ increased A by 11.6, 19.9, and 31.5% at AT, ET, and HS, respectively ($P<0.05$; Fig 4.2a). However, for C₄ species, elevated CO₂ increased A only at AT and ET by 8.2 and 23.3%, respectively, but had a negative effect on A (-27.9%) at HS ($P<0.01$). Elevated CO₂ had no significant effect on $Fᵥ/Fₘ$ at AT or ET, regardless of photosynthetic pathway (Fig 4.2b). However, at HS, $Fᵥ/Fₘ$ was decreased by elevated CO₂ for C₃ and C₄ species by -3.2 and -7.4%, respectively ($P<0.01$; Fig 4.2b). For C₃ species, $gₛ$ was decreased by elevated CO₂ at all temperature treatments, although the difference was not significant among plants exposed to different temperatures. For C₄ species, elevated CO₂ decreased $gₛ$ by -37.6 and -10.2% at AT and HS ($P<0.05$; Fig 4.2c).

Responses of $A$, $Fᵥ/Fₘ$, and $gₛ$ to elevated CO₂ also differed in legumes and non-legumes at AT, ET, and HS levels (Fig 4.3). For legumes, elevated CO₂ increased A at AT, ET, and HS levels by 9.8, 22.3, and 57.2%, respectively ($P<0.01$). However, for non-legumes, elevated CO₂ increased A only by 12.8, 19.2, and 4.4% (Fig 4.3a). Elevated
CO₂ did not significantly alter F₇₀/F₉₀ at ET for legume and non-legumes species (Fig 4.3b). At AT, F₇₀/F₉₀ was decreased by elevated CO₂ for non-legumes by -1.3%, but increased by 2.9% for legume species (P<0.05). At HS, however, elevated CO₂ decreased F₇₀/F₉₀ for legumes and non-legumes by -2.2 and -4.6%, respectively. For both legumes and non-legumes, gₛ was decreased by elevated CO₂ at AT, ET, and HS levels. For legumes, elevated CO₂ decreased gₛ by -34.0, -41.7 and -35.8% at AT, ET, and HS, respectively, but the difference was not significant (Fig 4.3c). For non-legumes, elevated CO₂ decreased gₛ by -27.3, -15.7, and -20.7% at AT, ET, and HS, respectively (Fig 4.3c).

In addition to photosynthetic physiology, N concentration was also affected by CO₂ differently at the different temperature treatments (Fig 4.4). Nₐₕ was decreased more by elevated CO₂ at HS (-15.2%) than at AT (-6.3%) or ET (-6.3%) (Fig 4.4a). In contrast, Nₖ was not impacted significantly by elevated CO₂ at ET and HS due to large variance, but was decreased at AT (-7.0%) (Fig 4.4b). Nₐₕ was decreased by elevated CO₂ for both C₃ and C₄ species at all temperature levels (Fig 4.5). For example, elevated CO₂ decreased Nₐₕ of C₃ species by -5.2, -5.7 and -16.5% at AT, ET, and HS levels, respectively. Nₐₕ of C₄ species tended to decrease under elevated CO₂ by -8.9, -13.0, and -13.4% at AT, ET, and HS, respectively, though the effect was not significant due to large variance (Fig 4.5a). Nₖ of C₃ species was decreased by elevated CO₂ by -3.1% at AT, but was increased by 8.5 and 8.7% at ET and HS, though the effect was not significant due to small sample size, however, Nₖ of C₄ species was decreased by -12.7 and -7.1% at AT and HS (Fig 4.5b).

Enhancement of Wₜ by elevated CO₂ was greater at AT (38.4%) and ET (37.0%) than at HS (25.2%), but the difference was not significant (Fig 4.6a). Elevated CO₂
increased $W_{AG}$ and $W_{BG}$ to a similar extent at AT, ET, and HS. For example, $W_{AG}$ was
stimulated 26.1, 22.8, 25.3% (Fig 4.6b), and $W_{BG}$ was stimulated 28.2, 24.7, and 28.2%
by elevated CO$_2$ at AT, ET, and HS, respectively (Fig 4.6c).

The response of plant growth to elevated CO$_2$ also differed in C$_3$ and C$_4$ species
(Fig 4.7). Enhancement of $W_T$ by elevated CO$_2$ in C$_3$ species decreased from 42.5% at
AT, to 36.7% at ET, and to 25.9% at HS, but the difference was not significant, while $W_T$
of C$_4$ species was not impacted by elevated CO$_2$ significantly at AT, ET, and HS due to
large variance (Fig 4.7a). Elevated CO$_2$ increased $W_{AG}$ of C$_3$ species by 33.8, 24.9, and
29.9% at AT, ET, and HS, respectively (Fig 4.7b). In C$_4$ species, elevated CO$_2$
significantly increased $W_{AG}$ at ET, but not at AT or HS (Fig 4.7b). Similar to $W_{AG}$,
elevated CO$_2$ increased $W_{BG}$ of C$_3$ species by 35.5, 23.3, and 19.2% at AT, ET, and HS,
respectively (Fig 4.7c). $W_{BG}$ of C$_4$ species was decreased by elevated CO$_2$ by -35.5% at
AT, but was increased by 36.7 and 47.0% at ET and HS, though the effect was not
significant (Fig 4.7c).

The response of plant growth to elevated CO$_2$ also varied in legume and non-
legume species (Fig 4.8). $W_T$ enhancement by elevated CO$_2$ in legume species decreased
from 38.7% at AT and 48.4% at ET, to 23.9% at HS, while $W_T$ of non-legumes species
was increased by elevated CO$_2$ by 37.7, 30.6, and 26.0% at AT, ET, and HS, respectively
(Fig 4.8a). Elevated CO$_2$ increased $W_{AG}$ of legume species by 42.8, 26.3, and 29.7% at
AT, ET, and HS, respectively, but the difference was not significant (Fig 4.8b). In non-
legume species, elevated CO$_2$ increased $W_{AG}$ by 18.4, 20.8, and 31.5% at AT, ET, and
HS (Fig 4.8b). Similar to $W_{AG}$, elevated CO$_2$ increased $W_{BG}$ of legume species by 54.6,
22.3, and 16.2% at AT, ET, and HS, respectively, but the difference was not significant
(Fig 4.8c). $W_{BG}$ of non-legume species was increased by elevated CO$_2$ by 12.0, 27.1, and 38.7% at AT, ET, and HS, respectively.
4.4 Discussion

