LIFE HISTORY AND RESOURCE ACQUISITION: PHOTOSYNTHETIC TRAITS IN SELECTED ACCESSIONS OF THREE PERENNIAL CEREAL SPECIES COMPARED WITH ANNUAL WHEAT AND RYE

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• Premise of the study: Few previous studies have considered how plant age affects photosynthetic physiology in herbaceous perennials or how photosynthetic capacity in annual cereals compares to perennial relatives. Newly developed perennial cereals offer novel systems for addressing these questions. Our study makes a novel contribution by considering how life history differences affect photosynthetic physiology.

• Methods: In two linked field studies, we evaluated effects of life history and plant age on photosynthetic rates (A), and related biochemical, morphological, and water-relations traits, comparing 1- and 2-yr-old cohorts of perennial wheat, intermediate wheatgrass, and perennial rye to close annual relatives (wheat and rye).

• Key results: Photosynthetic rates (A) were 10–50% higher in perennial cereals compared to annuals. In wheatgrass, elevated A was associated with higher carboxylation (Vc), triose phosphate utilization (TPU) and electron transport rates (J), and higher leaf soluble protein and chlorophyll. Younger wheatgrass plants maintained higher A, TPU, J, and Vc than older plants did. Perennial wheat and rye differed from annual relatives in some but not all of these parameters. Differences in stomatal limitation were not involved, while differences in stomatal conductance (gₛ) became evident under drier conditions.

• Conclusions: This study demonstrates that some perennial cereal species can maintain higher midseason A than their annual crop relatives. These changes are not fully explainable by increased access to soil water and may reflect trade-offs between allocation to reproduction and to resource acquisition. We also found evidence for age-related changes in photosynthetic physiology in a herbaceous perennial plant.

Key words: age; annual; perennial; Poaceae; photosynthesis; Triticum; Thinopyrum; whole plant.

Annual monocarpy and perennial polycarpy represent contrasting plant life history strategies, characterized by differences in resource allocation (Reekie and Bazzaz, 1987a; Aragón et al., 2009). Research is ongoing to characterize how perennials and annuals differ not only in patterns of resource allocation, but resource acquisition rates as well (van Noordwijk and DeJong, 1986; Houle, 1991). The trade-offs of these contrasting life histories can be expressed in terms of various limiting resources including carbon, nitrogen (Wheelwright and Logan, 2004), phosphorus (Zotz and Richter, 2006), and others. Reekie and Bazzaz (1987b), however, argued that carbon was the most generally applicable resource “currency” in plants, since plants can often accumulate other resources by investing carbon belowground.

Therefore, in this paper, we consider only the ability of perennials and annuals to accumulate carbon through photosynthesis. Our study investigates differences in photosynthetic traits between annual crop plants and their close perennial relatives, as well as between 1- and 2-yr-old perennial cereal plants. While some previous studies have compared photosynthetic traits in annual and perennial species, few have looked specifically at annual crop species as compared to perennial relatives, and very few have considered age effects on photosynthesis in herbaceous perennials.

Perennials might be expected, a priori, to have lower instantaneous photosynthetic rates (A) on the basis of evolutionary theory: plants with longer lifespans might adopt conservative resource-use strategies, trading off rapid resource accumulation and high seed yield for lower risk of mechanical damage and greater stress tolerance (Grime, 1977; Garnier, 1992; Ehleringer, 1994). However, empirical comparisons of closely related perennials and annuals indicate that while instantaneous photosynthetic rates in perennials are most commonly lower than in annual relatives, there are numerous cases in which they are higher (Ehleringer, 1994). Comparisons between annual agricultural plants and perennial relatives, in particular, might represent exceptions to the more commonly observed pattern because of the unusual nature of annual crops. Centuries of artificial selection have resulted in annual crop species becoming specialized for extremely high allocation to reproduction (Van Tassel et al., 2010) and rapid development of reproductive tissues. If high allocation to reproduction comes at the expense of carbon allocation to roots or nitrogen allocation to photosynthetic
machinery, as some have hypothesized (Nicotra et al., 2003; Thomas, 2002), then annual cereal crops could have lower photosynthetic rates than perennial relatives. Such patterns have been documented with perennial rice relatives (Zhao et al., 2010).

Newly developed perennial cereal species provide an ideal novel system to study links between life history and photosynthetic physiology. These species are being developed as perennial alternatives to annual cereals, which could potentially provide higher levels of ecosystem services than annual crops (DeHaan et al., 2007). Three potential perennial cereals for the cold temperate zone include perennial wheat, perennial ryegrass, and intermediate wheatgrass. The first two are hybrids of annual wheat and rye with closely related grasses: the third is a Eurasian steppe grass partially interfertile with annual wheat, used for forage and in wheat breeding programs. Relatively little work has been done to compare wheat with perennial wheatgrass species, in spite of their agricultural importance. Some researchers have translocated individual wheatgrass chromosomes or portions of chromosomes into wheat, generally with the purpose of increased disease resistance, and a few studies have considered how these transformed lines perform photosynthetically. Placido et al. (2013) found elevated photosynthetic rates in a line containing the 7EL chromosome segment from *Thinopyrum elongatum* compared with the wheat parent. Other studies, however, have found little effect (e.g., Reynolds et al. [2001] found that wheat lines containing genetic material from *Thinopyrum elongatum* did not show increased photosynthesis prior to flowering). Furthermore, there has been little work directly comparing annual wheat to wheatgrass species (rather than to transformed lines). Our study seeks to close this knowledge gap.

