Drivers of Natural Variation in Water-Use Efficiency Under Fluctuating Light Are Promising Targets for Improvement in Sorghum

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Improving leaf intrinsic water-use efficiency (iWUE), the ratio of photosynthetic CO\textsubscript{2} assimilation to stomatal conductance, could decrease crop freshwater consumption. iWUE has primarily been studied under steady-state light, but light in crop stands rapidly fluctuates. Leaf responses to these fluctuations substantially affect overall plant performance. Notably, photosynthesis responds faster than stomata to decreases in light intensity; this desynchronization results in substantial loss of iWUE. Traits that could improve iWUE under fluctuating light, such as faster stomatal movement to better synchronize stomata with photosynthesis, show significant natural diversity in C\textsubscript{3} species. However, C\textsubscript{4} crops have been less closely investigated. Additionally, while modification of photosynthetic or stomatal traits independent of one another will theoretically have a proportionate effect on iWUE, in reality these traits are interdependent. It is unclear how interactions between photosynthesis and stomata affect natural diversity in iWUE, and whether some traits are more tractable drivers to improve iWUE. Here, measurements of photosynthesis, stomatal conductance and iWUE under steady-state and fluctuating light, along with stomatal patterning, were obtained in 18 field-grown accessions of the C\textsubscript{4} crop sorghum. These traits showed significant natural diversity but were highly correlated, with important implications for improvement of iWUE. Some features, such as gradual responses of photosynthesis to decreases in light, appeared promising for improvement of iWUE. Other traits showed tradeoffs that negated benefits to iWUE, e.g., accessions with faster stomatal responses to decreases in light, expected to benefit iWUE, also displayed more abrupt losses in photosynthesis, resulting in overall lower iWUE. Genetic engineering might be needed to break these natural tradeoffs and achieve optimal trait combinations, e.g., leaves with fewer, smaller stomata, more sensitive to changes in photosynthesis. Traits describing iWUE at steady-state, and the change in iWUE following decreases in light, were important contributors to overall iWUE under fluctuating light.

Keywords: water-use efficiency, stomata, photosynthesis, non-steady-state gas-exchange, fluctuating light, dynamic light, sustainability, sorghum
INTRODUCTION

Water is the primary abiotic factor limiting crop productivity (Boyer, 1982), with agriculture consuming up to 85% of freshwater withdrawals (WWAP, 2015; D’Odorico et al., 2020). Breeding has almost tripled productivity of major crops over the last 60 years, without parallel improvement in the amount of water required to produce a ton of crop biomass (Sinclair et al., 1984; Ort and Long, 2014). With changing patterns of precipitation and increased drought frequency (Dai, 2013; Spinoni et al., 2018; Sun et al., 2018), rising atmospheric vapor pressure deficit due to global warming (Ort and Long, 2014), and decreasing groundwater supply around the world (Dalin et al., 2017), supplying sufficient water to crops is increasingly difficult and unsustainable (Lobell et al., 2014; WWAP, 2015). Therefore, improving crop water-use efficiency is important to achieve the crop productivity required to meet global demand (Bonsch et al., 2016; Flexas, 2016; FAO et al., 2018; Leakey et al., 2019).

The dependence of crop productivity on water supply derives from the interaction between leaf photosynthetic CO₂ assimilation (A) and transpiration. Stomatal pores on the leaf surface allow CO₂ diffusion into the leaf for A, but also allow water vapor to escape from the leaf. The inverse of the resistance to water vapor loss collectively imposed by the stomatal pores is measured as the stomatal conductance to water vapor (gₛ). The ratio of A to gₛ gives leaf intrinsic water-use efficiency (iWUE) (Leakey et al., 2019). Most inter- and intra-specific surveys of A, gₛ, and iWUE, and analyses of their limitations have concerned steady-state conditions (Galmes et al., 2007; Giuliani et al., 2013; Driever et al., 2014; Jahan et al., 2014; Sollenberger et al., 2014; Viswanathan et al., 2014; Tomeo and Rosenthal, 2017; Yabiku and Ueno, 2017; Leakey et al., 2019).

Although steady-state measurements are important to understand plant physiological performance (von Caemmerer and Farquhar, 1981; von Caemmerer, 2000), leaves in field-grown crop canopies are rarely in steady-state light conditions, and experience frequent fluctuations in incident photosynthetic photon flux density (PPFD) that cause substantial deviations from steady-state carbon and water fluxes (Peary, 1990; Zhu et al., 2004; Kaiser et al., 2015, 2018; McAusland et al., 2016; Vialet-Chabrand et al., 2017; Slattery et al., 2018; Acevedo-Siaca et al., 2020; De Souza et al., 2020; Wang et al., 2020). Evidence of increased water deficit stress (Sun et al., 2017), temperature stress (Leakey et al., 2003), and sensitivity of A to elevated CO₂ (Leakey et al., 2002; Tomimatsu and Tang, 2016) under fluctuating PPFD, and recent breakthroughs in improving A under fluctuating PPFD by manipulating photoprotection (Kromdijk et al., 2016; Hubbart et al., 2018), chlorophyll content (Gu et al., 2017), and stomatal light-sensing (Papanatsiou et al., 2019), have demonstrated the value of considering leaf behavior under non-steady-state lighting conditions.