In this meta-analysis, in contrast to the prediction that high temperature stress is expected to be alleviated by elevated CO₂ due to the improved photosynthesis under hot conditions (Long 1991), net photosynthesis remained unchanged by elevated CO₂ at HS. However, net photosynthesis (A) was enhanced by elevated CO₂ at AT and more at ET, in agreement with many previous studies (Long 1991; Morison & Lawlor 1999) (Fig 4.1a). Plants with different photosynthetic pathways responded differently to elevated CO₂ and temperature. Net photosynthesis was enhanced by elevated CO₂ at all temperatures, and greatest at HS for C₃ species. For C₄ species, elevated CO₂ had no effect at AT, positive effect at ET (Wand et al. 1999), and a negative effect at HS (Fig 4.2a), which contributed to an overall smaller effect of elevated CO₂ under heat stress (Fig 4.1a). In addition to no benefit from decreased photorespiration because C₄ species can avoid photorespiration and are CO₂-saturated at current CO₂, the negative effect of elevated CO₂ on net photosynthesis for C₄ species might be correlated to higher leaf temperature caused by decreased stomatal conductance (Wang et al. 2008). In this meta-analysis, I found decreased stomatal conductance (gs) at elevated CO₂ for both C₃ and C₄ or legume and non-legume species, regardless of different temperatures. These results are consistent with those from other reviews (Ainsworth & Long 2005; Ainsworth & Rogers 2007; Long et al. 2004). However, when I separated species into woody and herbaceous species, gs was not significantly decreased by CO₂ at all temperatures (data not shown) for woody species, which was consistent with other reviews (Saxe et al. 1998) in which no significant decreases in gs were found in trees, particular woody coniferous trees.

It was hypothesized that at supraoptimal temperature, electron transport capacity
or rubisco activase capacity is the limitation on photosynthesis at elevated CO2 (Sage & Kubien 2007). In this analysis, Photosystem II efficiency (Fv/Fm) was negatively affected by elevated CO2 at HS, regardless of photosynthetic pathways or ability for N2 fixation, indicating that elevated CO2 might enhance photoinhibition at higher temperatures (Roden & Ball 1996a). Rubiso activity was negatively affected by elevated CO2 at all temperature treatments (Fig 4.1d). This photosynthetic acclimation often closely matches the reduction in nitrogen content and increases in soluble carbohydrates (Korner 2006; Reich et al. 2006; Rustad 2006). Decreased RA could be caused by reduced Rubisco content or by decreased specificity of Rubisco (Aranjuelo et al. 2005; Gutschick 2007; Korner 2006). At HS, decreased RA might be caused by damage to rubisco activase, on which the activation of rubisco is dependent, because rubisco activase is more heat susceptible than rubisco (Crafts-Brandner & Salvucci 2000; Eckardt & Portis 1997) and can be significantly impaired by elevated CO2 because elevated CO2 can cause a shift in the ratio of ATP to a lower-energy compound called adenosine diphosphate, or ADP, which then leads to a reduction in activase activity (Streusand & Portis 1987). However, these responses may be more important for short-term regulation of photosynthesis; and in the long term, the reductions in RA are more correlated with reductions in Rubisco content (Moore et al. 1999; Stitt & Krapp 1999).

It has been well documented that availability of mineral nutrients, particularly N, can greatly modify plant growth responses to elevated CO2 (Diaz et al. 1993; Hebeisen et al. 1997; Luo et al. 2004; Reich et al. 2006). The negative effect of elevated CO2 on photosynthesis for C4 species at HS, including Fv/Fm and RA, might also be related to decreased nitrogen concentration, which could result in impaired synthesis of
photosynthetic enzymes and protectant systems (Aranjuelo et al. 2005; Bunce 2000). In this study, I found that nitrogen concentration in shoots, but not in roots, decreased at all temperatures by elevated CO₂, and was greatest at HS (Fig 4.4). I further tested this hypothesis by separating plants into legumes and non-legumes, on which elevated CO₂ imposed similar effects at AT and ET. But at HS, elevated CO₂ had significantly positive effect on legumes but no effect on non-legumes (Fig 4.3a), which indirectly suggested shoot N concentration might play a role in plants tolerating HS at elevated CO₂. However, the reduced N concentration could not explain the negative effect of elevated CO₂ on C₄ species, but positive effects on C₃ species at HS, as shown by decreased N concentration only for C₃ species in Fig 5a. In this meta-analysis, due to the smaller sample sizes for HS, no specific mechanisms can be generalized about the effects of elevated CO₂ on C₃ and C₄ species in extreme high temperatures at this stage.

The smaller enhancement on A in response to elevated CO₂ at HS might contribute to the smaller enhancement on Wₜ (Fig 4.6a). But it is also important to note that A per unit leaf area is not the most important factor for predicting overall plant growth (Korner 1991). The combination of carbohydrate production, which is determined by photosynthetic rate and leaf area, and the consumption of carbohydrates for growth, respiration, storage, root exudation accounted most for the overall growth (Morison & Lawlor 1999). In addition, the effect on aboveground and belowground biomass did not differ with temperature treatments and functional groups significantly (Fig 4.6, 4.7 and 4.8). The negative effect of elevated CO₂ on A for C₄ species at HS might be offset by the effect of elevated CO₂ on leaf area, respiration, water use efficiency (Hamerlynck et al. 1997; Owensby et al. 1993; Owensby et al. 1999) or the imbalance between source
(photosynthesis) and sink (growth). It has been found that increased CO₂ could reduce respiration (Bruhn et al. 2002; Bunce 2005; Gonzalez-Meller et al. 2004). Reduced stomatal conductance at high CO₂ will slow the rate of transpiration and therefore decrease the onset, severity and impact of water stress, which is often accompanied by high temperature stress (Morison 1993). The stimulation of leaf area, in particular, appears to be characteristic of the CO₂-response of C₄ species (Ackerly et al. 1992; Ghannoum et al. 2000; Wolfe et al. 1998). More experiments are needed to assess the balance of these processes and the extent and direction of acclimation of these processes to temperature and CO₂ interaction.

It is essential that potential confounding factors be considered in a meta-analysis, which synthesizes results from a large number of studies that were conducted under a variety of growing conditions on different plant species. In this analysis, I excluded studies in which plants were grown under environmental stresses, e.g., drought, low nutrients, light deficiency or elevated ozone. Great variation in CO₂ effects on unstressed plants, however, was still found, due to differences in experimental protocols and temperature regimes in the empirical studies. I found that length of CO₂ exposure was an important factor affecting plants responses to temperature changes. For example, photosynthetic enhancement by CO₂ enrichment in plants exposed to elevated CO₂ for < 30 days was over two times greater than for those that were exposed to elevated CO₂ for > 30 days at AT. At ET, photosynthesis was enhanced by 24.7% at elevated CO₂ when CO₂ exposure was less than one year and by 16.3% when CO₂ exposure was more than one year. At HS, elevated CO₂ increased photosynthesis by 10.4% when exposure was < 30 days and 24.5% when exposure was > 30 days. This temporal pattern suggests a
down-regulation of photosynthesis relative to initial capacity at high CO$_2$ at AT or ET, and is consistent with a model of sink regulation of photosynthesis over these time intervals under unstressed conditions (Aranjuelo et al. 2005). Another issue of concern about the effects of elevated CO$_2$ has been how much the effects are influenced by particular cultural or exposure systems used (Schulze & Mooney 1994). The exposure systems used in these studies in this meta-analysis mostly were closed system, including greenhouses and growth chambers. My study, however, found no significant effect of exposure systems on photosynthetic responses to CO$_2$ or temperature. Pot size was thought to be another important factor affecting the magnitude of A responses to elevated CO$_2$, by implicating root sink strength (Arp 1991). At AT, no elevated CO$_2$ effect was found when plants were grown in pots smaller than 10L and there was a greater CO$_2$ effect (24.1%) for plants grown in ground than in pots larger than 10 L (9.98%). At ET, A was enhanced by elevated CO$_2$ by 22.66% for plants grown in ground and 8.24% for plants grown in pots bigger than 10 L. At HS, no elevated CO$_2$ effect was found when plants were grown in pots smaller than 10L and there was a greater CO$_2$ effect (25.1%) for plants grown in ground than in pots bigger than 10 L (6.59%). Though the results were consistent with Arp (1991) in that no effect was found when plants were grown in pots smaller than 10 L, special caution should be given on how much the pot size/plant size ratio was and how much nutrients were flushed through the pots (Korner 2003).

