

Tree Physiology 38, 789–800  
doi:10.1093/treephys/tpy019



## Research paper

# Heterosis in poplar involves phenotypic stability: cottonwood hybrids outperform their parental species at suboptimal temperatures

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Received July 17, 2017; accepted February 5, 2018; published online March 2, 2018; handling Editor Sean Thomas

Heterosis or hybrid vigor is common in hybrid poplars, and to investigate its occurrence and physiological basis we compared narrowleaf cottonwoods, *Populus angustifolia* James, prairie cottonwoods, *Populus deltoides* Bartr. ex Marsh, and their native intersectional hybrids, *P. × acuminata* Rydb., from Alberta, Canada. Clonal replicates from 10 separate trees from each taxon were raised in growth chambers at different temperatures (T). Growth was similarly vigorous across the taxa at 20 and 24 °C, and morphological and physiological traits of the hybrids were generally intermediate between the parental species, or similar to the larger parent, demonstrating additive inheritance or dominance, respectively. Growth declined at 18 and 15 °C particularly in the parental species, and consequently hybrid vigor was displayed for root and especially leaf growth. Stomatal distributions and chlorophyll indices were intermediate in the hybrids and unaffected by T. Foliar nitrogen (N), net assimilation ( $A_{\text{sat}}$ ), stomatal conductance ( $g_s$ ) and transpiration ( $E$ ) per unit of leaf area were lower in the hybrids, but the hybrids generally had larger leaf areas. Water-use efficiencies ( $A_{\text{sat}}/g_s$ ) were similar across the taxa and reduced with warming, while nitrogen-use efficiencies ( $A_{\text{sat}}/N$ ) increased.  $\delta^{13}\text{C}$  was correlated with leaf mass per area, which varied across the taxa. Photosynthesis ( $A_{\text{sat}}$ ) was correlated with chlorophyll content index, N and/or  $g_s$  in *P. deltoides* and the hybrids, but not in *P. angustifolia*, indicating different physiological limitations. We conclude that heterosis in *P. × acuminata* results from the compound benefits from multiple dominant traits, and superior growth particularly at suboptimal conditions. This indicates phenotypic stability or environmental adaptability, whereby heterozygosity provides metabolic diversity that allows hybrids to thrive across a broader environmental range.

**Keywords:** gas exchange, growth allocation, hybrid poplar, hybrid vigor, *Populus deltoides*, *Populus angustifolia*.

## Introduction

Some interspecific poplar hybrids display heterosis or hybrid vigor, and thus outperform both of their parental species (Mather and Jinks 1971, Hinckley et al. 1989, Bradshaw et al. 2000). Heterosis is common in some other diploid plants and has contributed substantially to the yield increases of field crops and vegetables over the 20th century (Tollenaar et al. 2004). Despite the importance of heterosis in crops and trees, its physiological basis remains uncertain and this may reflect the involvement of multiple processes and correspondingly, multiple traits and genes (Birchler et al. 2003).

Poplars are deciduous and dioecious trees from the diverse genus *Populus*, which includes about 29 species around the

Northern Hemisphere and parts of Africa (Eckenwalder 1996). The poplars are grouped into sections, a taxonomic category between genus and species, with more continuous variation within sections, and greater morphological distance across sections (Eckenwalder 1996, Hamzeh and Dayanandan 2004). Some intersectional crosses are fertile and probably due to the greater genetic distance, some intersectional hybrids display substantial hybrid vigor (Hinckley et al. 1989, Li et al. 1998, Bradshaw et al. 2000).

Most intersectional hybrids that are commercially grown in plantations are the result of artificial crossing, and intersectional hybridization also occurs naturally in sympatric zones (Eckenwalder 1996, Gom and Rood 1999). Some natural hybrids may provide

opportunities for vigorous biomass production or display local adaptation suitable for reclamation or other plantings. Additionally, since these are native genotypes, they may be more acceptable for plantings on public lands than artificial hybrids, particularly with parental species that are not locally native. Natural hybrids may also provide insight into the physiological basis for hybrid vigor and contribute to analyses of poplar diversity, adaptation and evolution (Eckenwalder 1996, Whitham et al. 2008).

Southern Alberta, Canada, provides a global center of *Populus* biodiversity and hybridization, with five *Populus* species overlapping and four interbreeding (Brayshaw 1965, Gom and Rood 1999, Floate 2004, Cooke and Rood 2007). These include three section *Tacamahaca* species, including the narrowleaf cottonwood, *Populus angustifolia* James, the black cottonwood, *Populus trichocarpa* Torr. & Gray, and the closely related balsam poplar, *Populus balsamifera* L. Here, the section *Aigeiros* prairie cottonwood, *Populus deltoides* Bartr. ex Marsh, reaches its western and northern limit and interbreeds with the other species to produce natural intersectional hybrids. We have been studying these cottonwoods, or riparian poplars, in their native floodplain zones and in nursery and greenhouse environments (Rood et al. 2013, 2017, D.W. Pearce, S.G. Woodman, M.G. Letts and S.B. Rood, submitted) and in this study, we investigated their growth and physiology in growth chambers, which permitted investigation into their responses to temperature (T).

We compared a triplet of taxa, with *P. angustifolia*, *P. deltoides* and their natural intersectional hybrids of lanceleaf cottonwood, *P. × acuminata* Rydb. These taxa occur in an overlapping sequence along river valleys from *P. angustifolia* in the cooler foothills, *P. × acuminata* through the transition and *P. deltoides* in the warmer prairie ecoregion (Kalischuk et al. 2001, Floate 2004). We expected that the cottonwoods would differ in their T adaptation and to investigate this we included multiple, clonally replicate genotypes of each taxon and provided a sequence of T that would reflect spring warming with climate change (Beaubin and Freeland 2000). We expected growth rates to increase with warming from 15 °C at least to 20 °C (Domingo and Gordon 1974) providing an environmental (E) effect, and differences across the three taxa, providing a genetic (G) effect. Reflecting the trees' geographic distributions we expected a genotype × environment (G × E) interaction, with *P. angustifolia* demonstrating proportionally increased growth at cooler T, while *P. deltoides* would display more vigorous growth with the warmer T (Figure 1).