Further goals of our study included assessing whether photosynthetic physiology was affected by whole-plant age and to determine which biochemical and biophysical traits might contribute to differences in photosynthetic rate. While age-related effects on photosynthetic physiology have been documented in woody perennials, as yet they have been studied in relatively few herbaceous perennials (though see Amaya et al., 1995; Casper et al., 2006; Oñate and Munné-Bosch, 2009). Our study contributes to the growing literature on how photosynthetic traits may vary between seedlings and established plants in hemicaceous perennials. We also sought to identify specific mechanistic factors that might explain any observed photosynthetic differences between these two annual crop species and their perennial relatives. One explanation for high photosynthetic rates in perennial species might be that deeper root systems allow these species to avoid water stress and subsequent stomatal closure, which would allow a higher carbon dioxide concentration ($C_\text{L}$) within the leaf, and decreases in stomatal limitation ($L_s$). Alternatively, perennial cereal relatives might maintain high rates of photosynthesis due to changes in concentration or activity of key photosynthetic enzymes. In our study we attempted to test whether observed photosynthetic differences between our species can be fully explained by lower stomatal limitation or whether they involved changes in biochemical processes limiting photosynthesis as well.

In a 2012 field experiment, we compared 1- and 2-yr-old perennial wheat, intermediate wheatgrass, and perennial rye plants to close annual crop relatives (annual wheat in the first two cases, annual rye in the third). A second experiment in 2013 compared 1- and 3-yr-old wheatgrass plants to annual wheat. The key parameter of interest was midseason photosynthetic rate measured at ambient midmorning irradiance and carbon dioxide levels, henceforth referred to as $A_n$ (consistent with Farquhar and Sharkey, 1982). We also measured various biochemical and biophysical traits affecting photosynthesis. Our guiding hypothesis was that both changes in biochemical traits and in moisture access would explain observed annual vs. perennial physiological differences. Specifically, we predicted the following: (1) Perennial cereals will show higher photosynthetic rates than their annual analogues. (2) There will be no age-related differences in photosynthetic rates between older and younger perennials. (3) Perennial cereals, compared with annual relatives, will maintain higher leaf protein, chlorophyll content, triose phosphate utilization, maximum carboxylation rate, maximum electron transport capacity, and water use efficiency (reflecting higher biochemical capacity for photosynthesis) as well as less stomatal limitation (reflecting increased access to soil moisture).

**MATERIALS AND METHODS**

**Site**—We conducted this study at W. K. Kellogg Biological Station, located in southwest Michigan, United States, 50 km east of Lake Michigan (42°24′N, 85°24′W, elevation 288 m a.s.l.), within the oak–maple–hickory deciduous forest–oak savanna transition zone, on soils developed from glacial outwash deposited ca. 12 000 yr ago. Soils are loamy mixed mesic Typic Hapludalfs. Between 1987 and 2012, the site received ca. 900 mm of precipitation annually, half as snow, and the mean annual temperature was 9.2°C.

**Study species**—Our study involved five accessions in 2011–2012 (Experiment 1), and four in 2013 (Experiment 2). The perennial wheat ‘P-0019’, obtained from Washington State University, represented the F5 generation following a tall wheatgrass × annual wheat hybridization event, backcrossed to annual wheat and selected for adequate postsexual cycle regrowth: *Thinopyrum elongatum × Triticum aestivum* ‘Chinese Spring’ // *T. aestivum* ‘Madsen’ (Murphy et al., 2010). The annual wheat accession (*T. aestivum* ‘Frankenmuth’, PVP 8000165; Huebner et al., 1999) was bred at Michigan State University, lacks the dwarfining genes common to modern cultivars, and is used as a benchmark in Michigan breeding studies. The perennial wheatgrass accession (*Thinopyrum intermedium* ‘TLIC1’, syn. *Elyrigia intermedia* and *Agronopyrum intermedium*) was a breeding population that had undergone two cycles of selection for grain yield at The Land Institute (Salina, KS). The annual rye (*Secale cereale* ‘Wheeler’: Helsel and Thomas, 1987) was a forage cultivar developed in southern Michigan, while the perennial rye (*Secale cereale × montanum* ‘Rival’; Peters Seed Co., Myrtle Creek, Oregon, USA) had demonstrated perenniality in Michigan (Jaikumar et al., 2012). These species varied in ploidy level: annual bread wheat is hexaploid, wheatgrass is hexaploid, perennial wheat (approximately) octoploid, annual rye diploid, and perennial rye tetraploid (Cox et al., 2002).

To gain a broader perspective on how annual wheat and intermediate wheatgrass (the most vigorously perennial species) compared, we conducted a second experiment in 2013, comparing first-year and third-year intermediate wheatgrass plants (our most perennial species) to a set of three wheat accessions. These included ‘Frankenmuth’, ‘Caledonia’ (PI 610188; Sorrells et al., 2004) and ‘Aubrey’. ‘Caledonia’ and ‘Aubrey’ are cultivars adapted to the region, which accounted for 9% and 8% of Michigan soft white winter wheat acreage in 2011 (Nagelkirk and Black, 2012). ‘Aubrey’ is lower yielding than ‘Caledonia’ but often planted for its better resistance to some diseases.

**Experimental design**—Experiment 1 was laid out as a nested randomized complete block design with $n = 6$ blocks, each including one $2.30$-m$^2$ plot for each species and age-class. Plots were seeded in October 2010 at 175 seeds·m$^{-2}$. Following the harvest, perennial plants regrew into a second season, while a second set of plots of each species was planted in November 2011. Thus in 2012 the study included annuals and two cohorts of perennials (first-year and second-year). Experiment 2 was laid out in the same field as Experiment 1 (n = 4 blocks) and used some of the same plots. Second-year and first-year wheatgrass
plots from Experiment 1 were allowed to regrow into 2013, and each of the three wheat accessions was planted in each block. Thus, Experiment 2 included annual wheat and three cohorts of wheatgrass (first-year, second-year, and third-year). Both experiments were fertilized in October of 2010, 2011, and 2012 with 91 kg ha⁻¹ N (poultry manure). Both experiments were hand-weeded four times each season and irrigated in June–July 2012.