A and gₛ typically require several minutes to rise to a new steady-state following an increase in PPFD, as in a sun fleck within a crop canopy. The rate of increase in gₛ may be mechanically limited by stomatal opening kinetics and stomatal patterning. The rate of increase in A may be limited by the kinetics of biochemical processes, in particular Rubisco activation (Soleh et al., 2016, 2017; Acevedo-Siaca et al., 2020), or the kinetics of stomatal opening and the associated mitigation of intercellular CO₂ (cᵢ)-limitation to A (Lawson and Blatt, 2014; McAusland et al., 2016; De Souza et al., 2020). Because the response times of A and gₛ are similar following an increase in PPFD, the deviation of iWUE from steady-state is relatively modest (McAusland et al., 2016). In contrast, the response times of A and gₛ are more distinct following a decrease in PPFD, as A typically declines to a new steady-state within seconds while gₛ decreases over the course of several minutes as stomata close (Lawson et al., 2011; Way and Pearcy, 2012; Lawson and Blatt, 2014; McAusland et al., 2016; Lawson and Vialet-Chabrand, 2019). This lag in gₛ relative to A leads to continued water loss, resulting in reduced iWUE. While the decrease in A is often simplified as a step-change from one steady-state to the next (McAusland et al., 2016; Bellasio et al., 2017), physiological processes such as post-illumination decarboxylation of photorespiratory metabolites and slow relaxation of photoprotective mechanisms may temporarily suppress A following a decrease in PPFD (Doncova et al., 1989; Kaiser et al., 2015; Kromdijk et al., 2016; Wang et al., 2020).

Attempts to improve leaf physiology under fluctuating light have generally followed three axes: (1) accelerate induction of A following increases in PPFD (Soleh et al., 2016, 2017; Taylor and Long, 2017; Acevedo-Siaca et al., 2020; Wang et al., 2020), (2) limit the inhibition of A following decreases in PPFD (Kromdijk et al., 2016; Wang et al., 2020), (3) accelerate stomatal movement to reduce gₛ response time, leading to improved A following increases in PPFD and improved iWUE following decreases in PPFD (Wang et al., 2014; Papanatsiou et al., 2019; Lawson and Matthews, 2020). An essential component of this research has been the discovery of broad diversity in A, gₛ, and iWUE in fluctuating light across species (McAusland et al., 2016; Deans et al., 2019) and within C₄ species such as rice (Acevedo-Siaca et al., 2020), cassava (De Souza et al., 2020), and soybean (Soleh et al., 2016, 2017; Wang et al., 2020). In contrast, within-species diversity in C₄ species is not well understood, despite use of the C₄ pathway by some of the world’s most productive crops, including maize (Zea mays L.), sorghum (Sorghum bicolor (L.) Moench), and sugarcane (Saccharum officinarum L.). Further, while modification of A or gₛ traits independent of one another will theoretically have a proportionate effect on iWUE, in reality these traits are inter-dependent. It is unclear how interactions between these traits affect natural diversity in iWUE, and whether some traits might be more tractable drivers for improvement of iWUE. In C₃ crops, there is the added complexity of the carbon-concentrating mechanism, which may alter the relationship between A and gₛ relative to C₄ species (McAusland et al., 2016). Similarly, it is unclear whether steady-state and non-steady-state traits interact with one another and are predictive of overall leaf gas-exchange under fluctuating light.

Here, steady-state and non-steady-state iWUE were examined in the model C₄ crop sorghum. Sorghum is a key food crop for water-limited regions of the globe and fiber sorghum is among the most productive potential cellulosic bioenergy crops of the warm temperate zone, characterized by high drought-tolerance
water-use efficiency and productivity (Regassa and Wortmann, 2014; Hadebe et al., 2017). This study asked three questions:

1. Is there significant diversity in non-steady-state responses of $A$, $g_s$, and $iWUE$ to light fluctuations among diverse sorghum accessions?
2. What are the main drivers for natural variation in $iWUE$ in terms of steady-state $A$ and $g_s$, non-steady-state $A$ and $g_s$, and stomatal patterning?
3. Are steady-state and non-steady-state gas-exchange traits predictive of leaf performance under fluctuating PPFD?

We analyzed gas exchange and stomatal patterning in 18 diverse sorghum accessions under steady-state and fluctuating PPFD conditions. Our results identify drivers for improvement of $A$ and $iWUE$, including stomatal size and non-steady-state $A$ and $iWUE$ traits, and demonstrate that the sorghum pan-genome harbors significant potential for improving the already impressive $iWUE$ of sorghum via breeding.

