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Leaf temperature impacts canopy water use efficiency independent of changes in leaf level water use efficiency

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ABSTRACT

Canopy water use efficiency (above-ground biomass over lifetime water loss, WUE_{canopy}) can influence yield in wheat and other crops. Breeding for WUE_{canopy} is difficult because it is influenced by many component traits. For example, intrinsic water use efficiency (WUE_i), the ratio of net carbon assimilation (Anet) over stomatal conductance, contributes to WUE_{canopy} and can be estimated from carbon isotope discrimination (Δ). However, Δ is not sensitive to differences in the water vapor pressure deficit between the air and leaf (VPD_{leaf}). Alternatively, measurements of instantaneous leaf water use efficiency (WUE_{leaf}) are defined as A_{net} over transpiration and can be determined with gas exchange, but the dynamic nature of field conditions are not represented. Specifically, fluctuations in canopy temperature lead to changes in VPD_{leaf} that impact transpiration but not A_{net}. This alters WUE_{leaf} and in turn affects WUE_{canopy} . To test this relationship, WUE_{canopy} was measured in conjunction with WUE_i, WUE_{canopy}, and canopy temperature under well-watered and water-limited conditions in two droughttolerant wheat cultivars that differ in canopy architecture. In this experiment, boundary layer conductance was low and significant changes in leaf temperature occurred between cultivars and treatments that correlated with WUE_{canopy} likely because of the effect of canopy temperature on VPD_{leaf} driving T. However, deviations between WUE_i, WUE_{leaf}, and WUE_{canopy} were present because measurements made at the leaf level do not account for variations in leaf temperature. This uncoupled the relationship of measured WUE_{leaf} and WUE_i from WUE_{canopy} and emphasizes the importance of canopy temperature on carbon uptake and transpired water loss.

1. Introduction

The rising global demand for food and decreasing amount of arable land necessitates increases in crop yield per hectare (Tilman et al., 2011; Alexandratos and Bruinsma, 2012). Furthermore, it is necessary to improve crop performance when water is limiting as increases in global temperature place greater evaporative demand on plants and prolonged drought becomes more common (Fisher et al., 2017). Unfortunately, the large historical increases in yield accomplished by breeding programs may be plateauing as selection for traits such as the amount of grain produced per mass plant (harvest index, HI) and efficiency in fertilizer utilization appear to have reached physiological maximums (Zhu et al., 2010). Thus, crop breeding programs must identify traits such as improved carbon capture per unit water lost from transpiration, which have not previously been under strong selective pressure but can potentially improve crop performance and yield. For wheat, which is often grown under Mediterranean conditions, this is a primary concern because yields under these conditions are strongly correlated with water availability (Zhang and Oweis., 1999).

Yield in wheat and in other crops has been defined by the product of the total amount of water transpired by a plant (T) relative to total amount of water lost by the crop and from soil evaporation (evapotranspiration, ET), canopy water use efficiency (WUE_{canopy}, amount of above-ground biomass accumulated divided by total transpired water loss), and HI (Condon et al., 2004) as:

$$\text{Yield} = \text{ET} \times \frac{T}{\text{ET}} \times \text{WUE}_{\text{canopy}} \times \text{HI}$$
(1)

In dryland agriculture T can be increased by selecting for more efficient extraction of soil water by roots or more rapid expansion of leaves to reduce the loss of water through ET. Alternatively, the HI appears to have approached its physiological maximum with limited

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Received 15 July 2020; Received in revised form 22 December 2020; Accepted 23 December 2020 Available online 5 January 2021 0176-1617/© 2021 Elsevier GmbH. All rights reserved. opportunity for future improvement (Long et al., 2015). This suggests that increasing WUE_{canopy} maybe the best option for potential improvements in biomass that would translate to increased yields provided HI is constant. However, selection for WUE_{canopy} in traditional breeding programs is difficult even though variation in WUE_{canopy} has been observed across species and within a limited number of wheat cultivars (Farquhar and Richards., 1984; Galavi and Moghaddam, 2012). Direct measurements of WUE_{canopy} cannot be done in the field and it is a complex trait made up of many components so large-scale selection of genotypes with greater WUE_{canopy} has been difficult (Rengel, 2013).

That said, WUE_{canopy} defined by Farquhar et al., 1989 can increase with the ratio of net carbon assimilation (A_{nel}) over *T* or by decreasing respired carbon (ϕ_c) and nighttime water losses (ϕ_w) as shown by:

$$WUE_{canopy} = \frac{A_{net}(1 - \phi_c)}{T(1 + \phi_w)}$$
(2)

Where A_{net}/T is defined as the instantaneous leaf water use efficiency (WUE_{leaf}) and is determined by the difference between atmospheric and intercellular CO₂ concentrations (C_a and C_i respectively), the ratio of H₂O to CO₂ diffusivity in air (1.6) and by the water vapor pressure deficit between the air and leaf, *VPD*_{leaf} (Farquhar et al., 1989):

WUEleaf =
$$\frac{C_a - C_i}{1.6VPD_{leaf}}$$
 (3)