We predicted that the intersectional hybrids would typically display intermediate performance, thus displaying additive inheritance or for some traits, dominance, matching the superior parent (Mather and Jinks 1971, Lojewski et al. 2009). With a combination of dominant traits, the hybrids might display heterosis particularly at the favorable T with the view that the genotype defines the upper limit for growth rate, and heterosis

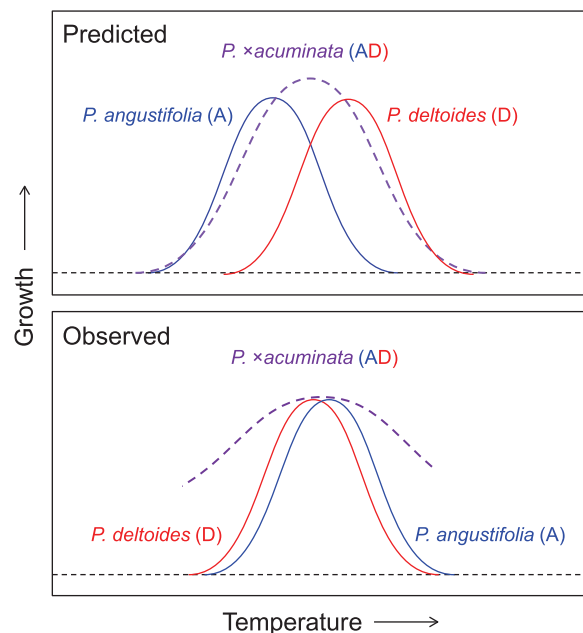


Figure 1. Schematics displaying the predicted (top) and observed (bottom) responses to temperature of two native *Populus* species and their intersectional hybrids.

would especially be expressed with favorable environmental conditions, providing another G × E interaction (Rood et al. 1985, Blum et al. 1990). We investigated growth and foliar physiological characteristics that might provide an ecophysiological basis for the G, E and G × E effects (Tschaplinski and Blake 1989, Gornall and Guy 2007, Kaluthota et al. 2015).

## Materials and methods

### Plant materials and growth conditions

Visually healthy shoots were cut from naturally occurring cottonwood trees in the Helen Schuler Nature Reserve along the Oldman River in Lethbridge, AB, Canada (49°70', 112°86' W, 829 m above sea level, Rood et al. 2013, Flanagan et al. 2017) in late March and early April 2012. Long shoots containing only the previous year's growth were cut from five female and five male trees that were spaced more than 50 m apart to avoid clonal ramets, from each of three taxa: the narrowleaf cottonwood, *P. angustifolia* James (A), the prairie cottonwood, *P. deltoides* Bartr. ex Marsh. (D), and their naturally occurring intersectional hybrids of lanceleaf cottonwood, *P. × acuminata* Rydb. (AD).

The cuttings were stored at 5 °C for ~8 weeks, the terminal buds were excised, and the branches were cut into 10 cm lengths with the uppermost bud ~1 cm below the cut. Cuttings were soaked in tap water for 20 h at 22 °C and 20 uniform cuttings from each genotype were selected. Groups of five were randomly selected and vertically stuck in each of four 15 cm pots containing water-saturated Sun Gro Sunshine Mix 1, with *Sphagnum* peat moss, perlite and vermiculite (Sun Gro Horticulture Canada Ltd,

Seba Beach, AB, Canada). These were placed in Conviron (Winnipeg, MB, Canada) E15 CMP4030 reach-in growth chambers programmed for day/night (16 h/8 h) lighting and T of 16 °C, for establishment at the same T.

After 2 weeks, a few saplings that displayed poor growth were replaced and if a second stem had emerged it was pruned to ensure a single dominant stem. One pot for each genotype was then placed in each of four growth chambers with day/night T of 15/14, 18/16, 20/18 or 24/20 °C; the average T in Lethbridge in May, during spring regrowth, is 18/4 °C. We refer to the treatments by the day T, since the night T would have limited influence (Frantz et al. 2004). The study design thus included 10 genotypes for each of three taxa, four T treatments, and five clonal replicates for each condition:  $10 \times 3 \times 4 \times 5$  replicates = 600 study saplings.

The 16 h photoperiod included 1 h dusk and dawn periods with one-half illumination. Lighting was provided by incandescent and metal halide lamps that delivered  $620 \mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetically active radiation at the soil surface, and relative humidity averaged 90%. Cuttings were watered to saturation daily to avoid drought stress and fertilized biweekly with Miracle-Gro 24-8-16 water-soluble fertilizer (Scotts Canada, Ltd, Mississauga, ON, Canada), commencing 3 weeks after sticking.

### Foliar physiology

Gas exchange was measured in a 5-day period after 8 weeks growth in the different T conditions and 2 weeks before harvest. Due to the time required for measurements, 8 of the 10 genotypes in each taxon were randomly selected, and for these one plant in each treatment was used and a fully expanded neo-formed lower leaf was measured. We used a LI-6400XT portable photosynthesis system, fitted with a CO<sub>2</sub> mixer and a 6400-40 LCF light source (LI-COR Biosciences, Lincoln, NE, USA). Light was set to  $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density, reference CO<sub>2</sub> was  $400 \mu\text{mol mol}^{-1}$ , and the leaf-to-air vapor pressure deficit was maintained at 0.9 kPa (based on leaf T) through automatic control of air flow. The cuvette block T was set at 15 °C, 17.5 °C, 20 °C or 22.5 °C for the different treatments, resulting in mean leaf T of 16.4 °C, 18.8 °C, 20.9 °C and 23.7 °C, respectively. Light-saturated net CO<sub>2</sub> assimilation ( $A_{\text{sat}}$ ), stomatal conductance of water vapor ( $g_s$ ) and transpiration ( $E$ ) were measured and internal CO<sub>2</sub> is expressed relative to the regulated ambient CO<sub>2</sub> ( $c_i/c_a$ ). Because the vapor pressure deficit was stabilized,  $g_s$  and  $E$  were tightly correlated. Instantaneous (intrinsic) water-use efficiency ( $\text{WUE}_i$ ) was calculated as  $A_{\text{sat}}/g_s$ .

On the same leaf, the chlorophyll content index (CCI; calculated from absorbance at 653 and 931 nm) was measured with a CCM-200 chlorophyll content meter (Opti-Sciences, Hudson, NH, USA). We had earlier determined that in *P. angustifolia* CCI was strongly correlated with foliar chlorophylls (Kaluthota et al. 2015).



Figure 2. Photographs of the 5 week old *Populus* saplings from two parental taxa, *P. angustifolia* and *P. deltoides* and their *P. xacuminata* hybrids grown at 24 °C (A), and *P. xacuminata* grown at four temperatures (B). Each pot contains five replicate saplings of a single, vigorous genotype.

### Growth

Observations, leaf counts and height measurements were made weekly, and after 10 weeks plants were harvested by removing the soil and root plug from each pot, placing this in water and gently separating the roots from the potting media. With rinsing, the roots were readily separated from the growth medium and while some fine roots would have been lost with harvesting, the values are probably proportionally representative. The saplings were then dissected into leaves, stems and roots, and leaf areas were measured with a LI-COR LI-3000 Area Meter with a transparent conveyer belt assembly (LI-COR Instruments, Lincoln, NE, USA). All organs were oven dried at 80 °C for 1 week and weighed to determine dry mass (biomass). The leaves that were used for leaf gas exchange and CCI measurements were harvested separately, digitally scanned and areas were determined with WinFOLIA Pro 2007 (Regent Instruments Inc., Québec, QC, Canada). Those leaves were air-dried and weighed, and values were added to complete leaf measures for each sapling.

