Elevated ozone reduces photosynthetic carbon gain by accelerating leaf senescence of inbred and hybrid maize in a genotype-specific manner

Running title: Maize photosynthetic response to ozone

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ABSTRACT

Exposure to elevated tropospheric ozone concentration ($[O_3]$) accelerates leaf senescence in many C_3 crops. However, the effects of elevated $[O_3]$ on C_4 crops including maize (*Zea mays* L.) are poorly understood in terms of physiological mechanism and genetic variation in sensitivity. Using Free Air gas Concentration Enrichment (FACE), we investigated the photosynthetic response of 18 diverse maize inbred and hybrid lines to season-long exposure to elevated $[O_3]$ (~100 nL L^{-1}) in the field. Gas exchange was measured on the leaf subtending the ear throughout the grain filling period. On average over the lifetime of the leaf, elevated $[O_3]$ led to reductions in photosynthetic CO_2 assimilation of both inbred (-22%) and hybrid (-33%) genotypes. There was significant variation among both inbred and hybrid lines in the sensitivity of photosynthesis to elevated $[O_3]$, with some lines showing no change in photosynthesis at elevated $[O_3]$. Based on analysis of inbred line B73, the reduced CO_2 assimilation at elevated $[O_3]$ was associated with accelerated senescence decreasing photosynthetic capacity, and not altered stomatal limitation. These findings across diverse maize genotypes could advance the development of more ozone tolerant maize, and provide experimental data for parameterization and validation of studies modeling how O_3 impacts crop performance.

Keyword index

Zea mays, Photosynthesis, Senescence, Stomatal conductance, Tropospheric ozone

INTRODUCTION

Tropospheric ozone (O_3) is an airborne pollutant that enters leaves through their stomata and generates reactive oxygen species (ROS) upon contact with intercellular leaf surfaces (Heath 1988). Subsequently, these ROS can cause oxidative damage to membranes and other cellular components. At very high concentrations, O_3 can elicit a hypersensitive response, but even at lower concentrations O_3 can impair cellular function (Krupa & Manning 1988). When exposed to elevated O_3 concentrations ($[O_3]$) throughout the growing season, many plants have reduced photosynthetic C assimilation and biomass production as well as increased antioxidant capacity and mitochondrial respiration rates (Fiscus *et al*. 2005; Ainsworth *et al.* 2012). As a cumulative result of these physiological responses, crops exhibit significant reductions in economic yield with increasing $[O_3]$ (Mills *et al.* 2007; Ainsworth 2017).

The effects of exposure to chronic, elevated $[O_3]$ on photosynthesis and stomatal conductance can vary with leaf age and canopy position (Tjoelker *et al*. 1995; Morgan *et al*. 2004; Betzelberger *et al*. 2010; Feng *et al*. 2011). Studies that repeatedly measured a cohort of soybean and wheat leaves found that photosynthesis and stomatal conductance were not significantly affected by elevated $[O_3]$ in recently mature leaves, but continued exposure over time accelerated the decline in photosynthetic capacity as leaves aged (Morgan *et al*. 2004; Feng *et al*. 2011; Emberson *et al*., 2017). Stomatal conductance can also become uncoupled from photosynthesis as leaves age under O_3 stress, often with greater measured reductions in photosynthesis than stomatal conductance (Lombardozzi *et al*. 2012), resulting in decreased water use efficiency at elevated $[O_3]$ (Tjoelker *et al*. 1995; VanLoocke *et al*. 2012). Additionally, stomata of plants exposed to elevated $[O_3]$ can show delayed opening or closing responses to

environmental or hormonal signals (Keller & Häsler 1984; Wilkinson & Davies 2009; Paoletti & Grulke 2010; Wagg *et al*. 2013).

Much of our understanding of the effects of elevated [O_3] on photosynthesis and stomatal function come from study of C_3 species (Reich 1987; Paoletti & Grulke 2005; Felzer *et al*. 2007; Wittig *et al*. 2007). A few experiments have shown that elevated [O_3] can negatively impact photosynthetic properties and biomass production of the model C_4 species, maize (*Zea mays* L.) (Pino *et al*. 1995; Leitao *et al*. 2007a; Leitao *et al*. 2007b; Bagard *et al*. 2015; Yendrek *et al*. 2017). However, current understanding of the mechanisms underlying photosynthetic responses to O_3 in maize is limited to a few genotypes, and often to juvenile growth stages. The high ratio of photosynthetic CO_2 assimilation relative to stomatal conductance and isolation of Rubisco carboxylation in the bundle sheath cells make it possible that C_4 photosynthesis will respond distinctly to C_3 photosynthesis at elevated [O_3]. Analysis of historical U.S. maize yields suggests that reported physiological responses to O_3 pollution drive annual yield reductions of approximately 10% from 1980-2011, amounting to $7.2 billion in lost profit per year on average (McGrath *et al.* 2015). Considering that O_3-mediated yield reductions were more prominent in hot and dry years (McGrath *et al.* 2015) and future climate models predict an increase in growing season temperature with altered frequency and magnitude of precipitation events (Christensen *et al.* 2007; Walsh *et al.* 2014), efforts to address the knowledge gap about mechanisms of O_3-sensitivity in maize are likely to become increasingly important.

In addition to being the most widely produced food crop, maize is a model C_4 species with considerable resources for analysis of genotype to phenotype associations. This includes the maize nested association mapping (NAM) population, which was developed to encompass much of the existing diversity in a relatively small number of inbred lines (Yu *et al.* 2008). Substantial

phenotypic diversity for many agronomic traits exists within the parental lines of the NAM (Flint-Garcia *et al.* 2005). In order to exploit these resources to locate genetic factors associated with complex traits such as O_3 tolerance, there is a need to first identify lines that respond differently to elevated [O_3] for key physiological traits. For understanding and modeling the impacts of O_3 on crops, photosynthesis and stomatal conductance are two key traits (Reich 1987; Emberson *et al*. 2000; Sitch *et al*. 2007). In this study, we investigated the effects of season-long growth at elevated [O_3] on maize photosynthetic gas exchange. Maize was exposed to elevated [O_3] in the field using Free Air gas Concentration Enrichment (FACE). In 2013 and 2014, we studied the inbred cultivar B73. We hypothesized that growth at elevated [O_3] would reduce photosynthetic capacity and daily C gain as leaves progressed more rapidly through senescence. In 2015, we investigated the response of photosynthesis and stomatal conductance to elevated [O_3] in 10 diverse inbred and 8 diverse hybrid lines in order to test for genetic variation in O_3 response.

MATERIALS AND METHODS

Field site and experimental conditions

Maize (*Zea mays*) inbred and hybrid lines (Table 1) were studied at the FACE research facility in Champaign, IL (www.igb.illinois.edu/soyface/) in 2013, 2014 and 2015. This facility is located on a 32 ha farm. Maize is grown on half of the land each year, and rotated annually with soybean. Each year, the portion of the field growing maize was fertilized with N (180 lbs per acre) and treated with pre-emergent and post-emergent herbicides at the recommended rates. Additional weeding was done inside the FACE rings as needed. Maize inbred and hybrid lines were planted with a precision planter in rows spaced 0.76 m apart and 3.35 m in length, at a

density of 8 plants m^{-1} in replicated blocks (n=4). Each block had one ambient plot and one elevated [O_3] plot, which were octagonal in shape and of 20-m diameter. Weather conditions were monitored at the FACE facility, and Supplemental Figure 1 shows the seasonal time courses of: (1) daily maximum, (2) daily minimum temperatures, and (3) daily total precipitation for the 2013, 2014 and 2015 growing seasons.

Air enriched with O_3 was delivered to the experimental rings with FACE technology, as described in Yendrek *et al*. (2017). The target fumigation set-point in the elevated [O_3] rings was 100 nL L^{-1}, which was imposed for ~8 h per d throughout the growing season except when leaves were wet or wind speeds were too low to ensure accurate fumigation (i.e., <0.5 m s^{-1}). Over the three years, when the fumigation was on, the averaged 1-min [O_3] within the treatment rings was within 10% of the 100 nL L^{-1} set point for 53.7% of the time and within 20% of the set point for 78% of the time. Season-long 8 hr average [O_3] in ambient rings was 40.8 nL L^{-1} in 2013, 40.1 nL L^{-1} in 2014, and 40.0 nL L^{-1} in 2015, and season-long elevated [O_3] was 71.0 nL L^{-1} in 2013, 70.8 nL L^{-1} in 2014 and 64.0 nL L^{-1} in 2015.

Leaf-level gas exchange measurements

In situ photosynthetic gas exchange of the leaf subtending the ear was measured using portable photosynthesis systems (LI-6400, LICOR Biosciences, Lincoln, NE) in a modified version of a previously published protocol (Leakey *et al.* 2006). The net rate of photosynthetic CO_2 assimilation (A), stomatal conductance (g_s), the ratio of intercellular [CO_2] (c_i) to atmospheric [CO_2] (c_a), and instantaneous water use efficiency (iWUE = A/g_s) were measured. Reproductive development (time to silking and anthesis) was monitored in all experimental rows, and all dates of gas exchange measurements are reported relative to the date of anthesis.

In 2013 and 2014, the diurnal time course of leaf photosynthetic gas exchange was assessed with measurements taken across all replicate plots every few hours throughout the day at three or four development stages over the growing season of the B73 inbred genotype. In 2015, *in situ* gas exchange measurements on 10 inbred and 8 hybrid lines were collected at midday approximately every 7 d starting when the plants reached anthesis and ending when the ear leaf of >50% plants in an individual row had fully senesced. After senescence of a genotype in a given treatment, we recorded zero for A and g_s for all subsequent weekly measurements until both ambient and elevated plots were fully senesced. In all years, two or three plants were measured as sub-samples within each replicate plot at either ambient [O_3] or elevated [O_3].