Mid season Aᵢ—Due to time limitations, we took “snapshots” of photosynthesis at 1–3 time points. We focused on midseason photosynthetic rates, since we believed this time of the season, when annuals are beginning to enter the reproductive phase, might be characterized by trade-offs between allocation to flowering structures vs. photosynthetic machinery. In Experiment 1, Aᵢ was measured on all species on 28–30 April 2012 and 12–15 May 2012. Annual wheat followed approximately on 29 May. Additional Aᵢ measurements were taken on annual wheat and wheatgrass (first-year and second-year) on 20 April 2012. In Experiment 2, measurements were taken on 24–26 May, approximately 14–14 d before annual wheat flowering.

Photosynthesis was measured on a leaf-area basis between 0830 hours and 1200 hours because wheat photosynthesis peaks at approximately 0900 hours and 1430 hours with a midday depression (Rai et al., 2011). Measurements were taken using a LI-6400 XT gas exchange system with an LED head (LI-COR, Lincoln, Nebraska, USA), on sunny days, at ambient CO₂ (400 ppm) and photosynthetically active radiation (PAR) = 1200 µmol·m⁻²·s⁻¹, equivalent to full sun at midmorning in May. Leaves were allowed approximately 2 min to equilibrate, at which point measurements were generally stable within approximately ± 5%. Wheat photosynthetic rates at 1200 µmol·m⁻²·s⁻¹ are commonly approximated 90% of their modeled theoretical maximum (Müller et al., 2005; Ye and Yu, 2008; Yu et al., 2002). Assuming that PAR is proportional to total irradiance and that midday PAR at our site is around 1800 µmol·m⁻²·s⁻¹, PAR should vary between the hours of 0830 and 1200 from 1050 to 1750 µmol·m⁻²·s⁻¹, or between ~90% and 145% of the setting we used. To correct for chamber effects on light, temperature, and humidity, which could strongly affect Aᵢ, gₑ, and E, we sampled plants sequentially by block so as to factor out diurnal variation in light levels and temperature, controlled humidity between 45–70%, and set leaf temperature within the ambient air temperature. We sampled fully expanded flag leaves of 1–3 randomly chosen, healthy plants.

Leaf soluble protein and chlorophyll—On 1 May 2012 and 10 May 2013 chlorophyll readings were taken on 10 flag leaves per plot, using the Minolta SPAD-502 chlorophyll meter. Chlorophyll readings (x) were converted to units of g·m⁻² (y) using y = 0.06e⁻⁰·⁵x (Eq. 1) following Uddling et al. (2007). On 5 June 2012, leaf samples were collected for soluble protein determination. Six leaf discs of 1 cm² each were collected from flag leaves of visibly healthy, deep-green plants using a leaf punch. These were flash-frozen in dry ice and stored in liquid nitrogen, and extracted four times in imidazole buffer at pH = −80 °C (with a protease inhibitor cocktail to suppress protein breakdown: 2 mM L-AESBF, 0.3 mM L-α-proline, 130 mM L-β-histatin, 1 mM EDTA, 14 mM L-ε-64, 1 µM L-leupeptin). Following centrifugation, the supernatant extracts were assayed for total soluble protein using the modified Lowry protein assay procedure. The assay used the Modified Lowry Assay Reagent and Folin-Ciocalteu Assay Reagent (Thermo Scientific 23240) and measured absorbance at 750 nm following a 30-min incubation (Weise et al., 2013).

Biochemical processes limiting photosynthesis—To quantify biochemical processes limiting photosynthesis, Aᵢ/Cᵢ curves were generated, depicting photosynthetic rate (A) as a function of intercellular carbon dioxide concentration (Cᵢ; Farquhar et al., 1980; Wullschleger, 1993). From these curves, five parameters were estimated: triose phosphate utilization (TPU), ribulose bisphosphate carboxylation capacity (Vᵢ), electron transport rate (J), carbon compensation point (Γ), and degree of stomatal limitation (Lᵢ). If perennial cereals show lower Lᵢ than annuals, this could indicate that differences in Aᵢ are due to differences in access to soil water and subsequent stomatal closure.

To estimate these parameters, we measured Aᵢ (on one leaf per plot) at Cᵢ = 0, 50, 100, 150, 200, 250, 300, 400 (twice), 600, 800, 1200, 1400, and 1600 ppm. Measurements were taken on 10–20 May 2012 using temperature, humidity, and irradiance settings specified above. TPU, Vᵢ, and J were estimated by modeling Aᵢ as sequentially limited by ribulose bisphosphate (RuBP) carboxylation (Eq. 1), RuBP regeneration (Eq. 2), and triose phosphate utilization (Eq. 3; Farquhar et al., 1980; Shaffer et al., 2007). These functions are expressed in terms of chloroplast carbon dioxide concentration (Cᵢ), which is related to Cᵢ through Eq. 5.

\[ A = V_i \left( C_i - \Gamma \right) \left[ \frac{1 + \left[ O_2 / K_o \right]}{C_i + K_c} \right] - R_i \]  
(Eq. 2)

at low Cᵢ,

\[ A = J \left[ C_i - \Gamma \right] \left/ \left( 4C_i + 8\Gamma \right) \right] - R_i \]  
(Eq. 3)

at moderate Cᵢ,

\[ A = 3\text{TPU} - R_i \]  
(Eq. 4)

at high Cᵢ,

\[ C_i = C_o - A/g_e. \]  
(Eq. 5)

Here K_c, K_o, and Γ are known constants. Stomatal limitation was calculated according to Eq. 6:

\[ L_s = 1 - A_o / A_i \]  
(Eq. 6)

where A_o corresponds to A at C_i = 400 ppm, and A_i corresponds to A at C_i = 400 ppm, obtained through interpolation (Farquhar and Sharkey, 1982). Carbon compensation point (Γ) was defined as the value of Cᵢ for which A_i = 0. In fitting A vs. Cᵢ data, we constrained mesophyll conductance (gₑ) between 0 and 10 mol·m⁻²·s⁻¹·Pa⁻¹, day respiration (R_o) between 0 and −3.00 µmol·m⁻²·s⁻¹, and standardized parameter values to 25°C, based on the measured temperature on that day and existing temperature response curves (Barnes et al., 2001; Sage and Kubien, 2007). Parameters were estimated so as to minimize the total sum of squares, and the data point closest to Cᵢ = 250 ppm was omitted from the model (assumed to be the zone of transition between carbon-bicarbonate-limited and RuBP regeneration-limited photosynthesis).