The measured leaf from each genotype and T treatment was finely ground and ~4.5 mg of the fine powder was sent for combustion and elemental analysis at the University of California, Davis, Stable Isotope Facility. Carbon and nitrogen (N) contents were determined along with the carbon isotope composition ( $\delta^{13}\text{C}$ ) with a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon, Ltd, Cheshire, UK) and values were calculated relative to international

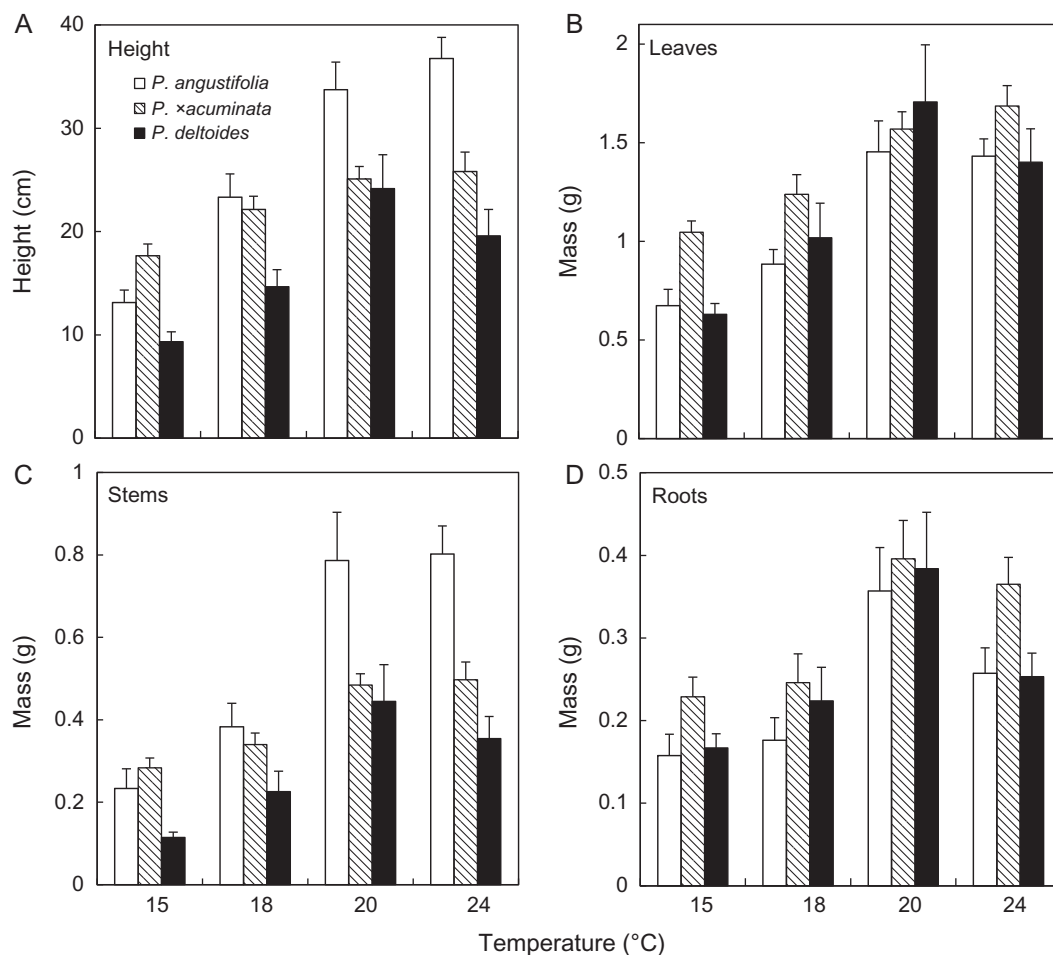


Figure 3. Growth characteristics of two *Populus* species and their hybrids at four temperatures (mean  $\pm$  SE).

standards of Vienna PeeDee Belemnite. Leaf N content was expressed as concentration and relative to leaf area ( $\text{g m}^{-2}$ ).

### Stomatal density

To assess stomatal density, a  $0.38 \text{ cm}^2$  disk was hole-punched from the widest portion of the dried sample leaf from each genotype and treatment, avoiding the midrib and major veins. The disk was dissected and the halves were mounted on aluminum stubs with double-sided adhesive tape, with either adaxial or abaxial (lower) leaf surfaces exposed. Observations were made at 300 $\times$  magnification using a Hitachi TM1000 Scanning Electron Microscope (Hitachi High Technologies America, Dallas, TX, USA). Digital images were compiled from three randomly selected fields of view for each leaf surface and uploaded into ImageJ (National Institutes of Health, Bethesda, MD, USA) for stomatal counts. Stomatal densities were determined for each surface, and the ratio of adaxial to abaxial stomatal density was calculated.

### Statistical analyses

For analyses of variance, the values for each characteristic were first averaged from the five or four clonal replicates for each genotype in each T treatment. This avoided pseudoreplication

and there were subsequently 10 values to represent the 10 genotypes for each taxon in each treatment ( $10 \times 3 \times 4 = 120$  values). Since the same genotypes were included across the T treatments our primary analyses were two-factor linear mixed model analyses of variance, with SPSS Statistics 19 (IBM Corp., Armonk, NY, USA). Genotypes were treated as 'subjects' in random intercept models, and T treatment and taxon were treated as factors with a factorial analysis to investigate three effects: taxon, T and the taxon  $\times$  T interaction.

Bivariate correlations were explored for all growth and foliar physiology characters, considering individual or combined taxa across the treatments. Subsequently, multiple linear regressions were undertaken to consider correspondences with two or more variables.

### Inheritance and heterosis

Inheritance patterns were quantified by two methods that compared the performance of the hybrids with that of the parents (Mather and Jinks 1971, Soliman et al. 2013). The potence ratio (PR) represents the overall degree of dominance as:

$$\text{PR} = (\text{hybrid} - mp) / ((P_2 - P_1) / 2)$$

Table 1. Results from mixed model two-factor ANOVAs with probabilities for effects of taxon, temperature and the taxon × temperature interaction. Trends are italicized ( $P < 0.1$ ), significant effects are in bold ( $P < 0.05$ ) or bold italics ( $P < 0.01$ ), and '0.001' generally indicates  $P < 0.001$ .

Characteristic	Effect		
	Taxon	Temperature	Taxon × Temp
<i>Morphology</i>			
Root mass (RM)	0.185	<b>0.001</b>	0.742
Stem mass (SM)	<b>0.001</b>	<b>0.001</b>	<b>0.001</b>
Leaf mass (LM)	0.102	<b>0.001</b>	0.354
Total mass (M)	0.238	<b>0.001</b>	0.305
Shoot/root (SM ± LM)/RM	<b>0.021</b>	<b>0.005</b>	0.066
Leaf number (LN)	<b>0.048</b>	<b>0.001</b>	<b>0.013</b>
Leaf area (LA)	<b>0.022</b>	<b>0.001</b>	0.143
Sapling height	<b>0.001</b>	<b>0.001</b>	<b>0.001</b>
Mortality	<b>0.001</b>	<b>0.016</b>	0.378
<i>Foliar physiology</i>			
Leaf area ratio (LA/RM)	0.146	<b>0.001</b>	<b>0.005</b>
Leaf mass per area (LMA; LM/LA)	<b>0.001</b>	<b>0.001</b>	0.332
Chlorophyll content index (CCI)	<b>0.001</b>	0.829	0.546
N/area	<b>0.001</b>	<b>0.001</b>	0.210
N concentration	<b>0.001</b>	<b>0.001</b>	0.609
Stomatal density (SD)	<b>0.001</b>	<b>0.001</b>	0.380
Stomatal conductance ( $g_s$ )	<b>0.001</b>	<b>0.001</b>	<b>0.010</b>
Net assimilation ( $A_{sat}$ )	<b>0.009</b>	0.557	0.080
CO <sub>2</sub> internal/ambient ( $c_i/c_a$ )	0.492	<b>0.004</b>	0.589
Nitrogen-use efficiency (NUE; $A_{sat}/(N/area)$ ) <sup>1</sup>	0.109	<b>0.001</b>	0.533
Water-use efficiency ( $WUE_i$ ; $A_{sat}/g_s$ )	0.582	<b>0.001</b>	0.686
$\delta^{13}C$	0.538	<b>0.001</b>	0.627

<sup>1</sup>Also referred to as photosynthetic NUE.

