Photosynthetic decline in aging perennial grass is not fully explained by leaf nitrogen

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Abstract

Aging in perennial plants is traditionally observed in terms of changes in end-of-season biomass; however, the driving phenological and physiological changes are poorly understood. We found that 3-year-old (mature) stands of the perennial grass Miscanthus × giganteus had 19–30% lower A$_{\text{net}}$ than 1-year-old M. × giganteus (juvenile) stands; 10–34% lower maximum carboxylation rates of Rubisco and 34% lower light-saturated A$_{\text{net}}$ (A$_{\text{sat}}$). These changes could be related to nitrogen (N) limitations, as mature plants were larger and had 14–34% lower leaf N on an area basis (Na) than juveniles. However, N fertilization restored Na to juvenile levels but compensated only 50% of the observed decline in leaf photosynthesis with age. Comparison of leaf photosynthesis per unit of leaf N (PNUE) showed that mature stands had at least 26% lower PNUE than juvenile stands across all N fertilization rates, suggesting that other factors, besides N, may be limiting photosynthesis in mature stands. We hypothesize that sink limitations in mature stands could be causing feedback inhibition of photosynthesis which is associated with the age-related decline in photosynthesis.

Keywords: Bioenergy, C$_4$ metabolism, nitrogen dilution, photosynthesis, plant aging, sink limitation, size effect.

Introduction

Aging in perennial plants is traditionally observed in terms of changes in end-of-season biomass. Perennial plants show a common growth pattern over growing seasons; an establishment phase where end-of-season biomass increases, a maturity phase when plants reach peak end-of-season biomass, and a reduced-yield phase associated with plant aging (Fig. 1). While this age response is well conserved across a wide range of perennial species [forest trees (Ryan et al., 1997, 2004; Bond, 2000), C$_4$ (e.g. Christian et al., 2008; Ramburan et al., 2013; Arundale et al., 2014) and C$_3$ (e.g. Angelini et al., 2009) perennial grasses, grasslands (Radrizzani et al., 2010; Jungers et al., 2015), and others (Howeler, 1991; Mantineo et al., 2009)], it is merely observational and does not indicate whether these changes in end-of-season biomass are driven by micro-environmental conditions that change along with the age of the stand (e.g. weed pressure, self-shading, or nutrient limitations), or are driven by intrinsic physiological changes associated with each phase. Understanding the physiological changes

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Abbreviations: A$_{\text{乖}},$ leaf photosynthetic net CO$_2$ assimilation; A$_{\text{sat}},$ light-saturated A$_{\text{乖}}$; N, nitrogen; N$_{\text{乖}}$ leaf N mass per unit leaf area; PNUE, photosynthetic nitrogen use; qPSII, PSII efficiency; R$_{\text{d}},$ leaf respiration in the dark; V$_{\text{cmax}},$ maximum carboxylation rate of Rubisco; V$_{\text{pmax}},$ maximum PEP carboxylation rate; Φ, maximum quantum yield.

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associated with each phase would help understand the nature of plant aging and is key to predicting when these phases are likely to occur, as well as what management or genetic interventions can be used to influence their timing.

Similar to plant biomass, leaf photosynthesis also changes in response to whole-plant age. Juvenile plants (i.e., those in the establishment phase) tend to have higher photosynthetic rates than mature individuals that are in the peak- or reducing-yield phase. This age-related decline has been observed in several tree species (Niinemets, 2002; Tang et al., 2014; Chondrogiani and Grammatikopoulos, 2016) and in warm- and cool-season perennial grasses (Boersma et al., 2015; Jaikumar et al., 2016). As photosynthesis represents the source of carbohydrates for plant growth (i.e., biomass) and metabolism, an age-related decline in photosynthesis could be a potential mechanism behind the observed decline in biomass. However, since leaf photosynthesis is highly dependent on environmental signals as well as internal regulation, it may be that variable growth conditions cause limitations that manifest over time, thus presenting as age effects.

Because plants grow larger as they age, changes in plant size (represented as plant biomass) could also underpin observed age-related changes in leaf photosynthesis. If plants have finite access to nutrients, then nutrient pools inside older, larger individuals would be diluted compared with their younger, smaller counterparts. For example, when given the same amount of soil-available nitrogen (N), older plants must allocate this N to a larger volume of biomass, which manifests as a reduction in the amount of N present per unit of biomass or area. If less N is available in leaves for allocation to photosynthetic enzymes, then observed age effects could be a mere consequence of N limitation. However, older plants have larger root systems with which to mine soil N, and larger N pools that can be transferred to new buds to support initial growth in early spring. Spring N mobilization ranges between 23 kg ha⁻¹ and 98 kg ha⁻¹ have been reported (Leroy et al., 2022), and these could be helpful to mitigate the size-dependent N limitation, but little is known about the effects of N reserves on leaf N content over years. It is also possible that lower photosynthetic rates result from older plants allocating a larger proportion of N to non-photosynthetic functions as they age, but that is also poorly understood.

Because previous research showed that N requirements are larger late in the season when plants are bigger (Zapater et al., 2017), we tested whether this larger N requirements in bigger plants could lead to larger N limitations in older larger plants compared with juvenile smaller plants. Since 50–70% of N is allocated to photosynthetic enzymes (Makino et al., 2003; Evans and Clarke, 2019), we hypothesized that this N limitation in older larger plants could explain the observed age-related decline in photosynthesis. Changes in leaf N concentrations are an indicator of N availability in the plant and have been shown to be tightly correlated with leaf photosynthetic net CO₂ assimilation (A_{net}) (Sinclair and Horie, 1989; Kattge et al., 2009; Sage et al., 2013; Walker et al., 2014). Classically, A_{net} shows an asymptotic response to leaf N concentration (expressed as mass per unit area; Nₐ): it increases linearly up to high Nₐ levels (0.5–1.5 g N m⁻²), then begins to saturate and plateau (Sinclair and Horie, 1989; Kattge et al., 2009; Sage et al., 2013; Walker et al., 2014). The slope of the linear portion of the A_{net}/Nₐ slope is known as the photosynthetic nitrogen use efficiency (PNUE) and can be used as an indicator to compare A_{net} relative to a unit of leaf N.

Key C₄ enzymes [phosphoenolpyruvate carboxylase (PEPC), pyruvate phosphate dikinase (PPDK)] and Rubisco abundances also increased linearly with Nₐ (Tazoe et al., 2016). Even when not directly measured, the performance of these enzymes and the photosynthetic apparatus can be inferred by measuring A_{net} at different internal CO₂ concentrations (i.e., Cᵢ curves; Von Caemmerer and Furbank, 2003; Sharkey et al., 2007; Sage et al., 2013) and different incident light levels (i.e., A–Q curves; Sage et al., 2013; Coe and Lin, 2018). If the potential N limitation in larger and older plants occurs at the leaf level, then the observed decline in A_{net} could be a physiological consequence of reduced leaf N limiting photosynthesis rather than changes associated with plant aging. Consequently, N supply becomes a key variable to control in order to accurately assess the nature of aging in perennial grasses. This is especially important in fast-growing perennial plants that can double or triple their size during establishment (Tejera et al., 2019).