A third potential confounding factor that I considered in my meta-analysis is the biased choice of species in the empirical studies and hence unbalanced composition of species in different categories in the analysis. For example, C$_4$ species, which are all non-legumes, might account for lower photosynthetic and growth responses to elevated CO$_2$.
in non-legumes, since $C_3$ species used in this meta-analysis included a number of legumes. I excluded all $C_4$ species from the non-legumes and performed another analysis on plant species that were all $C_3$ species. The results showed that at AT and ET, photosynthetic responses to elevated CO$_2$ were similar for legumes and non-legumes. But at HS, elevated CO$_2$ had a more pronounced effect (46.5%, $n = 15$) for legumes than for non-legumes (17.3%; $n=38$). These results demonstrate that inclusion of $C_4$ species did not obscure the comparison between non-legumes and legumes. Different growth forms might also differ in their responses to elevated CO$_2$ and temperature. I thus partitioned species into woody and herbaceous species (data not shown). I found that A of herbaceous species was significantly enhanced by elevated CO$_2$ at AT (12.5%, $n=68$), ET (17.2%, $n=69$), and HS (16.4%, $n=62$), but there were no significant differences among temperature treatments. For woody species, however, significant CO$_2$ effect was found only at ET (32.6%, $n=18$), presumably due to the smaller sample size for woody species. The finding of higher responsiveness to elevated CO$_2$ in woody than in herbaceous species at ET might due to the inclusion of the more responsive juvenile and exponentially-growing trees in many CO$_2$ studies (Wang 2007).
4.5 Conclusions

In summary, I found significant interactive effects of elevated CO$_2$ and temperature on a number of plant physiological and growth variables. The most surprising result from this meta-analysis was that at HS, elevated CO$_2$ had negative effect on photosynthesis for C$_4$ species, compared with positive effect for C$_3$ species. Compared with legumes, non-legumes showed less enhancement of photosynthesis in response to elevated CO$_2$, especially at HS, suggesting that the effects of elevated CO$_2$ was dependent on N status. In contrast to photosynthesis, W$_T$ responded positively to elevated CO$_2$ in C$_4$ species at all temperatures. The negative effects of elevated CO$_2$ to C$_4$ species on photosynthesis under HS may be alleviated by higher water-use efficiency of C$_4$ species at both the leaf and whole-plant level, especially in times with water stress; and the benefits of elevated CO$_2$ to C$_3$ species may be offset by likely changes in other environmental factors which influence thermotolerance differentially (e.g., changes in precipitation which might increase or decrease tolerance and increasing ozone which might decrease tolerance). My meta-analysis highlights the importance of improving mechanistic understanding of plant responses to the interactive effects of elevated CO$_2$ and other abiotic factors, particularly higher temperature, increased N deposition and altered pattern of precipitation in the future.
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## Appendix

Species, photosynthetic and functional types, experimental growth conditions (EC), for studies used in the analysis. GC, growth chamber; GH, greenhouse; G, ground; TGT, temperature gradient tunnel; AM, ambient temperature.