Table 2. Bivariate Pearson product–moment correlations for growth characteristics across all taxa and temperature combinations ( $n = 118$ ). \* $P < 0.05$  (bold); and \*\* $P < 0.01$  (bold italics), red or blue indicate positive or negative associations, respectively.

	SM	LM	M	Sh/R	LN	LA	Height	Mortality
Root mass	<b>0.663**</b>	<b>0.847**</b>	<b>0.869**</b>	<b>-0.328**</b>	<b>0.617**</b>	<b>0.734**</b>	<b>0.564**</b>	-0.180
Stem mass (SM)		<b>0.764**</b>	<b>0.872**</b>	0.095	<b>0.883**</b>	<b>0.624**</b>	<b>0.917**</b>	-0.127
Leaf mass (LM)			<b>0.977**</b>	-0.060	<b>0.756**</b>	<b>0.931**</b>	<b>0.743**</b>	<b>-0.221*</b>
Total mass (M)				-0.061	<b>0.832**</b>	<b>0.872**</b>	<b>0.826**</b>	<b>-0.195*</b>
Shoot/root (Sh/R)					0.021	-0.039	<b>0.211*</b>	0.053
Leaf number (LN)						<b>0.673**</b>	<b>0.824**</b>	-0.163
Leaf area (LA)							<b>0.633**</b>	<b>-0.420**</b>
Height								-0.096

where  $mp$  = the midparent value, or parental mean, and  $P_1$  and  $P_2$  represent the lower and higher value parents. PR values around 0 indicate incomplete dominance with the hybrids being intermediate between the parents and PR values around  $-1$  or  $1$  indicate dominance of the smaller or larger characteristic, respectively. Potence ratio values substantially below  $-1$  indicate hybrid break-down, and values exceeding 1 indicate overdominance or hybrid vigor.

Midparent indices, which are also referred to as midparent heterosis values, represent the percent increase or decrease in hybrid performance relative to the midparent value:

$$\text{Midparent index (\%)} = ((\text{hybrid} - mp)/mp) \times 100$$

## Results

### Growth patterns and correlations

The study involved two experimental factors: (i) three naturally occurring cottonwood taxa including two species and their native hybrids, and (ii) four temperature (T) treatments. Both experimental factors, taxon and T, produced major effects relative to sapling growth. By 5 weeks there were apparent differences across the taxa, including differences in leaf shapes and sizes (Figure 2A). At that time, there were clear differences in growth characteristics across the T treatments, with progressively increasing size with warming T (Figure 2B).

There was progressive growth over the study with consistent patterns across the taxa relative to heights, which were measured about weekly (see Figure S1 available as Supplementary Data at *Tree Physiology* Online). The heights displayed differences in the rankings of the taxa across the T treatments, with the hybrids being taller in the cool condition, while *P. angustifolia* was tallest with the warmer T (Figure 3A).

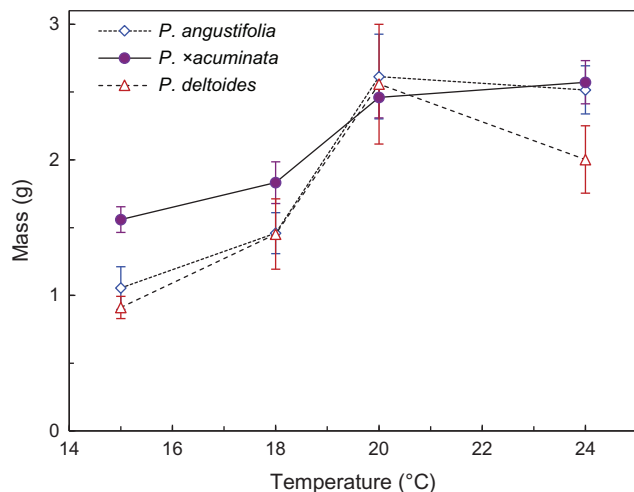


Figure 4. Whole plant dry masses (mean  $\pm$  SE) of two *Populus* species and their hybrids grown at four temperatures.

After 10 weeks, the harvest results confirmed major growth differences across the taxa, with the mixed model ANOVA revealing significant effects for six of the nine measures (Table 1). The T treatment was also very influential, with significant effects on all nine growth measures. There were also some significant taxon  $\times$  T (or G  $\times$  E) interactions, revealing that the different cottonwood types responded differently to the T relative to some growth measures (Table 1).

The growth patterns across taxa and T treatments displayed two primary patterns. Height, stem mass and leaf numbers were strongly correlated (Table 2) and displayed progressive increases with increasing T, particularly for *P. angustifolia*, which was taller and with larger stems (Figure 3A and C) and more leaves with the warm T (~12 leaves for all taxa at 15 °C, and 19 leaves for *P. angustifolia* at 20 and 24 °C vs 16 for the other taxa). For these traits, the hybrid *P. x acuminata* exceeded the parents at the lowest T and was then intermediate between the two parents at higher T (Figure 3).

The second, slightly different pattern, was displayed for the other primary growth traits including root mass and total leaf area and leaf mass. These traits were strongly correlated (Table 2) with slightly lower associations for the more variable hybrids (e.g., leaf mass vs root mass:  $r^2 = 0.673, 0.598$  and  $0.835$  for A, AD and D, respectively). These traits displayed common patterns, with increase from 15 to 20 °C and then levelling off or decreasing at 24 °C (Figure 3B and D). Across the

Table 3. Bivariate Pearson product-moment correlations for foliar physiological characteristics across all temperature treatments for the three taxa (A = *P. angustifolia*, D = *P. deltoides* and AD = *P. x acuminata*,  $n = 32$  to 38) with abbreviations as in Table 1. \* $P < 0.05$  (bold) and \*\* $P < 0.01$  (bold italics), red or blue indicate positive or negative associations, respectively.