Prior to a set of measurements being performed over a 1-2 h period of a day in all replicate plots, temperature and relative humidity (RH) in the leaf chamber cuvette were set to match prevailing ambient conditions as measured by an on-site weather station. A linear quantum sensor (AccuPAR LP-80; Decagon Devices, Pullman, WA) was used to measure the average light intensity at the position in the canopy for the leaf subtending the ear, which was used to set the PPFD incident on the leaf in the gas exchange cuvette. In 2015, a constant PPFD was used for all weekly midday measurements based on the measured PPFD at anthesis. For inbred cultivars, PPFD was set at 1,800 µmol m^{-2} s^{-1} and for hybrid cultivars PPFD was set at 450 µmol m^{-2} s^{-1}. Keeping light constant over the entire measurement period enabled comparison of changes in the response of A and g_s to leaf aging in ambient and elevated [O_3], and eliminated variation in those parameters due to PPFD. On all dates, four instruments were used simultaneously. All environmental conditions were held constant across instruments.

The response of A to c_i was measured on the leaf subtending the ear in 2013 and 2014 to examine the effects of elevated [O_3] on the maximum apparent rate of phosphoenolpyruvate

carboxylase activity (V_{pmax}) and on CO_2-saturated photosynthetic rate (V_{max}). Two to three leaves per ring were measured. Leaves were excised pre-dawn, immediately re-cut under water, and measured in a field laboratory. This approach minimized short-term decreases in water potential, chloroplast inorganic phosphate concentration, and maximum PSII efficiency that can occur in the field, and also enabled more leaves to be measured on multiple portable photosynthesis systems over a short period of time (Leakey *et al*. 2006). Measurements were initiated at ambient c_i, then the reference [CO_2] in the leaf cuvette was stepped down to 25µmol mol^{-1}, before it was increased stepwise to 1200 µmol mol^{-1} while keeping PPFD constant at 2,000 µmol m^{-2} s^{-1}. The initial slope of the A/c_i curve (c_i < 60 µmol mol^{-1}) was used to calculate V_{pmax} according to von Caemmerer (2000). A four parameter nonrectangular hyperbolic function was used to estimate V_{max} as the horizontal asymptote of the A/c_i curve. Stomatal limitation to A (l) was estimated from A/ci curves using the approach described by Farquhar and Sharkey (1982), and modified by Markelz *et al*. (2011). Mean values of V_{pmax} and V_{max} from A/c_i curves were used in combination with *in situ* values of c_i measured at midday to estimate the range of l and mean l for recently mature leaves (DOY 219 in 2013, DOY 213 in 2014) and senescing leaves (DOY 238 in 2013, DOY 245 in 2014) in ambient and elevated [O_3].

Statistical analysis

Diurnal gas exchange parameters (A, g_s) measured in 2013 and 2014 were analyzed with a repeated mixed model ANOVA with the Kenwood-Rogers option and compound symmetry covariance structure (SAS, version 9.4, Cary, NC). All analysis was done on the ring mean. Days were analyzed independently, time of day was a repeated measure and block was a random effect. A mixed model ANOVA with the Kenwood-Rogers specification was used to analyze

V_{pmax} and V_{max} in 2013 and 2014 (SAS, Version 9.4, Cary, NC). O_3 treatment and DOY were fixed effects in the model, block was a random effect, and years were analyzed independently. Statistical differences in least squared mean estimates were determined by linear contrasts with a threshold $p<0.05$.

A and g_s of the flag leaf measured in 2015 were fit with a quadratic equation: $y = y_o + \alpha x + \beta x^2$, where y was *A* or g_s, and x was days after anthesis (Proc Nlin, SAS). Other models (exponential decay, 3-parameter Weibull function) were tested, but did not fit all of the data for each genotype. Therefore, a simple quadratic equation was used. In order to test if there were differences in the decline in *A* or g_s over time in ambient and elevated [O_3], a single quadratic model was first fit to the *A* and g_s data for each genotype, then models were fit to each genotype and treatment combination. An F statistic was used to test if the model with genotype and treatment produced a significantly better fit to the data, i.e., if there were significant differences in the response of *A* or g_s over time in ambient and elevated [O_3]. Additionally the quadratic model was fit to each genotype within each ring in order to obtain parameter estimates for y_o, α and β. Parameter estimates as well as initial values of *A* and g_s measured shortly after anthesis were analyzed with a mixed model ANOVA with the Kenwood-Rogers specification. O_3 treatment and genotype were fixed effects in the model and block was a random effect. Statistical differences in least squared mean estimates between ambient and elevated [O_3] for each inbred or hybrid line were determined by linear contrasts with a threshold $p<0.05$.

RESULTS

Elevated [O_3] accelerates loss of photosynthetic capacity in inbred line B73

In 2013 and 2014, daily time courses of leaf photosynthetic gas exchange were measured *in situ* as the leaf subtending the ear aged in ambient and elevated [O_3] (Figs. 1, 2). When the leaf was recently fully expanded, shortly before or after anthesis, there was no significant effect of elevated [O_3] on *A* (Figs. 1d, 2e, 2f). However, as the leaf aged, growth at elevated [O_3] accelerated the decline in diurnal C gain, and later in the season *A* was significantly reduced by elevated [O_3] (Figs. 1e, 1f, 2g, 2h). Significant differences in *A* between ambient and elevated [O_3] were apparent earlier in the growing season than differences in g_s (Figs. 1e versus 1h, 2g versus 2k). But, there were no consistent differences in the ratio of intercellular [CO_2] to atmospheric [CO_2] (c_i/c_a) or instantaneous water use efficiency (iWUE = A/g_s) in ambient and elevated [O_3] in either year (Fig. 1j-o, Fig. 2m-t). On only one sampling date out of seven, which was characterized by low PPFD, elevated [O_3] led to significantly greater c_i/c_a and significantly lower iWUE (Fig. 1k, n).

Declines in *in situ A* over the leaf aging process were associated with decreased photosynthetic capacity, measured as V_{pmax} (Fig. 3a, b; Table 2) and V_{max} (Fig. 3c, d; Table 2). Declines in V_{pmax} from when the leaf was recently fully expanded to until ~30 days later were greater in elevated [O_3] (-64% in 2013 and -64% in 2014) than in ambient [O_3] (-51% in 2013 and -33% in 2014; Fig. 3a, b). Likewise, declines in V_{max} from when the leaf was recently fully expanded to until ~30days later were greater in elevated [O_3] (-40% in 2013 and -52% in 2014) than in ambient [O_3] (-25% in 2013 and -33% in 2014; Fig. 3c, d). Although an O_3 treatment effect was not significant in the mixed ANOVA model (Table 2), pair-wise comparisons of the means showed that V_{pmax} was significantly lower in elevated [O_3] on the final sampling date in 2014 (Fig. 3b) and V_{max} was significantly lower in elevated [O_3] on the final sampling date in both 2013 and 2014 (Fig. 3c, d).

225 *A*/c_i curves were also used to assess stomatal vs. biochemical limitations to *A* in aging
226 leaves grown at ambient and elevated [O_3]. Average *A*/c_i curves in ambient and elevated [O_3]
227 were plotted for the leaf subtending the ear shortly after anthesis, and then ~3-4 weeks later (Fig.
228 4). Shortly after anthesis, *l* was minimal in both ambient and elevated [O_3] in 2013 (2-3%), and
229 mean c_i was well above the inflection point of the *A*/c_i curve (Fig. 4a). In 2014, there was a wider
230 range of observed c_i shortly after anthesis, but mean *l* was still low (5-6%) in both ambient and
231 elevated [O_3], and the mean c_i was above the inflection point (Fig 4b). As leaves aged, *l*
232 increased in both ambient and elevated [O_3] to 20-30% (Fig. 4c, d). Both increased *l* and
233 decreased photosynthetic capacity result in lower *A* in aging leaves, but elevated [O_3] did not
234 appear to change *l*.
235
236 **Photosynthetic response to elevated [O_3] in diverse inbred and hybrid lines**
237 In 2015, *A* of the leaf subtending the ear was measured approximately weekly in 10
238 diverse inbred lines and 8 hybrid lines during the grain filling period. Initial values of *A*
239 measured shortly after anthesis differed among lines, but not between ambient and elevated [O_3],
240 and the interaction between inbred line and [O_3] was only marginally significant (Table 3).
241 Similarly, initial measurements of g_s varied among inbred lines, but not between ambient and
242 elevated [O_3] (Table 3). For 8 of the 10 inbred lines, there was significant acceleration in the
243 decline in *A* as the leaf aged under elevated [O_3], similar to what was observed in previous years
244 in B73 (Fig. 5). However, for two inbred lines (M37W and CML333), there was no significant
245 difference in the quadratic fit to the photosynthetic data between ambient and elevated [O_3] (Fig.
246 5). There were highly significant effects of [O_3], inbred line and their interaction on the
247 parameters describing the quadratic equation modeling the decline in *A* over time in the inbred

lines (Table 3). This reflected variation in the estimates of *A* at anthesis (y_o), the time to the start of a decline in *A* (α) and the rate of decline in *A* (β). The significant effect of $[O_3]$ on y_o is not consistent with the lack of an effect on initial rates of *A*, but some of the data were best fit by convex functions and some by concave functions which affected accurate prediction of y_o in the inbred lines (Fig. 5). Average α was 0.30 in ambient $[O_3]$ and -0.69 in elevated $[O_3]$, indicating that a decline in *A* started earlier in elevated $[O_3]$ (Fig. 5, Supplemental Table 1). Average β was -0.016 in ambient $[O_3]$ and -0.00025 in elevated $[O_3]$ indicating that the rate of decline in *A* was greater in ambient $[O_3]$ than elevated $[O_3]$ on average across inbred lines (Supplemental Table 1).