The value of WUE_{leaf} is influenced by A_{net} over stomatal conductance (g_s), termed intrinsic water use efficiency (WUE_i), and is dependent on VPD_{leaf} (WUE_{leaf} = WUE_i/ VPD_{leaf} ; Seibt et al., 2008). Both WUE_i and WUE_{leaf} respond to changes in g_s and A_{net} but decreases in g_s are often associated with greater WUEleaf, and generally decrease both Anet and plant biomass (Blum, 2009; Cernusak, 2020). Alternatively, WUEleaf can change when WUEi is constant through variation in VPDleaf. For example, improvements in WUE_{leaf} have been achieved by breeding for earlier sowing dates where air humidity is greater and temperature is lower, thus taking advantage of decreased VPD_{leaf} (Richards, 2006). Additionally, greater WUE_{leaf} can occur in plants that close stomata at midday to minimize water loss and open in the morning and evening when the air relative humidity is greater and VPDleaf is lower (Lawson and Blatt, 2014). Furthermore, because VPDleaf is a result of the water vapor concentration in the leaves relative to atmospheric air, cooler canopies have lower water vapor concentrations within the leaf and experience lower VPD_{leaf}, increasing WUE_{leaf} (Gates, 1968). In environments where plant growth is limited by water availability and not nutrient status, improving WUE_{leaf} could translate to greater biomass and yield provided that all available water is consumed and HI is constant because more carbon is captured during the use of limited available water.

Relatively rapid assessment of WUE_{leaf} is possible by determining carbon isotope discrimination by measuring the ratio of ¹³C to ¹²C in leaf tissue relative to atmospheric CO₂, (Δ , Richards, 2006). This is based on the fact that leaf tissue shows a reduced ¹³C to ¹²C ratio relative to atmospheric CO₂ because of fractionation against ¹³C as CO₂ moves into the leaf (4.4‰), as well as during the initial fixation of CO₂ by the photosynthetic enzyme Rubisco (27‰). Discrimination increases in proportion to the concentration of CO₂ within the leaf, described by the ratio of intercellular to atmospheric CO₂ concentrations (*C_i/C_a*), where Δ can be defined by a simplified equation as (Farquhar et al., 1989):

$$\Delta = 4.4 + (4.4 - 27)C_i/C_a \tag{4}$$

The dependency of both Δ and WUE_{leaf} (Eq. 3) on C_i/C_a allows the use of Δ as a time integrated estimate of WUE_{leaf} (Farquhar and Richards., 1984; Vadez et al., 2014; Cernusak, 2020), and as a proxy for selecting plants with increased WUE_{leaf} (Farquhar et al., 1989; Richards, 2006).

Measurements of Δ can indicate a decreased CO₂ concentration in the leaf but cannot distinguish if this occurs from a lower g_s or greater

 A_{net} (Seibt et al., 2008). An additional limitation of Δ is that correlations with WUE_{leaf} are dependent on a constant VPD_{leaf} , which can influence T independent of a C_i/C_a (Eq. 3). This potentially limits the applicability of Δ as a screen for WUE_{leaf} when VPD_{leaf} changes seasonally or diurnally due to differences in relative humidity and leaf temperature. For example, canopy temperature can change due to fluctuations in radiation loads and latent heat loss from transpiration (Nobel, 2009). Conversely, stomata tend to close under water stress reducing T and as a result Anet decreases while canopy temperature increases (Munns et al., 2010). Thermal imaging has been able to detect differences in leaf temperatures resulting from genetic manipulations of stomatal conductance that alter WUE_{canopy} (Yang et al., 2016), and in conjunction with Δ , thermal imaging may be able to be used to estimate WUE_{leaf} (Grant et al., 2012). Therefore, it is hypothesized that combining Δ with thermal imaging can be used identify traits that influence WUE_i, WUEleaf, and WUEcanopy.

To test this hypothesis, we measured WUE_{canopy} in conjunction with WUE_{leaf}, WUE_i, Δ , and canopy temperature in order to identify traits driving WUE_{canopy} in two drought-tolerant wheat cultivars that differ in canopy architecture. The goal was to resolve factors contributing to WUE_{leaf} and WUE_{canopy} in these two wheat cultivars under both well-watered and water-limited conditions.

2. Materials and methods

2.1. Plant growth, daily water use, and biomass accumulation

Seeds of spring wheat (Triticum aestivum) cultivars Alpowa and Louise were obtained from the spring wheat breeding program at Washington State University (gift of K. Kidwell, Pullman, WA, USA) and were germinated in petri dishes in the dark at 4 °C. One week old seedlings were transplanted into a commercial potting mix (Sunshine LC-1, Sun Gro Horticulture Inc., Bellevue, WA, USA) in four-liter pots. Pots were covered with black plastic with only a small opening for the seedling to grow through to minimize surface evaporation. As controls, plastic was also placed over six pots containing only potting mix to determine non-transpired water loss in each treatment. A total of 24 plants, six for each treatment and cultivar, were grown in the School of Biological Sciences greenhouse at Washington State University, Pullman Washington during July and August of 2017. Peak daytime temperatures were between 30–32 $^{\circ}$ C and low nighttime temperatures were 22–23 °C. Relative humidity in the greenhouse ranged between 50 % at night and 20 % during the day, averaging 30 % during light hours. Photosynthetically active radiation (PAR) from natural light averaged 550 μ mol m⁻² s⁻¹ during light hours, peaking at 800 for ~3 h during midday. Supplemental light was provided in the morning and evening when sunlight was limited, resulting in 14 h of light each day. The vapor pressure deficit ranged from 1.6 to 3.7 during daylight hours, averaging between 2.1 and 2.4 each day. Each pot with dried potting mix was weighed before planting in order to determine gravimetric water content (g water g⁻¹ soil). Well-watered and water-limited treatments were irrigated to a gravimetric water content of 3.5 and 1.1 each day respectively, beginning the day seedlings were transplanted into pots. The water-limited treatment value had been previously determined to provide substantial, but non-lethal, water limitation. All measurements were made during vegetative growth to avoid confounding interactions with changes in physiology associated with booting as plants begin heading. Plant accumulated biomass was ignored in the daily determination of gravimetric water content because fresh plant mass accounted for $\sim 2\%$ of pot mass for both treatments by the end of the experiment.