Water-relations traits limiting photosynthesis—In late April and mid-May (Experiment 1) and late May (Experiment 2), we measured transpiration (E), stomatal conductance (gₛ) and internal pCO₂ (Cᵢ) on each sampled plant, with the LI-6400 XT, simultaneously with measurements of Aᵢ. These measurements were used to calculate the C_i/C_o ratio (Ehleringer and Cerling, 1995) and instantaneous use efficiency (WUE = Aᵢ/E; McDowell, 2002). Both gₛ and Cᵢ/C_o are not simply “hydraulic” traits, as they are affected both by carbon “demand” (driven by biochemical processes within the chloroplasts) and water “supply” (driven by access to soil water).

LMA—LMA (leaf mass per unit leaf area) was measured on 2 June 2011 (Experiment 1: 6 d before annual wheat anthesis) and 18 June 2013 (Experiment 2: 10 d after annual wheat anthesis). Flag leaves from 1–4 plants of each species were clipped, scanned through a LI-COR LI-3100 leaf scanner to determine surface area, dried at 65°C to constant mass, and weighed. LMA was used to calculate A_max (photosynthesis per unit leaf mass) and A_max (photosynthesis per unit leaf mass, integrated over the expected leaf lifespan Lₑ). We used the guiding assumption, based on 27 C₃ grasses and sedges taken from the GLOPNET compilation of leaf economics data (Wright et al., 2004), that Lₑ varies with LMA: Lₑ = 0.34(LMA)^0.84 (Eq. 7) (r² = 0.58, P = 0.0001).

Thus,

\[ A_{\text{max}} = A_i / \text{LMA}. \]  
(Eq. 8)

\[ A_{\text{int}} = A_{\text{max}} / L_e = 0.34A_i / \text{LMA}^{0.16} \]  
(Eq. 9)

Statistical analysis—Both experiments were analyzed as nested mixed-model ANOVAs. In Experiment 1, life history was considered a fixed factor, block considered as a random factor, and both species and plant age were considered nested fixed factors:

\[ A_i = \mu + \text{Block} + \text{Life History} + \text{Species} \times \text{Life History} + \text{Age} \times \text{Life History}. \]
Life history was included as a factor to assess overall annual vs. perennial differences. In some cases, the life history effect was large enough to swamp species effects in the model (i.e., suggesting that species differences explained no additional variation once overall perennial vs. annual differences were taken into account). However, we still made our preplanned comparisons, to assess the exact magnitude of differences between closely related taxa (e.g., annual wheat vs. wheatgrass) controlling for phylogeny. If a significant age effect existed in the model, individual comparisons of first- and second-year plants were made using paired $t$ tests to determine which species showed an age effect. Where no age effect existed, measurements from first- and second-year plants were averaged.

In Experiment 2, species was a fixed factor, accession was a random factor nested within the annual species, plant age was a fixed factor nested within the perennial species, and block a random factor, e.g., $A_n = \mu + \text{Block} + \text{Species} + \text{Accession(Species)} + \text{Age(Species)}$.

Analyses were run using PROC MIXED in SAS 9.2 (SAS Institute, Cary, North Carolina, USA). $t$-values were log-transformed to homogenize variance. Individual comparisons were made by paired $t$ tests, following the Bonferroni correction for six comparisons (Experiment 1) or three comparisons (Experiment 2).

**RESULTS**

**Photosynthetic rates**—In Experiment 1, $A_n$ differed between annuals and perennials as well as between age-classes (Table 1). Perennial wheat and perennial rye maintained 18–50% higher $A_n$ than annual wheat and annual rye, respectively, in both April and May (Fig. 1). Plant age had a marginally significant effect on $A_n$ in perennial wheat ($t = 2.55, df = 5, P = 0.051$ without multiple-comparison correction) but not in perennial rye ($t = 0.11, df = 4, P = 0.91$).

Wheatgrass showed a similar pattern to the other two perennial species in mid-April 2012: $A_n$ was 20% higher in wheatgrass than in annual wheat and did not differ between first- and second-year wheatgrass plants (Table 1, Fig. 2). Unlike the other two perennial species, age-related differences became evident in late April ($t = 5.65, df = 5, P = 0.02$), and mid-May ($t = 2.87, df = 5, P = 0.035$), though both cohorts still maintained higher $A_n$ than annual wheat. $A_n$ for first-year wheatgrass was 61% higher than annual wheat in late April ($t = 6.11, df = 5, P = 0.011$), and 44% higher in mid-May. In contrast, second-year wheatgrass $A_n$ was only 28% higher than annual wheat in late April ($t = 7.23, df = 5, P = 0.005$), and 33% higher in mid-May ($t = 5.29, df = 5, P = 0.019$). Wheatgrass $A_n$ was the highest of the five species, approximately 19–34 $\mu$mol·m$^{-2}$·s$^{-1}$ for first-year plants.

The same trends were seen in Experiment 2: $A_n$ differed between species ($F_{5,23} = 45.22, P = 0.02$) and age-classes ($F_{5,23} = 16.00, P = 0.002$) but not among the three annual wheat accessions ($P = 1.00$). $A_n$ was 54% higher in first-year wheatgrass (between 27–49 $\mu$mol·m$^{-2}$·s$^{-1}$: $t = 5.22, df = 3, P = 0.041$) and 22% higher in second- and third-year wheatgrass (27–39 $\mu$mol·m$^{-2}$·s$^{-1}$: $t = 5.43, df = 3, P = 0.036$) compared with annual wheat (20–30 $\mu$mol·m$^{-2}$·s$^{-1}$ at the 95% confidence interval).