**MATERIALS AND METHODS**

**Plant Material and Growing Conditions**

Seeds of 18 sorghum accessions were planted on May 28, 2017 at the University of Illinois Energy Farm near Urbana, IL, United States (40°07'N, 5 88°21'W, 228 m above sea level) in 3 m rows at 25 seeds m$^{-1}$, in plots of four rows spaced 76 cm apart. The accessions were a subset of a biomass sorghum diversity harbors significant potential for improving the already impressive $iWUE$ of sorghum via breeding.

**Gas Exchange Measurements**

Leaf cuvettes of two portable photosynthetic gas-exchange systems (LI-6400XT; LI-COR, Inc., Lincoln, NE, United States with LI-6400-40 Leaf-Chamber-Fluorometer) were clamped onto each side of the midrib at the middle of each leaf. PPFD was set to 2000 $\mu$mol m$^{-2}$ s$^{-1}$, block temperature to 25$^\circ$C, flow rate to 700 $\mu$mol s$^{-1}$, [CO$_2$] in the sample cell set to 400 ppm and leaf-to-air VPD maintained <2 kPa. LEDs provided 10% blue and 90% red light. Leaves were acclimated to these conditions for an hour, then measurements began.

Because increases in PPFD are typically less impactful to $iWUE$ than decreases in PPFD, this study focused on extracting non-steady-state traits following decreases in PPFD. For each leaf, one cuvette measured steady-state PPFD response curves, which were used to obtain $A$, $g_s$, and $iWUE$ at steady-state, and to evaluate the transition from one steady-state to the next after decreases in PPFD. Curves were obtained by decreasing PPFD (2000, 1500, 1000, 500, 200, 100, and 0 $\mu$mol m$^{-2}$ s$^{-1}$) in 15 min steps, with data recorded every 10 s. Though useful for the purposes of quantifying steady-state and non-steady-state gas-exchange, this PPFD timecourse was highly artificial and provided limited information about repeated PPFD fluctuations, since it was only composed of lengthy step declines in PPFD. Therefore, in the second cuvette, additional measurements were used to determine whether the traits derived from steady-state PPFD response curves were predictive of performance in a repeatedly fluctuating PPFD environment. Fluctuating PPFD response curves were obtained by varying PPFD in 5.5 min steps in the following sequence: 2000, 1500, 2000, 1000, 2000, 500, 2000, 200, 2000, and 100 $\mu$mol m$^{-2}$ s$^{-1}$, with data recorded every 10 s.

Stomatal Phenotyping

Leaf tissue was mounted on glass slides and the abaxial surface was imaged using an optical topometer ($\mu$surf Explorer, NanoFocus, Karlsruhe, Germany) with 20$\times$ (0.8 mm$^2$ leaf surface, 20$\times$ M Plan APO, Olympus Corporation, Tokyo, Japan) and 50$\times$ air objectives (0.32 mm$^2$ leaf surface, 50$\times$ UM Plan FL N, Olympus Corporation). Reconstructions of the leaf epidermis were obtained from serial optical sections measured from surface to inside of leaf, with measurement depth of 40 $\mu$m and 20 $\mu$m for the 20$\times$ and 50$\times$ objectives, respectively ($\mu$surf Metrology, NanoFocus). Four images at 20$\times$, and one image at 50$\times$, were taken per leaf. Stomata on 20$\times$ images were counted to estimate stomatal density. The size, i.e., planar surface area, of the stomatal complex was measured by outlining relevant pixels of four stomata per 50$\times$ image using ImageJ (ImageJ1.51j8, NIH, United States).

**Gas-Exchange Data Analysis**

Analysis of Steady-State PPFD Response Curves

Steady-state net rate of leaf photosynthetic CO$_2$ uptake ($A_{2000}$), stomatal conductance to water vapor ($g_s_{2000}$) and intrinsic water-use efficiency ($iWUE_{2000}$) at $PPFD = 2000$ $\mu$mol m$^{-2}$ s$^{-1}$ were obtained as the average for the two cuvettes (i.e., two technical objectives) at each light level. For each leaf over the last 40 s of the initial 1 h acclimation period. Steady-state $A$, $g_s$, and $iWUE$ were also obtained for each PPFD in the steady-state PPFD response curve, as the average over the last 40 s of each PPFD

After each decrease in PPFD, $A$ declined from one steady-state to the next, often displaying a temporary inhibition as it decreased below steady-state (i.e., undershoot), then increased again to steady-state. This process was described by two traits: $t_{95A}$ and $A_{undershoot}$. $t_{95A}$ was the time required for $A$ to come within 5% of steady-state after a decrease in PPFD, where smaller $t_{95A}$ indicates that $A$ reached steady-state more rapidly. $A_{undershoot}$ was the difference between steady-state $A$ and the minimum $A$ reached at a given PPFD, where more negative $A_{undershoot}$ indicates more pronounced undershoot of steady-state. Corresponding traits were used to describe $g_s$ ($t_{95 g_s}$, $g_s_{undershoot}$).

