

# Seasonality and phenology alter functional leaf traits

Athena D. McKown · Robert D. Guy · M. Shofiul Azam ·  
Eric C. Drewes · Linda K. Quamme

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**Abstract** In plant ecophysiology, functional leaf traits are generally not assessed in relation to phenological phase of the canopy. Leaf traits measured in deciduous perennial species are known to vary between spring and summer seasons, but there is a knowledge gap relating to the late-summer phase marked by growth cessation and bud set occurring well before fall leaf senescence. The effects of phenology on canopy physiology were tested using a common garden of over 2,000 black cottonwood (*Populus trichocarpa*) individuals originating from a wide geographical range (44–60°N). Annual phenological events and 12 leaf-based functional trait measurements were collected spanning the entire summer season prior to, and following, bud set. Patterns of seasonal trait change emerged by synchronizing trees using their date of bud set. In particular, photosynthetic, mass, and N-based traits increased substantially following bud set. Most traits were significantly different between pre-bud set and post-bud set phase trees, with many traits showing at least 25 % alteration in mean value. Post-bud set, both the significance and direction of trait–trait relationships could be modified, with many relating directly to changes in leaf mass. In *Populus*, these dynamics in leaf traits throughout the summer season

reflected a shift in whole plant physiology, but occurred long before the onset of leaf senescence. The marked shifts in measured trait values following bud set underscores the necessity to include phenology in trait-based ecological studies or large-scale phenotyping efforts, both at the local level and larger geographical scale.

**Keywords** Bud set · Carbon flux · Ecophysiology · Gas exchange · *Populus*

## Introduction

Plant performance and survival are often quantified and compared using measurements of individual features termed “functional traits” (Violle et al. 2007). The extent that periodical phenomena, or seasonal phenology, directly affect plant functional traits (or trait–trait relationships) is generally overlooked in ecophysiology or assumed to be small (Ackerly 2004; Grime 2006; Pau et al. 2011). This interaction deserves examination due to changes in plant growth, productivity, and resource requirements throughout the green canopy period (Ceulemans and Isebrands 1996). A seasonality factor may be highly important in trait-based studies comparing plant communities found in different locations, sampled at different times within the summer period, or measured repeatedly between years (Wilson et al. 2001; Grassi et al. 2005; Stokes et al. 2010). In broad ecological studies or large-scale phenotyping efforts, it is essentially impossible to sample every plant on the same date; consequently sampling occurs at different times throughout a field season, and may represent trait assessments encompassing different seasonal phases of the plants being measured, especially if phases are subtle and not readily apparent in the canopy (Reich 1995).

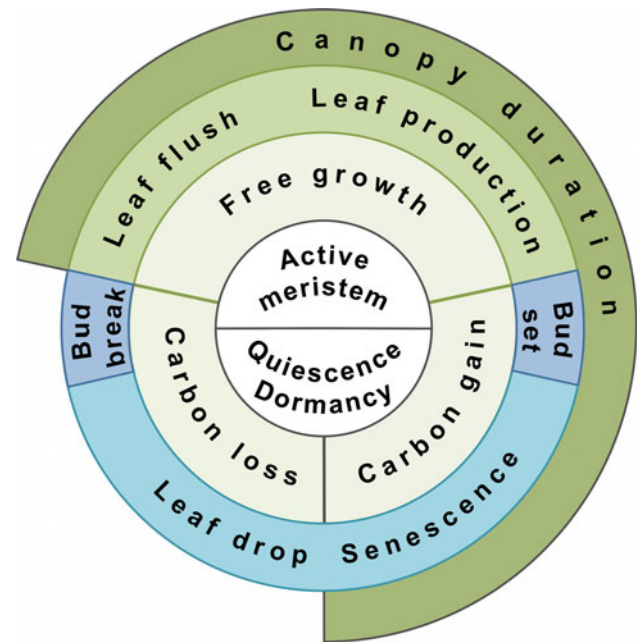
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A. D. McKown (✉) · R. D. Guy · M. S. Azam ·  
E. C. Drewes · L. K. Quamme  
Department of Forest Sciences, Faculty of Forestry, University  
of British Columbia, Forest Sciences Centre, 2424 Main Mall,  
Vancouver, BC V6T 1Z4, Canada  
e-mail: admckown@gmail.com

In a perennial plant, seasons are distinguished by phenological events that denote phases of the canopy cycle and are necessary for adaptation, particularly in northern or higher elevation environments (Díaz et al. 2004; Polgar and Primack 2011; Keller et al. 2011; Rohde et al. 2011). Among woody temperate plants, the canopy cycle generally is marked by observable phenological events of termination (breaking) of bud dormancy and quiescence, leaf flush, bud formation (setting), leaf senescence, and leaf drop (Fig. 1). The C and growth cycles occur alongside this canopy cycle, with shoot elongation following bud break and leaf flush, and height growth cessation preceding bud set. The switch from meristem activity to quiescence and dormancy is subtle, but visually observable as the formation and setting of a terminal bud. This generally occurs in mid-summer well before leaves begin senescing marking a “post-bud set” phase where C and nutrients are no longer allocated to height growth. The cycle of phenological events in temperate deciduous plants, such as poplar, requires different cues to initiate each response, and is highly conserved in different populations or ecotypes as a form of local adaptation (Chuine and Cour 1999; Ingvarsson et al. 2006; Luquez et al. 2008; Rohde et al. 2011). Cues involve both temperature and daylight length, and plants will not necessarily respond until a threshold level for response has been met (Farmer 1994; Chuine and Cour 1999; Kalcsits et al. 2009; Polgar and Primack 2011). In any given locale, phenological timing will vary from individual to individual due to inherent plasticity, variation in environmental cues, and varying genetic heritage (Kalcsits et al. 2009). As a result, trees can be shifted, or asynchronous, in the phases of their canopy and growth cycles (either in timing or duration) with respect to each other in a given growing season, or with themselves in different years (Reich 1995; Ma et al. 2011; Polgar and Primack 2011).

In both deciduous and evergreen perennials, traits may be dynamic and vary throughout the growing season in pattern and magnitude depending on the species, including gas exchange-based traits [e.g., assimilation, transpiration, stomatal conductance, intrinsic water use efficiency (WUE)], leaf-based traits (e.g., area, mass, foliar nutrients), and isotopic traits ( $\delta^{13}\text{C}$ ) (Morecroft and Roberts 1999; Wilson et al. 2001; Grassi et al. 2005; Chen and Chen 2007; Herbst et al. 2008; Maseyk et al. 2008; Stokes et al. 2010; Delagrangé 2011; Ma et al. 2011; Sang et al. 2011). In addition, seasonal differences between early spring versus early summer leaves, and summer versus late fall senescing leaves have been observed (Wilson et al. 2001; Grassi et al. 2005; Stokes et al. 2010). However, to our knowledge, no published study has explicitly investigated the effect of the summer phase marked by growth cessation and bud set (i.e., “post-bud set phase”), or the extent to which seasonality



**Fig. 1** Schematic representation of the annual cyclical activities in canopy cover, primary shoot growth, and C gain of deciduous woody plants. The central circle refers to the terminal meristem cycling between growth (active cell division) and dormancy/quiescence (no cell division). The innermost ring denotes free growth and C gain beginning at bud break and terminating at bud set, continued net C gain following bud set, and net C loss following leaf drop. The middle ring denotes the canopy cycle with bud break, followed by leaf flush (pre-formed leaves), and additional leaf production (neoformed leaves). Leaf formation halts with bud set, followed by leaf senescence. The outermost ring denotes full duration of the leaf canopy from bud break to leaf drop, and overlapping active and quiescent meristem phases

and asynchrony in canopy cycles contribute to trait variability. Comparisons of asynchronous plants may introduce unforeseen trait error leading to higher apparent intraspecific or “within-site” variability, which accounts for a non-negligible portion of trait variation (up to 30 %) in species comparisons (Cornwell and Ackerly 2009; Albert et al. 2010). In addition, asynchrony in canopy cycles is especially critical in common garden studies assessing plants of different origin (especially latitude) where phenological events are inherently altered (Gornall and Guy 2007; Luquez et al. 2008; Soolanayakanahally et al. 2009; Keir et al. 2011; Keller et al. 2011).