	Taxon	N/area	LMA	SD	$g_s$	$A_{sat}$	NUE	$WUE_i$	$\delta^{13}C$
CCI	A	-0.052	0.157	0.202	0.260	0.154	0.204	-0.192	-0.034
	AD	<b>0.544**</b>	<b>0.371*</b>	-0.151	-0.195	0.274	-0.334	<b>0.443**</b>	-0.206
	D	0.246	<b>0.391*</b>	0.203	<b>0.409*</b>	<b>0.694**</b>	0.302	-0.112	-0.073
N/area	A		<b>0.542**</b>	-0.305	<b>-0.440*</b>	0.341	<b>-0.687**</b>	<b>0.569**</b>	-0.109
	AD		<b>0.547**</b>	-0.134	-0.020	<b>0.632**</b>	<b>-0.480**</b>	<b>0.545**</b>	-0.210
	D		<b>0.689**</b>	-0.219	-0.050	<b>0.455**</b>	<b>-0.660**</b>	0.323	0.155
LMA	A			-0.301	<b>-0.364*</b>	0.223	-0.420*	<b>0.422*</b>	<b>-0.521**</b>
	AD			0.036	-0.035	<b>0.515**</b>	-0.320	<b>0.488**</b>	-0.188
	D			-0.037	-0.088	0.208	<b>-0.580**</b>	0.248	-0.273
SD	A				<b>0.749**</b>	-0.113	<b>0.386*</b>	<b>-0.547**</b>	<b>0.351*</b>
	AD				0.219	0.034	0.149	-0.112	<b>0.468**</b>
	D				<b>0.506**</b>	0.169	<b>0.381*</b>	<b>-0.398*</b>	0.004
$g_s$	A					0.002	<b>0.393*</b>	<b>-0.830**</b>	<b>0.381*</b>
	AD					<b>0.512**</b>	<b>0.583**</b>	<b>-0.634**</b>	<b>0.463**</b>
	D					<b>0.449**</b>	<b>0.455**</b>	<b>-0.852**</b>	0.182
$A_{sat}$	A						<b>0.397*</b>	<b>0.419*</b>	<b>0.402*</b>
	AD						<b>0.356*</b>	0.294	0.221
	D						0.341	0.004	0.256
NUE	A							-0.213	<b>0.504**</b>
	AD							-0.317	<b>0.452**</b>
	D							<b>-0.370*</b>	0.058
$WUE_i$	A								-0.156
	AD								-0.251
	D								-0.013

taxa, the hybrids had increased root mass and leaf areas at the lower T while the three taxa were similar at 20 °C. At the warmest T (24 °C), the hybrids had increased root mass and apparently larger leaf areas.

The sum of leaf, stem and root masses provided the total plant mass (Figure 4). This was tightly correlated with the leaf mass (Table 2), which provided the largest contribution to the harvested samples. The overall plant growth pattern (Figure 4) consequently largely matched the patterns for leaf mass, with increasing plant sizes from 15 to 20 °C and similar sizes at 24 °C for the hybrids and for *P. angustifolia* due to stem mass contributions (Figure 3C).

### Foliar physiology

The study revealed substantial differences in most of the foliar characteristics that included morphological traits, chemical composition, gas exchange rates and derived ratios (Table 1). The study outcomes for the foliar characteristics were fairly similar to that for the growth traits, with 7 of 12 foliar traits displaying significant effects across taxa, 9 of 12 demonstrating significant effects from the T treatments and 2 of the 12 traits indicating taxon × T treatment (G × E) interactions.

Foliar morphological traits varied across the conditions with significant differences in the leaf area ratio (leaf area/root mass) and leaf mass per area (LMA, Table 1), which would increase with increasing leaf thickness and/or density. Leaf mass per area was positively correlated with CCI for two taxa, and strongly correlated with foliar N in all taxa (Table 3). Stomatal density increased 35–41% with warming T, and varied consistently across taxa, being lowest in the hybrids and highest in *P. deltoides* (Figure 5B). Stomatal distribution between the leaf surfaces varied substantially across the taxa and was largely consistent across T, with *P. deltoides* having stomata equally distributed between surfaces (Figure 5A) while *P. angustifolia* and *P. × acuminata* were more hypostomatous, with the adaxial:abaxial ratio intermediate in the hybrids.

For foliar chemistry, CCI was quite consistent across T and varied across the taxa (Table 1), being higher in *P. angustifolia*, lower in *P. deltoides* and intermediate in the hybrids (Figure 6A). Foliar N content per unit leaf area was positively correlated with CCI (Table 3), varied across taxa and progressively declined with warming T (Table 1, Figure 6B). Since LMA varied across taxa and T, the N content per area differed somewhat from the N concentration within the leaves. Both representations demonstrated highly significant effects (Table 1) and we emphasize the patterns and correlations with N/area since gas exchange was assessed relative to leaf area.

From the gas exchange measurements, net assimilation ( $A_{\text{sat}}$ ) was highest in *P. angustifolia* and generally similar in *P. × acuminata* and *P. deltoides* (Figure 6C). However,  $A_{\text{sat}}$  was particularly reduced in *P. deltoides* at low T, providing an apparent taxon × T interaction (Table 1, Figure 6C). Across the study conditions,

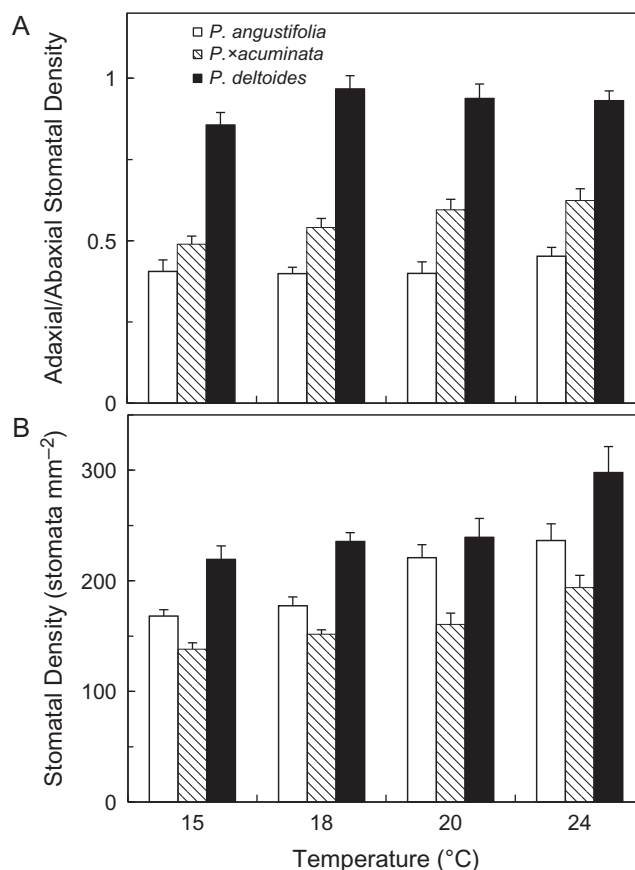


Figure 5. The (A) adaxial/abaxial stomatal density ratios (mean ± SE), and (B) total stomatal densities of two *Populus* species and their hybrids at four temperatures.

$A_{\text{sat}}$  was correlated with CCI only in *P. deltoides* and with foliar N in *P. deltoides* and the hybrids (Table 3, Figure 7). Stomatal conductance ( $g_s$ ) was correlated with  $A_{\text{sat}}$  in *P. deltoides* and the hybrids (Table 3).