In temperate areas of the USA and Europe, the warm-season perennial grass Miscanthus × giganteus (Greef et. Deu.; Hodkinson
A net than 3-year-old stands (Boersma et al., 2009) and low nutrient requirements. Because *M. × giganteus* is a clonally propagated perennial, establishment is relatively more challenging and expensive compared with seeded C₄ crops such as maize or sorghum, elevating the importance of understanding and accurately estimating yields over a multi-year life cycle (Edmonds et al., 2021). With minimal genetic variability, and therefore minimal genetic × environment interactions, this clonally propagated C₄ grass could also help elucidate age-related changes in perennial physiology and the interactive effects of N fertilization.

In a previous study, 1-year-old *M. × giganteus* stands had up to four times greater *A*ₙₒₜ than 3-year-old stands (Boersma et al., 2015). These results could suggest an aging response in *M. × giganteus*; however, N could have been a limiting factor as 3-year-old stands were notably bigger in size, and leaf N in 1-year-old stands was up to 2.4 times greater than in 3-year-old stands. In a different study, the response of *M. × giganteus* leaf photosynthesis to N fertilization was found to be smaller than that of leaf N. *A*ₙₒₜ and *V*ₘₐₓ were not affected by N fertilization in 4-year-old stands, even when fertilized stands had ~25% higher leaf N content than unfertilized stands, suggesting that factors other than N limited photosynthesis (Wang et al., 2012).

To our knowledge, no previous research has studied the combined effects of age and N fertilization on photosynthesis in perennial crops. Usually, age-related changes are studied from an ecological perspective on unfertilized co-existing stands of different ages. While representative of natural conditions, this approach may confound micro-environmental conditions (e.g. saplings grown in the understorey and mature trees with leaves in the upper canopy) or different site histories. We posit that these confounding effects have masked age effect differences, limiting the scope of earlier studies. Confounding environmental conditions are especially important when measuring photosynthetic traits as they rapidly change with the environment. To resolve the confounding effects of growing season and age, we suggest age effects should be studied in independent combinations of age and growing season. These combinations are impossible when studying multiple stand ages in one growing season, or one stand over multiple growing seasons. Staggered-start experiments, however, allow for such comparisons by replicating the planting year, effectively starting a new iteration of the experiment each year, to provide independent combinations of age and growing season (Casler, 1999; Loughin, 2006; Tejera et al., 2019).

To elucidate N limitation in older plants and overcome environmental limitations of previous experiments, we combined a staggered-start design (Tejera et al., 2019) with a wide range of N fertilization rates to study age-related changes in *M. × giganteus* photosynthetic performance in the field. The experiment was carried out during the first 3 years of *M. × giganteus* growth, when the plant transitions from juvenile to mature. We chose this period because previous research showed clear phenological and developmental differences between 1-, 2-, and 3-year old stands (Tejera et al., 2019, 2021). Overall, 2- and 3-year-old stands behaved similarly, indicating that they had reached maturity, in contrast to the juvenile 1-year-old stands.

In this experiment, we first tested how aging affects photosynthesis in the perennial grass *M. × giganteus*. Our hypotheses were:

Hypothesis (i) Photosynthesis by age: leaf photosynthesis in 2- and 3-year-old (mature) stands would be lower than in 1-year-old (juvenile) stands, and mature stands would not differ from each other. To test this, we compared *M. × giganteus* photosynthetic performance (specifically, *A*ₙₒₜ and *A*–*C*ᵣ and *A*–*Q* curve parameters), from unfertilized plots, between stand ages.

Hypothesis (ii) Leaf N by age: mature stands, which would be larger in size, would have lower leaf N than juvenile stands. We tested this by comparing leaf N content, on an area (*N*ₐ) and mass (*N*ₘ) basis, of unfertilized stands, across stand ages.

Hypothesis (iii) Photosynthesis by leaf N: leaf photosynthesis and leaf N would increase with N fertilization regardless of stand ages. We tested this by comparing *M. × giganteus* photosynthetic performance, *N*ₐ and *N*ₘ, averaged over stand ages, across the increasing levels of N fertilization.

Hypothesis (iv) Age by N fertilization: mature stands with reduced leaf photosynthesis and leaf N would reach juvenile leaf photosynthesis and N levels when fertilized with a high enough N fertilization rate. We tested this by comparing *M. × giganteus* photosynthetic performance from mature fertilized plots with unfertilized juvenile plots and by comparing PNUE across stand ages.

If hypothesis (iv) holds true, and there are no differences, then we would conclude that age-related changes in photosynthesis observed in the literature were probably driven by N limitations associated with reduced N per unit of biomass and aging effects would be minimal. Otherwise, if N fertilization does not arrest the aging effect and juvenile stands have higher PNUE, then we would conclude the nature of this age-related change is likely to be driven by ontogenic changes rather than the environment.

**Materials and methods**

**Experimental design**

This study was conducted in the field at the Iowa State University Sorenson Farm in central Iowa (42.0132 N, –93.7430 W), USA. *Miscanthus × giganteus* clone ‘Freedom’ (sourced from Repreve Renewables, now AGgrow Tech, Greensboro, NC, USA) was planted in 0.3 m rows at a density of ~11 rhizome m⁻² using a proprietary mechanized plot planter. The Freedom clone is genetically very similar to both the ‘Illinois’ clone widely used in the USA and the ‘EMI-1’ clone widely used in Europe (Głowacka et al., 2015). The experimental design was a staggered-start randomized split-plot with four blocked replications (*n*=4). Stand ages, implemented as planting years (2015, 2016, and
2017), were the main-plot (24 m×60 m) and N fertilization rates (0, 112, 224, 336, and 448 kg ha⁻¹) were the subplot (24 m×12 m). That is, 20 subplots (5 N rates×4 blocks=20) were planted each year during 2015–2017. By 2017, the design resulted in 4 replications×3 ages×5 N rates=60 experimental units (subplots) per site. Planting occurred in May, and N treatments were applied within a month. Fertilizer was applied in subsequent years within a month of crop emergence. Weeds were managed with herbicides when needed. See Tejera et al. (2019) for full details of plot management. Soils in the experimental site were classified as typic Endoaquolls; our site was characterized by lower soil N, P, and K, and low organic matter (Tejera et al., 2019). Soil N content in the top 0.15 m ranged from 11 kg ha⁻¹ to 92 kg ha⁻¹ across all N, P, and K, and low organic matter (Tejera et al., 2019). Soil N content in the top 0.15 m ranged from 11 kg ha⁻¹ to 92 kg ha⁻¹ across all plots; but showed no statistical differences prior to the establishment of the crop.