<table>
<thead>
<tr>
<th>Species</th>
<th>Photosynthetic and functional types</th>
<th>EC</th>
<th>Pot size (L)</th>
<th>Duration **</th>
<th>$\text{CO}_2$ (µmol mol$^{-1}$)</th>
<th>Temperature (°C)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Abutilon theophrasti</em></td>
<td>C3; herbaceous; non-legume</td>
<td>GC</td>
<td>0.5</td>
<td>26 d</td>
<td>400</td>
<td>400</td>
<td>AM+14-23</td>
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<tr>
<td><em>Sinapis alba</em></td>
<td>C3; herbaceous; non-legume</td>
<td>GC</td>
<td>1</td>
<td>80 d</td>
<td>400</td>
<td>700</td>
<td>AM+3</td>
</tr>
<tr>
<td><em>Amaranthus retroflexus</em></td>
<td>C3; herbaceous; non-legume</td>
<td>GC</td>
<td>&lt; 1</td>
<td>2 y</td>
<td>360</td>
<td>700</td>
<td>AM+1.5</td>
</tr>
<tr>
<td><em>Arachis glabrata</em></td>
<td>C3; herbaceous; legume</td>
<td>GH (TGT)</td>
<td>217 d</td>
<td>365</td>
<td>640</td>
<td>AM+1.5</td>
<td>Fritschi et al. 1999a</td>
</tr>
<tr>
<td><em>Paspalum notatum</em></td>
<td>C3; herbaceous; non-legume</td>
<td>GH (TGT)</td>
<td>217 d</td>
<td>365</td>
<td>640</td>
<td>AM+1.5</td>
<td>Fritschi et al. 1999b</td>
</tr>
<tr>
<td><em>Arachis hypogaea</em></td>
<td>C3; herbaceous; legume</td>
<td>GC</td>
<td>&gt; 10</td>
<td>4 m</td>
<td>350</td>
<td>700</td>
<td>32/22, 36/26, 40/30, 44/34</td>
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<td>360</td>
<td>720</td>
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<td>400</td>
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<td>370 700</td>
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<td>4 h 14 h</td>
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<td>700</td>
<td>25 45</td>
<td>Faria et al. 1996</td>
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<td>GC 10</td>
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Notes: * the first number indicated CO2 exposure duration and the second indicated temperature treatment duration; only one number was given if CO2 exposure time was equal to temperature treatment time; y, year; m, month; d, day; w, weeks; h, hours
Fig 4.1 Responses to elevated CO$_2$ in net photosynthetic rate (A), photosystem II efficiency ($F_v/F_m$), stomatal conductance ($g_s$) and Rubisco activity (RA) for plants grown under ambient temperature (AT), elevated temperature (ET), or heat stress (HS). ET = AM + 1.4-6 °C; HS = heat stress (AM + >8 °C). Each point is mean ± 95% confidence interval (CI). The number of observations for each category used in the analysis is shown under the corresponding CI. ** $P<0.01$ for comparison between temperature treatment categories whose CIs do not overlap.
Fig 4.2 Photosynthetic response to elevated CO₂ in C₃ and C₄ species at different temperature treatments. AT = ambient temperature; ET = elevated temperature (AM + 1.4-6 °C); HS = heat stress (AM + >8 °C). a, net photosynthetic rate (A); b, photosystem II efficiency (Fᵥ/Fₘ); c, stomatal conductance (gs). Each point is mean ± 95% confidence interval (CI). The number of observations for each category used in the analysis is shown under the corresponding CI. *P<0.05 and ** P<0.01 for comparison between temperature treatment categories whose CIs do not overlap.
Fig 4.3 Photosynthetic response to elevated CO$_2$ in legumes and non-legumes at different temperature treatment. AT = ambient temperature; ET = elevated temperature (AM + 1.4-6 °C); HS = heat stress (AM + >8 °C). a, net photosynthetic rate (A); b, photosystem II efficiency ($F_v/F_m$); c, stomatal conductance ($g_s$). Each point is mean ± 95% confidence interval (CI). The number of observations for each category used in the analysis is shown under the corresponding CI. *P<0.05 and ** P<0.01 for comparison between temperature treatment categories whose CIs do not overlap.
Fig 4.4 Responses of nitrogen concentration to elevated CO$_2$ for species at different temperature treatments. AT = ambient temperature; ET = elevated temperature (AM + 1.4-6 °C); HS = heat stress (AM + >8 °C). a, above-ground N concentration ($N_{AG}$); b, below-ground N concentration ($N_{BG}$). Each point is mean ± 95% confidence interval (CI). The number of observations for each category used in the analysis is shown under the corresponding CI.
Fig 4.5 Response of nitrogen concentration to elevated CO₂ in C₃ and C₄ species at different temperature treatment. AT = ambient temperature; ET = elevated temperature (AM + 1.4-6 °C); HS = heat stress (AM + >8 °C). a, above-ground N concentration (Nₐ₉G); b, below-ground N concentration (Nₜ₉G). Each point is mean ± 95% confidence interval (CI). The number of observations for each category used in the analysis is shown under the corresponding CI.
Fig 4.6 Biomass response to elevated CO₂ for species at different temperature treatment. AT = ambient temperature; ET = elevated temperature (AM + 1.4-6 °C); HS = heat stress (AM + >8 °C). a, whole biomass (WT); b, above-ground biomass (W_A); c, below-ground biomass (W_BG). Each data point is mean ± 95% confidence interval (CI). The number of observations for each category used in the analysis is shown under the corresponding CI.
Fig 4.7 Photosynthetic response to elevated CO₂ between C₃ and C₄ species at different temperature treatment. AT = ambient temperature; ET = elevated temperature (AM + 1.4-6 °C); HS = heat stress (AM + >8 °C). a, whole biomass (WT); b, above-ground biomass (WAG); c, below-ground biomass (WBG). Each point is mean ± 95% confidence interval (CI). The number of observations for each category used in the analysis is shown under the corresponding CI.
Fig 4.8 Photosynthetic response to elevated CO₂ between legumes and non-legumes at different temperature treatment: AT = ambient temperature; ET = elevated temperature (AM + 1.4-6 °C); HS = heat stress (AM + >8 °C). a, whole biomass (WT); b, above-ground biomass (WAG); c, below-ground biomass (WBG). Each point is mean ± 95% confidence interval (CI). The number of observations for each category used in the analysis is shown under the corresponding CI.
Chapter 5

Effects of N on plant response to heat-wave: a field study with prairie vegetation

Abstract

More intense, more frequent, and longer heat-waves are expected in the future due to global warming, which could have dramatic ecological impacts. Increasing nitrogen (N) availability and its dynamics will likely impact plant responses to heat stress and carbon (C) sequestration in terrestrial ecosystems. This field study examined the effects of N availability on plant response to heat-stress (HS) treatment in naturally-occurring vegetation. HS (5 days at ambient or 40.5 °C) and N treatments (± N) were applied to 16 1m² plots in restored prairie vegetation dominated by *Andropogon gerardii* (warm-season C₄ grass) and *Solidago canadensis* (warm-season C₃ forb). Before, during, and after HS, air, canopy, and soil temperature were monitored; net CO₂ assimilation (Pₙ), quantum yield of photosystem II (Φₚₛᵢᵢ), stomatal conductance (gₛ), and leaf water potential (Ψᵊ) of the dominant species and soil respiration (Rₛₒᵢᵢ) of each plot were measured daily during HS. One week after HS, plots were harvested, and C% and N% were determined for rhizosphere and bulk soil, and above-ground tissue (green/senescent leaf, stem, and flower). Photosynthetic N-use efficiency (PNUE) and N resorption rate (NRR) were calculated. HS decreased Pₙ, gₛ, Ψᵊ, and PNUE for both species, and +N treatment generally increased these variables (±HS), but often slowed their post-HS
recovery. Aboveground biomass tended to decrease with HS in both species (and for green leaf mass in *S. canadensis*), but decrease with +N for *A. gerardii* and increase with +N for *S. canadensis*. For *A. gerardii*, HS tended to decrease N% in green tissues with +N, while in *S. canadensis*, HS increased N% in green leaves. Added N decreased NRR for *A. gerardii* and HS increased NRR for *S. canadensis*. These results suggest that heat waves, though transient, could have significant effects on plants, communities, and ecosystem N cycling, and N can influence the effect of heat waves.

Key words: heat stress, global climate change, N resorption rate, photosynthesis, photosynthetic N-use efficiency.
Abbreviations

$C_g$: C\% of green leaf; $C_s$: C\% of senescent leaf; $C_{st}$, C\% of stem; $C_f$, C\% of flower; $N_g$: N\% of green leaf; $N_s$: N\% of senescent leaf; $N_{st}$, N\% of stem; $N_f$, N\% of flower; $g_s$, stomatal conductance to water vapor; HS, heat stress; $\Psi_w$, leaf water potential; NRR, nitrogen resorption rate; $P_n$, net photosynthesis; PNUE, photosynthetic N-use efficiency; $\Phi_{PSII}$, quantum yield of electron transport of photoystem II; SLA, specific leaf area, $R_{soil}$, soil respiration; $W_a$, aboveground biomass; $W_f$, biomass of flowers; $W_g$, biomass of green leaf; $W_s$, biomass of senescent leaf; $W_{st}$, biomass of stem.
5.1 Introduction

Global mean surface temperatures have risen by 0.6 °C from 1900 to 2000, mainly caused by increases in atmospheric CO$_2$ and other greenhouse gases, and are projected to increase by another 1.4-5.8 °C by year 2100 (Houghton et al. 2001; IPCC 2007). In addition to rising mean annual temperatures, there will also be increases in the frequency, duration, and severity of periods with exceptionally high temperatures (Wagner 1996). An increased trend in the frequency of extreme heat stress events has been reported in various parts of the world (Gaffen & Ross 1998; Gruza & Ran'kova 1999; Henderson & Muller 1997; Yan 2002). Thus, plants in the future will not only be exposed to higher mean temperatures, but will also likely experience more frequent heat stress, which can greatly impact ecosystem productivity (Ciais et al. 2005a) and biodiversity (Thomas et al. 2004). An extreme stress event is an episode in which the acclimatory capacities of an organism are substantially exceeded (Gutschick & BassiriRad 2003). Extreme events, in spite of their ephemeral nature, can cause shifts in the structure of plant communities. The environmental impacts from extreme events can be significantly greater than those associated with mean increases (Karl et al. 1997).