The response of $iWUE$ to each decrease in PPFD was different from that of $A$ and $g_s$, as $iWUE$ first abruptly declined, reflecting an instantaneous loss of $A$ while $g_s$ remained relatively high. In the following minutes, as $g_s$ declined, $iWUE$ increased toward...
steady-state. $t_{95iWUE}$ was calculated analogous to $t_{95A}$ and $t_{95g}$, and $iWUE_{undershoot}$ was calculated analogous to $A_{undershoot}$ and $g_s$ undershoot.

Analysis of Fluctuating PPFD Response Curves

Average $A$, $g_s$ and $iWUE$ were computed throughout the fluctuating PPFD response curves. If responses of $A$, $g_s$, and $iWUE$ to changes in PPFD were instantaneous, then gas-exchange under fluctuating PPFD would be equivalent to steady-state gas-exchange at each PPFD. Instead, $A$, $g_s$, and $iWUE$ showed substantial deviations from steady-state following changes in PPFD. Therefore, overall gas-exchange under fluctuating PPFD could be described as the sum of steady-state gas-exchange and the deviation of gas-exchange from steady-state following each PPFD fluctuation. To assess the importance of non-steady-state traits separate from steady-state, the deviation of gas-exchange from steady-state under fluctuating PPFD was calculated. Because steady-state and fluctuating PPFD response curves were measured on the same leaves, data from both types of curves were combined. Deviation of $A$ from steady-state was calculated as follows: (1) $A$ from the fluctuating PPFD response curves was normalized to $A_{2000}$, (2) steady-state $A$ from the steady-state PPFD response curve was obtained for each PPFD, and also normalized to $A_{2000}$, (3) The difference between (1) and (2) was calculated at each PPFD, yielding the deviation of $A$ from steady-state, normalized to $A_{2000}$. This deviation is positive when $A$ is greater than steady-state, negative when $A$ is less than steady-state, and 0 when $A$ is equal to steady-state. The average deviation of $A$ from steady-state was calculated following increases and decreases in PPFD. The same method was applied to $iWUE$.

To test whether leaves cut from plants and measured in the laboratory might behave differently from leaves still attached to plants, an experiment was performed comparing the two types of leaves. There was no substantial effect of leaf excision on steady-state or non-steady-state $A$, $g_s$, and $iWUE$ (Supplementary Figure 9).

Statistical Analysis

A summary of all traits analyzed in this study is given in Table 1. ANOVA was used to test the fixed effect of sorghum accession on gas-exchange and physiology traits with the aov() function in the package stats (R 3.6.1, R Core Team, 2017). There were 3–5 plants sampled per accession, with one plot per accession, such that each plant was a pseudo replicate. Time of day of measurements and leaf-to-air VPD were included as cofactors for gas-exchange measurements. If neither cofactor was significant for a given trait, then a simpler model testing only the effect of accession was used. $A_{2000}$, $g_s$ 2000, and $iWUE_{2000}$ were the average of two technical replicates per plant, and stomatal density and size were the average of four technical replicates per plant; all other measurements had one technical replicate per plant.

Homogeneity of variances was tested by the Levene test using function LeveneTest() in package DescTools (Signorell, 2020) and normality of studentized residuals tested by Shapiro–Wilk using function ols_test_normality in package olsrr (Hebbal, 2020) at $p = 0.01$ threshold. The assumption of normality was violated for $t_{95A}$, so the Kruskal–Wallis test was used to analyze this trait using kruskal.test() function in package stats (R Core Team, 2017).

Pairwise correlations were tested at $p < 0.05$ (significant) and $p < 0.1$ (marginally significant) thresholds between means per accession for the traits described above using cor.m.test() function in package corrplot (Wei and Simko, 2017).

RESULTS

Variation Among Accessions in Steady-State Gas Exchange and in the Transition From One Steady-State to the Next Following Decreases in PPFD

Two representative accessions, PI153852 and PI152636, exemplify the genetic variation that was observed in steady-state and non-steady-state $A$, $g_s$, and $iWUE$ (Figure 1). In PI153852, steady-state $A$ and $g_s$ at PPFD = 2000 µmol m$^{-2}$ s$^{-1}$ were less than in PI152636 (i.e., $A_{2000}$ and $g_s$ 2000, pink datapoints in Figures 1A–D, respectively). After each decrease in PPFD, $A$ and $g_s$ declined from one steady-state to the next. In PI153852, decline of $A$ occurred over the course of several minutes, during which $A$ decreased below steady-state (i.e., undershoot), then increased again to steady-state (Figure 1A). In contrast, in PI152636, $A$ reached a new steady-state in under a minute, with a less pronounced undershoot (Figure 1B). In PI153852, $g_s$ gradually decreased below steady-state (i.e., undershoot), then increased again to steady-state (Figure 1C). In contrast, in PI152636 $g_s$ declined more rapidly to steady-state, with a slightly less pronounced undershoot (Figure 1D).