Weather data

Historic and current weather data (Table 1) were obtained from the Iowa State University Soil Moisture Network (https://mesonet.agron.iastate.edu/). The weather station (Site ID: BOOI4) was 2.7 km from the experimental site. We considered the growing season to start \( T_{F} \) on the day of planting for new stands (juvenile stands) and the date of last hard spring frost for established stands (mature stands). Similarly, the end of the growing season \( T_{L} \) was defined as the date of the first hard autumn frost. We defined a hard frost as when the air temperature stayed below 0 °C for >12 h (Kaiser and Sacks, 2015). Thermal time was measured in growing degree days (GDD, °C d) as:

\[
GDD = \sum_{i} \left[ \frac{T_{\text{max}} + T_{\text{min}}}{2} \right] - T_{b}
\]

Where \( T_{\text{max}} \) and \( T_{\text{min}} \) are daily high and low temperatures, and \( T_{b} \) is a base growth temperature of 6 °C (Farrell et al., 2006).

Data collection

Gas exchange measurements

Photosynthetic gas exchange and chlorophyll fluorescence were surveyed with a portable infrared gas analyzer (LI-6400xt, Licor®, Lincoln, NE, USA) on the youngest, fully expanded leaf (as indicated by ligule presence) in the sunlit upper canopy (Dohleman and Long, 2009). Measurements were taken every 2 weeks beginning when leaves were big enough to fill the leaf cuvette (early summer), until a killing frost was recorded once values of \( A \) and stomatal conductance (\( g_{s} \)) reached steady state, typically within 30–90 s.

The responses of \( A \) to light (\( Q \)) and intercellular \( CO_{2} \) concentration (\( C_{I} \)) curves were measured every month on intact leaves in the field. For the \( A-Q \) curves, we decreased PPFD from 2000 \( \mu mol \text{ m}^{-2} \text{s}^{-1} \) to 0 \( \mu mol \text{ m}^{-2} \text{s}^{-1} \), while keeping the \( CO_{2} \) concentration in the cuvette constant at 400 \( \mu mol \text{ CO}_{2} \text{ mol}^{-1} \), in 10 steps (2000, 1500, 1000, 750, 500, 250, 150, 100, 50, and 0 \( \mu mol \text{ m}^{-2} \text{s}^{-1} \)). We measured and logged \( A \) measurements once stable. For \( A-C_{I} \) curves, \( A \) was measured at 10 \( CO_{2} \) concentrations inside the cuvette (400, 300, 200, 100, 0, 400, 400, 600, 800, 1000, 1200, and 1500 \( \mu mol \text{ CO}_{2} \text{ mol}^{-1} \)) in 2015 and eight (400, 300, 200, 100, 50, 400, 400, 600, 1000, and 1200 \( \mu mol \text{ CO}_{2} \text{ mol}^{-1} \)) in 2016 and 2017 to accommodate the date larger plot number. In all cases, PPFD inside the cuvette was matched to ambient values then kept constant for the duration of that day’s measurements. \( A-Q \) curves were then individually modeled with non-rectangular hyperbola to determine: the light-saturated rate of assimilation (\( A_{\text{max}} \)), the maximum efficiency of light-limited net CO₂ assimilation (\( q_{1} \)), and the rate of dark respiration (\( R_{d} \)) (Dohleman and Long, 2009; Archontoulis and Miguez, 2013). \( A-C_{I} \) data were modeled using a three-parameter asymptotic exponential equation to determine the \( CO_{2} \)-saturated rate of assimilation (\( V_{\text{max}} \)) and the compensation point (\( P \)) when net CO₂ assimilation is zero. The initial slope of the curve (\( C_{I} <100 \)) was used to estimate the maximum carboxylation capacity of phosphoenolpyruvate (\( V_{\text{max}} \), Markelz et al., 2011; Li et al., 2021).

As cuvette conditions were set to match environmental light and temperature conditions, the extra variability was introduced when observations were compared across sampling dates. Light conditions should not have caused large variability as the PPFD used in \( A-C_{I} \) curves was at least 1200 \( \mu mol \text{ m}^{-2} \text{s}^{-1} \), which represents 70% of a clear-sky PPFD value. In all cases, the \( A_{\text{sat}} \) value at ambient \( CO_{2} \) concentrations was at least 80% of the light-saturated \( A_{\text{sat}} \) values measured in the \( A-Q \) curves. Temperatures were more variable (Supplementary Fig. S1A), and to account for this variability we also estimated temperature-corrected \( A_{\text{sat}} \) values, both for survey and for \( A-C_{I} \) and \( A-Q \) curves. Temperature corrections were estimated based on the Beta equation (Bernacchi et al., 2003; Archontoulis and Miguez, 2013). Different temperature response functions were fitted for the survey \( A_{\text{sat}} \), \( CO_{2} \)- and light-saturated values. These values were then normalized to the predicted value at 25 °C (Supplementary Fig. S1B).

Table 1. Current and historic weather data in central Iowa (42.0132N, –93.743W), USA, for three Miscanthus giganteus growing seasons in a staggered-start experiment.

| Growing season | Monthly average temperature (°C) | Total GDDₙ | Total precipitation (mm) | Total incident PAR (MJ m⁻²) | Last spring frost | First autumnal frost | Growing season length (d) |
|----------------|----------------------------------|------------|-------------------------|----------------------------|-------------------|----------------------|------------------------|
| 2015           | 17.5                             | 2540       | 840                     | 1863                       | 27/03             | 20/11                | 238                    |
| 2016           | 18.6                             | 2779       | 713                     | 1884                       | 03/03             | 19/11                | 261                    |
| 2017           | 18.9                             | 2561       | 580                     | 1812                       | 15/03             | 10/28                | 227                    |
| 1986-2013      | 16.0                             | 2423       | 656                     | 1768                       | ---               | ---                  | ---                    |

购置 days (GDD) were calculated using 6 °C as the base temperature for growth (Farrell et al., 2006).

a Frost is considered as days with air temperatures below 0 °C for >12 h. Hourly weather data were not available to estimate historic events.
Modulated chlorophyll fluorescence was simultaneously measured with the other photosynthetic parameters. We estimated PSII efficiency (ϕPSII) as the ratio of absorbed photons used in photochemistry after a saturating light pulse (Genty et al., 1989), according to:

$$\phi_{PSII} = \frac{F_o - F'_m}{F_m'}$$

(2)

Where, $F_o$ is ‘steady-state’ fluorescence, and $F_m'$ is the maximal fluorescence during a saturating light flash. Decreasing ϕPSII has been previously correlated to $M. \times giganteus$ senescence (Boersma et al., 2015).