Stomatal conductance was also correlated with stomatal density, with somewhat different relationships for the three taxa (Figure 8). As a ratio of  $A_{\text{sat}}$  and  $g_s$ , which is correlated with  $E$ , the instantaneous water-use efficiency ( $WUE_i$ ) was correlated with those measures, especially  $g_s$  (Table 3). The primary influence on  $WUE_i$  was T condition (Table 1), with  $g_s$  increasing and  $WUE_i$  decreasing with warming T (Figure 6D and E).

Foliar  $\delta^{13}\text{C}$  was not correlated with  $WUE_i$  (Table 3), very similar across the taxa and more negative at the coolest T (Figure 6F). Foliar  $\delta^{13}\text{C}$  was most strongly correlated with LMA, with shifts in the associations across the taxa (Table 3). It was also correlated with stomatal density and with the gas exchange rates for  $A_{\text{sat}}$  and  $g_s$  (Table 3).

The correlations between foliar physiological characteristics and growth were of particular interest (Table 4). The strongest associations were with N and these negative correlations may be due to the dilution of N within larger plants, suggesting N limitation throughout the study. Some growth measures were

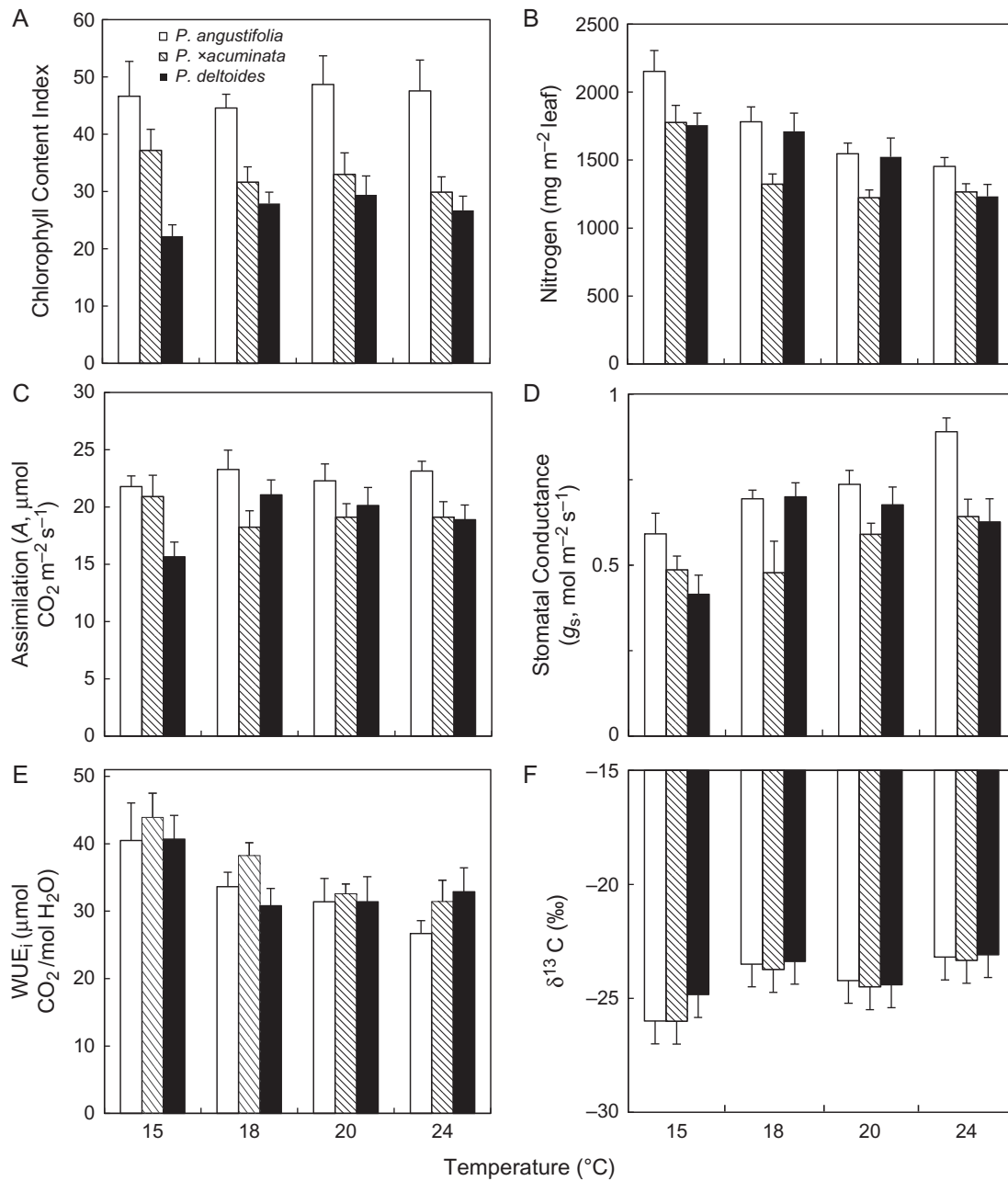


Figure 6. Foliar characteristics of two *Populus* species and their hybrids at four temperatures (mean  $\pm$  SE).

positively correlated with  $g_s$  and subsequently  $WUE_i$  was negatively correlated with some growth measures (Table 4). Chlorophyll content index was correlated with height (Table 4) and stem mass ( $r = 0.354$ ,  $P < 0.01$ ) and largely reflected *P. angustifolia* being taller and with darker green leaves (Figures 4 and 6A). Notably,  $A_{sat}$  was not significantly correlated with the growth measures across the experimental conditions (Table 4). Foliar  $\delta^{13}C$  was correlated with the shoot to root ratio (Table 4) and the leaf area ratio ( $r = 0.446$ ,  $P < 0.01$ ), measures that may relate to the balances of transpirational surface vs water uptake capacity.

### Inheritance and heterosis

The extent of heterosis or hybrid vigor was similarly assessed with the potency ratios (PR, Table 5) and midparent indices (Figure 9). The hybrids displayed heterosis for the morphological characters except leaf number, especially at 15 °C. Heterosis was also displayed at 18 °C but with reduced PR and midparent indices. Dominance was displayed at 20 °C and overdominance returned for leaf area and mass at 24 °C.

In contrast, the physiological characteristics for these hybrids displayed negative PR and midparent indices (Table 5, Figure 9). Across the T, the hybrids showed reduced physiological



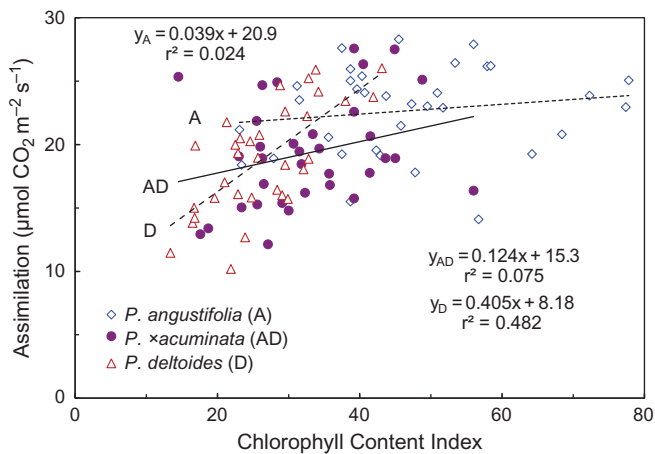


Figure 7. The relationships between assimilation vs chlorophyll content index for genotypes of two *Populus* species and their hybrid combination grown at four temperatures, with linear regressions plotted.