**Biochemical traits limiting photosynthesis**—Representative $A$ vs. $C_t$ curves for individual leaves within one block are depicted in Fig. 3A (perennial wheat), Fig. 3B (wheatgrass) and Fig. 3C (perennial rye). Visual inspection of the curves indicated that TPU limitation was present at high carbon dioxide levels. In Experiment 1, TPU capacity was higher in the perennial cereals than in the annuals (Table 2) but showed no effect of plant age. The largest species difference was seen in wheatgrass, where TPU was 40% higher than in annual wheat ($t = 4.46, df = 5, P = 0.04$). By contrast with TPU, maximum carboxylation and electron transport capacity differed between annual and perennial species and were also affected by plant age (Table 2). For example, first-year wheatgrass maintained 31% higher $V_c$ ($t = 7.71, df = 5, P = 0.004$) and 53% higher $J$ than annual wheat ($t = 4.84, df = 5, P = 0.028$), while second-year wheatgrass maintained only 17% higher $V_c$ ($t = 5.26, df = 5, P = 0.024$) and 24% higher $J$ ($t = 5.11, df = 5, P = 0.022$) compared to annual wheat (Table 2). In perennial wheat, while both age cohorts had higher $V_c$ than annual wheat, first-year plants also showed higher $J$ than annual wheat, while second-year plants did not. These observed trends in $J$ and $V_c$ support our findings that wheatgrass seedlings generally are more photosynthetically active than established plants. Carbon compensation point ($\Gamma$) did not vary between species (Table 2).

**Leaf soluble protein content and chlorophyll content on a per-area basis (LSP$_A$)**, in Experiment 1, was affected by life history but not by plant age (Table 3). LSP$_A$ was 44% higher in wheatgrass ($t = 7.41, df = 5, P = 0.004$) and 30% higher in perennial wheat than in annual wheat. When soluble protein was calculated on a per-mass basis (LSP$_M$), wheatgrass had 11% more protein per unit leaf mass than annual wheat ($t = 4.52, df = 5, P = 0.038$). Thus only 25% of the difference between annual wheat and wheatgrass, on a per-leaf-area basis, was due to higher protein concentration per unit mass, and 75% was due to thicker or denser leaves. Across the five species, leaf soluble protein was generally between 2.4 and 3.0%. Chlorophyll content per unit leaf area (Chl$_A$) in wheatgrass was 93% higher than annual wheat in Experiment 1 ($t = 9.51, df = 5, P = 0.0012$: Table 3), but was not affected by plant age.

**Water relations traits**—Stomatal conductance and water use efficiency, in Experiment 1, displayed opposite trends between late April and mid-May. In late April, species differences were seen in WUE but not $g_s$: specifically, wheatgrass plants had 67% higher WUE than annual wheat ($t = 5.34, df = 5, P = 0.018$: Table 4). By contrast, in mid-May, highly significant species differences were seen in $g_s$ but not in WUE. Stomatal conductance was 49% higher in wheatgrass ($t = 4.85, df = 5, P = 0.028$) and 51% higher in perennial wheat compared with annual wheat, while perennial rye $g_s$ was 59% higher than in annual wheat.

**Table 1.** $F$ values ($p$ values in parentheses) are presented for analyses of variance on photosynthetic rates at ambient light and carbon dioxide ($A_n$) in a 2011–2012 study of photosynthetic traits in 1-yr-old and 2-yr-old perennial cereals (perennial wheat, perennial rye, and intermediate wheatgrass) and closely related annual cereals (annual wheat and rye) at the Kellogg Biological Station (Hickory Corners, Michigan, USA).

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>20 April 2012</th>
<th>28 April 2012</th>
<th>12 May 2012</th>
</tr>
</thead>
<tbody>
<tr>
<td>df</td>
<td>2, 15 $^a$</td>
<td>7, 40</td>
<td>7, 39</td>
</tr>
<tr>
<td>Life history</td>
<td>23.76 (&lt;0.0001)</td>
<td>55.11 (&lt;0.0001)</td>
<td>1.77 (0.17)</td>
</tr>
<tr>
<td>Species</td>
<td>12.07 (0.0006)</td>
<td>3.33 (0.031)</td>
<td>1.43 (0.025)</td>
</tr>
<tr>
<td>Age</td>
<td>7.22 (0.23)</td>
<td>11.85 (0.002)</td>
<td>5.49 (0.025)</td>
</tr>
<tr>
<td>Block</td>
<td>1.51 (0.066)</td>
<td>1.43 (0.076)</td>
<td>0.86 (0.20)</td>
</tr>
</tbody>
</table>

$a$ At the first time point, measurements were taken only on two species (annual wheat and wheatgrass); at the second and third, all five species were included.

$b$ At the first time point, life history was not an informative variable since only one annual and one perennial were included.
more than hydraulic limitation. In Experiment 2, annual wheat and wheatgrass did not show differences in \( g_s \) (between 560 and 780 mol·m\(^{-2}\)·s\(^{-1}\) across accessions: \( P = 0.58 \)), WUE (between 170 and 250 units water lost per units carbon gained across accessions: \( P = 0.70 \)) or in \( C_i / C_a \) (between 74–76% across accessions: \( P = 0.58 \)). The changing importance of WUE and \( g_s \) might be explainable by weather conditions. Our site was relatively dry between 29 April–15 May 2012, with 27 mm of rain.