**Leaf nitrogen measurements**

Immediately after photosynthetic measurements were recorded, the measured leaf lamina was excised and used for specific leaf area (SLA; gDM m$^{-2}$) and leaf N content estimations. In 2015 and 2016, SLA for each plot was estimated based on a composite sample of 40 1.6 cm$^2$ leaf punches taken from all measured leaves within the plot and dried at 60 °C until constant weight. In 2017, excised lamina were kept at ~5 °C and leaf area was measured in the lab with a flatbed leaf scanner (LI-3100C; Area Meter; Licor®) and then dried at 60 °C to constant weight. SLA was then estimated as the ratio of average leaf mass to leaf area of all sub-samples. All dried leaves were then ground to a powder (<1 mm) and a 3–5 mg subsample was mixed with tungsten trioxide and combusted in an elemental analyzer (Elementar, Ronkonkoma, NY, USA) to determine the concentration of N ($N_{\mu}$; gN g$^{-1}$). Acetanilide was used as a standard N reference. Leaf N content on an area basis ($N_{\mu}$; gN m$^{-2}$) was estimated as the product of $N_{\mu}$×SLA.

**Data analysis**

For this analysis, 1-year-old stands were considered juvenile and 2- and 3-year-old stands mature. This determination is based on previous findings that suggested that the phonology of 1-year-old stands clearly differed from that of 2- and 3-year-old stands, indicating that they were developmentally distinct (Tejera et al., 2021). We began measuring gas exchange in mature stands in June, and in juvenile stands 2 months later, when the leaves of juvenile plants developed sufficient laminar tissue to fill the measurement cuvette. To avoid biased comparisons, we studied differences between juvenile and mature stands only on those sampling dates when all stands were present (co-occurring dates; August–November).

To facilitate data analysis and interpretation, we used subsets of the full dataset to test the different hypotheses:

Hypothesis (i) Photosynthesis by age: we compared photosynthetic parameters (e.g. $A_{\text{net}}$, $V_{\text{max}}$, $\Gamma$, $V_{\text{cmax}}$, $\phi_{\text{CO2max}}$, $A_{\text{max}}$, and ϕPSII) of only unfertilized plots of all planting years within co-occurring dates.

Hypothesis (ii) Leaf N by age: we compared $N_{\mu}$ of only unfertilized plots of all planting years within co-occurring dates.

Hypothesis (iii) Photosynthesis by leaf N: we compared $A_{\text{net}}$ and $N_{\mu}$ of all N rates and planting years averaged over stand ages.

Hypothesis (iv) Age by N fertilization: we compared the photosynthetic parameters and $N_{\mu}$ of fertilized mature stands to juvenile unfertilized stands. Further, we calculated the PNUE as the ratio of $A_{\text{net}}$ ($PNUE_{\text{net}}$), $V_{\text{max}}$ ($PNUE_{\text{vmax}}$), and $A_{\text{net}}$ ($PNUE_{\text{in}}$) over $N_{\mu}$.

We used R statistical software (R Core Team, 2017) for all analyses; the specific models, datasets, and procedures depended on the hypothesis and variables considered. For $A_{\text{net}}$ and $N_{\mu}$, we used mixed effect linear models to test the different hypotheses. Stand age, N rate, and sampling date were considered as fixed effects. As in Tejera et al. (2019), the full random structure that adjusted best to our experimental design (full model; subplots nested in main plots nested in blocks) had many near-zero variance terms.

A simpler random structure, with blocks as a random term, was the one that minimized the number of parameters and maximized goodness of fit [using the Akaike information criterion (AIC), Bayesian information criterion (BIC), and log-likelihood]. We used lmer() in the lme4 package (Bates et al., 2014) to fit the mixed models, Anova() in the car package (Fox and Weisberg, 2011) for analysis of deviance using type II Wald $\chi^2$ test, and emmeans() in the emmeans package (Lenth et al., 2018) for mean comparison.

The temporal dynamics of $A_{\text{net}}$ in mature stands showed two distinct phases; a phase with constant $A$ in summer (phase I) followed by steady decline until the first hard frost (phase II, Fig. 2). We compared mature stands based on the average $A_{\text{net}}$ value of the constant phase ($\bar{A}$), the slope (Rate) of $A$ during the declining phase ($\bar{\Delta}$), and the day of the year when the declining phase started (Breakpoint; Fig. 2).

We modeled this dynamic assuming a segmented relationship with time. A segmented relationship is defined by the slope parameters of each line segment, and the breakpoints are where the straight lines connect and the linear relation changes. We used the segmented() function in the segmented package in R (Muggeo, 2008) to model $A$ over day of the year and estimate break-points and slope of the two phases. Stand age and N fertilization rates were considered fixed effects for each growing season.

We used non-linear mixed models to model $A$–$C_i$ and $A$–$Q$ data and test stand age and N effects on model parameters. We modeled $A$–$C_i$ data with an asymptotic exponential three-parameter model (Pinheiro and Bates, 2000; Archontoulis and Miguez, 2013):

$$A_{\text{net}} = V_{\text{cmax}} \left( 1 - e^{-\theta_1(C_i - C_{\text{sat}})} \right)$$

(3)

Where net CO$_2$ assimilation ($A_{\text{net}}$) response to internal CO$_2$ concentration ($C_i$) is a function of the maximum rate of Rubisco carboxylation ($V_{\text{cmax}}$) and the CO$_2$ compensation point ($\Theta$). The lrC parameter is related to the initial slope of the response ($V_{\text{cmax}}$) but to simplify interpretation we directly estimated $V_{\text{cmax}}$ as the slope of the $A$–$C_i$ curve when $C_i$ is <100 μmol CO$_2$. We modeled $A$–$Q$ data using a four-parameter non-rectangular model (Johnson et al., 2010; Archontoulis and Miguez, 2013):

$$A_{\text{net}} = A_{\text{sat}} + \phi_{\text{CO2}} Q - \sqrt{(A_{\text{sat}} + \phi_{\text{CO2}} Q)^2 - 4\phi_{\text{CO2}} Q A_{\text{sat}}} - R_d$$

(4)

Where net CO$_2$ assimilation ($A_{\text{sat}}$) response to light intensity ($Q$) is a function of light-saturated net CO$_2$ assimilation ($A_{\text{sat}}$), the maximum efficiency of light-limited net CO$_2$ assimilation ($\phi_{\text{CO2}}$), a curvature parameter ($\Theta$), and the y-intercept interpreted as leaf respiration in the dark ($R_d$).