Plants were grown in the greenhouse for 37 days, where all gas exchange measurements were performed on the youngest fully expanded leaves within 14 days prior to harvest. Nighttime transpiration was determined on five consecutive days prior to harvest by measuring total pot mass at dusk and at dawn. Pots were randomly moved around the greenhouse bench each day when pots were weighed and watered. On the five separate days, pot mass was determined every hour to evaluate diurnal patterns in water use. At harvest above-ground dry biomass was compared to total transpired water to calculate water use efficiency of the plant canopy (WUE_{canopy}; g dry above-ground biomass per kg of transpired water).

2.2. Leaf thermal imaging and gas exchange

Canopy temperature was determined using an uncooled infrared camera (A655sc, FLIR Systems Inc., Wilsonville, Oregon, USA) connected to a laptop computer to control image capture and collection. The camera was placed one meter above plants and emissivity was set at 0.95 for all measurements. While the error range of the camera was $\pm 2\%$ of the reading, giving individual measurements an average error of $\sim \pm 0.5$ °C, observed variation within treatments and genotypes was typically less than this value. Diurnal thermal images were collected every hour from 0600 h to 2100 h during the same five consecutive days as pot mass was determined hourly. These measurements were performed 30-34 days after plants were transplanted into pots. Canopy temperature was estimated using thresholding, where all pixels above a manually specified value were removed from the image. This was effective because the black plastic on the surface of the pot and black cloth that the pot was sitting on were warmer than the leaves of the canopy. Following removal of background pixels through thresholding, remaining pixels representing leaves were averaged following visual examination of the image to ensure fair representation of all plant leaves.

All leaf gas exchange was measured using a LI-6400XT Portable Photosynthesis System (LI-COR Biosciences, Lincoln, NE, USA) with a 2 \times 3 cm LED light source chamber head (6400–02B) on a single fully expanded leaf. A_{net} - C_i curves (A_{net} in response to increasing C_i) were measured on five plants of each cultivar and treatment group. Curves began at 40 Pa CO₂, decreased to 10 Pa CO₂, and then increased stepwise until reaching 200 Pa CO₂. Photon flux density (PFD) was keep at 2000 μ mol m⁻² s⁻¹ during A_{net} - C_i curves and VPD_{leaf} was maintained between 1.25 and 1.75 kPa. Stomatal responses to light were made by allowing the leaf to stabilize at 1000 PFD for 20 min, then turning the light source off for 20 min, and then turning the light back on at 1000 PFD for a final 20 min. Dark respiration rates were taken from the A_{net} measurements at the end of the 20 min dark period. Stomatal responses to VPDleaf were also made on five plants in each group in the same manner at 1000 PFD, where VPDleaf was maintained at 1.5, 2.0, and 0.8 kPa for 20, 35, and 45 min respectively. Instantaneous "spot" measurements were made by setting environmental conditions to match those in the greenhouse and then clamping a leaf in the chamber. Stomatal conductance and photosynthetic rate were then collected every 3 s and averaged over the following 30 s. These measurements were made on six plants per cultivar and treatment five times during the day on the 30th, 32nd and 34th days after transplanting starting at 0700 and every 3 h until 1900 h. Calculations of WUE_i and WUE_{leaf} were calculated by dividing Anet</sub> by gs and T respectively for all spot measurements.

2.3. Carbon isotope composition

Leaf tissue of each plant (24 plants total) was collected during the final harvest, dried at 50 °C for one week, ground and homogenized, and then analyzed for carbon and nitrogen content using an elemental analyzer (ECS 4010, Costech Analytical, Valencia, CA). A continuous flow mass spectrometer (Delta PlusXP, Thermofinnigan, Bremen) was simultaneously used to measure the stable isotope ratios of the combusted CO₂ gas. Stable isotope ratios of carbon were calculated heavy over light, denoted with small delta: δ , following Farquhar and Sharkey (1982). Carbon isotope discrimination (Δ) was calculated from the isotope composition of the reference (atmospheric air, -11.0‰) and the sample (plant tissue) as described by Farquhar et al. (1989).

2.4. Canopy architecture and projected leaf area

Average leaf angle of each canopy was determined from visible images taken using an 8megapixel camera phone (iPhone 5c, Apple, Cupertino California, USA) of the profile of each plant. ImageJ software (Schneider et al., 2012) was used to manually fit a line to leaves on either side of the pot. Horizontal and vertical leaves had leaf angle values of 0° and 90° respectively. Leaves oriented directly toward or away from the camera were not included because the angle could not be determined. The average leaf angle was determined from 8 to 20 leaves for water-limited plants and between 18–34 leaves per each well-watered plant.

Projected leaf area for each canopy was determined from visible images captured using the same camera phone mounted one meter directly above each pot sitting on black cloth. Image J was used to perform manual thresholding so only pixels containing green leaves remained and then conversion to binary was done in order to automatically count pixels. Pixels were converted to square centimeters using the known area of the top of the pot. Overlap of leaves was not corrected for in order to better represent leaf area exposed to the radiation intensity driving transpiration.

2.5. Modeling Anet-Ci curves and leaf energy balance calculations

The maximum carboxylation rate (V_{cmax}) and maximum rate of electron transport (J_{max}) were estimated *in vivo* using the R package "plantecophys" (Duursma, 2015) from measurements of A_{net} - C_i curves. Following Sharkey et al. (2007), the temperature response of photosynthetic parameters determining assimilation rate including V_{cmax} , Ko, Kc, and gamma star, were modeled for temperatures between 25 and 30 °C using A_{net} - C_i data collected at 30 °C for all plants. These parameters we used to predict RuBP saturated assimilation rates following von Caemmerer (2000) for 25–30 °C.