Measurements of g_s in the aging flag leaf in inbred lines differed from the response of *A* over time. For 7 of the 10 genotypes, there was no evidence for accelerated decline in g_s over time (Fig. 6). Of the remaining 3 genotypes, MS71 and C123 displayed more rapid decline in g_s over time, while NC338 had initially higher g_s in elevated $[O_3]$. The influence of this small set of genotypes meant that there were significant effects of $[O_3]$, inbred line and their interaction on the shape of the quadratic fit to the decline in g_s over time, especially on parameters α and β (Table 3; Supplemental Table 1). Apparent differences in the response of *A* and g_s to elevated $[O_3]$ over the grain filling period did not impact the iWUE, which was not different in ambient and elevated $[O_3]$ (Supplemental Fig. 2).

All 8 of the hybrid lines showed acceleration in the decline of *A* in the leaf subtending the ear over the grain filling period (Fig. 7). Unlike the inbreds, the hybrids had similar initial values of *A* and g_s shortly after anthesis (Table 3). These measurements were taken at a lower light level than the inbred measurements, which was representative of the light measured in the canopy at the leaf subtending the ear in hybrids vs. inbreds, but may explain why there was less variation

among hybrid lines. There was a significant effect of [O_3] and a significant [O_3] x hybrid line interaction on the parameters describing the decline in *A* over time (Table 3; Supplemental Table 1). Consistent with the inbred lines, α was positive in ambient [O_3] (1.06) and negative in elevated [O_3] (-0.033), indicating a more immediate decline in *A* in elevated [O_3], and β was more negative in ambient [O_3] (-0.02) than elevated [O_3] (-0.006) indicating more rapid decline in *A* in ambient [O_3] (Supplemental Table 1). Only 3 of the 8 hybrid lines showed significant changes in g_s at elevated [O_3] over the grain filling period (Fig. 8), and consistent with the inbred lines, the apparent differences in the response of *A* and g_s to elevated [O_3] over time did not impact iWUE (Supplemental Fig. 3). Notably, the hybrids of MS71 and C123 crossed with B73 were among this sensitive group, matching the greater than average sensitivity of these genotypes as inbreds.

DISCUSSION

Tropospheric [O_3] continues to increase in many of the world's crop growing regions as a result of inconsistent regulation of precursor pollutants around the globe and short- and long-distance transport of pollutants (Lin *et al*. 2017). Early studies of maize reported that it was significantly more tolerant to increasing [O_3] than C_3 crops like soybean or peanut (Heck *et al*. 1983). However, more recent experimental studies using open top chambers reported that maize leaf physiology is sensitive to chronic O_3 exposure in the cultivar Chambord (Leitao *et al.* 2007a; Leitao *et al.* 2007b). Furthermore, multiple regression analysis of county-level yield data suggests that there is significant yield loss to O_3 in the Midwest U.S. growing region (McGrath *et al*. 2015). Here, we examined how season-long fumigation with elevated [O_3] using FACE technology in the primary area of maize production affected gas exchange of the leaf subtending

294 the ear. We concentrated on this leaf and time period because previous research has established
295 that most of the photosynthate used for grain filling in maize is provided by mid-canopy leaves
296 after anthesis (Borrás *et al*. 2004), and those leaves are the last on the plant to senesce
297 (Valentinuz & Tollenaar 2004). Diurnal measurements of B73 showed that ear leaf C
298 assimilation was not affected by elevated [O_3] around the time of anthesis when the leaf was
299 recently mature; however, loss of photosynthetic capacity of that leaf was accelerated by
300 elevated [O_3] (Figs. 1 & 2).

301 The seasonal pattern of elevated [O_3] effects on photosynthetic carbon gain over the diurnal
302 period was reflected in measurements of *A* at midday (Figs. 1 & 2). Therefore, screening of
303 midday *A* was used to explore genotypic variation in sensitivity to elevated [O_3] among diverse
304 hybrid and inbred lines of maize. On average, the sum of midday CO_2 assimilation by the leaf
305 subtending the ear over the period from anthesis to complete leaf senescence was reduced by
306 22% in inbred lines and 33% in hybrid lines (Table 4). However, sensitivity to elevated [O_3] in
307 inbred lines ranged from no loss in photosynthetic CO_2 assimilation (CML333, M37W) to 59%
308 lower CO_2 assimilation (C123; Fig. 5, Table 4). Likewise, sensitivity to elevated [O_3] in hybrids
309 ranged from 13% lower photosynthetic CO_2 assimilation (B73 x M37W) to 44% lower CO_2
310 assimilation (B73 x MS71; Fig. 7, Table 4). All ozone sensitive lines showed the same
311 developmentally dependent pattern of response as B73, with similar *A* in young leaves at
312 ambient and elevated [O_3], but more rapid decline in *A* over time (Fig. 5, 7). Thus, acceleration
313 of senescence rather than decreased initial investment in photosynthetic capacity is a key
314 response of maize to elevated [O_3] at the leaf level. But, there is genetic variation in response
315 that might be exploited to breed maize with improved tolerance to ozone pollution, and possibly
316 even broader spectrum oxidative stress tolerance.

317 Accelerated loss of photosynthetic capacity in elevated [O_3] has been documented in FACE
318 experiments with soybean (Morgan *et al*. 2004; Betzelberger *et al*. 2010), wheat (Feng *et al*.
319 2011; 2016), rice (Pang *et al*. 2009) and now maize. In rice and wheat, accelerated senescence of
320 the flag leaf at elevated [O_3] shortened the grain filling duration and reduced seed yields (Gelang
321 *et al*. 2000; Pang *et al*. 2009; Feng *et al*. 2011). Additionally, genetic variation in the response of
322 seed yield to elevated [O_3] was attributed in part to variation in leaf senescence (Pang *et al*. 2011;
323 Feng *et al*. 2011) as well as to variation in detoxification and antioxidant capacity (Feng *et al*.
324 2010; 2016). Acceleration of senescence in the ear leaf of maize is associated with maize yield
325 loss under a number of environmental stresses (Wolfe *et al*. 1988; Bänzinger *et al*. 1999), and
326 greater yield potential of newer maize varieties has been associated with maintenance of
327 photosynthetic capacity of the ear leaf for a longer period of time following anthesis (Ding *et al*.
328 2005). Thus, the acceleration of senescence reported here under elevated [O_3] for both inbred and
329 hybrid maize has the potential to contribute to grain yield losses.
330 We found that growth at elevated [O_3] both accelerated the speed at which flag leaves lost
331 photosynthetic capacity (represented by the β parameter in our quadratic model) and decreased
332 the length of time that leaves maintained optimum photosynthetic capacity (α term in quadratic
333 model). It would be interesting to test the response of maize lines with delayed leaf senescence
334 or stay-green phenotypes for O_3 response. There are a variety of genetic and physiological
335 mechanisms that enable a stay-green phenotype (Thomas & Howarth 2000), and there may be
336 mechanisms that are more promising under O_3 stress than others. For example, stay-green lines
337 with altered chlorophyll catabolism may be less O_3-tolerant as the build-up of phytotoxic
338 degradation products might compete with O_3-induced reactive oxygen species for detoxification
339 by the anti-oxidant system. However, stay-green lines with perennial tendencies or lines where

senescence is initiated later, but progresses at the normal rate and with typical catabolism may have the potential to retain higher rates of photosynthesis later in the growing season, and therefore yield better in higher [O_3].

Studies in a number of C_3 species have indicated that biochemical limitation, and not stomatal limitation, are responsible for the sustained effects of elevated [O_3] on *A* (Farage *et al*. 1991; Morgan *et al*. 2004; Paoletti & Grulke 2005; Feng *et al*. 2011). In C_3 species, accelerated decline in *A* in aging leaves was associated with decreased maximum carboxylation capacity and electron transport capacity (Zheng *et al*. 2002; Morgan *et al*. 2004; Feng *et al*. 2011). We found a similar response in the maize inbred line B73 with the decline in *A* at elevated [O_3] associated with decreased photosynthetic capacity (V_{pmax} and V_{max}), and not with any change in limitation imposed by stomata. Stomatal limitation increased from 2-5% in the ear leaf when it was recently mature to 20-30% when it was 4-5 weeks older, which is consistent with the loss of stomatal control in aging leaves previously described for other species (Reich 1984). This increase in stomatal limitation occurred in both ambient and elevated [O_3], and so there does not appear to be evidence for an uncoupling of *A* and g_s in elevated [O_3] that has been described in other studies (Paoletti & Grulke 2005). Across the diverse inbred and hybrid lines grown at elevated [O_3], both *A* and g_s declined with ear leaf age (Figs. 5-8), although the reduction in A was often greater than g_s. Still iWUE remained relatively constant (Supplemental Fig. 2, 3), providing further evidence from diverse maize lines that growth at elevated [O_3] does not result in uncoupling of *A* and g_s.

In addition to testing for cultivar variation in response to elevated [O_3], we were also able to identify significant variation in *A* and g_s in recently mature flag leaves of inbred maize lines (Table 3). The hybrid lines, which all contained B73 as the female parent, did not show variation

363 in *A* and g_s (Table 3). Previous studies have also reported that inbred lines show greater variation
364 in photosynthetic traits compared to hybrid lines (Albergoni *et al*. 1983; Ahmadzadeh *et al*.
365 2004), but that hybrid lines maintain higher rates of *A* over a leaf lifespan compared to inbred
366 lines (Ahmadzadeh *et al.* 2004). We found similar results in our studies of diverse maize lines,
367 however, it should be duly noted that due to the recurrence of B73 as the female parent, there
368 was more limited genetic diversity in the hybrids used in this study. Additionally, the light
369 environment at the ear leaf at anthesis was different in inbred and hybrid canopies. *A* and g_s were
370 monitored at higher PPFD in the inbred lines (1,800 µmol m^{-2} s^{-1}) compared to the hybrid lines
371 (450 µmol m^{-2} s^{-1}), which may have also contributed to the greater variation observed amongst
372 inbred lines.