As with linear models, our non-linear model-building strategy was to start with the models that better described our experimental design, with random effects on all curve parameters, and then dropped near-zero variance terms. We chose simpler models based on fewer numbers of parameters and goodness of fit (AIC, BIC, and log-likelihood) (Pinheiro and Bates, 2000). Then we included fixed effect terms (i.e. stand age and Nrate) and assessed their effect on the model parameters. We used the nlme R package (Pinheiro and Bates, 2000) for model construction and testing, and the emmeans package to compare model parameters.

**Results**

During the first 3 years of growth, the age of the stand was the main driver of differences in $M. \times giganteus$ photosynthesis and leaf N. Two- and three-year-old (mature) stands had ~20% lower leaf-level photosynthesis (i.e. $A_{\text{net}}$, $V_{\text{cmax}}$, and $A_{\text{in}}$) and those leaves had 34% lower $N_{\mu}$ content (Figs 3, 4).
than leaves of 1-year-old (juvenile) stands. Nitrogen fertilization increased photosynthetic parameters and N content, regardless of the stand age, and A and N content leveled off at higher fertilizer rates (Table 2). Fertilized mature stands reached similar photosynthesis and N content to unfertilized juvenile stands (Figs 2 and 3). A net during the constant phase (Rate), and the day when stands started declining phase (breakpoint), to compare treatment effects. See Fig. 3 for comparison of juvenile and mature rates.

Mature stands had 10–34% lower leaf photosynthesis than juvenile stands

When considering only unfertilized stands, mature stands had 19–30% lower A net than juvenile stands in 2016 and 2017 (when measured on co-occurring dates August–November; P<0.05; Fig. 3A), and 11–33% lower ΦPSII (P<0.05; Fig. 3B). Similarly, mature stands had 10–34% lower V cmax (P<0.05; Fig. 4A) and 34% lower A sat (P<0.05; Fig. 4B). Other A–C i and A–Q parameters showed some trending results but, due to large variability, age and N effects were not significant at 0.05. A–C i and A–Q parameters for all sampling dates are presented in Supplementary Tables S1 and S2, respectively.

Mature stands showed a bi-phase dynamic throughout the growing season of A net across ages and years (Fig. 3A). In 2016, the segmented model achieved convergence after two iterations with an R 2=0.65. In 2017, convergence was achieved after nine iterations with an R 2=0.60. Two- and three-year-old stands had very similar A net values during the constant phase (P>0.05; A net, 2016–2yr=30.5 ± 1.36 µmol CO 2 m −2 s −1; A net, 2017–2yr=32.5 ± 2.2 µmol CO 2 m −2 s −1; Fig. 3A), and similar declining rates during the declining phase (P>0.05; Rate, 2016–2yr= −0.4 ± 0.15 µmol CO 2 m −2 s −1 d −1; Rate, 2017–2yr= −0.14 ± 0.056 µmol CO 2 m −2 s −1 d −1; Rate, 2017–3yr= −0.19 ± 0.081µmol CO 2 m −2 s −1 d −1; Fig. 3A). A net transitioned from the constant to the declining phase on 5 September (± 11 d) in 2016, and on 8 July (± 35 d) and 1 August (± 26 d) for 2-year-old and 3-year-old stands in 2017, respectively.

Temperature-corrected A net values did not show the bi-phase trend, and steadily declined during the growing season (Supplementary Fig. S2). In spite of the large variability, averaged over growing seasons, the temperature-corrected A net rate of decline was 1.5× and 2.5× faster in 2- and 3-year-old stands than in 1-year-old stands. The temperature-corrected A net rate of decline over the growing season was the only parameter not different from zero (P>0.05). For the other photosynthetic parameters, mature stands had variable responses. Two-year-old stands had 20% lower ΦPSII (Fig. 3B), and 3-year-old stands had 20% higher V cmax (Fig. 4A), but none was significantly different (P>0.05). Two- and three-year-old stands also had very similar A sat values (P>0.05; Fig. 4C). Temperature-corrected V cmax and A sat also declined during the season, but in these cases, none of the slopes was significantly different from zero (P>0.05; Supplementary Fig. S3).

Mature stands had 14–34% lower leaf nitrogen on an area basis than juvenile stands

Unfertilized mature stands had 14–34% lower N content than unfertilized juvenile stands in 2016 and 2017 when measured on co-occurring dates (P<0.05; Fig. 5). No differences were found between mature 2- and 3-year-old, stands (P>0.05). N content remained almost constant across stand ages during the growing season, especially during the co-occurring period, with a significant but near-zero slope (P<0.05; Fig. 5). Similarly, SLA also declined very slightly over the growing season with a slope near zero and very low variance (Supplementary Fig. S4). Juvenile and mature stands had very similar SLA values in all years (P>0.05).

Nitrogen fertilization increased leaf photosynthesis by 8–27% and leaf N by 30% across stand ages

N fertilization increased leaf photosynthesis during the growing season across all stand ages. Compared with the unfertilized stands, the N rate with the largest effect increased A net by 8%, V cmax by 27%, and A sat by 14% (P<0.05; Table 2). Similarly, N increased by 30% in fertilized stands relative to the unfertilized stands averaged over all stand ages (P<0.05; Table 2). Along with A sat, other A–Q parameters also showed a clear response to N fertilization; φ CO2 and R d increased up to 15% and 25%, respectively. ΦPSII, Γ, and θ were not affected by N fertilization (P>0.1).
In spite of the wide range of N fertilization tested in this experiment, leaf photosynthesis showed a rather discrete response to N fertilization, with differences being mainly between no fertilizer and any fertilizer. In the vast majority of cases, the unfertilized treatment was only significantly different at the higher N fertilization rates (i.e. 336 kgN ha $^{-1}$ or 448 kgN ha $^{-1}$), while intermediate N rates did not differ from either high or low extremes (Table 2). In contrast, N$_{a}$ showed an asymptotic response to N fertilization, where the 112 kg N ha$^{-1}$ treatment had significantly higher N$_{a}$ than the unfertilized treatment, and the 336 kgN ha$^{-1}$ or 448 kg N ha$^{-1}$ were also statistically higher than all other treatments. This suggest that both N$_{a}$ and photosynthesis are maximized between 112 kgN ha$^{-1}$ and 224 kg N ha$^{-1}$, since 224 kg N ha$^{-1}$ is not different from 448 kg N ha$^{-1}$ for any parameter.