Modeling leaf temperature, transpiration rate, and VPD_{leaf} across a range of stomatal conductance values was done by setting environmental and leaf parameters in R v. 4.0.1 (R Core Team, 2020) using the R package "tealeaves" (Muir, 2019). All parameters remained at default values with the exception of atmospheric pressure (93 kPa), wind speed (0.01 m/s), relative humidity (0.3), air temperature (305 K), leaf characteristic dimension (0.01 m), and incident short-wave radiation flux (500 W m⁻²).

Assimilation rate was modeled following Farquhar and Sharkey (1982) by fitting a linear regression to the initial slope of A_{net} - C_i curves, where that regression was used to determine total leaf conductance (g_t: boundary, stomatal, and mesophyll conductance) for a range of A_{net} and C_i values by rearranging the equation A_{net} - $g_t(C_a - C_c)$ where C_c is the concentration of CO₂ in the chloroplast. Assimilation values for any given leaf conductance were then determined from the logarithmic relationship between A_{net} and g_t . Changes in WUE_{leaf} across a range of leaf conductance were calculated as this assimilation rate over transpiration rate determined from tealeaves as described above.

2.6. Statistical analysis

Statistical analysis was done using R statistical software, where normality was first tested for all variables measured and two-way *ANOVA* were performed to identify significant variation, cultivar or treatment effects, and interactions. *Fishers* least significant differences (*LSD*) test was used to identify significant variation between treatments and cultivars when an interaction was significant (P < 0.05).

3. Results

3.1. Whole-plant carbon gain and water loss

Differential water treatments (Fig. 1A) kept the well-watered



Fig. 1. Daily water use (A) and gravimetric soil water content (B, grams of water per gram of soil) were measured each day that plants were grown in the greenhouse. Soil water content was maintained in each treatment during the lifetime of the plants as daily water use increased. Six biological replicates were measured for each cultivar and treatment combination. Open symbols represent water-limited plants while closed symbols represent the well-watered treatment. Triangles represent the cultivar Louise and circles denote Alpowa.

treatment between 2.69 and 3.58 g of water g^{-1} of soil, and the waterlimited treatment between 1.13 and 1.39 g of water g^{-1} of soil until plants were harvested (Fig. 1B). Above-ground biomass accumulation and water use in the water-limited treatment were significantly reduced to 22.7 % ± 6.2 and 32.9 % ± 10.8 of the values for the well-watered treatment, respectively (Fig. 2 A and B, P < 0.01). Despite this treatment effect, for both cultivars the well-watered plants had greater WUE_{canopy} (above-ground biomass at harvest over total transpired water losses) than water-limited plants, and Louise had greater WUE_{canopy} than Alpowa in both treatments (Fig. 2C).

Nighttime transpiration accounted for 5% of total daily water loss for both cultivars under both water treatments and was significantly greater in well-watered plants compared to watered-limited plants but there was no difference between cultivars under either water treatment (Table 1). Leaf level dark respiration rates were not significantly different between cultivars or treatment, ranging from 2.5 to 3.8 μ mol CO₂ m² s⁻¹ (Table 1).

3.2. Leaf water use efficiency determined using gas exchange

Midday spot measurements of assimilation rate (A_{net}) were not significantly different between cultivars or treatments, but stomatal conductance (g_s) was reduced by 50 % in water-limited plants compared to well-watered plants (Fig. 3 A and B) while no difference in g_s was observed between cultivars. Leaf water use efficiency (WUE_{leaf}, A_{net}/T) did not show significant differences between cultivars under either treatment but was two-fold higher in water-limited plants relative to well-watered plants (Fig. 3C). Intrinsic water use efficiency (WUE_i; A_{net} over g_s) determined from the same gas exchange measurements showed



Fig. 2. Total above ground dry biomass collected at harvest (A) was divided by lifetime water use (B) to calculate whole plant water use efficiency (WUE_{plant}, C). A two-way ANOVA was performed for each variable. Dry mass and water use showed significant treatment effects while WUE_{plant} showed both cultivar and treatment effects, where well-watered plants had greater WUE_{plant} than water-limited plants regardless of cultivar and Louise had greater WUE_{plant} than Alpowa regardless of treatment.

the same trends as $\mathsf{WUE}_{\mathsf{leaf}}$ with no differences in statistical significance between groups.

Transient responses of stomatal conductance to light and humidity were not significantly different between cultivars or treatment (Supplementary Figs. S1 and S2). Furthermore, gas exchange measurements made at five points during the day repeated on three days showed a strong diurnal pattern that was consistent amongst all cultivars and treatments, with greater WUE_{leaf} in water-limited relative to the wellwatered plants but no difference between cultivars (Supplementary

Table 1

Physiological measurements of spring wheat cultivars Louise and Alpowa under well-watered and water-limited treatments.