373 Our study showed that accelerated loss of photosynthetic capacity was the basis for
374 photosynthetic sensitivity to elevated [O_3] in both inbred and hybrid maize lines. Drought and
375 high temperature stress also negatively impact photosynthesis in maize (Bänzinger *et al*. 1999;
376 Neiff *et al*. 2016), and those stresses often co-occur with high O_3 events, and will likely increase
377 in the future (Cook *et al*. 2014). Further experimental studies are needed to understand the
378 interactions of rising [O_3] with intensifying drought and heat stress, but our work suggests that a
379 key trait for robust improvement of maize response to elevated [O_3] is maintenance of
380 photosynthetic capacity during the grain filling period. By testing a diverse panel of maize
381 genotypes under field conditions in the world's primary area of production, this study provides a
382 foundation on which to investigate the genetic variation in maize oxidative stress tolerance, and
383 possibly develop more stress tolerant germplasm. Under elevated [O_3], both inbred and hybrid
384 maize lines were shown to have reduced carbon gain of the leaf subtending the ear, which
385 heavily influences yield. In contrast to many previous studies of crop responses to atmospheric

386 change using FACE technology (e.g. Markelz *et al.* 2011; Gillespie *et al.* 2012), this study
387 describes the average and variance in treatment effects across diverse genotypes, and so should
388 provide experimental data that is a stronger basis for parameterization or validation of studies
389 attempting to model regional crop performance.
390

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396

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TABLES

Table 1. Planting date (day of year), inbred and hybrid lines examined in each field season, and the date that anthesis was recorded for each line.

Year	Planting (DOY)	Line	Anthesis (DOY)
2013	136, 137	B73	208
2014	139	B73	210
2015	139	B73	205
		C123	203
		CML333	214
		Hp301	205
		IL14H	201
		M37W	211
		Mo17	204
		MS71	202
		NC338	214
		OH43	201
		B73xC123	202
		B73xCML333	207
		B73xHP301	202
		B73xIL14H	201
		B73xM37W	206
		B73xMo17	202
		B73xMS71	201
		B73xOH43	201

Table 2. Analysis of variance (*F*, p) of the maximum apparent rate of phosphoenolpyruvate carboxylase activity (V_{pmax}) and CO_2-saturated photosynthetic rate (V_{max}) measured in inbred line B73 grown at ambient and elevated [O_3] in 2013 and 2014. Years were analyzed independently.

		2013		2014	
		F	p	*F*	p
V_{pmax}	O_3	0.39	0.5754	2.46	0.1296
	DOY	129	<0.0001	8.57	0.0005
	O_3 x DOY	2.22	0.1867	0.92	0.4457
V_{max}	O_3	2.97	0.1358	3.02	0.1327
	DOY	67.9	0.0002	18.3	<0.0001
	O_3 x DOY	4.01	0.0921	0.94	0.4411

Table 3. Analysis of variance (*F*, p) of photosynthesis (*A*), stomatal conductance (g_s) and the quadratic parameters describing the decline in *A* and g_s over time. Measurements were made in in 10 inbred and 8 hybrid lines grown at ambient and elevated [O_3] in 2015, and inbreds and hybrids were analyzed in separate models. *A* and g_s represent the first measurement taken shortly after anthesis in Figs. 5-8.

	Inbred Lines		***A***			**g_s**		
	A (μmol m^{-2} s^{-1})	g_s (mol $m^{-2}s^{-1}$)	y_o	α	β	y_o	α	β
[O_3]	1.33, 0.254	0.26, 0.609	13.1, 0.0006	28.8, <0.0001	28.0, <0.0001	2.95, 0.091	6.92, 0.011	7.65, 0.007
Line	2.93, 0.006	2.89, 0.007	12.9, <0.0001	13.0, <0.0001	9.19, <0.0001	8.19, <0.0001	7.67, <0.0001	5.96, <0.0001
[O_3] x Line	1.87, 0.076	1.77, 0.094	3.12, 0.004	5.51, <0.0001	5.76, <0.0001	1.23, 0.296	2.29, 0.028	2.58, 0.014
	Hybrid Lines		***A***			**g_s**		
	A (μmol m^{-2} s^{-1})	g_s (mol $m^{-2}s^{-1}$)	y_o	α	β	y_o	α	β
[O_3]	0.00, 0.965	0.00, 0.953	55.9, 0.017	66.4, 0.015	61.8, 0.016	1.82, 0.310	4.15, 0.179	4.72, 0.095
Line	1.04, 0.429	0.39, 0.902	1.52, 0.202	1.67, 0.157	4.50, 0.002	0.77, 0.618	1.80, 0.128	3.98, 0.004
[O_3] x Line	0.58, 0.765	1.19, 0.340	5.72, 0.0004	8.16, <0.0001	10.4, <0.0001	1.60, 0.177	1.97, 0.095	2.36, 0.050

Table 4. The percentage change in elevated [O_3] for the sum of midday photosynthesis (*A*) and average stomatal conductance (g_s) of the leaf subtending the ear measured during grain filling in 2015. Data and quadratic fits for *A* and g_s in inbred and hybrid lines are shown in Figs. 5-8.

Line	***A***	g_s
B73	-21%	0%
C123	-59%	-13%
CML333	0%	0%
Hp301	-17%	0%
IL14H	-34%	0%
M37W	0%	0%
Mo17	-16%	0%
MS71	-46%	-5%
NC338	-2%	-12%
OH43	-28%	+14%
B73xC123	-39%	-31%
B73xCML333	-22%	0%
B73xHP301	-32%	0%
B73xIL14H	-39%	0%
B73xM37W	-13%	0%
B73xMo17	-43%	0%
B73xMS71	-44%	-43%
B73xOH43	-28%	-27%

FIGURE LEGENDS

Figure 1. The diurnal response of net assimilation (A) and stomatal conductance (g_s) of maize inbred line B73 in 2013. Measurements were made on the leaf subtending the ear on three days of the year (DOY). The number of days from anthesis is shown in parenthesis after measurement DOY. The conditions in the leaf cuvette including leaf temperature (T_{leaf}), vapor pressure deficit (VPD), and photosynthetically active radiation (PAR) are given for each date in panels a, b and c. Diurnal A is shown for each DOY in panels d, e and f, and g_s is shown in panels g, h and i. The least squared means ± 1 standard error are shown. The p-value represents the O_3 effect for each DOY.

Figure 2. The diurnal response of net assimilation (A) and stomatal conductance (g_s) of maize inbred line B73 in 2014. Measurements were made on the leaf subtending the ear on four days of the year (DOY). The number of days from anthesis is shown in parenthesis after measurement DOY. The conditions in the leaf cuvette including leaf temperature (T_{leaf}), vapor pressure deficit (VPD), and photosynthetically active radiation (PAR) are given for each date in panels a, b, c and d. Diurnal A is shown for each DOY in panels e, f, g and h, and g_s is shown in panels i, j, k and l. The least squared means ± 1 standard error are shown. The p-value represents the O_3 effect for each DOY.

Figure 3. Maximum carboxylation capacity of PEPC (V_{pmax}) (a, b) and CO_2-saturated photosynthetic rate (V_{max}) (c, d) of maize inbred line B73 measured in 2013 (a, c) and 2014 (b, d). Measurements were made on the leaf subtending the ear. The least squared means ± 1

standard error are shown. Significant differences between ambient and elevated [O_3] (p<0.05) for individual days are indicated with an asterisk.

Figure 4. Summary of A/c_i response curves (solid lines) and CO_2 supply functions (dashed lines) for maize inbred line B73 grown at ambient [O_3] (black lines) and elevated [O_3] (grey lines). The supply functions show the maximum and minimum c_i observed at midday in the field during diurnal measurements of A, and the points on the curves show the mean observed c_i. Measurements were made on the leaf subtending the ear shortly after anthesis (a, b) and ~4-5 weeks after anthesis (c, d). Estimates of mean stomatal limitation (l) are reported in each panel for ambient and elevated [O_3].

Figure 5. Photosynthetic rate (A) of the leaf subtending the ear measured during grain filling in 2015 for 10 inbred lines grown at ambient (open circles) and elevated [O_3] (filled circles). The best fit quadratic curves for ambient (dashed line) and elevated [O_3] (solid line) are shown if the statistical test revealed that there was a better fit to the data using two quadratic curves (i.e., one for each treatment) rather than a single curve for all of the data. Grey lines represent the best quadratic fit through all of the data when the statistical test revealed no difference between ambient and elevated [O_3]. Each point represents the mean value for an individual replicate ring for each measurement date. Parameters describing these curves (y_o, α, β) are reported in Supplemental Table 1.

Figure 6. Stomatal conductance (g_s) of the leaf subtending the ear measured during grain filling in 2015 for 10 inbred lines grown at ambient (open circles) and elevated [O_3] (filled circles). The

best fit quadratic curves for ambient (dashed line) and elevated [O_3] (solid line) are shown if the statistical test revealed that there was a better fit to the data using two quadratic curves (i.e., one for each treatment) rather than a single curve for all of the data. Grey lines represent the best quadratic fit through all of the data when the statistical test revealed no difference between ambient and elevated [O_3]. Each point represents the mean value for an individual replicate ring for each measurement date. Parameters describing these curves (y_o, α, β) are reported in Supplemental Table 1.

Figure 7. Photosynthetic rate (*A*) of the leaf subtending the ear measured during grain filling in 2015 for 8 hybrid lines grown at ambient (open circles) and elevated [O_3] (filled circles). The best fit quadratic curves for ambient (dashed line) and elevated [O_3] (solid line) are shown if the statistical test revealed that there was a better fit to the data using two quadratic curves (i.e., one for each treatment) rather than a single curve for all of the data. Grey lines represent the best quadratic fit through all of the data when the statistical test revealed no difference between ambient and elevated [O_3]. Each point represents the mean value for an individual replicate ring for each measurement date. Parameters describing these curves (y_o, α, β) are reported in Supplemental Table 1.

Figure 8. Stomatal conductance (g_s) of the leaf subtending the ear measured during grain filling in 2015 for 8 hybrid lines grown at ambient (open circles) and elevated [O_3] (filled circles). The best fit quadratic curves for ambient (dashed line) and elevated [O_3] (solid line) are shown if the statistical test revealed that there was a better fit to the data using two quadratic curves (i.e., one for each treatment) rather than a single curve for all of the data. Grey lines represent the best

quadratic fit through all of the data when the statistical test revealed no difference between ambient and elevated [O_3]. Each point represents the mean value for an individual replicate ring for each measurement date. Parameters describing these curves (y_o, α, β) are reported in Supplemental Table 1.