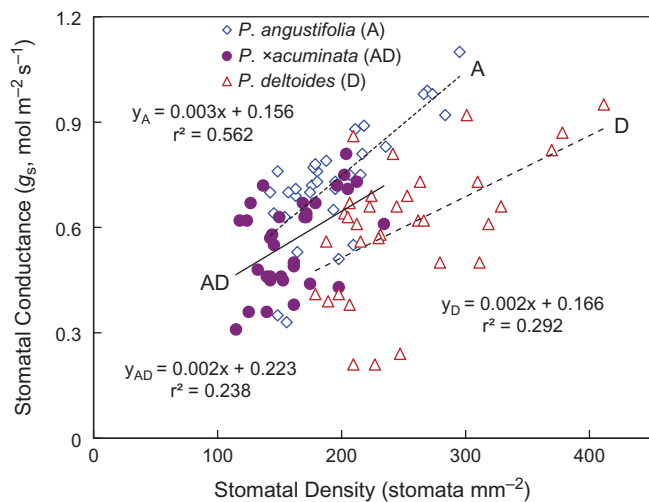


Figure 8. Stomatal conductance ( $g_s$ ) vs stomatal density for genotypes of two *Populus* species and their hybrid combination, grown at four temperatures, with linear regressions plotted.

performance relative to the midparent values, with the exception of similar or slightly increased conductance and assimilation rates at 15 °C (Figure 9). Thus, in this study, these hybrids displayed heterosis for growth at the cool T but not for the foliar ecophysiological characteristics that were assessed on a per unit leaf area basis.

## Discussion

In assessing the patterns across this study, it should be recognized that each T treatment was provided by a separate growth chamber, introducing vulnerability from pseudoreplication (Hurlbert 1984). With this study design, unanticipated variation in a particular growth chamber could confound the effects from the T treatment. Conversely, the plants appeared similarly healthy in the different growth chambers and the common characteristics for

some traits, and progressive growth patterns for other traits, suggests uniformity. There could have been an artifact if, with limited air exchange, the warmer treatments might have partially depleted  $\text{CO}_2$  due to the larger plants (Bernier et al. 1994). This could provide an indirect and artificial influence on foliar  $\delta^{13}\text{C}$ , complicating the interpretation of slight differences among treatments (Edwards et al. 2009).

The results may be considered relative to the two predicted  $G \times E$  interactions. We expected *P. deltoides* to display a different T response than *P. angustifolia*, with a shift to reflect adaptation to warmer T (Figure 1), reflecting its occurrence in the warmer prairie region (Gom and Rood 1999, Kalischuk et al. 2001, Floate 2004). Opposing this prediction, the two species displayed very similar T responses, and there was even a slight contrary trend, with the *P. angustifolia* clones apparently demonstrating greater growth at the warmest T, 24 °C (Figure 4). As a likely contributing influence, the source trees all occurred in the same woodland and might thus be adapted to the same T regime. Within poplar species, there is substantial localized clinal variation (Bassman and Zwier 1991, Dunlap et al. 1993, Rood et al. 2007, Keller et al. 2011, Soolanayakanahally et al. 2015) and thus rather than indicating that these two species display common T responses, this study may indicate that cottonwood genotypes of different species that occur in a common location could be similarly adapted to the associated T regime.

While the two parental species displayed similar growth responses, there were differences in foliar physiological characteristics. There were differences in stomatal distributions and densities and these were somewhat correlated with stomatal conductance, which influences  $\text{CO}_2$  availability and transpiration. Photosynthesis also requires the light harvesting complex that includes chlorophylls that are indicated with the CCI and the Calvin Cycle enzymes including RuBisCO, which could be correlated with foliar N. The observed correlations across the genotypes within the taxa suggest that these were limiting for *P. deltoides* and the hybrids but not in *P. angustifolia*. Thus, there are some common features but some important ecophysiological differences across these cottonwood species, consistent with D.W. Pearce, S.G. Woodman, M.G. Letts and S.B. Rood (submitted).

Our second prediction anticipated another  $G \times E$  interaction, relating particularly to the intersectional hybrids. We expected the hybrids to display an intermediate T response and further anticipated that some hybrids might thrive particularly around the optimal T and thus demonstrate hybrid vigor (Figure 1). This followed from the view that heterozygosity could provide a higher growth rate capacity, but that this would be particularly expressed with favorable conditions since unfavorable environmental conditions could similarly limit the growth of hybrids and their parents (Rood et al. 1985, Blum et al. 1990, Betrán et al. 2003).

The study results opposed that prediction, and contrarily, demonstrated superior growth of the hybrids particularly under the less favorable, suboptimal T. With the cooler T, the hybrids

Table 4. Bivariate Pearson product–moment correlations between growth characteristics and foliar physiological characteristics across temperature treatments by taxon. Taxon and character abbreviations as in Tables 1 and 3,  $n = 32–38$ ,  $*P < 0.05$  (bold) and  $**P < 0.01$  (bold italics), red or blue indicate positive or negative relationships, respectively.

	Taxon	CCI	N/area	$g_s$	$A_{sat}$	NUE	$WUE_i$	$\delta^{13}C$
Root mass	A	-0.037	<b>-0.424**</b>	0.006	-0.137	0.174	-0.079	-0.218
	AD	0.023	-0.299	0.149	-0.148	0.227	-0.270	0.076
	D	0.198	<b>-0.373*</b>	<b>0.346*</b>	0.025	<b>0.436*</b>	<b>-0.381*</b>	0.059
Stem mass	A	0.115	<b>-0.557**</b>	0.248	-0.093	0.353	-0.243	0.073
	AD	-0.102	<b>-0.474**</b>	0.210	-0.205	0.326	<b>-0.399*</b>	0.149
	D	0.177	<b>-0.399*</b>	0.291	0.107	<b>0.535**</b>	-0.287	0.150
Leaf mass	A	0.150	<b>-0.601**</b>	<b>0.349*</b>	-0.151	0.352	<b>-0.367*</b>	0.057
	AD	-0.117	<b>-0.390*</b>	0.237	-0.099	0.338	-0.322	0.232
	D	0.236	<b>-0.414*</b>	0.324	0.125	<b>0.543**</b>	-0.309	0.106
Total mass	A	0.143	<b>-0.598**</b>	0.298	-0.142	0.356*	-0.330	0.029
	AD	-0.119	<b>-0.403**</b>	0.246	-0.114	0.356*	<b>-0.348*</b>	0.213
	D	0.227	<b>-0.412*</b>	0.322	0.112	<b>0.532**</b>	-0.314	0.099
Leaf area	A	0.106	<b>-0.580**</b>	<b>0.404*</b>	-0.133	0.352	<b>-0.397*</b>	0.185
	AD	-0.266	<b>-0.450**</b>	0.296	-0.111	0.434*	<b>-0.400*</b>	<b>0.366*</b>
	D	0.224	-0.299	0.266	0.214	<b>0.484**</b>	-0.196	0.149
Leaf area ratio	A	0.055	0.084	<b>0.560**</b>	0.064	0.250	<b>-0.419*</b>	<b>0.594**</b>
	AD	-0.277	-0.104	0.033	0.012	0.094	-0.035	0.219
	D	-0.052	0.082	-0.108	<b>0.355*</b>	0.128	0.329	<b>0.394*</b>

Table 5. Potence ratios (PR), quantifying the inheritance pattern for growth and physiological characteristics across (day) temperatures. Recognizing variation, values between  $-0.5$  and  $1.5$  indicate dominance, with the hybrids similar to the larger (+ve, red) or smaller parent (-ve, blue,  $-1.5$  to  $-0.5$ ). Values  $>1.5$  or  $<-1.5$  indicate overdominance, with the hybrids being larger or smaller than either parent (bold).