In this study, we investigate how seasonality, and particularly late summer phenology, affects leaf-based functional traits within a perennial temperate plant species. We assessed *Populus trichocarpa*, a common poplar and foundation species in western North America, comparing a large number of genotypes originating from a wide latitudinal range grown within a common garden environment, recording phenological events, and measuring numerous commonly assessed leaf traits. We explore (1) whether

timing of phenological events are correlated within or between years to estimate their predictability. We determine (2) the extent that leaf traits change through a particular phase (cf. Grassi et al. 2005), and assess patterns of trait change in relation to date of collection, age of canopy, or proximity to bud set. We predict that asynchrony of tree canopy cycles obscures seasonal trends when aligning traits by calendar date of sampling, whereas biologically meaningful trends are observed when aligning traits by canopy age and/or proximity to bud set. We calculate (3) the magnitude of difference in traits measured between early vs. late growing season and pre- versus post-bud set trees, and expect plants in different phases to show significant differences in traits reflecting growth and productivity requirements prior to leaf senescence (cf. Ceulemans and Isebrands 1996). In addition, we test (4) whether well-established global trait–trait relationships (cf. Wright et al. 2004, 2005) remain constant or are modified by phenological phase.

## Materials and methods

### Field materials

Original branch cuttings from native tree genotypes of black cottonwood poplar (*Populus trichocarpa* Torr. and Gray) were collected from 180 provenances throughout western North America by the British Columbia (BC) Ministry of Forests and planted in common gardens within BC in 2000 (Xie et al. 2009). Samples from 136 provenances (spanning 44–60°N) were taken from the Surrey garden in 2008 for use at the University of British Columbia (UBC) in a large-scale poplar phenotyping experiment. The new cuttings were rooted and grown as stecklings at UBC under greenhouse conditions, and a total of 456 genotypes, with at least four replicates of each genotype, were out-planted in June 2008 in a common garden at Totem field, UBC. As a result, all trees in this study represent material rooted, grown, and out-planted at the same time providing a common garden with a variety of tree genotypes of similar age and condition. Within the Totem field common garden, replicates were assigned using a random, block design for the clones and all trees were planted with 1.5 × 1.5 m spacing. Trees were watered daily throughout 2008 and 2009 from rainfall or using a drip-piping irrigation system, and field maintenance performed by mowing to limit weed overgrowth. No fertilizers or other soil supplements were provided at any time during the study. Individual tree identifications were confirmed through extensive genetic single nucleotide polymorphism analyses using Illumina iSelect Infinium sequencing (Geraldes et al., unpublished data).

### Field monitoring

All trees in the Totem field common garden were assessed for the onset of spring, summer, and fall seasonal phases throughout the green canopy seasons of 2008–2011. Calendar dates of phenological events were recorded for each individual using visual observations of the terminal bud on the leading shoot or canopy as a whole, including: terminal bud break, canopy leaf flush, terminal bud set, and canopy leaf drop events. For each year, the green cover period (canopy duration) was calculated as the time (days) from terminal bud break to leaf drop. This overestimates the actual duration of fully greened leaves by a month, but is highly correlated to other definitions of canopy duration [e.g., bud flush to 50 % canopy yellowing cf. Soolanayakanahally et al. (2012)] within these trees ( $r_s = 0.7$ , data not shown). All trees were measured throughout the progression of the 2009 season for height growth to determine the period of free growth, and to estimate date of height growth cessation. Daily local field conditions including minimum, maximum, and mean daily temperatures (°C), minimum, maximum, and mean humidity values (%), mean wind speed ( $\text{km h}^{-1}$ ), and total precipitation (mm) variables were obtained using weather records (Online Resource Fig. 1).

### Trait measurement

Individual trees ( $n = 1,726$ ) were sampled throughout the green canopy period of 2009 for 12 ecophysiological leaf traits, including gas exchange, mass-based, nutrient, and isotopic traits. These functional traits are often measured as they reflect the investment of resources and potential output for leaves, or are used to explain global plant patterns and trait trade-offs, forming the basis of the “leaf economics spectrum” (Cornelissen et al. 2003; Wright et al. 2004, 2005; Shipley et al. 2006). Samples for leaf traits were taken once from each individual tree throughout the summer season before canopy showed signs of senescence (late May up to and including early August). Some pre-formed leaves were used in early season measurements prior to the formation of neoformed leaves; however, the majority of measurements were taken using neoformed leaves. During 2009, the range of phenological sampling occurred throughout 10 months to fully encompass and date canopy events, while leaf traits were sampled for 67 days total within mid-season (Julian date 149–216), and from 24 to 72 % of the canopy age ( $\sim 50$  % of green canopy time). A limited number of individual trees ( $n = 413$ ) were re-sampled for trait repeatability in 2010.

All gas exchange measurements were taken on warm, clear days between 0800 and 1400 hours on each sampling date (Online Resource Fig. 1). Prior to measurements, trees

were irrigated for at least 30 min in the morning and throughout the day as necessary to prevent drought-stress effects. All traits were measured on an individual leaf per tree, selecting the first fully expanded, undamaged leaf from the leading shoot (usually the 5th leaf) to compare young, healthy, exposed leaves between all trees. Using LI-COR 6400 and LI-COR 6400 XT portable gas exchange systems (LI-COR Biosciences, Lincoln, NE), gas exchange traits measured included maximum photosynthetic rate ( $A_{\max}$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), stomatal conductance ( $g_s$ ,  $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ), and instantaneous WUE as determined by photosynthetic rate over transpiration ( $\mu\text{mol CO}_2 \text{ mmol}^{-1} \text{ H}_2\text{O}$ ). Each sample leaf was placed within the LI-COR chamber and allowed to equilibrate to a photosynthetic photon flux density of 2,000 ( $\mu\text{mol m}^{-2} \text{ s}^{-1}$ ), 400 p.p.m.  $\text{CO}_2$  ( $\mu\text{mol mol}^{-1}$ ), and 25 °C. Measurements were recorded when vapor pressure difference (VPD; 1.45–1.55 kPa), intercellular  $\text{CO}_2$  to ambient  $\text{CO}_2$  ratios (0.6–0.9), and rates of photosynthesis and conductance had stabilized for at least 2 min. Following these conditions, three readings were recorded separated by 20–30 s each, and an average value for each gas exchange trait was calculated per sample.