Supplemental Figure 1. Seasonal maximum and minimum temperatures and precipitation in 2013, 2014 and 2015 measured at the FACE experimental site in Champaign, IL.

Supplemental Figure 2. Instantaneous water use efficiency (iWUE = A/g_s) of the leaf subtending the ear measured during grain filling in 2015 for 10 inbred lines grown at ambient (open circles) and elevated [O_3] (filled circles).

Supplemental Figure 3. Instantaneous water use efficiency (iWUE = A/g_s) of the leaf subtending the ear measured during grain filling in 2015 for 8 hybrid lines grown at ambient (open circles) and elevated [O_3] (filled circles).

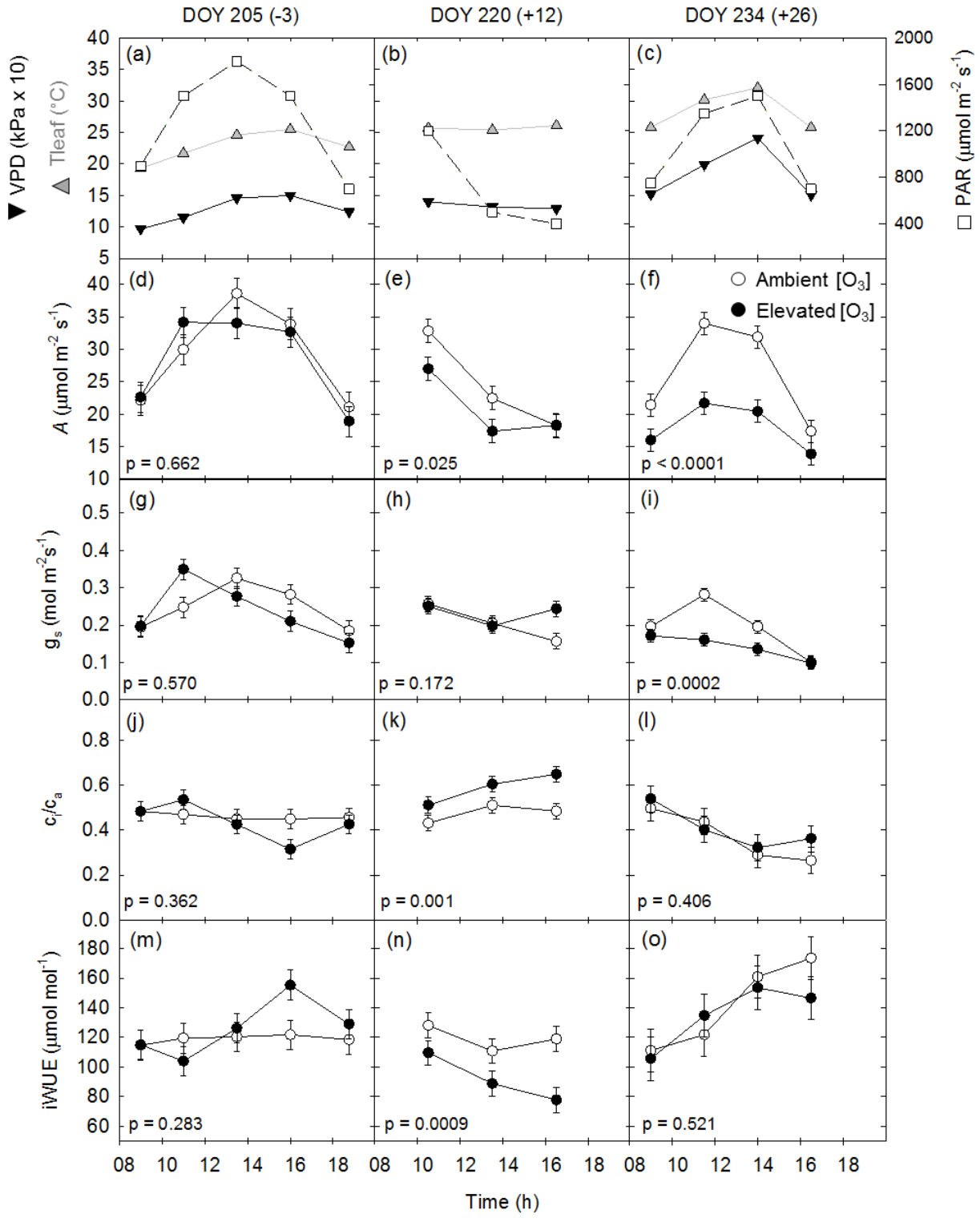


Figure 1.

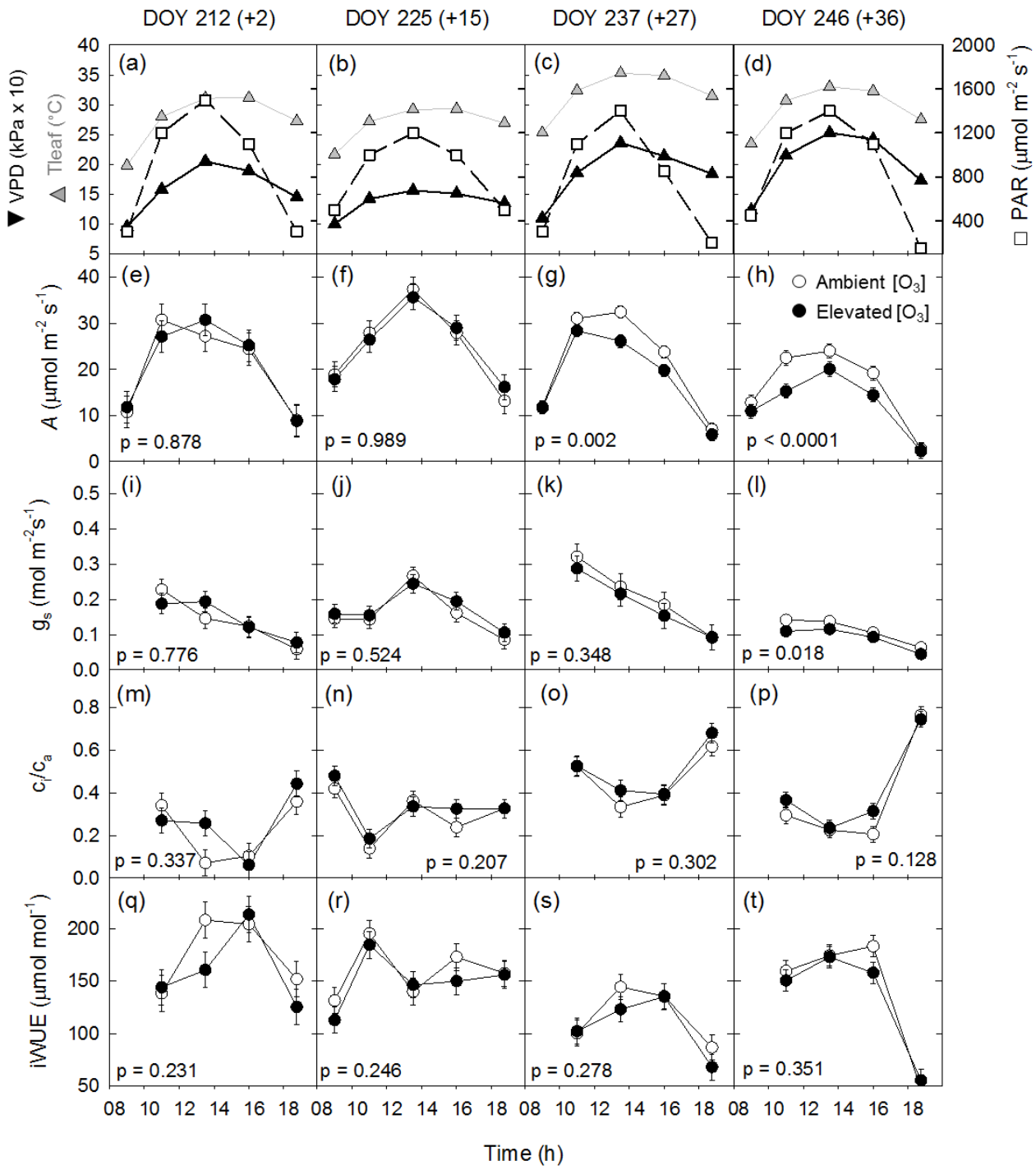


Figure 2.