Fertilizing mature stands reduced the age decline in leaf photosynthesis by 50% despite similar leaf nitrogen to unfertilized juveniles

N fertilization increased mature stand $A_{\text{net}}$ by 11–15%, $V_{\text{max}}$ by 2–45%, and $A_{\text{sat}}$ by 26–34% during 2016 and 2017 when measured on co–occurring dates ($P$–value $<0.05$; Fig. 6; Supplementary Fig. S5). The $\Phi$PSII response to N fertilization was smaller in mature stands ($\sim$ 8–16%). N fertilization of mature stands reduced the magnitude of age-related decline in photosynthetic parameters that had been observed between unfertilized juveniles and mature stands by $\sim$50%. For example, in 2017, unfertilized mature stands had 27% lower $A_{\text{net}}$ than unfertilized juvenile stands, but this difference dropped to 15% when fertilized mature stands were compared with unfertilized juveniles. In this case, N fertilization reduced the age effect by 56% ($15/27=0.56$). Overall, the photosynthetic values observed in fertilized mature stands were not statistically different from those seen in unfertilized juvenile stands ($P>0.05$; Fig. 6), and in some cases N fertilization restored photosynthesis values to those seen in juvenile stands.

The fertilization effect on N$_{a}$ was more consistent across years and stand ages; it increased N$_{a}$ by 23–36% (Fig. 6). This increase in N$_{a}$ caused fertilized mature stands to have very similar, or even higher, N$_{a}$ values than unfertilized juvenile stands. Accordingly, mature stands showed a larger N$_{a}$ response to N fertilization than leaf photosynthesis.
Fig. 4. Changes in Miscanthus × giganteus maximum carboxylation rate of Rubisco ($V_{\text{c,max}}$; A) and light-saturated net CO$_2$ assimilation ($A_{\text{sat}}$; B) at different stand ages during the 2015, 2016, and 2017 growing seasons. Data are averages from unfertilized plots ($n=4$). Error bars correspond to ±1 SEM. Inset graphs compile all net CO$_2$ assimilation response curves to internal CO$_2$ concentration ($C_i$) and PPFD during the period of the year when all stand ages occurred at the same time.

Table 2. Nitrogen (N) fertilization effect on Miscanthus × giganteus leaf N content on an area basis (N$_a$) and leaf photosynthetic parameters [i.e. net CO$_2$ assimilation ($A_{\text{net}}$), PSII efficiency ($\phi$PSII), and $A$–$C_i$ and $A$–$Q$ response curve parameters].

| Nitrogen fertilization rate (kg N ha$^{-1}$) | 0 | 112 | 224 | 336 | 448 |
|--------------------------------------------|---|-----|-----|-----|-----|
| Survey                                    |   |     |     |     |     |
| N$_a$                                      | 1.4 ± 0.055 c* | 1.6 ± 0.055 b | 1.7 ± 0.055 ab | 1.8 ± 0.055 a | 1.7 ± 0.055 a |
| $A_{\text{sat}}$                            | 26 ± 0.59 b | 28 ± 0.59 ab | 28 ± 0.59 a | 28 ± 0.59 a | 28 ± 0.59 a |
| $\phi$PSII                                 | 0.2 ± 0.0063 | 0.21 ± 0.0063 | 0.21 ± 0.0063 | 0.21 ± 0.0063 | 0.21 ± 0.0063 |
| A–C$_i$ curve                              | $V_{\text{c,max}}$ | 34 ± 1.5 b | 36 ± 1.5 ab | 41 ± 2 ab | 36 ± 1.5 ab | 43 ± 2.5 a |
|                                            | $l_{\text{rc}}$ | −4.7 ± 0.075 a | −4.8 ± 0.072 a | −5.1 ± 0.078 b | −4.8 ± 0.07 a | −5.2 ± 0.081 b |
|                                            | $\Gamma$ | 5.5 ± 5.2 a | 6.7 ± 4.5 a | −20 ± 7.6 b | 10 ± 4.2 a | −37 ± 9.9 b |
| A–Q$_Q$ curve                              | $A_{\text{sat}}$ | 29 ± 1.1 b | 33 ± 1.1 ab | 33 ± 1.1 ab | 33 ± 1.1 a | 32 ± 1.1 ab |
|                                            | $\phi_{\text{CO}_2}$ | 0.059 ± 0.0013 b | 0.061 ± 0.0015 a | 0.058 ± 0.0014 ab | 0.056 ± 0.0014 ab | 0.057 ± 0.0015 ab |
|                                            | $\theta$ | 0.79 ± 0.021 | 0.7 ± 0.027 | 0.75 ± 0.022 | 0.72 ± 0.026 | 0.73 ± 0.026 |
|                                            | $R_g$ | 2.4 ± 0.11 b | 3 ± 0.11 a | 2.8 ± 0.11 ab | 2.8 ± 0.11 ab | 2.8 ± 0.11 ab |

$A$–$C_i$ parameters were estimated using a three-parameter asymptotic exponential regression. $A$–$Q$ parameters were estimated using a four-parameter non-rectangular hyperbola regression. See the Materials and methods for model equation and parameter interpretation. Values (mean ± SE) are the average over four replicates and all stand ages.

* Different lower case letters indicate significant differences ($P$-value < 0.05) between nitrogen rates for each parameter.
Leaf photosynthesis relative to Na was 35–86% higher in juvenile than in mature stands when estimated based on $A_{\text{net}}$ (PNUE$_{A_{\text{net}}}$), and 26–58% higher based on $A_{\text{sat}}$ (PNUE$_{A_{\text{sat}}}$) during 2016 and 2017 co-occurring dates, regardless of N fertilization rates (Table 3). The PNUE$_{V_{\text{cmax}}}$ was rather similar across stand ages; 1-year-old stands had 7% higher PNUE$_{V_{\text{cmax}}}$ than 2-year-old stands ($P<0.05$), but 3-year-old stands had 7% higher PNUE$_{V_{\text{cmax}}}$ than 1-year-old stands ($P<0.05$). There was a small, but significant, effect of N fertilization on all NUE indices; all indices showed a slight decline with increasing N fertilization rates (Fig. 7). The age by N fertilization interaction was also significant; however, all NUE indices remained fairly constant across stand ages and N fertilization rates (Fig. 7).

Discussion

We studied changes in M.×giganteus leaf-level photosynthesis as the crop aged during the establishment phase of the crop (first three growing seasons), and the potential interactive effects of N fertilization with age. We found that older M.×giganteus stands had 19–30% lower photosynthetic rates during the establishment phase of the crop (first three growing seasons). These changes could be related to N limitation as mature plants were 2.5× larger in size and Na was 14–34% lower than in juvenile plants. N fertilization increased leaf N up to 30% in older stands and allowed photosynthetic performance of fertilized mature stands to reach similar values to unfertilized juvenile stands; however, PNUE was higher in juvenile stands in 2016 and 2017. That is, 1-year-old stands had higher photosynthetic rates with the same leaf N content as older stands, suggesting that other factors were limiting photosynthesis in mature stands.