After gas exchange sampling, two discs (30.5 mm<sup>2</sup> each) were taken from the leaf using a single-hole, hand-held punch. Discs were selected from healthy green tissue of the central portion of the leaf with care to avoid major leaf veins. All samples were stored in small tubes on ice, then oven dried at 50 °C for 48 h, and weighed to determine leaf mass per area (LMA,  $\text{mg mm}^{-2}$ ) and photosynthetic rate per mass ( $A_{\max/\text{mass}}$ ,  $\mu\text{mol CO}_2 \text{ g}^{-1} \text{ s}^{-1}$ ). Between 2 and 2.5 mg of dried tissue per sample was packed in individual tin foil capsules and analyzed for C isotopes and total C and N at the UC Davis Stable Isotope Facility (Davis, CA). Foliar C and N contents were expressed per unit mass ( $C_{\text{mass}}$ ,  $N_{\text{mass}}$ ,  $\text{mg mg}^{-1}$ ) and area ( $C_{\text{area}}$ ,  $N_{\text{area}}$ ,  $\text{mg mm}^{-2}$ ), and used to calculate nutrient stoichiometry (C:N,  $\text{mg mg}^{-1}$ ) and photosynthetic N use efficiency (NUE,  $\mu\text{mol CO}_2 \text{ mg}^{-1} \text{ N s}^{-1}$ ). Foliar  $^{13}\text{C}/^{12}\text{C}$  isotope ratios ( $\delta^{13}\text{C}$  ‰) were expressed relative to Vienna-Pee Dee belemnite (V-PDB):

$$\delta^{13}\text{C}_{\text{leaf}} = \left[ \frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{V-PDB}}} - 1 \right] \times 1,000.$$

Assuming a well-mixed atmosphere,  $\delta^{13}\text{C}_{\text{leaf}}$  values were compared with the  $\delta^{13}\text{C}$  of source air ( $\delta^{13}\text{C}_{\text{air}}$ ) to calculate net discrimination ( $\Delta$  ‰), an inverse index of the longer-term, assimilation-averaged WUE (Farquhar et al. 1989):

$$\Delta = \delta^{13}\text{C}_{\text{air}} - (\delta^{13}\text{C}_{\text{leaf}}) / (1,000 + \delta^{13}\text{C}_{\text{leaf}}) \times 1,000.$$

We accounted for seasonal variation in  $\delta^{13}\text{C}_{\text{air}}$  using atmospheric  $\text{CO}_2$  records from May–September 2008 from

Point Barrow, Alaska and La Jolla Pier, California stations in the SIO Air Sampling Network (<http://cdiac.ornl.gov>). A fitted curve from averaged month values from both sites was used to determine the  $\delta^{13}\text{C}_{\text{air}}$  values on a daily basis for Vancouver, British Columbia, where  $x$  = day of year: [ $f(x) = -1.46 \times 10^{-6}x^3 + 7.98 \times 10^{-4}x^2 - 0.139x - 0.908$ ].

In addition to field monitoring, we performed further measurements to test the effect of controlling for leaf age. We compared a range of genotypes using seedlings started in 4-L pots under field conditions and transferred to a greenhouse in late summer to provide stable environmental conditions. At the onset of the experiment, trees were categorized by phenological phase: (1) continual growth (no bud set), (2) growth followed by bud set, and (3) early stage bud set. Three consecutive, full-sized leaves similar in aspect and age were tagged on each tree and monitored for either gas exchange ( $A_{\max}$ ,  $g_s$ , WUE) or LMA over 3 consecutive weeks.

#### Season time metric assessment

Sampling dates for traits and seasonal events were initially recorded in Julian date of year (day 1–365). We calculated four other time metrics for synchronizing tree sampling to a “biological” scale, including canopy age (day or %), and proximity to growth cessation or bud set (in days). To determine canopy age, we calculated: (1) the difference in number of days between date of sampling and bud break, or (2) the canopy age (day) at date of sampling divided by the canopy duration (see above). Time scales for proximity to growth cessation or bud set were measured by the difference in number of days between dates of sampling and either growth cessation or bud set. In determining patterns of traits throughout the summer and assessing the extent of canopy cycle asynchrony, trait data were plotted against each sampling time metric. Traits were binned for each time point (day or %) to calculate mean values (and variance), and plotted against Julian sampling date, canopy age (day or %), and proximity of sampling to growth cessation or bud set.

#### Statistical analyses

All statistical tests were performed with R Project version 2.14.0 (GNU freeware; <http://cran.r-project.org>). To understand seasonal patterns, we determined average Julian dates for each phenological event (bud break, leaf flush, growth cessation, bud set, leaf drop) and the variance around each event. We tested for potential linkage and relationships of phenological events (within and between years) with Spearman’s rank correlations and Bonferroni correction for multiple comparisons using both average



**Table 1** Mean calendar date of seasonal canopy events for *Populus trichocarpa* trees from 2008 to 2011 (Julian date  $\pm$  SD in parentheses), and significance of Spearman's rank correlations between seasonal events

Date	Bud break	Leaf flush	Growth cessation <sup>a</sup>	Bud set	Leaf drop
Calendar date (Julian day)	8 March (67 $\pm$ 9)	3 April (93 $\pm$ 8)	16 August (228 $\pm$ 21)	1 September (244 $\pm$ 26)	19 November (323 $\pm$ 14)
Leaf flush	***	–			
Growth cessation	n.s.	n.s.	–		
Bud set	n.s.	n.s.	***	–	
Leaf drop	n.s.	n.s.	***	***	–

\*\*\*  $P < 0.001$ , n.s. not significant<sup>a</sup> Data available only for 2009

Julian dates calculated within individual years, and averages generated from multiple years.

To assess trait differences between pre-bud set and post-bud set phases, we split trees into two groups (by phase), both of which might be reasonably measured in a given summer field season. Pre-bud set trees included all trees measured to within 1 week of a noted terminal bud formation, and post-bud set trees included all trees within this range and trees with a fully formed terminal bud. Where observed, lammas trees (a 2nd leaf flush following an initial terminal bud set) were not included in our data groups (Soolanayakanahally et al. 2012). Trait data were compared between the pre-bud set and post-bud set tree groups using Wilcoxon's non-parametric two-sample test. Because pre- and post-bud set groups showed unequal sampling by latitude (post-bud set trees tended to average higher in latitude than pre-bud set trees), we re-tested traits using a smaller data set of pre-bud set trees from similar latitudes to the post-bud set tree group, considering only trees from 51.5 to 60° N.

To gauge the effect of putative trait trends, we compared the amount of change (%) in traits between early and late-growing-season plants (i.e., within the growing season prior to bud set). We selected trait data from our data set and compared canopy cycle asynchrony (measured by Julian date), or canopy cycle synchrony (measured by growing season). Trait data from early summer (Julian days 151–153) were compared to later summer (Julian days 212–214), and growing season start (114–121 days to bud set) compared to growing season termination (8–14 days to bud set). Initial and final mean values from these data sets were used to determine the percent change a trait might be predicted to have within a summer season using both time metrics. Data were further tested to ensure no major latitudinal differences between groups. Finally, we compared these estimates for magnitude of trait change in early versus late summer season with pre- versus post-bud set trees.