In addition to temperatures, human activities are increasing global N availability (IPCC 2007). N availability is likely to affect plant, community, and ecosystem responses to increasing heat stress, which will then impact ecosystem C sequestration. Understanding effects of N on the responses of vegetation to heat stress requires insight into how stress physiology and community structure interact. While the influence of plant N status on response to acute heat stress has been previously examined, past studies have largely focused on laboratory experiments examining physiological responses.
(Heckathorn et al. 1996a, b; Lu & Zhang 2000). Further, because of the difficulties of imposing heat stress on naturally-occurring vegetation, little experimental work has been conducted on response to acute heat stress in field-grown plants (Morison & Lawlor 1999; Weis & Berry 1987). To date, there have been only a handful of studies in which plant communities were exposed to extreme high temperatures, and these focused on community processes (e.g., recolonization, competition, invasion, and the role of species richness during extreme events) and were conducted on grassland (Van Peer et al. 2004; White et al. 2001) or arctic species (Marchand et al. 2006; Marchand et al. 2005). Also, N availability had significant effects on plant N-relations responses to moderate warming (rather than acute heat stress) in a tallgrass prairie (An et al. 2005). Thus, little is known as to how heat stress in general, and N interactions with heat stress in particular, will affect natural plant communities. In this study, I concentrate on physiological and growth responses of two dominant warm-season tall-grass prairie species with contrasting photosynthetic pathways (a C4 grass and a C3 forb) in experimental field plots receiving heat and N treatments.

C4 species typically have higher temperature optima for photosynthesis than C3 species (Sage & Monson 1999) as a consequence of lower photorespiration, which increases with temperature. This may contribute to greater tolerance to heat waves for C4 species than co-occurring C3 species (Coleman and Bazzaz 1992, Ehleringer et al. 1997, Wang et al. 2008). Thus, heat stress can potentially affect the relative distribution of C4 and C3 species. In natural systems, the significance of climate warming for C4 vegetation can depend less on the mean increase in global temperature, and more on the spatial and temporal variation of the temperature increase (Sage & Kubien 2003). In New Zealand,
for example, episodic heat events inhibit $C_3$ plants more than $C_4$ grasses, and as a result, facilitate $C_4$ grass invasion of $C_3$-dominated grasslands (White et al. 2000, 2001). On the other hand, because of their greater nitrogen (N) investment in rubisco and photorespiratory enzymes, $C_3$ plants have lower N-use efficiency of photosynthesis (PNUE) than $C_4$ plants (Li 1993; Sage & Pearcy 1987). The ecological consequences of greater PNUE in $C_4$ species have been studied to only a limited degree. For example, in grasslands, when soils are low in N, $C_4$ grasses can be superior competitors to $C_3$ grasses and can dominate (Wedin & Tilman 1996). When soils become N-enriched, the advantage in PNUE is offset and $C_3$ species can match the photosynthetic potential of $C_4$ species, and thereby increase in cover. However, whether N availability interacts with heat stress differently in $C_3$ vs. $C_4$ species remains to be determined, but will have a bearing on the relative impact of global environmental change on $C_3$ and $C_4$ species abundance and distribution.

To examine the influence of N on plant response to heat stress in naturally-occurring mixed $C_3$-$C_4$ vegetation, I conducted a field study with the following three major objectives: (1) to determine how heat stress affects the ecophysiological and morphological variables of naturally-occurring co-dominant $C_4$ and $C_3$ species; (2) to determine the effect of N on resistance and resilience of each species to heat stress; (3) to investigate how heat stress affects plant C and N concentration, N-use efficiency, and N resorption rate. I predicted that (1) heat stress will have a more pronounced negative effect on the $C_3$ than the $C_4$ species; (2) supplemental N will help both the $C_3$ and $C_4$ species to better tolerate heat stress, especially for the $C_3$ species; and (3) heat stress will increase leaf N concentration and decrease N-use efficiency, as a result of decreased leaf
expansion and photosynthesis, but more so for the C3 species.
5.2 Materials and methods

5.2.1 Field site and treatments

The experiment site was located within restored prairie vegetation at the University of Toledo’s Stranahan Arboretum (Toledo, Ohio, USA), which is located within the oak-savannah glacial-sand ecosystem referred to as the “Oak Openings” region (http://oakopen.org/). *Andropogon gerardii* (big bluestem), a warm-season C₄ perennial grass, and *Solidago canadensis* (goldenrod), a warm-season C₃ perennial herbaceous dicot, are the two dominant plant species in this field site. The experiment design was a 2x2 factorial (±heating x ±added N; with n=4 replicates per treatment combination), utilizing 16x1m² randomly selected and assigned-treatments plots. Eight of the plots received heat treatment for five days from 17 to 21 August 2006, and eight of the plots received added N treatment (NH₄NO₃) applied twice (one and two weeks) before heat treatment at a rate of 5g N/m²/year. Heat treatment was applied by using eight top-vented 1m³-chambers made with transparent plastic attached to a wooden frame. A portable electric heater with a maximum capacity of 1500W was installed in the chamber to increase air temperature and an electric fan was used to circulate warm air inside the chamber. The target treatment temperature was 41 °C, which is 10 °C higher than average daytime temperature for August in Toledo, and 2-3 °C higher than the typical maximum temperature in the summer season in this area. Heat treatments were imposed during daytime for five days and for 10-h per day (8:00 am to 6:00 pm). Control plots were not covered by chambers and experienced ambient temperature. The air temperature and leaf temperatures inside and outside the chambers were monitored using data-loggers and fine-wire thermocouples during heat treatments; soil temperature at 10-cm depth was
measured with a thermometer.

5.2.2 Physiological Measurements

Before, during, and after heat stress, net photosynthesis, stomatal conductance to water vapor, quantum yield of electron transport of photosystem II (PSII), and leaf water potential were measured daily on randomly-chosen recently-expanded fully-lit leaves. Steady-state net photosynthesis ($P_n$; net CO$_2$ exchange) and stomatal conductance ($g_s$) of single leaves was measured with a portable photosynthesis system containing an infrared gas analyzer (model 6400, LiCOR, Lincoln, NE, USA), equipped with a 250-mm$^3$ leaf chamber as in (Heckathorn et al. 1997). Measurements were made at ambient light and temperature within one min of insertion of leaves into the cuvette (immediately after stabilization of CO$_2$ and H$_2$O fluxes). Quantum yield of PSII electron transport ($\Phi_{PSII}$) was measured with a pulse-amplitude-modulated (PAM) fluorometer (Model PAM 101/103, Walze, Germany), as in Wang et al. (2008). A pressure chamber (Model 600, PMS Instruments Co., Corvallis, Oregon) was used to measure midday leaf water potential ($\Psi_w$).