	Water-Limited		Well-Watered		Two Way ANOVA		
	Alpowa	Louise	Alpowa	Louise	С	W	C x W
Midday Canopy	$27.6~\pm$	$27.1~\pm$	$25.4 \pm$	$25.0~\pm$	*	*	
Temperature (°C)	0.3	0.2	0.1	0.1			
Canopy Leaf Area	167 \pm	$143 \pm$	419 \pm	408 \pm		*	
(cm ²)	28	21	30	39			
Canopy Height (cm)	11.1 \pm	$23.8~\pm$	12.8 \pm	$24.4~\pm$	*		
	2.5	1.6	0.9	1.6			
Projected Leaf Angle	35.4 \pm	45.7 \pm	37.5 \pm	48.0 \pm	*		
(°)	3.5	3.3	0.9	2.5			
Specific Leaf Area	$273~\pm$	$232~\pm$	299 ± 3	$265 \pm$	*	*	
$(cm^2 g^{-1})$	12	11		8			
Nighttime	20.4 \pm	$22.5~\pm$	30.1 \pm	$24.8~\pm$		*	
Transpiration (mg cm ⁻²)	1.1	1.2	2.4	1.7			
Dark Respiration	$2.5 \pm$	$3.29 \pm$	$3.8 \pm$	$3.3 \pm$			
$(\mu mol CO_2 m^2 s^{-1})$	0.4	0.4	0.3	0.2			
Chlorophyll Content	$482 \pm$	517 \pm	$380 \pm$	$480 \pm$	*	*	
$(mg m^{-2})$	25	18	29	39			
Δ ‰	$20.0~\pm$	18.7 \pm	$20.8~\pm$	$20.0~\pm$	*	*	
	0.1	0.1	0.1	0.2			
In vivo V _{cmax} 25 °C	118 ± 7	106 \pm	103 ± 8	94 ± 4		*	
$(\mu mol CO_2 m^2 s^{-1})$		5					
In vivo J _{max} 25 °C	$264 \pm$	$230~\pm$	$217~\pm$	$210~\pm$		*	
$(\mu mol CO_2 m^2 s^{-1})$	24	13	20	6			
Maximum	56.3 \pm	52.8 \pm	47.1 \pm	$49.2~\pm$		*	
Photosynthetic	3.7	.0	3.7	1.4			
Rate (μ mol CO ₂ m ² s ⁻¹)							

A two-away ANOVA was performed using treatment (W) and cultivar (C) to test significance, where * represents P < 0.05.

Fig. S3).

3.3. Canopy characteristics

Canopy temperatures were similar in the morning and evening but there were significant differences between cultivars and treatments near midday from 1200 to 1500 h on the three days with minimal cloud cover (Supplementary Fig. S4, Table 1). Well-watered plants of both cultivars had significantly reduced midday canopy temperatures, 2.0 $^\circ \mathrm{C} \pm 0.3$ lower, than water-limited plants (Table 1). Additionally, midday canopy temperature was significantly different between the cultivars (Table 1) with a 0.4 $^\circ\text{C}\pm$ 0.1 lower temperature in Louise than Alpowa under wellwatered conditions (P < 0.05), and 0.5 $^\circ C$ \pm 0.2 lower in Louise than Alpowa under water-limited conditions (P < 0.05, Table 1). Canopy height and leaf angle were visibly and significantly greater in Louise compared to Alpowa, where Louise was 24.1 \pm 1.6 cm tall with an average leaf angle of 36.5 \pm 2.2 $^{\circ}$ and Alpowa was 11.9 \pm 1.7 cm and 46.9 \pm 2.9 °C respectively (Fig. 7, Table 1). There was no treatment effect for either of these traits (Table 1). Lifetime loss was closely associated with projected leaf area of the canopy ($R^2 = 0.94$, Supplementary Fig. S6)

3.4. Variation in photosynthetic parameters and carbon isotope discrimination

In vivo carboxylation capacity (V_{cmax}) and electron transport (J_{max}) modeled from A_{net} - C_i curves (Supplementary Fig. S5) showed no differences between cultivars, but water-limited plants had a significantly greater V_{cmax} at 25 °C of 112 ± 6 compared to 98 ± 5.5 µmol CO₂ m² s⁻¹ in the well-watered plants. Water-limited plants had a significantly greater J_{max} of 247 ± 19 relative to well-watered plants at 214 ± 13 µmol CO₂ m² s⁻¹ (Table 1). Carbon isotope discrimination (Δ) estimated



Fig. 3. Gas exchange was measured at midday on three days where photosynthetic rate (A) was divided by stomatal conductance (B) to calculate intrinsic water use efficiency (C). A two-way ANOVA showed all three variables had significant treatment effects with no cultivar effects. An interaction was present for photosynthetic rate only where a Fishers LSD test showed Alpowa under water-limited conditions was significantly greater than all other treatments and Louise.

from dried leaf and atmospheric CO₂ isotope compositions indicated that the water-limited plants had a 1.1‰ ± 0.1 lower Δ relative to well-watered plants. Additionally, Louise had 1.0‰ ± 0.2 lower Δ than Alpowa across both treatments (Table 1). Examining each cultivar separately there were positive correlations between Δ and g_s (R² = 0.51 and 0.49, P = 0.05) while WUE_{leaf} was negatively correlated with Δ (R² = 0.50 and 0.63, P = 0.05 and <0.05 respectively) (Fig. 4).

3.5. Energy balance and assimilation modeling

Leaf temperatures estimated using tealeaves energy balance



Fig. 4. Relationship between Δ and average midday stomatal conductance of three days in response to treatment for Louise and Alpowa (A and C respectively). Panels B and D show average midday leaf water use efficiency (WUE_{leaf}) of three days for Louise and Alpowa respectively. Closed symbols represent the well-watered treatment, open symbols represent the water-limited treatment, and circles and triangles represent Alpowa and Louise respectively.

modeling showed that an increase in g_s from 0.23 to 0.5 µmol m⁻² s⁻¹ resulted in a 1.8 °C reduction in leaf temperature. This same change in g_s increased predicted WUE_{leaf} as a result of a 19.5 % increase in photosynthetic rate while transpiration rates only increased by 14.1 % due to the accompanied reduction in leaf temperature that decreased VPD_{leaf} . Using Lamberts Cosine Law (Campbell and Norman, 2012) to manipulate incident radiation received as a result of leaf angle resulted in a 0.45 °C increase in leaf temperature as a result of increasing leaf angle from 37° to 47° at a g_s of 0.5 µmol m⁻² s⁻¹. Increasing boundary layer conductance by manipulating wind speed to be larger than 0.15 m s⁻¹ resulted in substantial decreases in WUE_{leaf} with any increase in g_s .