In estimating the biological effect of season on trait relationships, we compared traits between pre- and post-

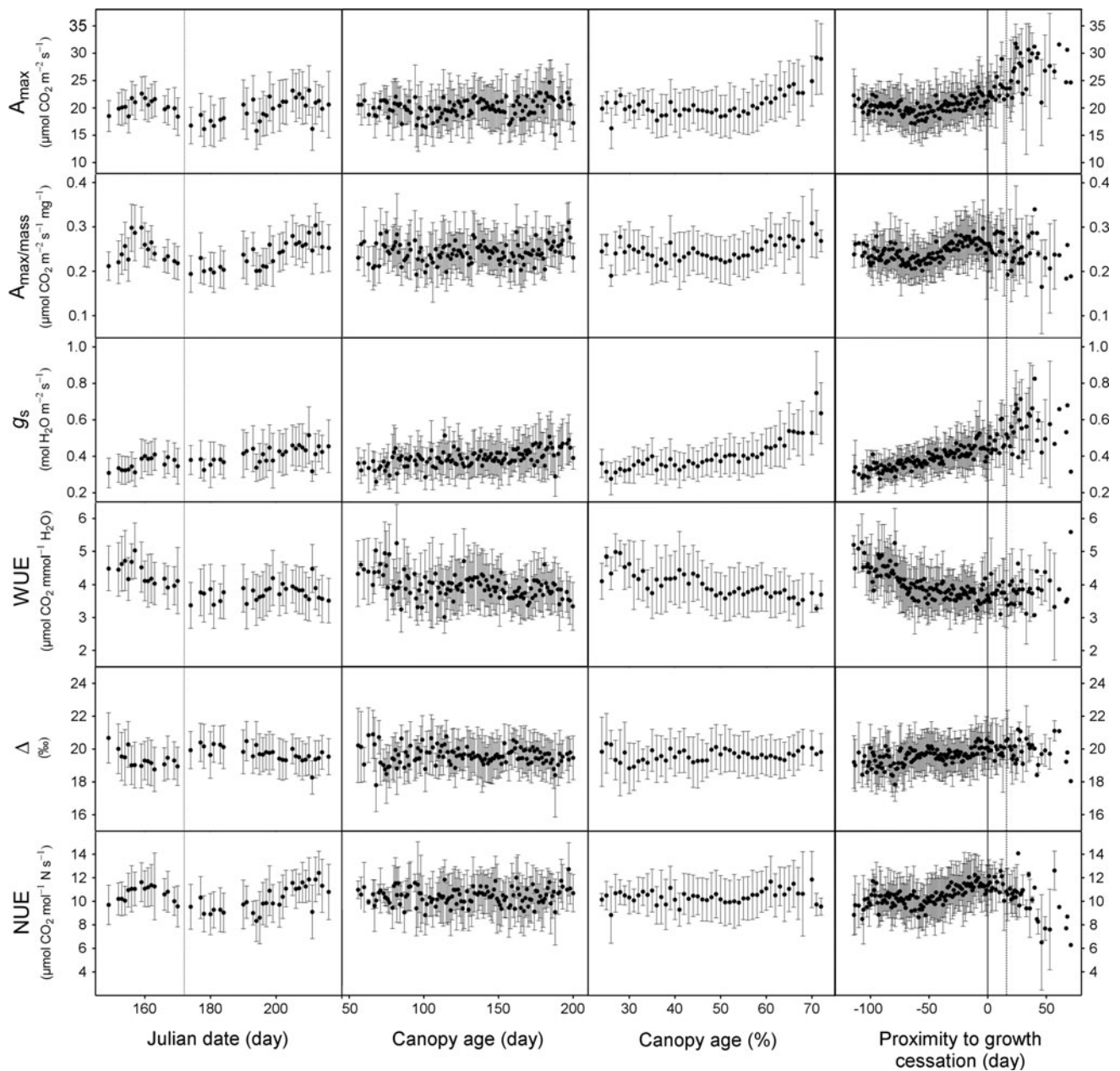
bud set trees using Spearman's rank correlations and Bonferroni correction for multiple comparisons. Trait comparisons were calculated with binned data (by proximity to bud set) for pre-bud set trees, and un-binned data for post-bud set trees to correct for inequality in sampling and generated groups of fairly equal size for assessments (pre-bud set = 128, post-bud set = 121). Trait relationships were noted for correlations significant in only one phase, or when the direction of the relationship changed.

## Results

Within the *P. trichocarpa* collection, variability around dates for different phenological events across years was lower early in the season, and higher towards the end of the season (Table 1). Both spring phenological events of bud break and leaf flush had less variability than the late summer events of growth cessation, bud set, or autumn leaf drop when considering data averaged from all years (or on a year by year basis). Analyses using average dates across all years indicated that events within seasons were correlated, while events between seasons were not necessarily associated. In general, growth cessation and bud set were relatively early compared to autumn leaf drop. Height growth cessation occurred when the canopy age was approximately 65 % ( $\pm 7.7$  SD), or 177 days ( $\pm 37$  SD), and the transition between growth cessation and terminal bud set was relatively quick at 16 days ( $\pm 8$  SD). No visible changes in the canopy, such as health or greenness of leaves, were observed apart from formation of the terminal bud.

### Variability in traits throughout the canopy duration

Individual analysis of traits showed different patterns of change depending on the season time metric (Figs. 2, 3). Many traits that showed no trend using certain metrics (especially Julian date) were observed to have strong patterns when synchronized by “biological” time. These