5.2.3 Biomass and C, N measurements

One-week after heat stress, 40x50 cm$^2$ of each plot was harvested. The clipped plants were sorted into different categories (green and senescent leaves, stems and flowers), oven-dried at 65 °C for one week and weighed. N and C concentration for different plant parts, as well as rhizosphere and bulk soil, was measured with a Perkin Elmer CHN Analyzer (Model 2400). N respiration rate (NRR) for each species was calculated by $\text{NRR} = (N_g-N_s)/N_g$, where $N_g$ was the green leaf N concentration and $N_s$ was the senescent leaf N concentration. Photosynthetic N-use efficiency (PNUE) was
based on net photosynthesis per unit plant N.

5.2.4 Statistical analysis

Analyses were conducted within each species to determine whether the physiological variables differed as a function of different treatments. For daily-measured variables like $\Psi_w$, $P_n$, $g_s$, PNUE, $\Phi_{PSII}$, $R_{soil}$, and leaf temperature, three-way analysis-of-variance (ANOVA) (SAS 9.1) was used to test for significant effects of days, heat, N, and their interaction during heat stress (day 1-day 5). A two-way ANOVA was used to test for significant effects of heat, N, and their interaction on $P_n$, $g_s$, PNUE, $\Phi_{PSII}$, $R_{soil}$, and leaf temperature after heat stress (i.e., on day 12), and on biomass, C%, and N% of plants and soil. In order to test for species effect, three-way ANOVA was conducted on biomass, C% and N%, with species, heat, N and their interactions as independent factors. Days, heat, and N were all treated as fixed effects.
5.3 Results

During heat stress (HS), air temperature in the heated plots was increased on average to $40.5 \pm 2.8 \, ^\circ C$. During the five days of heat treatment, leaf temperature of *A. gerardii* and *S. canadensis* in heated plots was higher than that in control plots, but returned to control levels after heat stress (Fig 5.1, Table 5.1). Nitrogen treatment had no effect on leaf temperature for *A. gerardii*, but for *S. canadensis*, plants with N treatment had a lower leaf temperature. Soil temperature (at 10-cm) was not altered by heat or N treatment (not shown). C% of both rhizosphere and bulk soil was not changed by heat stress, and N% of both rhizosphere and bulk soil was not impacted by heat stress, but was increased by N treatment ($P<0.01$) (not shown).

Leaf water potential ($\Psi_w$) was decreased for heated plants, but N had little effect on $\Psi_w$; and $\Psi_w$ was recovered one week post heat-stress (day 12) and was similar among treatments (Fig 5.2, Table 5.1). Soil (=soil+root) respiration ($R_{soil}$) was decreased by both heat and N, but there was no interactive effect of N and heat, and post-heat-stress $R_{soil}$ was similar for ±HS in +N plants but still lower in +HS vs. –HS plants with no added N (Fig 5.3, Table 5.1). There was an overall negative effect of heat treatment on net photosynthesis ($P_n$). For both *A. gerardii* and *S. canadensis*, $P_n$ was significantly lower in heated plots than in control plots during heat stress. N had no significant effect on $P_n$ for *A. gerardii*, but for *S. canadensis*, N increased $P_n$ and there was a significant heat x N interaction (Fig 5.4, Table 5.1). Further, $P_n$ remained depressed one week after HS in +HS plants with added N, relative to un-heated controls, but this was not observed in plants receiving no added N. Variation in stomatal conductance to water vapor ($g_s$) was a function of both heat and N. For *A. gerardii* and *S. canadensis*, $g_s$ was lower in heated
plots and higher at N-treated plots. There was also a significant interactive effect of heat and N on $g_\text{s}$ for *S. canadensis* (Fig 5.4, Table 5.1). Also, $g_\text{s}$ remained depressed one week after HS in +HS plants (more so in +N), relative to un-heated controls, especially for *S. canadensis*. Quantum yield of electron transport ($\Phi_{\text{PSII}}$) was not decreased by heat stress for *A. gerardii* and *S. canadensis*, but N had a significant positive effect on $\Phi_{\text{PSII}}$ for *A. gerardii* and *S. canadensis* (Fig 5.4, Table 5.1).

For *A. gerardii*, N treatment increased specific leaf area (SLA), but heat did not affect SLA, while for *S. canadensis*, neither N nor heat affected SLA (Fig 5.5, Table 5.2, 5.3). Total aboveground biomass ($W_a$) was not significantly affected by heat or N in either species, but there was a decrease in $W_a$ with heat in both species (ANOVA including both species; $P=0.024$), and increases in $W_a$ with added N in *S. canadensis* and decreases in $W_a$ with added N in *A gerardii* (Fig 5.6, Table 5.2 & 5.3). Biomass of green leaf, stem, and senescent leaf was not significantly altered by either heat or N treatment for both *A. gerardii* and *S. canadensis*. However, flower biomass was increased significantly by N treatment for *S. canadensis* and decreased by heat stress for *A. gerardii*. And for *A. gerardii* without N treatment, the percent of senescent leaf was significantly higher in heated plots (18.2%) than at control plots (12.3%).

Carbon concentration of plant tissue (C%) was altered by both heat and N treatment, but the effects differed with species and among different plant parts (Fig 5.7, Table 5.2 & 5.3). For *A. gerardii*, C% of N-treated plants was lower in green leaves and flowers, but higher in non-heated senescent leaves. Heat decreased C% only in senescent leaves for *A. gerardii* with N treatment. C% was not altered by either heat or N in green leaf, senescent leaf, stem, and flower for *S. canadensis*. In general, nitrogen concentration
(N%) was increased in N-treated plant tissues (excluding flowers) for both *A. gerardii* and *S. canadensis* (Fig 5.7, Table 5.2 & 5.3). Heat had little effect on N% in *A. gerardii*, though there was a tendency for decreases in N% in N-treated plants, while in *S. canadensis*, heat increased N% in green leaves, and for *A. gerardii*, there was also an interactive effect of heat and N on N% in green leaves.