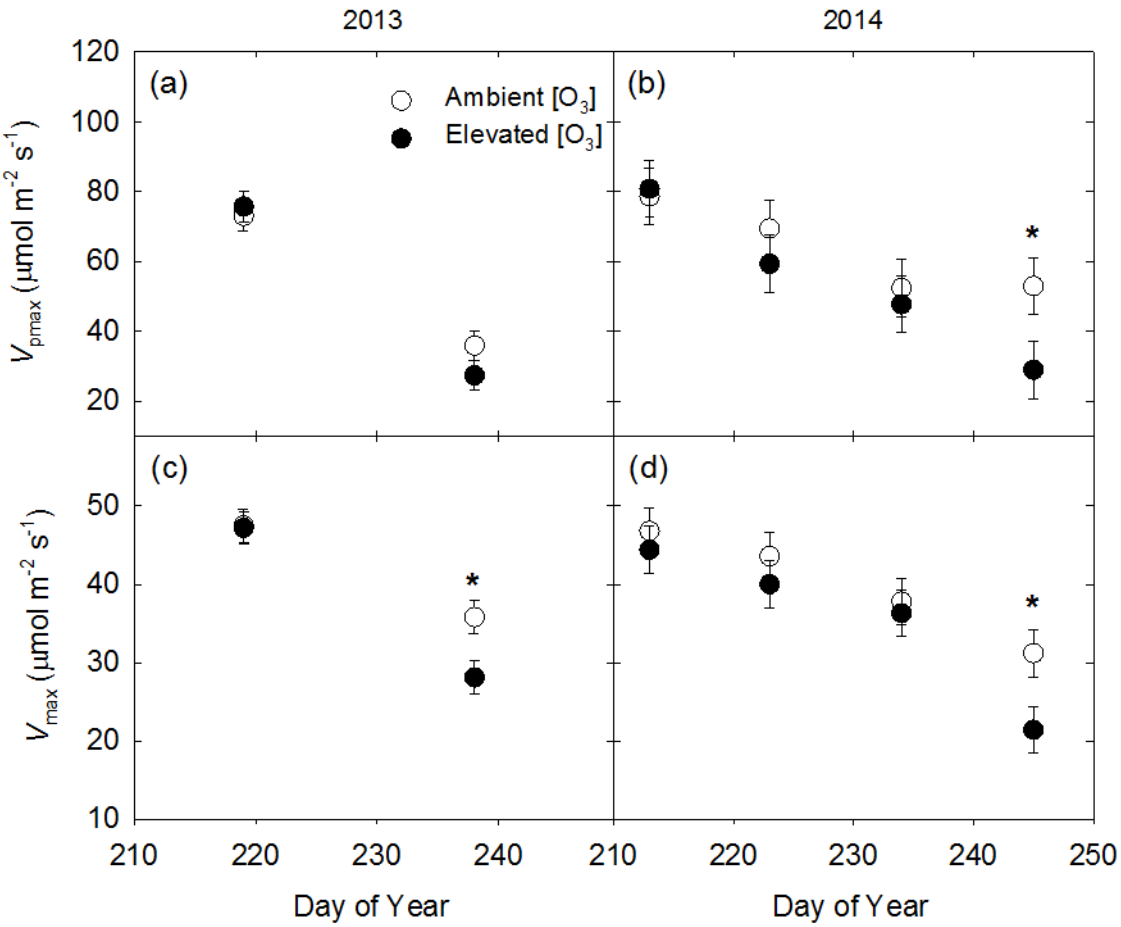


Figure 3.

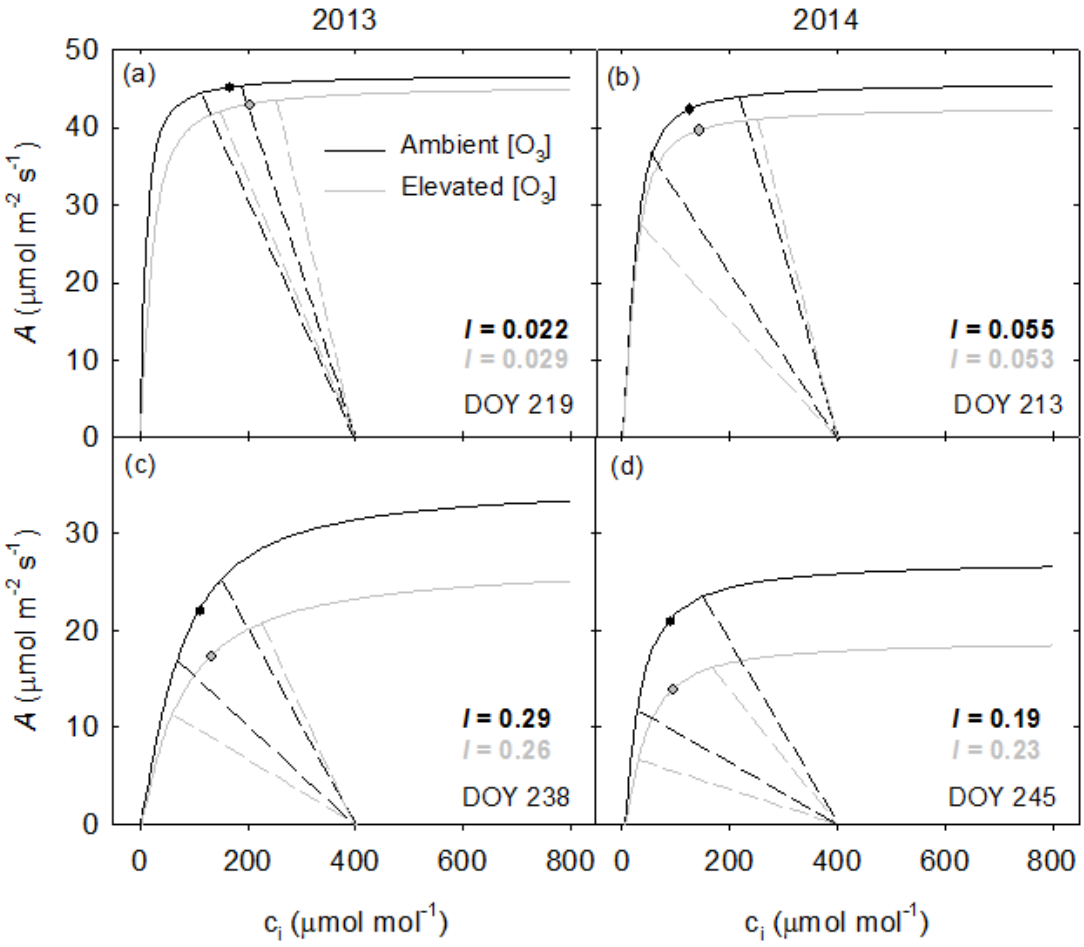


Figure 4.

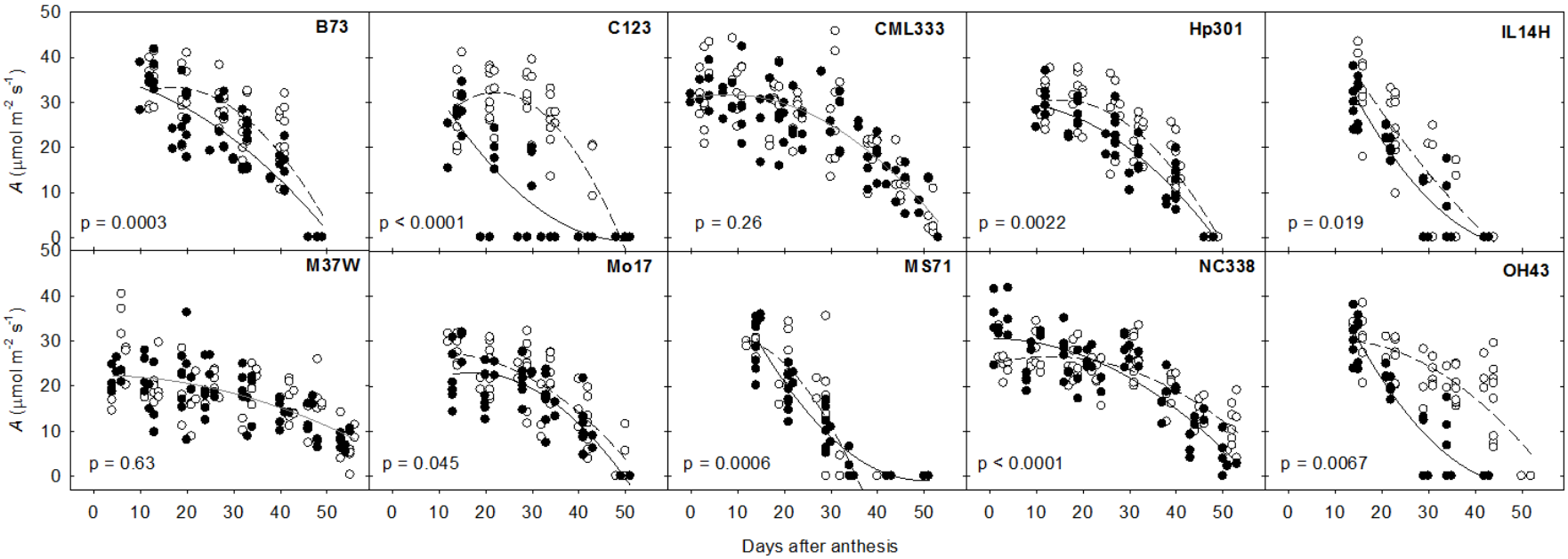

B73
C123
CML333
Hp301
IL14H
M37W
Mo17
MS71
NC338
OH43
p = 0.0003
p < 0.0001
p = 0.26
p = 0.0022
p = 0.019
p = 0.63
p = 0.045
p = 0.0006
p < 0.0001
p = 0.0067
A (µmol m-2 s-1)
Days after anthesis

Figure 5.