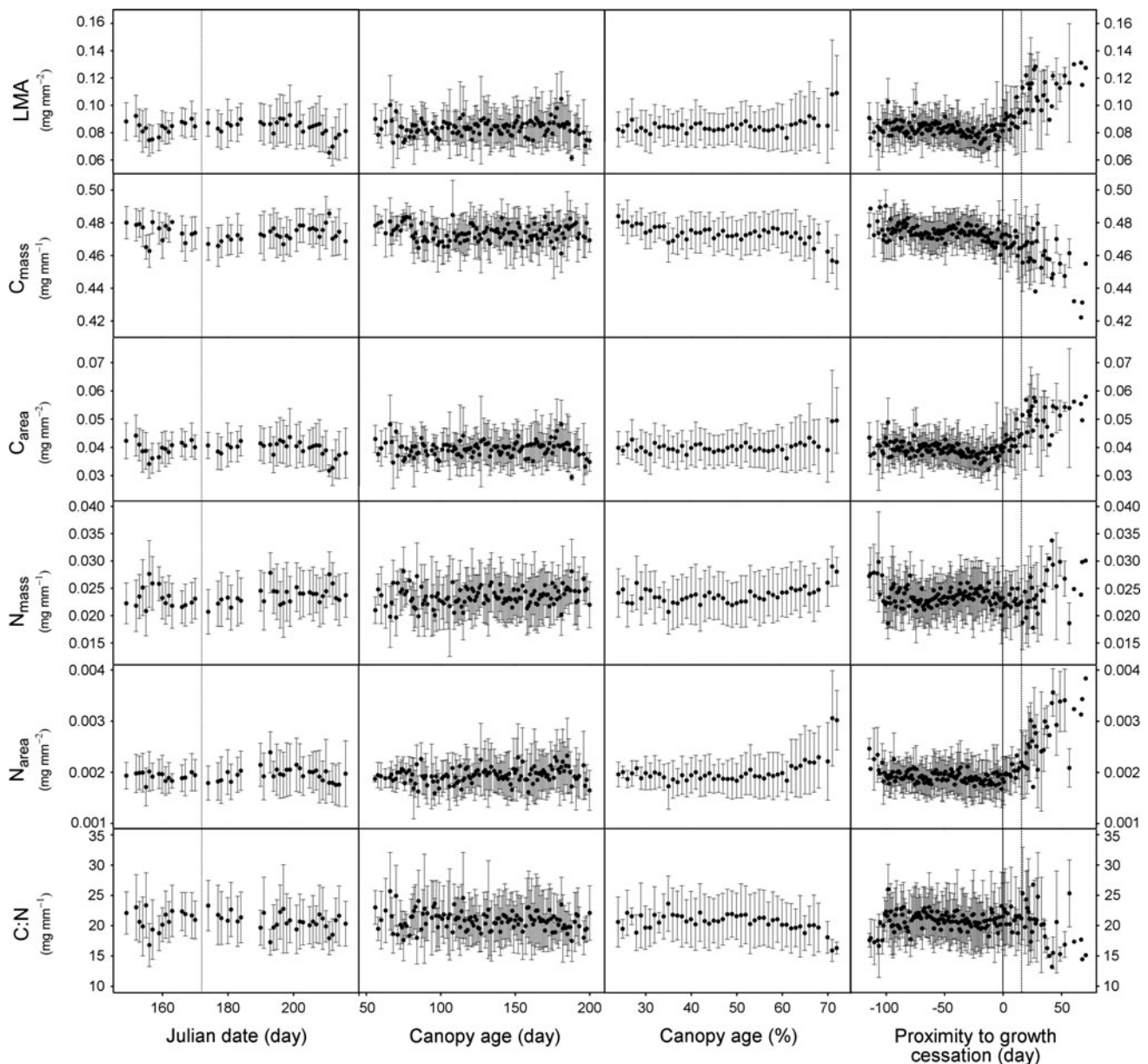


**Fig. 2** Alignment of gas exchange and photosynthetic efficiency traits in *Populus trichocarpa* trees using different time metrics. Data are binned by date according to each time metric showing mean value ( $\pm$ SD). Measured traits shown include: maximum photosynthetic assimilation rate per unit area ( $A_{\max}$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), photosynthetic assimilation rate per unit mass ( $A_{\max/\text{mass}}$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ mg}^{-1}$ ), stomatal conductance ( $g_s$ ,  $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ), intrinsic water use efficiency ( $WUE$ ,  $\mu\text{mol CO}_2 \text{ mmol}^{-1} \text{ H}_2\text{O}$ ), leaf

C isotope discrimination ( $\Delta$  ‰), and photosynthetic N use efficiency ( $NUE$ ,  $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ N s}^{-1}$ ). Time metrics shown include: Julian sampling date (day), canopy age (day), canopy age (%), and proximity to growth cessation and bud set (day). In Julian date graphs, the summer solstice (21 June, day 172) is represented by a *dashed line*. In proximity to growth cessation graphs, growth cessation (day 0) is represented by a *hard line* and average bud set date (day 16) by a *dashed line*.

trends observed with different time metrics were repeated using traits measured in 2010 (data not shown). In general, patterns of change were similar when traits were aligned by Julian date and canopy age (day), or canopy age (%) and proximity to growth cessation or bud set. Results for traits aligned by growth cessation and bud set were highly comparable; data presented apply to both phenomena.

Gas exchange and photosynthetic efficiency traits showed different patterns depending on the season time metric (Fig. 2). Using Julian date of sampling, measured traits showed no substantive trends. While patterns observed in these traits appeared superficially similar to the weather in Totem field (Online Resource Fig. 1, temperature wave pattern), none of these traits showed significant



**Fig. 3** Alignment of leaf composition traits in *P. trichocarpa* trees using different time metrics. Data are binned by date according to each time metric showing mean value ( $\pm$ SD). Measured traits shown include: leaf mass per area ( $LMA$ ,  $\text{mg mm}^{-2}$ ), C mass per leaf mass ( $C_{\text{mass}}$ ,  $\text{mg mg}^{-1}$ ), C mass per leaf area ( $C_{\text{area}}$ ,  $\text{mg mm}^{-2}$ ), N mass per leaf mass ( $N_{\text{mass}}$ ,  $\text{mg mg}^{-1}$ ), N mass per leaf area ( $N_{\text{area}}$ ,  $\text{mg mm}^{-2}$ ), and C mass: N mass ( $C:N$ ,  $\text{mg mg}^{-1}$ ). Time metrics

correlations with daily temperature, humidity, wind speed, or precipitation ( $P > 0.05$ ; data not shown). Trends generally showed no relationship to the summer solstice (Fig. 2, dashed line on Julian date). Distinct patterns were not observed aligning traits by canopy age (day), and results appeared similar to those using Julian date. By comparison, clearer trends were observed in most traits using canopy age (%) or proximity to growth cessation,

shown include: Julian sampling date (day), canopy age (day), canopy age (%), and proximity to growth cessation and bud set (day). In Julian date graphs, the summer solstice (21 June, day 172) is represented by a *dashed line*. In proximity to growth cessation graphs, growth cessation (day 0) is represented by a *hard line* and average bud set date (day 16) by a *dashed line*.

with the strongest patterns observed using the latter time metric. Assimilation-related traits ( $A_{\text{max}}$ ,  $A_{\text{max/mass}}$ ,  $\text{NUE}$ ) showed substantial shifts in mean trait values later in the season (Fig. 2, solid line indicating growth cessation), where  $A_{\text{max}}$  increased, and  $A_{\text{max/mass}}$  and  $\text{NUE}$  decreased. A steady increase in  $g_s$  and early decrease in  $\text{WUE}$  were observed using these time metrics, while no apparent trends in  $\Delta$  were observed from early to late season. Notably,

**Table 2** Mean values of sampling data (latitude, sample size), functional traits in pre- and post-bud set *P. trichocarpa* trees ( $\pm$ SD), and significance of Wilcoxon's comparisons between pre- and post-bud set trees