After each decrease in PPFD, $iWUE$ abruptly declined below steady-state (i.e., undershoot), reflecting an instantaneous loss of $A$ while $g_s$ remained relatively high. This decline was less pronounced in PI153852 (Figure 1E) than PI152636 (Figure 1F). In the following minutes, $iWUE$ increased to reach steady-state, reflecting a decline of $g_s$ to steady-state. This occurred slightly more rapidly in PI153852 (Figure 1E) than in PI152636 (Figure 1F). This suggests that the differences between these two accessions in $A$ and $g_s$ at steady-state and non-steady-state translated to differences in $iWUE$. Steady-state $iWUE$ was relatively stable from PPFD = 500–2000 µmol m$^{-2}$ s$^{-1}$, but began to decline at lower PPFD (Figures 1E,F and Supplementary Figure 1).

Among all 18 accessions, there was significant variation in $A_{2000}$ ($p < 0.001, 1.63$-fold variation among accessions, Figure 2A), $g_s$ 2000 ($p < 0.001, 1.78$-fold variation among accessions, Figure 2B), and $iWUE_{2000}$ ($p = 0.001, 1.29$-fold variation among accessions, Figure 2C), as well as the time required for $A$ ($t_{95A}, p = 0.010, 11$-fold variation among accessions, Figure 2D) and $g_s$ ($t_{95g}, p = 0.041, 1.98$-fold variation among accessions, Figure 2E) to come within $5\%$ of steady-state after a decrease in PPFD. However, there was no significant variation in the time required for $iWUE$ to come within $5\%$ of steady-state after a decrease in PPFD ($t_{95iWUE}, p = 0.318, 1.65$-fold variation among accessions, Figure 2F). There was significant variation in the undershoot of steady-state by $A$ ($A_{undershoot}$.
TABLE 1 | Trait abbreviations and definitions.

| Trait                                                                   | Abbreviation | Measurements used                      |
|-------------------------------------------------------------------------|--------------|----------------------------------------|
| Steady-state A at PPFD = 2000 μmol m⁻² s⁻¹                             | A2000        | Steady-state PPFD curve                 |
| Steady-state gs at PPFD = 2000 μmol m⁻² s⁻¹                            | g2 2000      | Steady-state PPFD curve                 |
| iWUE at PPFD = 2000 μmol m⁻² s⁻¹                                       | iWUE2000     | Steady-state PPFD curve                 |
| Time required for A to come within 5% of steady-state after a decrease in PPFD | tA5A         | Steady-state PPFD curve                 |
| Time required for gs to come within 5% of steady-state after a decrease in PPFD | tgs          | Steady-state PPFD curve                 |
| Time required for iWUE to come within 5% of steady-state after a decrease in PPFD | tgiWUE       | Steady-state PPFD curve                 |
| Undershoot of steady-state A after a decrease in PPFD                  | AUndershoot  | Steady-state PPFD curve                 |
| Undershoot of steady-state gs after a decrease in PPFD                 | gUndershoot  | Steady-state PPFD curve                 |
| Undershoot of steady-state iWUE after a decrease in PPFD               | iWUEUndershoot | Steady-state PPFD curve              |
| Average A under fluctuating PPFD                                        | AverageA     | Steady-state PPFD curve                 |
| Average iWUE under fluctuating PPFD                                    | AverageiWUE  | Steady-state PPFD curve                 |
| Average deviation of A from steady-state following increases in PPFD   | DevA         | Steady-state and fluctuating PPFD curves|
| Average deviation of A from steady-state following decreases in PPFD    | DevA         | Steady-state and fluctuating PPFD curves|
| Average deviation of iWUE from steady-state following increases in PPFD | DeviWUE      | Steady-state and fluctuating PPFD curves|
| Average deviation of iWUE from steady-state following decreases in PPFD | DeviWUE      | Steady-state and fluctuating PPFD curves|
| Stomatal density                                                       | Density      | Stomatal profiles                      |
| Stomatal size                                                           | Size         | Stomatal profiles                      |

p = 0.010, 2.50-fold variation among accessions, Figure 2G), gs (tUndershoot, p = 0.019, 2.51-fold variation among accessions, Figure 2H), and iWUE (iWUEUndershoot, p < 0.001, 1.98-fold variation among accessions, Figure 2I) after each decrease in PPFD. A approached steady-state within seconds in most accessions (tA5A < 1 min, Figure 2D), with the slowest decline of A in PI153852 (tA5A = 2.86 min, Figures 1A, 2D). gs and iWUE approached steady-state more slowly than A (tgs and tgiWUE ranging from 2.22 to 4.40 min, Figures 2E,F).