Fig. 1. Photosynthetic rates (\( A_o \)) measured in late April and mid-May 2012, on first-year and second-year perennial wheat (\( Triticum aestivum \times Thinopyrum elongatum \)), and perennial rye (\( Secale cereale \times montanum \)) compared with annual wheat (\( T. aestivum \)), and annual rye (\( S. cereale \)). Data were collected at the Kellogg Biological Station (Hickory Corners, Michigan, USA). Abbreviations for species and cohorts are as follows: PW – 1 yr = first year perennial wheat, PW – 2 yr = second year perennial wheat, AW = annual wheat, PR – 1 yr = first year perennial rye, PR – 2 yr = second year perennial rye. Error bars represent standard errors. Significant differences from annual wheat or rye following the Bonferroni correction for six comparisons: *\( p < 0.05 \), **\( p < 0.01 \).

Fig. 2. Photosynthetic rates (\( A_o \)) measured at three time points in spring 2012, on first-year (IWG – 1 yr) and second-year plants of intermediate wheatgrass (IWG – 2 yr: \( Thinopyrum intermedium \)) compared with its close relative annual wheat (AW: \( Triticum aestivum \)). Data were collected at the Kellogg Biological Station (Hickory Corners, Michigan, USA). Error bars represent standard errors. Significant differences relative to annual wheat following the Bonferroni correction for six comparisons: *\( p < 0.05 \), **\( p < 0.01 \).
Fig. 3. Photosynthetic rate ($A$) as a function of intercellular CO$_2$ concentration ($C_i$) in perennial and annual cereals. (A) Annual wheat and first- and second-year intermediate wheatgrass (*Thinopyrum intermedium*). (B) Annual wheat and first- and second-year perennial wheat (*Thinopyrum elongatum* × *T. aestivum*). (C) Annual rye (*Secale cereale*) and first- and second-year perennial rye (*S. montanum* × *S. cereale*). Data were collected at the Kellogg Biological Station (Hickory Corners, Michigan, USA). Each curve is based on measurements from a single leaf, all within one block of the experiment. IWG – 1 yr = 5-mo-old wheatgrass, IWG – 2 yr = 17-mo-old wheatgrass.
compared to 99 mm in the previous 2 wk, and one might expect differences in stomatal conductance to become more important under the drier conditions in the late spring, if perennials do indeed have greater capacity to tap into soil moisture at deeper layers. In 2013, compared to 2012, the 10 April–28 May period was much wetter (271 mm compared to 107 mm precipitation), presumably with lower water stress. This might explain why the species differences in $g_s$ that developed in 2012 were not seen in 2013.

**LMA**—In Experiment 1, LMA varied between species ($F_{7,40} = 4.27, n = 6, P = 0.01$). Wheatgrass and perennial wheat LMA were higher than in annual wheat (7.23 ± 0.35 mg·cm$^{-2}$, compared to 5.69 ± 0.08 mg·cm$^{-2}$), but annual and perennial rye did not differ (6.74 ± 0.25 and 7.60 ± 0.65 mg·cm$^{-2}$). Similarly, in Experiment 2, LMA was 30% higher in wheatgrass compared to annual wheat, but was not affected by plant age ($t = 6.29$, df = 3, $P = 0.025$: Table 2).

**DISCUSSION**

As predicted, each perennial cereal had higher midseason $A_o$ than their annual analogues did. Differences between annual wheat and wheatgrass held across at least three accessions, as established in Experiment 2. Our findings complement previous studies that found that translocation of individual *Thinopyrum* chromosomes into wheat led to elevated $A_o$ (Placido et al., 2013). Some recent studies, similarly, have found higher $A_o$ in some perennial plants compared with annual relatives: for example, in *Oryza rufipogon* compared to *O. sativa* (Zhao et al., 2010), in perennial vs. annual *Lesquerella* spp. (González-Paleo and Ravetta, 2011b), in perennial vs. annual Panicum (Taylor et al., 2010) and in perennial vs. annual races of *Machaeranthera gracilis* (Monzon and Szarek, 1981). By contrast, many phylogenetically controlled annual vs. perennial contrasts show higher rates in annuals (Zangerl and Bazzaz, 1983; Ehleringer, 1994; Sobrado, 2011; Van Auken and Bush, 2011).

Our second prediction, that photosynthetic physiology would not differ between seedlings and established plants, was only partially supported. Perennial wheat and perennial rye, as predicted, showed few age-related changes in photosynthetic parameters. However, wheatgrass displayed pronounced age effects, with second-year plants having lower $A_o$, $J$, WUE, and $V_c$ than young seedlings did. Changes in photosynthetic physiology with plant aging have rarely been investigated in herbaceous perennials, though age-related declines in photosynthetic parameters are found in sugarcane (Amaya et al., 1995) and *Urtica dioica* (Onate and Munné-Bosch, 2009). By contrast, Casper et al. (2006) found that larger (presumably older) individuals of the herbaceous desert perennial *Cryptantha flava* had equal or higher photosynthetic rates than in younger plants. These contrasting, species-specific patterns are consistent with the literature on woody perennials. Many woody perennials including giant sequoia (Grulke and Miller, 1994), black cherry (Fredericksen et al., 1996), and numerous conifers, maintain higher $A_o$ per unit leaf area during the seedling establishment phase compared to established plants, while many deciduous trees show opposite trends (Rijkers et al., 2000; Steppe et al., 2011). Our study shows that similar age-related effects exist in a perennial grass.

In woody plants, various hypotheses have been offered to explain declines or increases in $A_o$ with plant age. Perennial plants might experience more nitrogen limitation as they age, leading to lower concentration of key proteins. Alternatively, they might experience higher $A_o$ due to greater access to water and reduced $L_s$. Some have argued that young seedlings have a higher risk of mortality in response to environmental fluctuations than established plants and should therefore follow a high-risk strategy characterized by distinctive “primary” foliage specialized for high $A$ (Bond, 2000). Field observations suggested that younger wheatgrass leaves were softer to the touch than older ones and broke more easily. Finally, if photosynthesis in these perennial cereals is sink-limited under the conditions of our study and if older plants are mostly relying on an established root network rather than constructing new root tissues, it is possible that lower belowground sink demand could explain lower $A$ and WUE in older plants (Flore and Layne, 1999), potentially mediated by downregulation of $J$ or $V_c$. As our data suggest that older wheatgrass plants have lower WUE, equivalent stomatal limitation and equivalent leaf soluble protein to first-year wheatgrass seedlings, we found no evidence for the first two hypotheses. It is possible that changing source to sink ratios and leaf morphology may provide the explanatory mechanism, as source–sink changes could cause changes in activity of key proteins without affecting overall leaf soluble protein.