The temperature response of photosynthetic parameters calculated following Sharkey et al. (2007) included V_{cmax} , the Michaelis-Menten kinetic constants for carboxylation and oxygenation by Rubisco (K_c and K_o respectively), the CO₂ compensation point in the absence of respiration (Γ^*) and were modeled for temperatures between 25 and 30 °C using A_{net} - C_i curves collected for all plants at 30 °C. While V_{cmax} decreased with temperature from 30 to 25, corresponding to reductions in K_o and Γ^* , this resulted in non-significant changes in A_{net} modeled across 25–30 °C for C_i values below 400 µmol mol⁻¹. Therefore, temperature responses of photosynthesis were not included when modeling predicted changes in WUE_{leaf} across stomatal conductance values that affect leaf temperature.

4. Discussion

4.1. WUE_{canopy}

Two spring wheat cultivars that are resistant to low soil water conditions (Li et al., 2011) but differ in phenology and growth habitat were compared to evaluate the relationships between canopy and leaf water use efficiency (WUE_{canopy} and WUE_{leaf}, respectively). In the current study WUE_{canopy} increased under well-watered conditions where plants accumulated more biomass relative to the amount of water used as compared to the water-limited conditions (Fig. 2). These changes in WUE_{canopy} may result from differences in WUE_{leaf}, however, there are other component traits such as nighttime transpiration or carbon lost through respiration that can also influence WUE_{canopy} (Eq. 2, Farquhar et al., 1989). In the current study nighttime transpiration accounted for only 5% of total water loss in both treatments and didn't differ between cultivars (Table 1). Additionally, leaf level dark respiration, one component of total carbon loss from the plant, was not significantly different between treatments or cultivars (Table 1). Thus, because nighttime water losses and dark respiratory carbon losses do not appear different between cultivars or treatments, the observed variation in WUE_{canopy} likely resulted from some other component trait. To further evaluate the factors influencing WUE_{canopy}, leaf gas exchange and carbon isotope discrimination (Δ) were used to directly and indirectly estimate WUE_{leaf} and WUE_i.

4.2. Leaf water use efficiency

Gas exchange measurements of WUE_{leaf} and WUE_i are often correlated with WUEcanopy; however, in this study water-limited plants had greater WUE_{leaf} and WUE_i (Fig. 3) but reduced WUE_{canopy} compared to well-watered plants. This lack of coordination implies that steady-state gas exchange measurements may not provide an integrated estimate of WUE_{leaf} that is representative over the lifetime of the plant. This can occur due to diurnal changes in physiological status as well as differences in conditions between the growth environment and the measurement chamber. During gas exchange there may also be differences in the rate of stomatal response to stimuli. While differences in stomatal responses were not observed here (Supplementary Figs. S1 and S2), the speed at which stomata open and close in response to environmental fluctuations can have significant effects on WUE_{leaf} and WUE_i (Faralli et al., 2019). Therefore, to potentially minimize these gas exchange limitations, Δ has been used as a time-integrated estimate of WUE_{leaf} and WUE_i (Condon et al., 1990; Ehdaie et al., 1991).

Following previous observations in wheat (Farquhar and Richards., 1984; Araus et al., 1997; Royo et al., 2002), Δ measured here was reduced under water-limitation relative to well-watered plants (Fig. 4). Reduced Δ indicates a lower C_i/C_a , suggesting that water-limitation decreased gs or increased Anet and would lead to an increase in WUEi. These data are supported by the gas exchange measurements that also showed a greater WUE_i and reduced g_s in water-limited plants relative to the well-watered treatment (Fig. 3). In addition to reduced g_s, greater Anet can result from greater photosynthetic investment per leaf area and may have contributed to the increased WUE_i of water-limited plants. In the water-limited plants there was an increase in chlorophyll content and reduced specific leaf area (Table 1). Additionally, increased A_{net} is supported by increased in vitro Rubisco activities as well as Anet-Ci curves that had a greater A_{net} for a given C_i in water-limited plants relative to the well-watered treatment (S5, Table 1). This reduction in leaf area with greater photosynthetic rate per leaf area in response to drought can increase carbon capture relative to water loss (Liu and Stützel, 2004). Both gas exchange and Δ data presented here show that WUE_i was greater in the water-limited plants relative to well-watered plants. However, WUE_{canopy} had the opposite response suggesting other factors may influence WUE_{canopy} besides WUE_i. For example, the estimates of WUE_i do not take into account potential differences in transpiration (*T*) that in addition to g_s, can be influenced by VPD_{leaf}. Furthermore, gas exchange measurements of WUE_{leaf} eliminate the impact of canopy structure on leaf temperature and boundary layer conductance.