Variation Among Accessions in Gas Exchange Under Fluctuating PPFD

Fluctuating PPFD response curves were used to determine whether the traits derived from steady-state PPFD response curves were predictive of performance in a fluctuating PPFD environment. Following decreases in PPFD, A rapidly declined to steady-state, while increases in PPFD triggered more gradual increases of A toward steady-state (Figure 3A, B). As a result, A was slightly above steady-state following decreases in PPFD, but substantially below steady-state following increases in PPFD (Figures 3C, D). As in the steady-state PPFD response curves, in accessions such as PI152636 there was little deviation of A from steady-state following decreases in PPFD, whereas in PI153852, A remained above steady-state for several minutes (Figures 3C, D).

Following decreases in PPFD, iWUE abruptly declined, then gradually increased toward steady-state (Figures 3E, F). In contrast, increases in PPFD caused iWUE to rise slightly above steady-state, then return to steady-state (Figures 3E, F). In other words, iWUE was slightly above steady-state following increases in PPFD, but substantially less than steady-state following decreases in PPFD (Figures 3G, H). This resulted from the fact that A declined faster than gs following decreases in PPFD, whereas A and gs increased at a similar rate following increases in PPFD (Supplementary Figures 5–7). In accessions such as PI152636, there was substantial loss of iWUE relative to steady-state following decreases in PPFD, and a slight gain of iWUE relative to steady-state following increases in PPFD, compared to the less pronounced deviation of iWUE from steady-state in accessions such as PI153852 (Figures 3G, H).

Among all accessions, there was significant or marginally significant variation in average A under fluctuating PPFD (p = 0.002, 1.50-fold variation among accessions, Figure 4A), average gs under fluctuating PPFD (p = 0.037, 1.78-fold variation among accessions, Figure 4B), and average iWUE under fluctuating PPFD (p = 0.069, 1.22-fold variation among accessions, Figure 4C), as well as the deviation of A from steady-state following increases in PPFD (p = 0.010, 2.50-fold variation among accessions, Figure 4D), the deviation of iWUE from steady-state following increases in PPFD (p = 0.088, 4.02-fold variation among accessions, Figure 4E), and the deviation of A from steady-state following decreases in PPFD (p = 0.078, 3.07-fold variation among accessions, Figure 4F). However, the experiment could not resolve significant differences among accessions for the deviation of iWUE from steady-state following decreases in PPFD (p = 0.121, 3.15-fold variation among accessions, Figure 4G). Finally, there was significant variation among accessions in stomatal density (p < 0.001, 1.72-fold variation among accessions, Figure 4H) and stomatal size (p < 0.001, 1.59-fold variation among accessions, Figure 4I) determined from optical topometry of the epidermis (Figure 5).

Correlations Between A, gs, iWUE, and Stomatal Patterning Traits

Traits Correlated With Steady-State A, gs, and iWUE

Steady-state gs 2000 was positively correlated with A2000 (p < 0.001, R² = 0.87, Figure 6) and negatively correlated with iWUE2000 (p = 0.0019, R² = 0.46, Figure 6). After a decrease in PPFD, iWUE increased toward steady-state more slowly in accessions with greater A2000 and gs 2000 (positive correlation
FIGURE 1 | Timecourses of steady-state PPFD response curves in two accessions: (A,C,E) PI153852 and (B,D,F) PI152636. For each curve, leaves were acclimatized to PPFD of 2000 µmol m$^{-2}$ s$^{-1}$ for 1 h, then PPFD declined in steps every 15 min. (A,B) A, (C,D) $g_s$, and (E,F) iWUE were logged throughout. Each point is a mean ± s.e. of 4 plants. The final measurements at each PPFD were used to estimate steady-state A, $g_s$, and iWUE at each PPFD. Timecourses were also used to describe the transition from one steady-state to the next after each decrease in PPFD, including the time for A, $g_s$, and iWUE to come within 5% of steady-state, and the undershoot of steady-state by A, $g_s$, and iWUE.

of $A_{2000}$ with $t_{95iWUE}$, $p = 0.094$, $R^2 = 0.17$; positive correlation of $g_s_{2000}$ with $t_{95iWUE}$, $p = 0.059$, $R^2 = 0.2$, (Figure 6). After a decrease in PPFD, undershoot of steady-state iWUE was more pronounced in accessions with greater $A_{2000}$ and $g_s_{2000}$ (negative correlation of $A_{2000}$ with iWUE$_{undershoot}$, $p = 0.03$, $R^2 = 0.26$; negative correlation of $g_s_{2000}$ with iWUE$_{undershoot}$, $p = 0.074$, $R^2 = 0.19$, (Figure 6). Accordingly, deviation of iWUE from steady-state following decreases in PPFD was more negative in accessions with greater $A_{2000}$ ($p < 0.001$, $R^2 = 0.61$, (Figure 6) and greater $g_s_{2000}$ ($p < 0.001$, $R^2 = 0.52$, (Figure 6).