Sampling data	All pre-bud set trees	Latitude subset of pre-bud set trees	Post-bud set trees <sup>a</sup>	All pre- versus post-bud set trees Wilcoxon <i>P</i>	Subset pre- versus post-bud set trees Wilcoxon <i>P</i>
Latitude of tree origin (°)	51.2 ( $\pm$ 2.7)	53.8 ( $\pm$ 1.7)	55.8 ( $\pm$ 2.1)	***	n.s.
Sample size ( <i>n</i> )	1,606	607	121	—	—
$A_{\max}$ ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )	19.7 ( $\pm$ 3.8)	21.1 ( $\pm$ 3.8)	25.2 ( $\pm$ 5.1)	***	***
$A_{\max/\text{mass}}$ ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ mg}^{-1}$ )	0.242 ( $\pm$ 0.052)	0.258 ( $\pm$ 0.054)	0.246 ( $\pm$ 0.056)	n.s.	n.s.
$g_s$ ( $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ )	0.386 ( $\pm$ 0.098)	0.423 ( $\pm$ 0.11)	0.538 ( $\pm$ 0.16)	***	***
<i>NUE</i> ( $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ N s}^{-1}$ )	10.5 ( $\pm$ 1.9)	10.8 ( $\pm$ 1.9)	10.5 ( $\pm$ 2.3)	n.s.	n.s.
<i>WUE</i> ( $\mu\text{mol CO}_2 \text{ mmol}^{-1} \text{ H}_2\text{O}$ )	3.92 ( $\pm$ 0.77)	3.88 ( $\pm$ 0.73)	3.80 ( $\pm$ 0.72)	n.s.	n.s.
$\Delta$ (‰)	19.6 ( $\pm$ 1.3)	19.7 ( $\pm$ 1.4)	20.0 ( $\pm$ 1.1)	***	*
<i>LMA</i> ( $\text{mg mm}^{-2}$ )	0.0826 ( $\pm$ 0.013)	0.0831 ( $\pm$ 0.014)	0.106 ( $\pm$ 0.021)	***	***
$C_{\text{mass}}$ ( $\text{mg mg}^{-1}$ )	0.474 ( $\pm$ 0.011)	0.472 ( $\pm$ 0.011)	0.461 ( $\pm$ 0.014)	***	***
$C_{\text{area}}$ ( $\text{mg mm}^{-2}$ )	0.0391 ( $\pm$ 0.0063)	0.0392 ( $\pm$ 0.0064)	0.0489 ( $\pm$ 0.0093)	***	***
$N_{\text{mass}}$ ( $\text{mg mg}^{-1}$ )	0.0234 ( $\pm$ 0.0045)	0.0241 ( $\pm$ 0.0045)	0.0236 ( $\pm$ 0.0053)	n.s.	n.s.
$N_{\text{area}}$ ( $\text{mg mm}^{-2}$ )	0.00190 ( $\pm$ 0.00036)	0.00198 ( $\pm$ 0.00037)	0.00251 ( $\pm$ 0.00074)	***	***
C:N ( $\text{mg mg}^{-1}$ )	21.0 ( $\pm$ 4.2)	20.2 ( $\pm$ 3.9)	20.2 ( $\pm$ 4.9)	**	n.s.

$A_{\max}$  maximum photosynthetic assimilation rate per unit area,  $A_{\max/\text{mass}}$  assimilation rate per unit mass,  $g_s$  stomatal conductance, *LMA* leaf mass per area, *NUE* photosynthetic N use efficiency, *WUE* intrinsic water use efficiency,  $\Delta$  leaf C isotope discrimination

\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$

<sup>a</sup> Post-bud set data same for both paired-samples Wilcoxon's comparisons

most traits showed higher apparent variability following bud set (Fig. 2, dashed line indicating bud set) compared to earlier within the season.

Leaf composition traits showed similar discrepancies in patterns of trait change using different time metrics (Fig. 3). No directional trends were observed for any trait throughout the season using Julian date of sampling and canopy age (day). These traits also did not correlate to daily weather ( $P > 0.05$ ; data not shown), and there were no patterns in traits prior to the summer solstice (Fig. 3, dashed line on Julian date). Using canopy age (%) or proximity to growth cessation showed that most traits had a distinct directional trend late in the summer season, similar to the photosynthetic traits, and these patterns were strongest by aligning traits to growth cessation. Most traits showed little change during the growing season, but strongly increased (*LMA*,  $C_{\text{area}}$ ,  $N_{\text{mass}}$ ,  $N_{\text{area}}$ ) or decreased ( $C_{\text{mass}}$ , C:N) following growth cessation (Fig. 3, solid line indicating growth cessation). Trait variability generally increased following bud set (Fig. 3, dashed line indicating bud set).

#### Effects of seasonality and bud set

Pre-bud set trees showed lower variance in all traits, excepting the *WUE* traits, compared to post-bud set trees

(Table 2). More than half of the functional traits were significantly different between pre-bud set and post-bud set trees. Trait comparisons using a subset of pre-bud set trees (accounting for possible effects from latitude of origin, see “Materials and methods”; Online Resource Table 1) with post-bud set trees showed that many of the trait differences were still observed. Traits differing between pre- and post-bud set trees did not necessarily show the same magnitude in trait change (Table 2). Box plot representation showed that the overall variability in trait data tended to be greater in post-bud set trees, although the absolute range for many traits was relatively similar between both pre- and post-bud set trees (Online Resource Fig. 2).

Comparisons of traits prior to bud set (measured in early vs. late-summer growing season) showed differences not necessarily observed in the overall trait patterns, or between pre- and post-bud set trees (Table 3). Using a Julian calendar date it was estimated that seven traits would show at least 15 % change prior to bud set. By contrast, using proximity to bud set it was estimated that only six traits would change, and none of these involved leaf composition traits. The discrepancy between both time metrics in assessments of  $A_{\max}$  and *LMA* during the progression of the summer season was notable. Direction and significance of trait change in trees prior to bud set was the same with either time metric; however, the extent and



**Table 3** Percent change in mean trait values for *P. trichocarpa* trees from early to late growing season (prior to bud set) using two time scales (Julian date, proximity to bud set), compared to overall percent change between mean trait values for pre- and post-bud set trees

Trait	Julian date time scale (% change)	Bud set time scale (% change)	Pre- versus post-bud set trees (% change)
$A_{\max}$	2.6	<b>22.2</b>	<b>27.7</b>
$A_{\max/\text{mass}}$	<b>18.4</b>	<b>24.1</b>	1.7
$g_s$	<b>22.7</b>	<b>58.5</b>	<b>39.4</b>
NUE	<b>15.6</b>	<b>25.5</b>	0.2
WUE	<b>−23.0</b>	<b>−19.4</b>	−3.1
$\Delta$	−0.16 <sup>a</sup>	<b>0.83<sup>a</sup></b>	<b>0.45<sup>a</sup></b>
LMA	<b>−21.2</b>	−0.1	<b>28.4</b>
$C_{\text{mass}}$	−1.2	−1.4	−2.7
$C_{\text{area}}$	<b>−22.6</b>	−1.6	<b>24.8</b>
$N_{\text{mass}}$	4.5	−1.7	0.9
$N_{\text{area}}$	<b>−14.9</b>	−2.6	<b>31.4</b>
C:N	−6.6	1.4	−3.5

**Bold highlights** >15 % change, and statistical significance comparing groups using Wilcoxon's comparisons

For abbreviations and units, refer to Table 2

<sup>a</sup> Value represents subtraction of trait averages (%)

**Table 4** Correlation and significance of trait–trait relationships in *P. trichocarpa* trees prior to, and following, bud set

Trait	Phase	$A_{\max}$	$A_{\max/\text{mass}}$	$g_s$	WUE	$\Delta$	NUE	LMA	$C_{\text{mass}}$	$C_{\text{area}}$	$N_{\text{mass}}$	$N_{\text{area}}$
$A_{\max/\text{mass}}$	Pre-bud set	***										
	Post-bud set	***										
$g_s$	Pre-bud set	***	***									
	Post-bud set	***	***									
WUE	Pre-bud set	n.s.	n.s.	***								
	Post-bud set	n.s.		***								
$\Delta$	Pre-bud set	n.s.	n.s.	***	***							
	Post-bud set	n.s.	n.s.	n.s.	**							
NUE	Pre-bud set	***	***	***	***	+						
	Post-bud set	n.s.	***	n.s.	n.s.	**						
LMA	Pre-bud set	n.s.	*	n.s.	n.s.	n.s.	n.s.					
	Post-bud set	+	***	n.s.	n.s.	n.s.	***					
$C_{\text{mass}}$	Pre-bud set	n.s.	n.s.	*	+	*	*	n.s.				
	Post-bud set	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.				
$C_{\text{area}}$	Pre-bud set	n.s.	*	n.s.	n.s.	n.s.	n.s.	***	n.a.			
	Post-bud set	n.s.	*	n.s.	n.s.	n.s.	***	***	n.a.			
$N_{\text{mass}}$	Pre-bud set	**	***	n.s.	+	***	n.s.	***	+	***		
	Post-bud set	**	***	+	n.s.	***	***	n.s.	n.s.	n.s.		
$N_{\text{area}}$	Pre-bud set	***	n.s.	n.s.	***	***	*	+	n.s.	+	n.a.	
	Post-bud set	***	n.s.	+	n.s.	***	***	***	n.s.	***	n.a.	
C:N	Pre-bud set	*	***	n.s.	*	***	n.s.	***	n.a.	***	n.a.	***
	Post-bud set	*	***	*	n.s.	***	***	n.s.	n.a.	n.s.	n.a.	***