4.3. Effects of boundary layer conductance in balancing carbon gain and water loss

The conductances of boundary layer, stomtata, and mesophyll influence the movement of CO_2 into the leaf. The primary mechanism plants use to regulate both the flux of CO_2 into the leaf and water vapor out of the leaf is through stomatal conductance (g_s). However, in addition to g_s , the concentration gradients of these gasses also drive the net flux rates (Nobel, 2009). While greater g_s can increase net carbon assimilation rates, the rate of water loss from the leaf also increases, ususally more rapidly because the rate of assimilation can become saturated and diminish the concentration gradient driving CO_2 into the

leaf. This is especially relevant under low light or temperature when maximum assimilation rates are relatively low (Yu et al., 2004; Avola et al., 2008). Alternatively, the rate of water loss for a given g_s depends on the evaporative demand between the leaf and atmosphere (VPD_{leaf}). Under conditions where the air surrounding the plant is well mixed, there is a linear relationship between g_s and transpiration (Nobel, 2009). Therefore, under these conditions a significant amount of water loss can occur when photosynthetic rates are CO₂ saturated and do not increase with g_s (*i.e.* A_{net}/g_s decreases). When boundary layer conductance is low, the leaf and the airspace surrounding it can diverge from ambient conditions due to reduced convection that normally maintains leaf temperature close to air temperature. When boundary layer conductance is low, as observed in the current experiment due to stagnant air, an increase in g_s can substantially reduce leaf temperature, especially when air temperature is high (Gates, 1968). This reduction in leaf temperature can decrease VPDleaf by reducing water vapor pressure within the leaf, thereby reducing the concentration gradient driving water out of the leaf. Additionally, when boundary layer conductance is low the water lost through stomata remains near the surface of the leaf and increases the humidity within this airspace further decreasing VPD_{leaf}. Under these conditions transpiration rates can become uncoupled from g_s (Fig. 7B, Meinzer et al., 1997) and a greater g_s can increase rates of A_{net} indepent of T (Fig. 7C), increasing WUE_{leaf} (Cowan and Farquhar, 1977; Meinzer et al., 1997; Buckley et al., 1999).

4.4. Limitations of gas exchange and carbon isotope discrimination measurements

As discussed above, conditions where boundary layer conductance is low can alter the dynamics of carbon gain and water loss as a result of the effects on leaf temperature and *VPD*_{leaf}. It is important to note that these dynamics are likely not detected during gas exchange measurements because environmental conditions within the leaf chamber are controlled to minimize the boundary layer and regulate leaf temperature. Additionally, carbon isotope discrimination (Δ) has been used as proxy of WUE_{leaf} because of their shared dependence on C_i/C_a (Eqs. 3 and 4). Theory predicts that measurements of Δ will negatively correlate with WUE_i (Farquhar et al., 1989) and predict WUE_{canopy} (Farquhar and Richards., 1984) provided it is not offset by other components of WUE_{canopy}, including *VPD*_{leaf} (Eq. 2). Here Δ negatively correlated with WUE_{leaf} only within each cultivar, and these measurements did not reflect WUE_{canopy}.

Negative correlations between Δ and WUE_{leaf} have long been experimentally observed (Wright et al., 1988) following theory where Δ is reduced in response to decreasing C_i/C_a . Both Δ and photosynthetic rate depend on the CO₂ concentration in the leaf, a result of the conductance allowing CO₂ to diffuse into the leaf. And because this conductance also partly determines water flux out of the leaf, T, Δ can correlate with WUE_{leaf} at constant VPD_{leaf} . However, T and thereby WUE_{leaf}, is influenced by VPD_{leaf} (Eq. 3) but Δ is not. In summary, because Δ is only sensitive to C_i/C_a and not VPD_{leaf} .

4.5. Improvements in WUE_{canopy} between treatments and cultivars

Here the 35 % increase in WUE_{canopy} of the well-watered compared to water-limited treatment was likely a result of greater g_s reducing the temperature of leaves by 2 °C and decreasing the evaporative demand on those leaves (Fig. 5, Table 1). The concurrent increase in water use and WUE_{canopy} in well-watered plants relative to water-limited plants seen here is not typical (Fig. 6), but has been predicted and observed when photosynthetic capacity is high and the rate of water loss becomes uncoupled from g_s due to low boundary layer conductance (Cowan and Farquhar, 1977; Condon et al., 1987; Meinzer et al., 1997; Buckley et al., 2014; Schymanski and Or, 2016). To support observed experimental data, we parameterized energy balance and assimilation models with



Fig. 5. Relationship between midday canopy temperature averaged over three days and lifetime water use (A), and midday stomatal conductance averaged over three days (B). Closed symbols represent the well-watered treatment, open symbols represent the water-limited treatment, and circles and triangles represent Alpowa and Louise respectively.



Fig. 6. WUE_{plant} *versus* midday canopy temperature average over three days (A). Correlations between WUE_{plant} and canopy temperature were similar each day, while absolute canopy temperature varied. Panel B shows the relationship between WUE_{plant} and dry mass, both determined at harvest. Closed symbols represent the well-watered treatment, open symbols represent the water-limited treatment, and circles and triangles represent Alpowa and Louise respectively.

environmental and gas exchange data to quantify the effect of increased g_s on canopy temperature reductions that decreased VPD_{leaf} and increased WUE_{canopy} between treatments in this experiment.