Our third prediction was that both hydraulic and biochemical factors would play a role in explaining higher $A_o$ in the perennial species, and here we found partial support. We did find that changes in leaf biochemical traits, as well as leaf morphology, played an important role in explaining differences in $A_o$ between the annual crops and their perennial relatives. The perennial cereals showed a coordinated increase in all three biochemical processes limiting photosynthesis ($J$, $V_C$, and TPU). In other words, differences in $A_o$ were at least in part, independent of $C_i$ and not fully explainable by increased stomatal opening. In contrast to our expectation, however, we did not find evidence of reduced stomatal limitation in the perennial species. In addition, the low values for $L_s$ suggest that stomatal resistance played a minor part in determining photosynthetic rate. The lack of major stomatal limitation higher $A_o$ in the perennial cereals was not primarily due to improved water relations. By contrast, Placido et al. (2013) found that translocation of single chromosomes from *Thinopyrum elongatum* into wheat improved photosynthetic rate primarily through alleviating drought stress. Perennial cereals did exhibit higher $g_s$ in late spring than annual wheat and rye, which could suggest they were taking advantage of greater access to soil water. However, changes in $g_s$ could also be driven by increasing demand for CO$_2$ (due to faster carboxylation or electron transport) rather than by increased water supply. Similarly, the fact that $C_i/C_n$ did not vary between species could simply indicate that plants were tightly regulating $g_s$ so as to keep carbon supply and demand in balance. Thus, our observations of $g_s$ and $C_i/C_n$ cannot be clearly classified as evidence for biochemical or stomatal limitation.

Higher carboxylation and electron transport rates in perennials could have various causes. Perennial cereals might allocate more nitrogen to leaves than annuals, at the cost of lower allocation to seeds. Some have argued that reproductive tissues and photosynthetic machinery may compete for nitrogen (McGinley and Charnov, 1988; Karlsson, 1994; Obeso et al., 1998; Nicotra et al., 2003) and that high nitrogen demands by developing reproductive tissues in annuals lead to increased breakdown of leaf protein (Thomas, 2002). For example, perennial *Lesquerella*
Table 2. Means (±SE) and F values (p values) are presented for stomatal limitation (Ls), carbon compensation point (Γ) maximum RuBP carboxylation capacity (Vc), electron transport rate (J) and triose phosphate utilization (TPU) rates, calculated for annual wheat, annual rye, and on first-year and second-year plants of intermediate wheatgrass, perennial wheat and perennial rye in May 2012 at Kellogg Biological Station (Hickory Corners, MI).

<table>
<thead>
<tr>
<th>Species (Age)</th>
<th>Ls (%)</th>
<th>Γ (ppm)</th>
<th>Vc (µmol·m⁻²·s⁻¹) CO₂</th>
<th>J (µmol·m⁻²·s⁻¹) electrons</th>
<th>TPU (µmol·m⁻²·s⁻¹) triose phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annual wheat</td>
<td>4.9 ± 0.3</td>
<td>4.8 ± 0.3</td>
<td>102.3 ± 6.2*</td>
<td>191.3 ± 17.9</td>
<td>15.78 ± 1.76</td>
</tr>
<tr>
<td>Perennial wheat (1 yr)</td>
<td>9.4 ± 2.0</td>
<td>48.0 ± 5.0</td>
<td>136.0 ± 5.1*</td>
<td>271.0 ± 24.1*</td>
<td>21.92 ± 2.3*</td>
</tr>
<tr>
<td>Perennial wheat (2 yr)</td>
<td>19.7 ± 4.3</td>
<td>42.3 ± 2.1</td>
<td>117.9 ± 8.5*</td>
<td>222.5 ± 20.5</td>
<td>18.68 ± 2.35*</td>
</tr>
<tr>
<td>Wheatgrass (1 yr)</td>
<td>14.3 ± 3.2</td>
<td>43.6 ± 3.8</td>
<td>139.2 ± 7.9*</td>
<td>293.3 ± 31.8*</td>
<td>23.43 ± 2.83*</td>
</tr>
<tr>
<td>Wheatgrass (2 yr)</td>
<td>18.7 ± 4.4</td>
<td>46.6 ± 4.6</td>
<td>120.2 ± 4.76*</td>
<td>237.5 ± 24.6*</td>
<td>20.95 ± 3.51*</td>
</tr>
<tr>
<td>Annual rye</td>
<td>13.5 ± 4.4</td>
<td>41.9 ± 1.6</td>
<td>95.2 ± 8.7</td>
<td>204.5 ± 14.1</td>
<td>18.62 ± 1.28</td>
</tr>
<tr>
<td>Perennial rye (1 yr)</td>
<td>4.9 ± 2.0</td>
<td>39.0 ± 2.5</td>
<td>114.7 ± 3.8</td>
<td>279.2 ± 11.4*</td>
<td>25.8 ± 1.23*</td>
</tr>
<tr>
<td>Perennial rye (2 yr)</td>
<td>10.0 ± 3.5</td>
<td>43.1 ± 4.1</td>
<td>131.5 ± 6.0</td>
<td>281.8 ± 11.8*</td>
<td>24.25 ± 1.53*</td>
</tr>
</tbody>
</table>

Sources of variation
- df: 7, 39
- Life history: 0.82 (0.37)
- Species: 2.78 (0.055)
- Age: 3.88 (0.056)
- Block: 1.09 (0.14)

Table 3. Means (±SE) and F values (p values) are presented for leaf soluble protein on a per-mass (LSPₘ) and per-area (LSPₐ) basis, chlorophyll content (on a per area basis) and photosynthetic rate under ambient midday conditions on a per-mass basis (Aₘₜₐₚₚ) calculated for annual wheat, annual rye, and on first-year and second-year plants of intermediate wheatgrass, perennial wheat, and perennial rye, in 2012 at Kellogg Biological Station (Hickory Corners, MI).