n.a. Not applicable; for other abbreviations and units, refer to Table 2

\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$

magnitude of trait change was generally much higher when using the proximity to bud set time scale (Table 3; Online Resource Table 2). Comparing directional trends of traits from pre- and post-bud set trees showed that some traits maintained a single directional trend, whereas others could reverse following growth cessation and bud set (Table 3). This resulted in some traits as appearing invariant because

different seasonal phases reversed the trend direction (particularly  $A_{\max/\text{mass}}$ , WUE, NUE; Table 2; Fig. 2).

#### Effect on trait–trait relationships

Correlative relationships between different functional traits were not necessarily the same in pre- and post-bud set trees

(Table 4). Approximately two-thirds of trait comparisons remained unaltered between pre- and post-bud set trees (both in significance and direction of correlation), while the remaining comparisons were found significant either in one seasonal phase or the other. All traits, excepting  $A_{\text{max/mass}}$ , showed at least one altered relationship with another trait depending on season. Changes to trait relationships occurred in either phase. In most cases, the relationship was significant only in pre-bud set trees, but in a number of others (especially relating to NUE) trait–trait relationships were only significant in post-bud set trees. Three traits in particular ( $WUE$ ,  $\Delta$ ,  $C_{\text{mass}}$ ) lost a number of trait relationships following bud set, and in two instances, trait relationships ( $g_s$  vs.  $N_{\text{area}}$ , NUE vs.  $C_{\text{mass}}$ ) showed a change in the direction of the correlation (Online Resource Table 3).

## Discussion

Detailed time-course analyses using a broad range of genotypes from the northern, deciduous tree *P. trichocarpa* showed that within a foundation species, numerous functional traits changed throughout the progression of the green canopy season. This was particularly apparent with the end of height growth and the onset of bud set, altering mean trait values and some trait relationships. Trends in traits occurred throughout the summer, both within the growing season to some extent, and following growth cessation and bud set, but many of these patterns were only apparent by synchronizing, or aligning trees to phenological events in the canopy cycle. These results demonstrated that many commonly assessed leaf trait values were directly affected by the phenology of the trees, and the specific seasonal phase in which the trait was measured.

### Estimating trait change throughout a season

The dates of most phenological events in our *P. trichocarpa* collection were not necessarily the same between years, meaning that the timing and duration of any seasonal phase might shift from year to year affecting multi-year comparisons of traits. In addition, the early and late phenological events observed in *P. trichocarpa* trees were not correlated within any year measured indicating the apparent length of a growing season was not fixed. This highlighted an inherent plasticity in *P. trichocarpa* to adjust its growing season and respond to different yearly conditions, and flexibility in timing is not entirely unexpected (Chuine and Cour 1999; Luquez et al. 2008). Bud set dates had greater variability in timing than spring bud break, strengthening the importance of a combination of certain cues to initiate a response, such as day length which does

not vary year to year, and temperature or other stressors which do vary (Kalcisits et al. 2009; Soolanayakanahally et al. 2012). This plasticity within the system means that it is impossible to accurately “match” measurements of trees or synchronize trait sampling without knowing the timing of phenology for a particular year.

A number of traits showed some trends throughout the green canopy period, but many were only apparent when incorporating the timing of growth cessation and bud set. Within our study, using the calendar date of sampling was found to be inappropriate because asynchronized trees were in different phenological seasons masking inherent biological differences (Figs. 2, 3). Using calendar dates alone would indicate that many traits did not necessarily change to a great extent throughout the summer, and suggested no differences between pre- and post-bud set trees. Changing the time metric to canopy age, as counted by days since bud break, did not correct the problem because the duration of a growing season was different for each genotype. Aligning traits using canopy age by percent corrected for this factor, and revealed some seasonal change in traits; however, the clearest trends were obtained by synchronizing trees in relation to growth cessation and bud set. This became especially apparent as the physiological differences (as indicated by leaf traits) preceding and following bud set were significantly different (Table 2). Thus, while canopy age (%) was a relatively good biological metric for traits by reflecting an inherent limitation on the duration of the canopy, using growth cessation or bud set was more biologically accurate by synchronizing the cycles of different trees to a particular event. Because our trees spanned different latitudes of origin, northern trees showed a curtailed growing season compared to southern trees, and generally tended to set bud at an earlier date (A. D. McKown, unpublished results). The effect of this growing season variation within a species was underscored by using calendar date of sampling, which masked seasonal changes in traits. These situations are most obvious in large-scale common gardens (this study; Farmer 1994; Ingvarsson et al. 2006; Luquez et al. 2008; Soolanayakanahally et al. 2009; Keller et al. 2011; Rohde et al. 2011). However, an equivalent might be finer-scale comparisons of temperate species from widely different locales, or equating studies of individuals across different years, especially where the seasonal phases could be modified depending on the conditions of each year (cf. Pau et al. 2011), thus contributing to higher intraspecific variability (see below).

### Influence of bud set versus leaf age on traits

A number of traits showed a change in pattern up to and following bud set resulting in trait differences between

trees in both phases. In some traits ( $A_{\max}$ ,  $g_s$ , LMA,  $C_{\text{area}}$ ,  $N_{\text{area}}$ ), significant differences between pre- and post-bud set trees equated to at least 25 % change in average trait value (Table 3). Other traits ( $A_{\max/\text{mass}}$ , NUE, WUE) showed a change in direction of the trait trend following bud set, resulting in apparent “non-significance” due to the directional shift (Table 3). Trait correlations between both pre- and post-bud set phases showed that the nature of these relationships could also change in significance (Table 4). The degree of variability in traits was relatively stable throughout the growing season, suggesting that a certain amount of intraspecific variation is predictable at a given time of data collection (Figs. 2, 3), possibly relating to leaf age (see below) whereas trait variability tended to be greater in post-bud set trees (Table 2). Our tests using a subset of pre-bud set trees originating from a similar range in latitude as the post-bud set trees (i.e., 51.5–60°N) showed that within the species, seasonal effects, especially from bud set were still observable (Table 2). Numerous traits are known to vary with latitude of origin in *Populus* (Gornall and Guy 2007; Soolanayakanahally et al. 2009; Keller et al. 2011; A. D. McKown, unpublished results); however, the results clearly demonstrated that seasonal phases still outweighed these inherent differences.