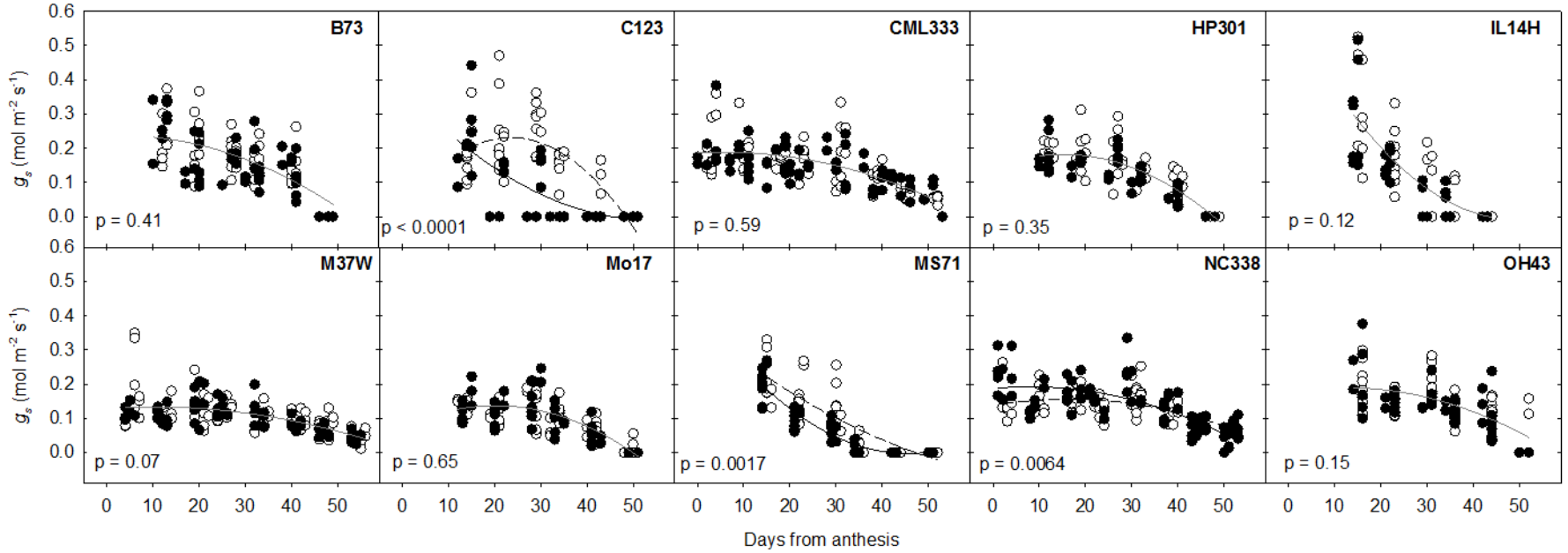

B73
C123
CML333
HP301
IL14H
M37W
Mo17
MS71
NC338
OH43
p = 0.41
p < 0.0001
p = 0.59
p = 0.35
p = 0.12
p = 0.07
p = 0.65
p = 0.0017
p = 0.0064
p = 0.15
g_s (mol m^{-2} s^{-1})
Days from anthesis

Figure 6.

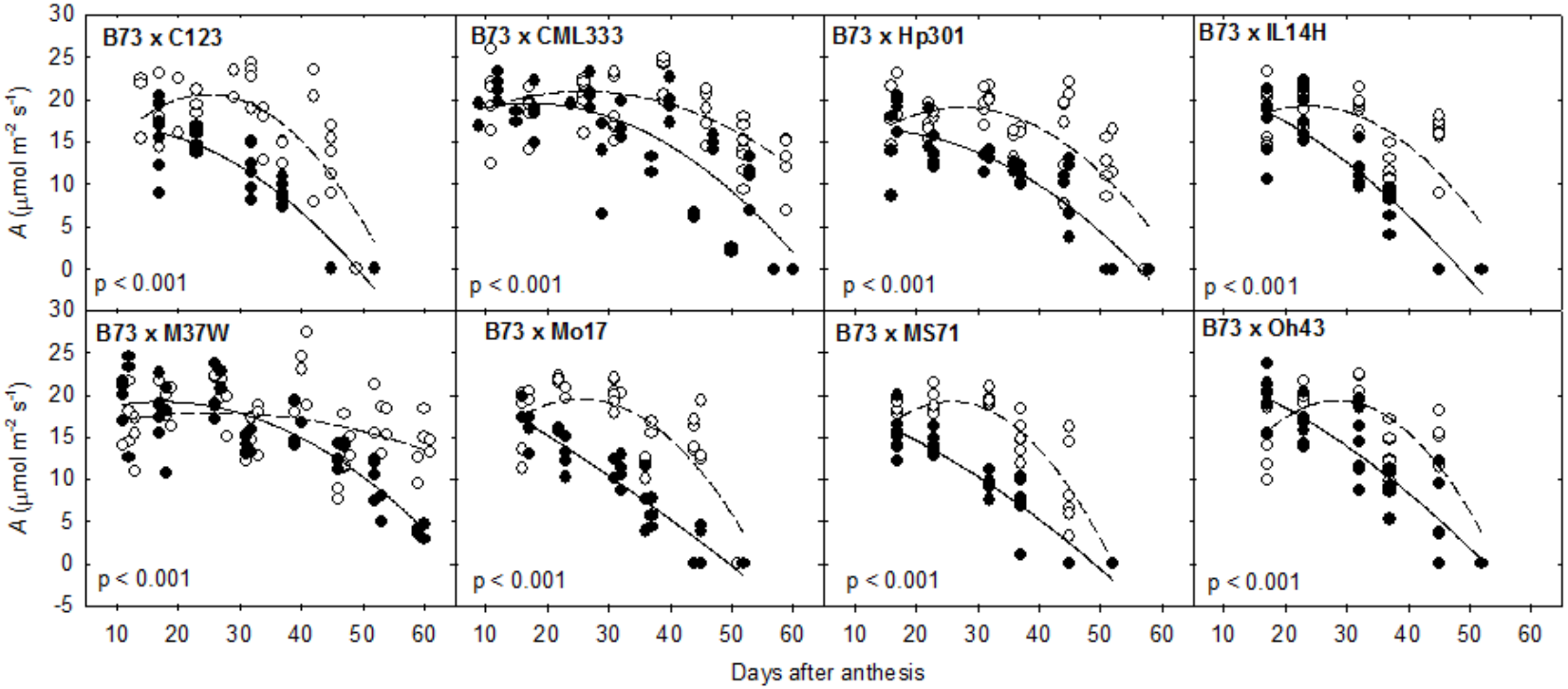

B73 x C123
B73 x CML333
B73 x Hp301
B73 x IL14H
B73 x M37W
B73 x Mo17
B73 x MS71
B73 x Oh43
p < 0.001
A (µmol m-2 s-1)
Days after anthesis

Figure 7.

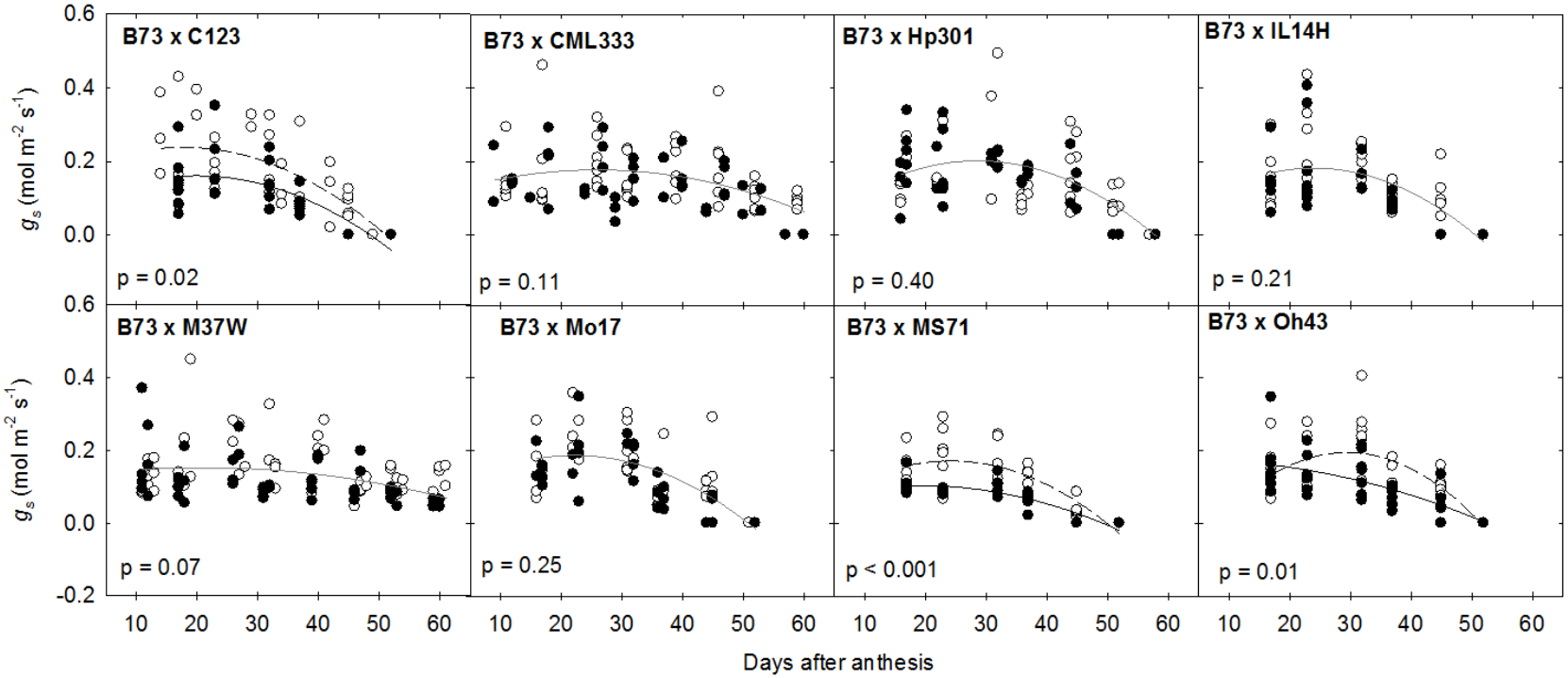
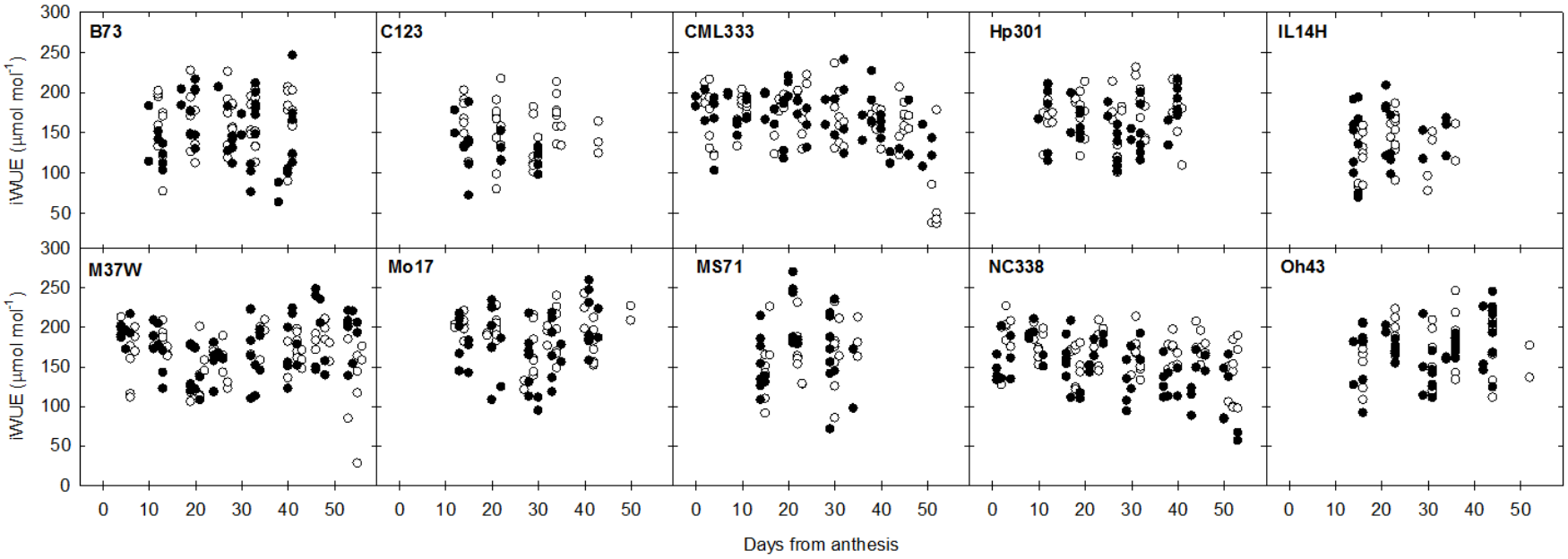