Several thorough analyses have been performed examining the relationship between g_s and WUE_{leaf} in response to varying boundary layer conductance (Meinzer et al., 1997; Buckley et al., 1999, 2014; Schymanski and Or, 2016). These studies have shown that

humidification of the air surrounding the leaf and cooler leaf temperatures can increase WUE_{leaf} with greater gs. Energy balance models combine environmental parameters such as wind speed air temperature, and relative humidity with plant characteristics including g_s, and leaf width. Using the R package "tealeaves" (Muir, 2019), expected changes in leaf temperature for a given g_s for the experimental conditions were determined. We also quantified the assimilation rates for the same set of gs values following Farquhar and Sharkey (1982) from measured Anet-Ci curve data in order to calcuate WUE_{leaf}. The energy balance model showed that the 0.27 μ mol m⁻² s⁻¹ change in average g_s from 0.23 to 0.5 μ mol m⁻² s⁻¹ between water-limited and well-watered treatments, respectively, would change leaf temperature by 1.8 °C, similar to observations made with thermal imaging. The increase in g_s in the well-water plants would reduce leaf temperatures as a result of latent heat loss but would also humidify the air surronding the leaf, both reducing VPDleaf. This uncoupling of gs and transpiration at high gs has

been previously shown and predicted when boundary layer conductance is low as was the case in this experiment due to stagnant air (Meinzer et al., 1997; Buckely et al., 1999). The energy balance model shows that the ratio of net carbon assimilation to transpiration (WUE_{leaf}) increases with g_s greater than 0.3 µmol m^{2 -1} (Fig. 7C) under conditions of low boundary layer conductance where transpiration plateaus as g_s increases (Fig. 7B). This modeled increase in leaf water use efficiency with g_s agrees with experimental data where water limited plants had a lower g_s and WUE_{canopy} relative to well-watered individuals with a much larger g_s and WUE_{canopy}.

It is worth noting however, that modeled increases in WUE_{leaf} predicted with rising g_s depend on a low boundary layer conductance. Under modeled conditions with higher boundary layer conductance, results show a reduction of WUE_{leaf} with greater g_s (not shown). This is likely a result of leaf temperatures remaining closer to air temperature, keeping *VPD_{leaf}* constant with changes in g_s resulting in a linear relationship between leaf transpiration and g_s . Additionally, high air temperature and low humidity conditions in the greenhouse during this experiment were key environmental components allowing for large leaf temperature depression. As described above for boundary layer conductance, under low temperature conditions air and leaf temperatures are more closely coupled resulting in a linear response between g_s and transpiration. High humidity however will likely result in transpiration rates that are independent from g_s .

While variation in g_s explains the temperature difference between treatments, Louise had greater WUE_{canopy} and cooler leaves than Alpowa despite a lack of significant difference in g_s . The canopy temperature of Louse was 0.5 °C cooler than Alpowa and was not attributed to increased total water loss or water loss per leaf area, as these were not different between cultivars. Therefore, differences in leaf temperatures were attributed to variation in canopy structure, which can have a large impact on absorption of incident radiation. The upright and taller canopy structure of Louise (36.5° average leaf angle and 24.1 cm tall canopy compared to 46.9° average leaf angle, and 12 cm tall canopy of Alpowa, Table 1, Fig. 8) relative to the shorter and more horizontally positioned leaves of Alpowa would impact received radiation. Energy balance



Fig. 7. Canopy temperature predicted from tealeaves energy balance modeling across a range of stomatal conductance values (A). Transpiration rates also modeled from tealeaves show a platueing with greater stomatal conductance due to reduced VPD_{leaf} as canopy temperature decreases with greater stomatal conductance (B). The result of the combined response of both assimilation and transpiration shows that increases in stomatal conductance above 0.3 µmol m⁻² s⁻¹ raise the ratio of assimilation relative to transpiration (C).



Fig. 8. Images of Louise (left column) and Alpowa (right column) under waterlimited (top row) and well-watered (bottom row) conditions.

modeling of leaf angle suggests that leaf angle can have a significant impact on received incident radiation and leaf temperatures. For example, modeling predicts that a leaf angle of 47° relative to 37° at a g_s of 0.5 µmol m⁻² s⁻¹ would increase leaf temperatures by 0.45 °C. The response experimentally observed and modeled here using an energy balance has been described in previous research where vertically positioned leaves receive less direct radiation and are cooler as a result of a reduced thermal load, especially at midday (Lovelock and Clough, 1992; King, 1997; Flexas et al., 2010; Rebetzke et al., 2013). These reductions in leaf temperature are beneficial for balancing carbon uptake and water loss because reductions in leaf temperature reduce *VPD*_{leaf} and decrease transpiration rate for a given g_s .

5. Conclusions

These data illustrate the importance of canopy temperature in the balance between carbon gain and water loss. While different mechanisms were responsible for changes in canopy temperature between cultivars and treatments, reduced canopy temperatures consistently increased WUE_{leaf} and contributed to greater WUE_{canopy} . These experimental observations were supported by energy balance and net carbon assimilation models showing an improved ratio of carbon gain to water loss resulting from either greater gs or reduced leaf angle that decreased leaf temperature and VPDleaf. Additionally, this study demonstrated that under certain conditions greater gs increased net carbon assimilation with smaller effects on transpiration rate. These observations should be tested in the field in order to evaluate the contribution of canopy temperature to WUEplant when variables such as radiation, VPDleaf, and wind speed vary. However, the association between canopy temperature and plant performance has previously been observed, where cooler canopies had reduced water loss and were associated with increases in WUE_{canopy} (Richards et al., 1986), grain yield (Roohi et al., 2015; Thapa et al., 2018; Zhang et al., 2018), and biomass (Gautam et al., 2015; Guo et al., 2016). Data presented here is in agreement with this previous work and strongly suggest that under conditions where canopy temperatures significantly differ, cooler canopies increase WUE_{plant} and above-ground biomass production.

Author contribution statement

Thomas performed experiments, data collection, and analysis. Interpretation of data and writing of this manuscript was conducted and agreed upon by both authors.

CRediT authorship contribution statement

Thomas M. Sexton: Investigation, Formal analysis, Writing - original draft. Camille M. Steber: Conceptualization, Writing - review & editing, Funding acquisition. Asaph B. Cousins: Conceptualization, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

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