Mature stands had lower leaf photosynthesis than juvenile stands across a wide range of photosynthesis indices and growing conditions

Our results support our hypothesis (i) Photosynthesis by age: that M.×giganteus leaf photosynthesis in 2- and 3-year-old stands would be lower than in 1-year-old (juvenile) stands and mature stands will not differ from each other. We found a 19–30% age-related decline in $A_{\text{net}}$ in mature stands, which is within the range of other studies: in the cool season perennial grass Thinopyrum intermedium, the age-related decline in $A_{\text{net}}$ was ~25% (Jaikumar et al., 2016); in another M.×giganteus study, it was 38–68% (Boersma et al., 2015), and in pine trees (Pinus abies and Pinus sylvestris) it was ~40% (Niinemets, 2002). Similarly, the age-related decline on ΦPSII (11–33%) is also similar to another M.×giganteus study (Boersma et al., 2015). Our study complements
previous research on age-related changes in leaf photosynthesis in perennial grasses (e.g. Boersma et al., 2015; Jaiku- 
mar et al., 2016) by quantifying age effects on a wider range of leaf photosynthetic parameters using A–C– and A–Q re- 
response curves. Parameters such as $\nu_{\text{cm}}$ and $A_{\text{at}}$ are commonly used in terrestrial biosphere models and could help 
modelers include age-related changes in their models. We 
found that age-related effects are larger on CO$_{2}$- and light- 
saturated $A_{\text{net}}$ than any of the other parameters of the curves. Juvenile $M. \times$ giganteus stands had 10–34% higher $\nu_{\text{cm}}$ and 
$A_{\text{at}}$, which suggests that the $A_{\text{net}}$ differences were not driven by CO$_{2}$ or light limitations.

**Mature stands showed a biphasic dynamic on $A_{\text{net}}$ that 
did correspond to other autumnal leaf senescence 
changes in $\phi PSII$ or leaf nitrogen**

Among the mature stands, 2- and 3-year-old stands showed a 
similar $A_{\text{net}}$ pattern during the growing season; a constant phase 
where $A_{\text{net}}$ varied along a constant mean and a declining phase 
where $A_{\text{net}}$ steadily declined until a hard frost. The observed bi- 
phasic trend is a novel finding for $M. \times$ giganteus; however, late- 
season photosynthesis decline occurred in other perennial grasses 
(Boersma et al., 2015; De Souza et al., 2018; Stavridou et al., 2020; 
Kar et al., 2020). This biphasic dynamic could be partly driven by 
temperature differences during the season, as temperature-corrected 
$A_{\text{net}}$ did not show this dynamic. However, temperature-corrected observations still showed a steady decline, suggesting 
that other factors continue to limit photosynthesis during the 
growing season. Both $\phi PSII$ and $N_{L}$ showed a steady decline 
throughout the growing season (Figs 3B, 5), relating this decline to 
autumnal leaf senescence (Boersma et al., 2015).

**Mature stands had lower leaf $N$ and, while fertilized 
plants had higher leaf $N$ and photosynthetic rates, $N$ 
fertilization did not fully compensate for age-related 
$A_{\text{net}}$ decline**

These differences between stand ages could be driven by $N$ 
limitation. Mature stands had almost 2.5× more biomass than 
juvenile stands (Tejera et al., 2019), then for a finite $N$ supply 
from the soil, $N$ would need to be distributed in a larger volume 
of biomass in the larger plants, leading to less $N$ per unit of bi-
omass. Older larger plants have larger $N$ reserve pools in the 
rhizomes, and a larger root system that could potentially com-
penstate the $N$ limitation. We found that $N_{L}$ was 14–34% lower in mature plants than in juvenile plants, supporting hypothesis 
(ii) Leaf $N$ by age, and did not differ between 2- and 3-year- 
old stands (Fig. 5). This suggests that the increase in biomass of 
mature plants relative to juvenile plants is greater than the 
covering increase in leaf $N$ of the youngest ligulated leaf. 
Previous research shows that $N$ requirements are larger late in 
the season when plants are bigger (Zapater et al., 2017); here 
we show this increased $N$ requirement with size also occurs at 
the same time during the growing season, when stands are of 
different ages. Given the tight correlation between $A_{\text{net}}$ and $N_{L}$ 
(Sinclair and Horie, 1989; Sage et al., 2013; Walker et al., 2014), 
the decline in leaf photosynthesis in mature stands could be a 
consequence of the corresponding decline in $N_{L}$.

Both $N_{L}$ and $A_{\text{net}}$ positively responded to $N$ fertilization, 
supporting our hypothesis (iii) Photosynthesis by leaf $N$. N fer-
itilization increased $N_{L}$ by ~30% in fertilized stands over stand 
ages and years (Table 2); however, $N$ fertilization effect on leaf 
photosynthesis was not as large and was more variable; its effect 
increased $A_{\text{net}}$ by 8%, $\nu_{\text{cm}}$ by 27%, and $A_{\text{at}}$ by 14% (Table 2). 
The disproportional leaf $N$ response to $N$ fertilization com-
pared with the leaf photosynthesis response is rather common 
in $M. \times$ giganteus; 4-year-old fertilized $M. \times$ giganteus had 15% 
higher $N_{L}$, but had similar $A_{\text{net}}$ compared with unfertilized 
plants (Wang et al., 2012). Similarly, in two greenhouse-grown 
studies, 1-year-old fertilized $M. \times$ giganteus had ~2× and 2.5× 
higher $N_{L}$ than unfertilized plants, while their $A_{\text{at}}$ was only 
20% and 60% higher, respectively (Feng et al., 2012; Ma et al., 
2017).

Our experimental design allowed a direct test for the role 
of $N$ dilution on perennial grass aging; fertilized mature stands 
against unfertilized juveniles. Fertilized $M. \times$ giganteus mature 
stands had similar, or even higher, $N_{L}$ values than unferti-
lized juvenile stands (Fig. 6), supporting our hypothesis (iv) 
Age by $N$ fertilization. However, the $N$ fertilization effect on 
leaf photosynthesis of mature $M. \times$ giganteus stands was not as
Tejera et al. showed that N fertilization increased leaf photosynthesis, but did not completely close the age gap. N fertilization reduced the age decline on $A_{net}$ and $\Phi_{PSII}$ by ~50% and actually stimulated an increase in $V_{cmax}$ levels ~10% greater than those seen in juveniles (Fig. 6; Supplementary Fig. S5). Ultimately, a comparison of leaf photosynthesis per unit of N (i.e. NUE) among stand ages controls much of the variability and shows that juvenile stands had at least 26% higher PNUE$_{A_{net}}$ or PNUE$_{A_{sat}}$ than mature stands across all N fertilization rates (Fig. 7). This implies that while N fertilization increased leaf photosynthesis in mature stands, in some cases up to juvenile levels, this increment was largely driven by a disproportionally larger change in leaf N, which could indicate a luxury consumption.