Traits Correlated With Non-Steady-State A
For non-steady-state A to be maximized following a decrease in PPFD, A should slowly approach steady-state (i.e., high $t_{95A}$) with minimal undershoot (i.e., greater, i.e., less negative, $A_{undershoot}$). This non-steady-state A may also be related to non-steady-state $g_s$. Accordingly, accessions with a more positive deviation of A from steady-state following decreases in PPFD, also had greater $t_{95A}$ ($p = 0.0088$, $R^2 = 0.36$, Figure 6), greater $t_{95g_s}$ ($p = 0.0062$, $R^2 = 0.38$, Figure 6), and less negative $g_s_{undershoot}$ ($p = 0.078$, $R^2 = 0.18$, Figure 6). In accessions in which A and $g_s$ approached steady-state more slowly (i.e., greater $t_{95A}$ and $t_{95g_s}$), the undershoot of steady-state A and $g_s$ was less pronounced (i.e., less negative $A_{undershoot}$ and $g_s_{undershoot}$). This was evidenced by the positive correlation of $t_{95A}$ with $A_{undershoot}$ ($p = 0.063$, $R^2 = 0.2$, Figure 6) and $g_s_{undershoot}$ ($p = 0.0033$, $R^2 = 0.43$, Figure 6) and the positive correlation of $t_{95g_s}$ with $A_{undershoot}$ ($p = 0.0081$, $R^2 = 0.36$, Figure 6) and $g_s_{undershoot}$ ($p < 0.001$, $R^2 = 0.54$, Figure 6).

Non-steady-state A and $g_s$ were coordinated following a decrease in PPFD. In accessions in which A approached steady-state more slowly (i.e., greater $t_{95A}$) with a less pronounced undershoot (i.e., less negative $A_{undershoot}$), the same was also seen for $g_s$ (i.e., greater $t_{95g_s}$ and less negative
also had a smaller decrease of \( iWUE \) and a more pronounced undershoot of steady-state with \( g_s \). This was evidenced by the negative correlation of \( t_{95iWUE} \) with \( t_{95g} \) (\( p \leq 0.001, R^2 = 0.63, \text{Figure 6} \)) and the positive correlation of \( A_{\text{undershoot}} \) with \( g_{\text{undershoot}} \) (\( p < 0.001, R^2 = 0.66, \text{Figure 6} \)).

**Traits Correlated With Non-Steady-State \( iWUE \)**

For non-steady-state \( iWUE \) to be maximized following a decrease in PPFD, \( iWUE \) should rapidly approach steady-state (i.e., small \( t_{95iWUE} \)) with minimal undershoot (i.e., greater, i.e., less negative \( iWUE_{\text{undershoot}} \)). Accordingly, accessions with a less negative deviation of \( iWUE \) from steady-state following decreases in PPFD also had smaller \( t_{95iWUE} \) (\( p = 0.051, R^2 = 0.21, \text{Figure 6} \)) and greater \( iWUE_{\text{undershoot}} \) (\( p = 0.0074, R^2 = 0.37, \text{Figure 6} \)).

Non-steady-state \( iWUE \) traits were associated with \( A \) rather than \( g_s \). Specifically, in accessions in which \( iWUE \) approached steady-state more slowly (i.e., greater \( t_{95iWUE} \)), \( A \) approached steady-state more rapidly with a more pronounced undershoot (i.e., smaller \( t_{95A} \) and more negative \( A_{\text{undershoot}} \)). This was evidenced by the negative correlation of \( t_{95iWUE} \) with \( t_{95A} \) (\( p = 0.085, R^2 = 0.17, \text{Figure 6} \)) and \( A_{\text{undershoot}} \) (\( p = 0.035, R^2 = 0.25, \text{Figure 6} \)). Additionally, accessions which displayed a less pronounced undershoot of steady-state by \( iWUE \) following decreases in PPFD (i.e., greater, i.e., less negative \( iWUE_{\text{undershoot}} \)) also had a slower decrease of \( A \) and \( g_s \) to reach steady-state (positive correlation of \( iWUE_{\text{undershoot}} \) with \( t_{95A} \), \( p < 0.001, R^2 = 0.65 \); positive correlation of \( iWUE_{\text{undershoot}} \) with \( t_{95g} \), \( p = 0.0021, R^2 = 0.46, \text{Figure 6} \)). Further, in accessions which displayed a less pronounced undershoot of steady-state by \( iWUE \), the same was seen for \( A \) and \( g_s \) (positive correlation of \( iWUE_{\text{undershoot}} \) with \( A_{\text{undershoot}} \), \( p < 0.001, R^2 = 0.51 \); positive correlation of \( iWUE_{\text{undershoot}} \) with \( g_{\text{undershoot}} \), \( p = 0.0034, R^2 = 0.42, \text{Figure 6} \)). Finally, accessions in which \( iWUE \) approached steady-state more slowly (i.e., greater \( t_{95iWUE} \)) had a more pronounced undershoot of steady-state \( iWUE \) (negative correlation of \( t_{95iWUE} \) with \( iWUE_{\text{undershoot}} \), \( p = 0.0068, R^2 = 0.38, \text{Figure 6} \)).