Character	Temperature ( $^{\circ}C$ )			
	15	18	20	24
<b>Morphology</b>				
Root mass	<b>14.0</b>	<b>1.93</b>	-1	<b>53.5<sup>2</sup></b>
Stem mass	<b>1.85</b>	-	-0.77	-
Leaf mass	<b>17.8</b>	<b>4.30</b>	-	<b>17.5<sup>2</sup></b>
Total mass	<b>8.06</b>	<b>115<sup>2</sup></b>	-	1.22
Leaf number	-	-	-1.17	0.51
Leaf area	<b>3.29</b>	0.86	-0.60	<b>13.2</b>
Height	<b>3.39</b>	0.73	-	-
<b>Foliar physiology</b>				
CCI	-	-0.56	-0.63	-0.70
N/area	-0.90	<b>-12.24<sup>2</sup></b>	-	-
Stomatal density	<b>-2.2</b>	<b>-1.9</b>	<b>-7.5</b>	-1.2
$g_s$	-	-	-	-0.88
$A_{sat}$	0.72	-	-	-

<sup>1</sup>No significant difference across taxa or PR between  $-0.5$  and  $0.5$ .

<sup>2</sup>Similar parental values inflate the PR.

produced larger leaf areas than the parental species and we conclude that these larger photosynthetic surfaces, rather than photosynthetic rates, were especially associated with hybrid vigor, consistent with findings by Guet et al. (2015). While contrary to our prediction, the finding of increasing heterosis at low T is consistent with prior studies with maize and rice (McWilliam and Griffing 1965, Kaw and Khush 1985).

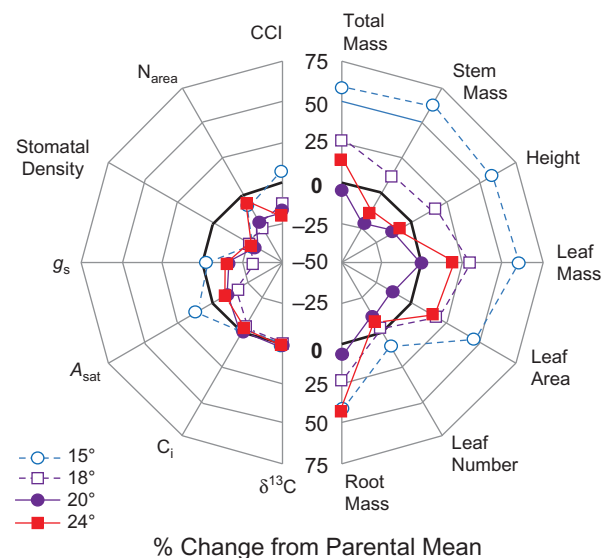


Figure 9. Midparent indices, or midparent heterosis values for morphological and physiological characters of *P. x acuminata* hybrids relative to its parental species, grown at four temperatures. The values indicate the % change of the hybrids relative to the midparent value, the mean of the two parents. The '0' position with the darker ring indicates that the hybrid mean equaled the parental mean, while values outside or inside indicate higher or lower mean hybrid values, respectively.

Prior observations of hybrid superiority in unfavorable environments led to the proposal that this provides a general foundation for heterosis (Pedersen 1968). This theory was supported with subsequent studies with maize and *Arabidopsis* and persists as one primary theory for hybrid vigor (Griffing and Zsiros 1971, Groszmann et al. 2015), although the collective literature lacks consensus (Blum 2013). As a physiological foundation, it was

proposed that the hybrid superiority across varying or stressful environments would reflect the enzymatic diversity that accompanies heterozygosity and this principle of enzymatic polymorphism has some conceptual and empirical support (Berger 1976, Brown 1979). However, while this may contribute, it is likely that multiple physiological processes combine to enable heterosis in hybrid poplars and other plants (Rood et al. 1988, Bradshaw et al. 2000, Birchler et al. 2003).

While the molecular basis is uncertain, superior hybrid performance under suboptimal environmental conditions has been repeatedly observed and has been described as phenotypic stability (Lewis 1954, Becker 1981, Fridman 2015). This probably applies to T and water status, but not nutrient availability, which apparently similarly limits growth in hybrids and parents (Pedersen 1968, Betrán et al. 2003), somewhat similar to our findings. Relative to the collective outcomes, while growth may be more vigorous in hybrids from genetically diverse parents, there can also be negative consequences of interspecific hybridization, which may be regarded as hybrid break-down, and in poplars this commonly represents increased vulnerability to pests and disease (Whitham 1989, Kalischuk et al. 1997). We thus conclude that there may be physiological benefits vs ecological costs of intersectional hybridization in poplars.

### Climate change

A final consideration relates to the prospective future woodland populations and the importance of natural interspecific *Populus* hybrids. These include intrasectional hybrids that display continuous variation and limited heterosis or hybrid break-down (Campbell et al. 1993), and the more distant intersectional hybrids that can provide fast-growing hybrid poplars (Bradshaw et al. 2000). If interspecific poplar hybrids commonly display broader ranges of environmental adaptation than parental species, they might be better able to withstand the progressive changes in T and water regime accompanying anthropogenic climate change (Stromberg et al. 2010, Perry et al. 2012, Rood et al. 2013). With phenotypic stability from heterozygosity, the native hybrids might thus be more tolerant of changing environments and this could broaden hybrid zones and increase introgression and gene flow across the poplar species (Keim et al. 1989, Whitham et al. 2008). This might benefit future adaptation and the migrations of poplar species and hybrids toward higher latitudes and altitudes in association with global warming (Landhäusser and Wein 1993, Grady et al. 2011, Kremer et al. 2012).

### Supplementary Data

Supplementary Data for this article are available at *Tree Physiology* Online.

### Acknowledgments

We extend thanks to Samuel Woodman, Evan Hillman and Soba Kaluthota for assistance with data collection, and to two anonymous reviewers for their thorough assessments.

### Conflict of interest

None declared.

### Funding

This study was supported through funding to S.B.R. from Alberta Innovates, Alberta Environment and Parks, and the Natural Sciences and Engineering Research Council of Canada.

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