Late-season sink limitations could drive the mature stands’ decline in photosynthesis

If N fertilization alone did not compensate for the age decline in photosynthesis, what other factors could limit photosynthesis in mature stands, and not as strongly limit photosynthesis in juvenile plants? We found that temperature was an important driver of the bi-phasic dynamic; however, temperature-corrected $A_{net}$ still showed a steady decline over the growing season, suggesting that other factors may also limit photosynthesis during the season. The current understanding of temperature correction assumes that temperature is the only limiting factor. This assumption is valid when changes in CO$_2$ assimilation are studied over a range of sequential temperatures (e.g. temperature response curves). It is worth pointing out that in the field setting when changes in temperature occur during the growing season, they involve changes in development and reserve status. We hypothesize that sink limitations may play an important role in the photosynthesis of mature stands of perennial grasses. Sink limitations occur when the main carbohydrate sinks (e.g. rhizomes and growing points) have ceased their activity, and recently assimilated carbohydrates are consumed at a lower rate than they are produced. Ultimately, carbohydrates accumulate in the leaf, trigger feedback limitations on photosynthesis, and reduce $A_{net}$. Sink limitations have already been suggested to limit photosynthesis and late-season growth of perennial grasses, (McCormick et al., 2006, 2009). Here we propose that sink limitations could help explain the photosynthetic decline observed in mature stands late in the season, and the photosynthetic decline observed in mature stands compared with juveniles.

From Tejera et al. (2021), we know that mature $M. \times giganteus$ plants reached a steady number of tillers in mid-June, and completed vegetative development by the end of August. Since growth represents a major sink of carbohydrates, mature plants could experience sink limitations around late summer when vegetative development had ceased. Accordingly, mature stands showed that $A_{net}$ started declining around the same
time; early September in 2016 and late July in 2017. While this marked decline was not observed in temperature-corrected $A_{\text{net}}$, it still showed a steady decline, suggesting other limiting factors. Altogether this suggests that the $A_{\text{net}}$ decline observed in mature stands late in the season could be related to sink limitations driven by reduced growth. When comparing stand ages, growth may remain active, both above- and below-ground, in juvenile stands for longer in the season. Juvenile stands did not reach reproductive stages in 2016 or 2017, and continued to grow until the late autumn. In turn, mature stands ceased vegetative growth and transition to reproductive growth much earlier in the growing season (Tejera et al., 2021). In addition, below-ground biomass is known to increase in juvenile stands and remains constant after the third year of growth, when plants reached the mature phase (Nassi o Di Nasso et al., 2013). Considering that $M. \times gigan\text{teus}$ does not produce seeds and biomass allocation to reproduction is minimal, older stands by the end of the growing season may lack major sinks to which to direct photosynthates, ultimately limiting photosynthesis, leading to the observed photosynthetic decrease in mature stands.

Complementing leaf photosynthesis and N seasonal observations with seasonal sampling of carbohydrate reserves would help test this hypothesis. In sugarcane, leaf photosynthesis steadily declined during the growing season, while leaf sucrose and starch, and root sucrose reached a maximum concentration towards the end of development, suggesting a sink limitation late in the season when plants ceased growth (De Souza et al., 2018). It is worth highlighting that, while leaf-level photosynthesis was lower in older plants, on the whole they were bigger and have more leaves (Tejera et al., 2019, 2021); therefore, they would still have greater canopy-level carbon assimilation.

$M. \times gigan\text{teus}$ spp. breeding programs usually focus on traits such as later flowering to extend the vegetative growth season and achieve higher yields. Here, we suggest that prolonging vegetative growth could keep sink organs active, maintaining higher photosynthesis for longer in the season. In addition, breeding for genotypes that are more tolerant to sink limitations could be a successful strategy to maintain higher rates of photosynthesis for longer in the season and reduce the yield decline after peak yields.

Conclusion

The study of age-related changes in perennial physiology includes many confounding factors that limit our understanding of the underlying mechanisms. We add to previous research on the role of micro-environmental conditions on aging (e.g. Vitasse, 2013), by directly testing whether age-related changes in photosynthesis are due to N dilution in larger older plants under field conditions. Our staggered-start experimental design allowed us to conduct this work in the field while also controlling for stochastic and environmental conditions. Mature stands had lower photosynthetic rates and leaf N contents, suggesting potential N dilution in older larger plants. However, N fertilization increased mature $M. \times gigan\text{teus}$ N, up to the levels of juveniles, but the photosynthetic response per unit of N was much lower, suggesting that N was not a limiting factor. Considering that mature stands cease growth much sooner in the growing season than juvenile stands, and that juvenile stands allocate more biomass to below-ground organs, we hypothesize that sink limitations could drive the age-related difference between juvenile and mature $M. \times gigan\text{teus}$ plants. More research is needed to understand how leaf photosynthesis scales to canopy-level photosynthesis in $M. \times gigan\text{teus}$, and whether this age-related change holds at the canopy level.

Supplementary data

The following supplementary data are available at JXB online.

Table S1. Parameters of net CO$_2$ assimilation response to light response curves.

Table S2. Parameters of net CO$_2$ assimilation response to internal CO$_2$ concentration response curves.

Fig. S1. Leaf temperature distribution and net CO$_2$ assimilation response to leaf temperature.

Fig. S2. Temperature-corrected net CO$_2$ assimilation over the growing season.

Fig. S3. Temperature-corrected maximum carboxylation rate of Rubisco ($V_{\text{cmax}}$) and light-saturated net CO$_2$ assimilation ($A_{\text{sat}}$).

Fig. S4. Specific leaf area over the growing season.

Fig. S5. Nitrogen effect on leaf CO$_2$ assimilation, PSII efficiency, and leaf nitrogen age decline.

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Author contributions

All authors contributed ideas; MT: collected and analyzed data, coordinated field work, and led the writing of the manuscript; NB: designed the experiment and helped with data collection; SA: provided important feedback on photosynthesis and leaf nitrogen; FEM: made substantial contributions to data analysis; AV: critically reviewed all versions of the manuscript; EAH: supervised MT and NB, designed the experiment, and critically reviewed all versions of the manuscript. All authors contributed to the drafts and gave final approval for publication.
Conflict of interest
None declared

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Data availability
The datasets generated and/or analyzed during the current study are available in the Mendeley Data repository at https://data.mendeley.com/datasets/tr37gmjnmy (Tejera and Heaton, 2022).

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