Photosynthetic nitrogen-use efficiency (PNUE) was significantly lower for *S. canadensis* than *A. gerardii* and was decreased by heat for both *A. gerardii* and *S. canadensis* (Fig 5.8, Table 5.1). In heated plots, plants with N treatment tended to have a higher PNUE for *A. gerardii*, though the effect was not statistically significant. Further, recovery of PNUE was incomplete after one week post-HS, relative to un-heated controls, for both species and N levels. Nitrogen resorption rate (NRR) was decreased by N treatment, but not changed by heat, for *A. gerardii*, while for *S. canadensis*, NRR was significantly higher for heated plants, but was not different due to N treatment (Fig 5.9, Table 5.2 & 5.3).
5.4 Discussion

During heat stress, both *A. gerardii* (C4) and *S. canadensis* (C3) experienced decreased P$_{n}$, g$_{s}$, Ψ$_{w}$, PNUE, and soil (=soil+root) respiration decreased too; decreases in P$_{n}$, g$_{s}$, PNUE, and soil respiration were still evident one week after heat treatment ended (day 12). In general, N addition affected these physiological variables in both heated and unheated-plants (increasing P$_{n}$, g$_{s}$, Ψ$_{w}$, PNUE, but decreasing soil respiration). With few exceptions, during heat stress, N did not alter the nature of the heat-stress effect on these variables (i.e., there was no significant heat x N interaction). In contrast, after one week post-heat-stress recovery, residual heat-stress-related decreases in P$_{n}$ and g$_{s}$ were evident only (P$_{n}$), or greater (g$_{s}$), in high-N plants, and decreases in soil respiration were only evident in low-N plants. Both species exhibited trends of decreasing aboveground biomass with heat treatment, while added N tended to increase biomass in *S. canadensis* but decrease biomass in *A. gerardii*. Carbon concentration (C%) of tissues was affected only by heat treatment in *A. gerardii* (leaves and stems), while N% of green leaves in *A. gerardii* decreased with heat stress (+N only) but increased with heat stress in *S. canadensis*. Lastly, heat treatment increased N resorption rate (NRR) in *S. canadensis*, but not in *A. gerardii*, while added N decreased NRR for *A. gerardii*, but not in *S. canadensis*.

Collectively, these results indicate the heat waves imposed here were of moderate severity (as evidenced by the magnitude of HS effects), yet such moderate heat waves can affect plant C and N relations and biomass growth and allocation, and the heat effects can still be evident after one week of post-heat recovery. Further, many plant responses to the heat treatment were influenced by N availability and differed between the C$_{3}$
species, *S. canadensis*, and the C4 species, *A. gerardii*. Specifically, these results suggest that in a future warmer world with increasing N availability, *S. canadensis* may be affected less by heat waves than *A. gerardii*, but in the absence of more N, the reverse may be true. It is also worth noting that heat and N effects in this study may be smaller than likely to occur, as my heat treatment was a single heat wave (and plants in Northwest Ohio experience ca. 3-5 heat waves per summer) and my N treatment was initiated only 2 weeks prior to heat stress. Thus, predictions of N effects on heat-stress responses and differences between C3 and C4 plants based on this study may be conservative.

Any N-related influence on heat-induced changes in plant physiology, growth, biomass allocation, and tissue C and N concentration, and any such differences in N × heat effects between C3 and C4 species, will have important implications for plant herbivory and decomposition, and thus, for ecosystem N and C dynamics. For example, heat-related increases in tissue senescence and changes in C or N% will have a direct impact on herbivore feeding preference and growth rate, and on litter quantity and quality, and hence on decomposition rates. A shift in the ratio of C3:C4 species with increasing heat waves in the presence/absence of higher N would have dramatic impact on ecosystem N or C cycling also, as C4 foliage is characterized by a higher carbon-to-nitrogen (C:N) ratio and higher fiber content than C3 foliage, thus contributing high C:N litter to soil organic matter, which results in low N mineralization rates (Sage & Monson 1999; Wedin & Tilman 1996). High C:N ratios also reduce decomposition rates, such that proportionally more N on a site may reside in the soil organic matter pool (Aerts 1997); thus N availability often declines when a C4-dominated sward replaces C3 vegetation.
Plants appear to be more susceptible to high day or night-time temperatures during later flower-to-early seed developmental stages (Cross et al. 2003). Notably, in this study, flower biomass (Wf) was significantly reduced for A. gerardii by heat stress, and this decrease was somewhat smaller in high-N plants. Heat stress had no effect on Wf for S. canadensis, suggesting that increases in heat waves in the future may affect the seed bank of this plant community, which might affect community structure in the longer term. Heat-stressed plants can compensate for decreases in flower production by producing later flowers on existing inflorescences (Cross et al. 2003; Sato et al. 2000), but whether plants can still compensate for decreased flower production after a late-growing-season heat stress remains to be investigated.

The heat-treatment effects on plant C and N relations observed in this study differ somewhat compared to results from previous studies. For example, while I observed increased N% in green leaves with heat stress in S. canadensis, past studies applying long-term warming observed decreases in N% of green leaves with warming (Tjoelker et al. 1999, An et al. 2005). In contrast, other warming studies showed that elevated temperature increased leaf N concentration due to enhanced soil N mineralization and increased plant N uptake (Luomala et al. 2003; Nijs et al. 1996). I also observed unique effects on N resorption (NRR) from senescing leaves in this study, compared to previous studies. Here, NRR was decreased by N treatment but not changed by heat stress for A. gerardii; but for S. canadensis, NRR was increased by heat stress but was not altered by N treatment. The decreased NRR of A. gerardii due to N treatment in my study is consistent with the observation that species from nutrient-poor environments often have a
higher N resorption rate than species from nutrient-rich environments (Aerts 1997). The partitioning of N compounds between soluble and structural compounds is an important regulator of N resorption (Norby et al. 2001; Yuan et al. 2005). The increased NRR of *S. canadensis* in the heated plots might be caused by acceleration of the normal senescence and resorption process. Warming has been observed to accelerate the senescence of leaves, such that warmed plants completed the normal senescence and resorption process faster than those in un-warmed controls, resulting in plant litter in the warmed plots either having a lower N concentration or lower fraction of N in soluble compounds (Norby et al. 2000).
5.5 Conclusions

The present experiment showed that heat stress, though ephemeral, can potentially modify community composition and impact ecosystem nutrient cycling, via effects on plant growth and tissue N and C content. Further, increases in N availability may influence plant response to heat stress; e.g., as slowing recovery of heat-related damage to photosynthesis, and benefiting C$_3$ species more than C$_4$ species during heat stress. This study only examined short-term plant responses to acute heat stress within one generation of perennial plants, but the results indicate that the impact of acute heat stress on plant communities and ecosystems should be studied more extensively, particularly in combination with other potentially-interactive aspects of global environmental change (e.g., CO$_2$, O$_3$, and precipitation).
References


Yan YY (2002). Extreme temperature days in Hong Kong. *Physical Geography* 23: 476-
Table 5.1 Degrees of freedom (numerator and denominator \( df \)) and F-statistics from ANOVA on individual plant variables in response to heat and nitrogen treatment in a restored prairie in Toledo, OH, with days (day1-day5), heat, and nitrogen and their interactions as independent factors. F-statistics with a single asterisk indicated significance at \( P < 0.10 \), whereas a double asterisk indicates \( P < 0.05 \).

See text for abbreviations.

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<th>( S. ) canadensis</th>
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<td></td>
<td></td>
<td>( T_{\text{leaf}} )</td>
<td>( \Psi_w )</td>
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<td>35.44**</td>
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Table 5.2 Degrees of freedom (numerator and denominator $df$) and F-statistics from ANOVA on individual plant variables for *A. gerardii*, in response to heat and nitrogen treatment in a restored prairie in Toledo, OH, with heat, N and their interactions as independent variables. F-statistics with a single asterisk indicated significance at $P<0.10$, whereas a double asterisk indicates $P<0.05$. See text for abbreviations.