B73 x C123
B73 x CML333
B73 x Hp301
B73 x IL14H
B73 x M37W
B73 x Mo17
B73 x MS71
B73 x Oh43
p = 0.02
p = 0.11
p = 0.40
p = 0.21
p = 0.07
p = 0.25
p < 0.001
p = 0.01
g_s (mol m^{-2} s^{-1})
Days after anthesis

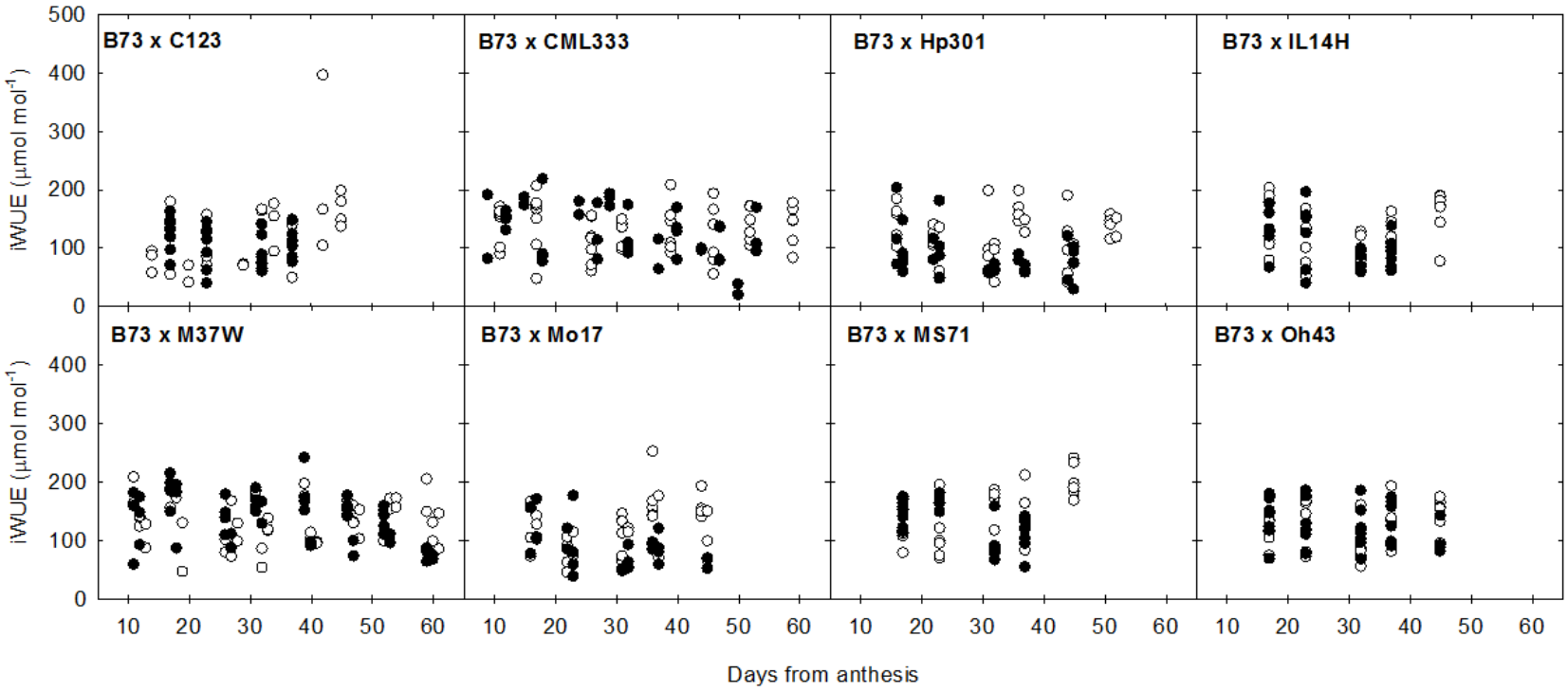
Figure 8.

Supplemental Table 1. Parameters describing the quadratic function describing the decline in *A* and g_s over time in the leaf subtending the ear, for 10 inbred lines and 8 hybrid lines grown at ambient and elevated [O_3]. Significant differences in the quadratic fits between ambient and elevated [O_3] for each inbred or hybrid line are shown in bold font.

		A			g_s		
Line	[O_3]	y_o	α	β	y_o	α	β
B73	Ambient	**25.6**	**0.91**	**-0.027**	0.16	0.0056	-0.00018
	Elevated	**35.9**	**-0.15**	**-0.011**	0.16	0.0056	-0.00018
C123	Ambient	**10.2**	**1.99**	**-0.045**	**0.026**	**0.015**	**-0.00037**
	Elevated	**49.4**	**-1.99**	**0.020**	**0.37**	**-0.014**	**0.00013**
CML333	Ambient	30.8	0.22	-0.014	0.19	0.00078	-0.00007
	Elevated	30.8	0.22	-0.014	0.19	0.00078	-0.00007
HP301	Ambient	**24.5**	**0.80**	**-0.026**	0.14	0.0052	-0.00017
	Elevated	**29.6**	**0.13**	**-0.015**	0.14	0.0052	-0.00017
IL14H	Ambient	**61.5**	**-1.89**	**0.011**	0.59	-0.025	0.00025
	Elevated	**63.4**	**-2.62**	**0.026**	0.59	-0.025	0.00025
M37W	Ambient	22.3	0.0077	-0.0046	0.13	0.00087	-0.00004
	Elevated	22.3	0.0077	-0.0046	0.13	0.00087	-0.00004
Mo17	Ambient	**25.9**	**0.30**	**-0.015**	0.073	0.0064	-0.00016
	Elevated	**16.6**	**0.75**	**-0.022**	0.073	0.0064	-0.00016
MS71	Ambient	**48.6**	**-1.19**	**0.0035**	**0.37**	**-0.011**	**0.000066**
	Elevated	**60.4**	**-2.47**	**0.025**	**0.42**	**-0.018**	**0.00020**
NC338	Ambient	**24.8**	**0.28**	**-0.011**	**0.096**	**0.0046**	**-0.00012**
	Elevated	**30.6**	**0.012**	**-0.010**	**0.047**	**0.0084**	**-0.00019**
OH43	Ambient	**20.0**	**0.97**	**-0.022**	0.17	0.0025	-0.00009
	Elevated	**28.8**	**0.28**	**-0.014**	0.17	0.0025	-0.00009
B73 x C123	Ambient	**4.72**	**1.28**	**-0.026**	**0.18**	**0.0071**	**-0.00021**
	Elevated	**17.0**	**0.10**	**-0.0089**	**0.099**	**0.0066**	**-0.00017**
B73 x CML333	Ambient	**15.7**	**0.41**	**-0.0078**	0.11	0.0051	-0.00010
	Elevated	**16.7**	**0.34**	**-0.0097**	0.11	0.0051	-0.00010
B73 x HP301	Ambient	**6.35**	**0.93**	**-0.017**	0.0063	0.014	-0.00024
	Elevated	**15.5**	**0.19**	**-0.0082**	0.0063	0.014	-0.00024
B73 x IL14H	Ambient	**7.52**	**0.98**	**-0.020**	0.041	0.012	-0.00025
	Elevated	**24.0**	**-0.22**	**-0.0054**	0.041	0.012	-0.00025
B73 x M37W	Ambient	**15.4**	**0.19**	**-0.0038**	0.13	0.0022	-0.00005
	Elevated	**16.8**	**0.28**	**-0.0083**	0.13	0.0022	-0.00005
B73 x Mo17	Ambient	**3.37**	**1.27**	**-0.025**	0.075	0.010	-0.00023
	Elevated	**24.1**	**-0.41**	**-0.0014**	0.075	0.010	-0.00023
B73 x MS71	Ambient	**1.02**	**1.42**	**-0.028**	**0.033**	**0.0116**	**-0.00024**
	Elevated	**21.4**	**-0.28**	**-0.0031**	**0.071**	**0.0037**	**-0.00010**
B73 x OH43	Ambient	**-4.85**	**1.71**	**-0.030**	**-0.15**	**0.023**	**-0.00039**
	Elevated	**24.4**	**-0.19**	**-0.0053**	**0.17**	**0.00060**	**-0.00007**



Supplemental Figure 2.



Supplemental Figure 3.