Trait variability may have corresponded to modified leaf physiology relating to some age effect of the leaves. Our large field study measured leaves of a similar canopy position and age among pre-bud set trees; however, it was impossible to match growing season leaf ages in post-bud set trees because the leading shoot meristem was no longer producing new leaves, thus measurements for bud set trees also incorporated an unavoidable, increasing leaf age effect. In additional studies controlling for leaf age, we found that overall, the variability between consecutive leaves on a given tree at any time point measured averaged 9 % in gas exchange ( $A_{\max}$ ,  $g_s$ , WUE) and 12 % in LMA, regardless of phenological phase (data not shown). In general, leaf ageing is considered to have a negative effect on photosynthesis with older leaves showing lower assimilation rates (Reich 1995; Wilson et al. 2001). Within *P. trichocarpa*, this was observed in leaves from growing trees prior to bud set, but was not the case following bud set as both  $A_{\max}$  and  $g_s$  increased (Online Resource Table 4). Repeated measurements of  $A_{\max}$ ,  $g_s$ , WUE, and LMA from trees initiating bud set or already fully set showed distinct patterns over time mirroring field observations (Online Resource Fig. 2) and indicating that the bud set effect is superimposed on an age effect in leaves.

#### Influence of phenology on whole plant physiology

The results of this study emphasized that commonly assessed functional traits indicative of whole plant

physiology are modified throughout the progression of the green canopy season showing marked changes with growth cessation and bud set. During the growth portion of the summer season, trees allocate C for vegetative biomass (height, volume) and generating new canopy leaves (Ceulemans and Isebrands 1996; Soolanayakanahally et al. 2012). Alongside summer growth in this study, many traits relating to assimilation were progressively increasing ( $A_{\max}$ ,  $A_{\max/\text{mass}}$ ,  $g_s$ , NUE) by more than 20 %, while WUE was decreasing (Table 3). By comparison, traits related to leaf composition and leaf nutrients were less affected by the summer growth phase and showed less directional trend, indicating that investment in leaves and nutrient balance was relatively stable and consistent throughout the growing season. Following the cues for bud set, both height growth and new leaf production cease while C allocation to other sinks continues (Ceulemans and Isebrands 1996; Kuptz et al. 2011; Soolanayakanahally et al. 2012).

In this study, aboveground growth cessation and bud set showed an apparent “ramping up” of photosynthetic activity in *P. trichocarpa* leaves. Both  $A_{\max}$  and  $g_s$  greatly increased, and N that might otherwise be allocated to growing new shoot or leaf tissues appeared to accumulate in existing leaves, increasing  $N_{\text{area}}$  (Table 3). In coordination with these changes, the mass and C content of the leaf (LMA,  $C_{\text{area}}$ ) increased, accounting for the apparent lack of change in  $A_{\max/\text{mass}}$  and NUE observed between pre- and post-bud set trees. This phase occupied approximately one-third of the green canopy period, and occurred before leaves showed any signs of fall senescence. Visually, leaves were indistinguishable between pre- and post-bud set phases, although some leaves appeared darker green. Similar results of increasing  $A_{\max}$ /photosynthate production and leaf greening following bud set have also been observed in common garden studies of other *Populus* species (Ceulemans and Isebrands 1996) and growth chamber studies (M. Song, unpublished results). In closely related *Populus balsamifera*, belowground root growth continues following bud set and soil resources continue to be acquired (Soolanayakanahally et al. 2012). Mechanistically, the shoot sink strength is presumably weaker; however, the continuation to grow in girth and total plant size, despite a relatively static leaf area, places increased demands on the existing leaves. The observed increase in leaf N (and  $A_{\max}$ ) after bud set is supported by continued acquisition of N from the soil (data not shown), whereas remobilization of leaf N from older leaves to younger leaves is unlikely to be a contributing factor prior to leaf senescence.

The effect of shifting C assimilation and allocation strategies during the green canopy period suggests the potential for trait relationships to likewise change. Several traits measured in this study ( $A_{\max}$ ,  $A_{\max/\text{mass}}$ , LMA,  $N_{\text{mass}}$ ,

$N_{\text{area}}$ ) have well-documented relationships in the leaf economics spectrum (Wright et al. 2004, 2005; Shipley et al. 2006). Most correlations between traits measured in *P. trichocarpa* trees, regardless of phase, agreed with the previously established trait relationships determined at the global scale; however, two important trait relationships did not correspond with these associations following bud set. Prior to bud set,  $A_{\text{max}}$  and LMA showed no significant relationship and LMA and  $N_{\text{mass}}$  were negatively correlated, as predicted by the leaf economics spectrum (Table 4). These trait–trait relationships altered following bud set, and  $A_{\text{max}}$  and LMA showed positive correlation, whereas LMA and  $N_{\text{mass}}$  were not significantly correlated. Although not necessarily part of the global leaf economics spectrum, a number of nutrient-related traits (NUE,  $C_{\text{mass}}$ ,  $N_{\text{mass}}$ ,  $N_{\text{area}}$ , C:N) showed altered relationships with other leaf traits depending on phase.

The magnitude of trait difference between early and late growing season or pre- and post-bud set phase varied from ~20 to 60 % depending on the trait (Table 3). This is in agreement with Albert et al. (2010) showing that the extent of intraspecific variability depends on the trait. Within a seasonal context, however, the phenological phase may account for a substantial portion of intraspecific variability observed in a particular trait. Thus, trait interpretations for finer-scale ecophysiological investigations, such as species-level comparisons and local interpretations of plant function, or larger-scale ecosystem, phenotyping, and plant breeding studies might be highly affected by a seasonally-variable trait. For instance, this could lead to misinterpretations of productivity (at the individual or community level), as a difference of 30 % in  $A_{\text{max}}$  would affect modeling efforts to understand rates of C flux, or stand productivity. Alterations to C flux and productivity during a particular phenological phase in a dominant tree species may also influence community dynamics by affecting dry matter production, nutrient cycling, and C storage (Grime 2006; Ma et al. 2011). Comparatively, a difference of 20–25 % in NUE or WUE in phenotyping efforts might incorrectly recommend individuals for crop improvement/breeding purposes.

## Conclusion

Findings from our detailed, time-course studies of *P. trichocarpa* throughout the duration of the green canopy season showed that physiological traits and the nature of trait–trait relationships could change, particularly following height growth cessation and bud set. We expect phenological effects on functional traits to occur in other woody species, especially other members of the same genus and family (i.e., cottonwoods, aspens and willows). In addition, because many temperate/boreal trees have wide ranges in latitude (or elevation), phenology, and its

effects on physiology, should be pronounced in these species, especially in more extreme environments. Detailed studies in other deciduous tree taxa have addressed overall patterns of change or trait plasticity throughout the green canopy period and shown that trait trends exist prior to leaf senescence (Morecroft and Roberts 1999; Grassi et al. 2005; Chen and Chen 2007; Stokes et al. 2010; Delagrangé 2011; Sang et al. 2011); however, the extent to which the bud set phase affects these taxa is unknown, despite occurring in each of them. Tracking phenology in individuals is time-consuming, but monitoring, adjustments to timing of field work (e.g., early vs. late summer), and visual indicators of an individual's phenology (i.e., leaf production vs. terminal bud formation) are achievable in most studies. In speculating on the broader biological and ecological relevance of our findings, we predict that some intraspecific and “within-site” variability is directly attributable to seasonal differences in the canopy, and that future detailed studies relating traits to phenological events may infer broader implications of phenological phase on water and C balance in individuals and plant communities.

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