<table>
<thead>
<tr>
<th>Species (Age)</th>
<th>LSPₘ (% fresh mass)</th>
<th>LSPₐ (µg·cm⁻²)</th>
<th>Chl (g·m⁻²)</th>
<th>Aₘₜₐₚₚ (28 Apr) (µmol·mg⁻¹·s⁻¹)</th>
<th>Aₘₜₐₚₚ (20 Apr) (µmol·mg⁻¹·s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annual wheat</td>
<td>2.44 ± 0.16</td>
<td>445 ± 27</td>
<td>0.48 ± 0.03</td>
<td>2.99 ± 0.31</td>
<td>2.78 ± 0.33</td>
</tr>
<tr>
<td>Perennial wheat (1 yr)</td>
<td>2.57 ± 0.18</td>
<td>593 ± 48**</td>
<td>0.74 ± 0.06</td>
<td>3.45 ± 0.32</td>
<td>—</td>
</tr>
<tr>
<td>Perennial wheat (2 yr)</td>
<td>2.85 ± 0.17</td>
<td>564 ± 32*</td>
<td>0.51 ± 0.02</td>
<td>2.81 ± 0.28</td>
<td>—</td>
</tr>
<tr>
<td>Wheatgrass (1 yr)</td>
<td>2.82 ± 0.32*</td>
<td>639 ± 58**</td>
<td>0.84 ± 0.11**</td>
<td>3.81 ± 0.23</td>
<td>2.45 ± 0.22</td>
</tr>
<tr>
<td>Wheatgrass (2 yr)</td>
<td>2.61 ± 0.14*</td>
<td>644 ± 56**</td>
<td>0.96 ± 0.04**</td>
<td>3.10 ± 0.30</td>
<td>2.85 ± 0.37</td>
</tr>
<tr>
<td>Annual rye</td>
<td>2.66 ± 0.27</td>
<td>497 ± 37</td>
<td>0.58 ± 0.02</td>
<td>2.47 ± 0.27</td>
<td>—</td>
</tr>
<tr>
<td>Perennial rye (1 yr)</td>
<td>2.95 ± 0.68</td>
<td>640 ± 99</td>
<td>0.50 ± 0.02</td>
<td>2.72 ± 0.24*</td>
<td>—</td>
</tr>
<tr>
<td>Perennial rye (2 yr)</td>
<td>2.44 ± 0.82</td>
<td>492 ± 121</td>
<td>0.58 ± 0.12</td>
<td>2.81 ± 0.35*</td>
<td>—</td>
</tr>
</tbody>
</table>

Sources of variation
- df: 7, 34
- Life history: 5.59 (0.025)
- Species: 0.09 (0.96)
- Age: 0.10 (0.75)
- Block: 0.71 (0.24)

Table 2. Mean ± SE values are presented for stomatal limitation (Ls), carbon compensation point (Γ) maximum RuBP carboxylation capacity (Vc), electron transport rate (J) and triose phosphate utilization (TPU) rates, calculated for annual wheat, annual rye, and on first-year and second-year plants of intermediate wheatgrass, perennial wheat and perennial rye in May 2012 at Kellogg Biological Station (Hickory Corners, MI).

Table 3. Mean ± SE values are presented for leaf soluble protein on a per-mass (LSPₘ) and per-area (LSPₐ) basis, chlorophyll content (on a per area basis) and photosynthetic rate under ambient midday conditions on a per-mass basis (Aₘₜₐₚₚ) calculated for annual wheat, annual rye, and on first-year and second-year plants of intermediate wheatgrass, perennial wheat, and perennial rye, in 2012 at Kellogg Biological Station (Hickory Corners, MI).

**mendocina** shows higher N allocation to leaves than in annual *L. fendleri* (Ploschuk et al., 2005). Changes in nitrogen allocation play some role in explaining photosynthetic differences in our system. On a leaf-area basis, two of the perennial species showed higher protein content, and one showed higher chlorophyll content. These trends are consistent with previous literature, which found that in wheat, leaf soluble protein concentration (up to approximately 700 mg·cm⁻², which spans the range in our study) is linearly correlated with Vc and TPU (Lawlor et al., 1989) and with A (Evans, 1989). In wheatgrass, higher LMA accounted for ~75% of the difference in protein and 48% of the difference in chlorophyll, with the rest presumably reflecting differences in leaf biochemical composition.

Leaf mass per area also affects carbon accumulation rates, and the integrated carbon gain from a leaf, per unit mass, over the expected life of the leaf (Aₑ). Older wheatgrass plants compensate for slightly lower Aₑ than annual wheat, by an increased leaf lifespan and subsequent greater seasonal productivity (the difference is even greater with 1-yr-old wheatgrass). Specifically, Aₑ would be 20% higher in older wheatgrass plants, 40% higher in first-year wheatgrass, and 27% higher in perennial wheat compared with annual wheat. Equivalently, wheatgrass and perennial wheat have higher Aₑ (relative...
to annual wheat) than would be expected purely on the basis of their thicker leaves.

Our study, in conclusion, illuminates how life history differences are reflected in differences in resource acquisition processes and in biochemical processes limiting photosynthesis. Age-related changes in photosynthetic physiology were also identified, in one of the few investigations of this phenomenon in an herbaceous perennial.

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