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<th>$W_f$</th>
<th>$W_a$</th>
<th>$C_g$</th>
<th>$N_g$</th>
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<th>$N_s$</th>
<th>$C_{st}$</th>
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<th>$C_f$</th>
<th>$N_f$</th>
<th>NRR</th>
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<td>0.06</td>
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<td>1.23</td>
<td>5.05**</td>
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<td>6.29**</td>
<td>5.79**</td>
<td>4.67**</td>
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Table 5.3 Degrees of freedom (numerator and denominator df) and F-statistics from ANOVA on individual plant variables for *S. canadensis*, in response to heat and nitrogen treatment in a restored prairie in Toledo, OH, with heat, N and their interactions as independent variables. F-statistics with a single asterisk indicated significance at $P<0.10$, whereas a double asterisk indicates $P<0.05$.

See text for abbreviations.

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<th>Variables</th>
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<th>$N_g$</th>
<th>$C_s$</th>
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<th>$C_{st}$</th>
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<th>$C_f$</th>
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<th>NRR</th>
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<td>15.68**</td>
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Fig 5.1 Effects of heat wave (open symbols - heat stress; dark symbols - without heat stress) and N (circle symbols - without N treatment; triangle symbols - with N treatment) on leaf temperature of *A. gerardii* and *S. canadensis*. Day 1-5 refers to the five days during heat treatment; day 12 refers to one week after the end of heat treatment when plants were in recovery. C, CH, N, and NH indicate plants with no treatment, heat, N and heat*N treatment, respectively. Values are means ± 1 SE; n=4.
Fig 5.2 Effects of heat wave (open symbols - heat stress; dark symbols - without heat stress) and N (circle symbols - without N treatment; triangle symbols - with N treatment) on leaf water potential ($\Psi_w$) of *A. gerardii* and *S. canadensis*. Day1-5 refers to the five days during heat treatment; day 12 refers to one week after the end of heat treatment when plants were in recovery. C, CH, N, and NH indicate plants with no treatment, heat, N and heat*N treatment, respectively. Values are means ± 1 SE; n=4.

<table>
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<td>Leaf water potential (mPa)</td>
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<td>-2.0</td>
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<tr>
<td>Leaf water potential (mPa)</td>
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</table>
Fig 5.3 Effects of heat wave (open symbols - heat stress; dark symbols - without heat stress) and N (circle symbols - without N treatment; triangle symbols - with N treatment) on soil respiration ($R_{\text{soil}}$). Day1-5 refers to the five days during heat treatment; day12 refers to one week after the end of heat treatment when plants were in recovery. C, CH, N, and NH indicate plants with no treatment, heat, N and heat*N treatment, respectively. Values are means ± 1 SE; n=4. Inserted is F-Statistics from ANOVA.
Fig 5.4 Effects of heat wave (open symbols - heat stress; dark symbols - without heat stress) and N (circle symbols - without N treatment; triangle symbols - with N treatment) on net photosynthesis ($P_n$), stomatal conductance ($g_s$) and quantum yield of photosystem-II electron transport ($\Phi_{PSII}$) of *A. gerardii* and *S. canadensis*. Day1-5 refers to the five days during heat treatment; day12 refers to one week after the end of heat treatment when plants were in recovery. C, CH, N, and NH indicate plants with no treatment, heat, N and heat*N treatment, respectively. Values are means ± 1 SE; n=4.
Fig 5.5 Effects of heat wave and N on specific leaf area (SLA) for *A. gerardii* and *S. canadensis*. Bars labeled by C, CH, N, and NH indicate plants with no treatment, heat, N and heat*N treatment, respectively. Values are means ± 1 SD; n=4.
Fig 5.6 Effects of heat wave and N on total above-ground, green leaves, stem, flower and senescent leaves biomass for *A. gerardii* and *S. canadensis*. Bars labeled by C, CH, N, and NH indicate plots with no treatment, heat, N and heat*N treatment, respectively. n=4.
Fig 5.7 Effects of heat wave and N on C\% and N\% of green leaf (GL), stem (ST), flower (FL) and senescent leaf (SL) for *A. gerardii* and *S. canadensis*. Bars labeled by C, CH, N, and NH indicate plots with no treatment, heat, N and heat*N treatment, respectively. Values are means ± 1 SD; n=4.
Fig 5.8 Effects of heat wave (open symbols - heat stress; dark symbols - without heat stress) and N (circle symbols - without N treatment; triangle symbols - with N treatment) on photosynthetic nitrogen use efficiency (PNUE) of *A. gerardii* and *S. canadensis*. Day1-5 refers to the five days during heat treatment; day12 refers to one week after the end of heat treatment when plants were in recovery. C, CH, N, and NH indicate plants with no treatment, heat, N and heat*N treatment, respectively. Values are means ± 1 SE; n=4.
Fig 5.9 Effects of heat wave and N on nitrogen resorption rate (NRR) for *A. gerardii* and *S. canadensis*. Bars labeled by C, CH, N, and NH indicate plots with no treatment, heat, N and heat * N treatment, respectively. Values are means ± 1 SD; n=4.
CO₂ had a significant effect on plant tolerance to heat stress. The effects differed among different species and functional groups. Thermotolerance of Pₙ in elevated (vs. ambient) CO₂ increased in C₃, but decreased in C₄ (especially) and CAM (high growth temperature only), species. The limitation of Pₙ during heat stress was caused mainly by biochemical limitations, such as electron transport, not by stomatal closure. The effects of elevated CO₂ on plant tolerance to heat stress were also dependent on plant N status. At high N, CO₂ had a positive effect on plant tolerance to heat stress for C₃ and negative effect for C₄ species. But at low N, CO₂ always had a negative effect for both C₃ and C₄ species. The negative effect of elevated CO₂ on plant tolerance to heat stress was correlated with lower production of heat-shock proteins (Hsps). The effects of elevated CO₂ on plant tolerance to heat stress also depended on the temperature treatments. When at moderate heat stress, CO₂ always had a positive effect on net photosynthesis for both C₃ and C₄ species, but at severe heat stress, CO₂ had a positive effect on C₃ but negative effect for C₄ species. Thus, benefits of elevated CO₂ to photosynthesis at normal temperatures may be partly offset by negative effects during severe heat stress, especially for C₄ species and at low N, so effects of elevated CO₂ on acute heat tolerance may contribute to future changes in plant productivity, distribution, and diversity. In the field, we found that short-term heat stress can significantly decrease plant biomass, but only for C₃ species. However, with N addition, the C₃ species gained more advantage than the C₄
species. The manipulated heat event also altered plant N resorption rate. For the C₃ species, N resorption rate was decreased by N treatment, but was not changed by heat stress; for C₄ species, N resorption rate was increased by heat stress, but was not changed by N treatment. The effect of heat stress on plant biomass and N resorption rate suggests that heat waves, though transient, could have significant effects on plants, communities, and ecosystem N cycling, and with N addition, the negative effect of heat wave might be reduced for some species. More research is needed to investigate the interactive effect of CO₂ and N on plants community responses to heat stress, considering more frequent and severe heat stress will happen in the future.