**FIGURE 2** | Bar graphs of traits derived from steady-state PPFD response curves: (A) steady-state \( A \) at PPFD = 2000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) \( (A_{2000}) \), (B) steady-state \( g_s \) at PPFD = 2000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) \( (g_s_{2000}) \), (C) steady-state \( iWUE \) at PPFD = 2000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) \( (iWUE_{2000}) \), (D) time required for \( A \) to come within 5\% of steady-state after a decrease in PPFD \( (t_{95A}) \), (E) time required for \( g_s \) to come within 5\% of steady-state after a decrease in PPFD \( (t_{95g}) \), (F) time required for \( iWUE \) to come within 5\% of steady-state \( (t_{95iWUE}) \), (G) undershoot of steady-state by \( A \) after a decrease in PPFD, i.e., difference between steady-state \( A \) and the minimum \( A \) reached at each PPFD \( (A_{\text{undershoot}}) \), (H) undershoot of steady-state by \( g_s \) after a decrease in PPFD, i.e., difference between steady-state \( g_s \) and the minimum \( g_s \) reached at each PPFD \( (g_{\text{ undershoot}}) \), (I) undershoot of steady-state by \( iWUE \) after a decrease in PPFD, i.e., difference between steady-state \( iWUE \) and the minimum \( iWUE \) reached at each PPFD \( (iWUE_{\text{undershoot}}) \). Bars are mean ± s.e. \( p \)-values are from ANOVA testing the fixed effect of accession on each trait.
These surprising findings are exemplified in accessions PI153852 and PI152636 (Figure 1). PI152636 displayed faster declines in \(g_s\), after each decrease in \(PPFD\) (i.e., smaller \(t_{0.5\%}\), Figure 1D), which theoretically would lead to increased \(iWUE\), when compared to the slower \(g_s\) response of PI153852 (Figure 1C). However, in PI153852 the stronger response of \(g_s\) was
FIGURE 4 | Bar graphs of traits derived from fluctuating PPFD response curves and leaf stomatal profiles: (A) Average \( A \) under fluctuating PPFD, (B) Average \( g_s \) under fluctuating PPFD, (C) Average \( iWUE \) under fluctuating PPFD, (D) Deviation of \( A \) from steady-state following increases in PPFD, expressed as a% of \( A_{2000} \), (E) Deviation of \( iWUE \) from steady-state following increases in PPFD, expressed as a% of \( iWUE_{2000} \), (F) Deviation of \( A \) from steady-state following decreases in PPFD, expressed as a% of \( A_{2000} \), (G) Deviation of \( iWUE \) from steady-state following decreases in PPFD, expressed as a% of \( iWUE_{2000} \), (H) stomatal density, (I) stomatal size, i.e., planar surface area of the stomatal complex. Bars are mean ± s.e. \( p \)-values are from ANOVA testing the fixed effect of accession on each trait.

also associated with slower, more gradual declines in \( A \) after each decrease in PPFD (i.e., greater \( \tau_{95A} \), Figure 1A), which would also theoretically lead to increased \( iWUE \), when compared to the faster \( A \) response of PI152636 (Figure 1B). The net result in terms of \( iWUE \) yielded a benefit to PI153852 (Figure 1E), with a less pronounced undershoot of steady-state \( iWUE \) (i.e., less negative \( iWUE_{undershoot} \)) and a faster return to steady-state \( iWUE \) (i.e., smaller \( \tau_{95iWUE} \)) when compared to PI152636 (Figure 1F). In other words, when comparing PI153852, with slow responses of \( A \) and \( g_s \) to decreases in PPFD, to PI152636, with fast responses of \( A \) and \( g_s \) to decreases in PPFD, PI153852 showed the greatest \( iWUE \) at non-steady-state.

Correlations With Stomatal Density and Size
Stomatal density and size were negatively correlated (\( p < 0.001, R^2 = 0.54 \), Figure 6). Accessions with more numerous, smaller stomata had greater steady-state \( g_s \) and more rapid responses of \( A \) and \( g_s \) to decreases in PPFD. Specifically, accessions with greater stomatal density had greater \( g_s_{2000} \) (\( p = 0.074, R^2 = 0.19 \), Figure 6), lower \( iWUE_{2000} \) (\( p = 0.032, R^2 = 0.26 \), Figure 6), smaller \( \tau_{95A} \) (\( p = 0.073, R^2 = 0.19 \), Figure 6), smaller \( \tau_{95g_s} \) (\( p = 0.069, R^2 = 0.19 \), Figure 6), and more negative \( g_{undershoot} \) (\( p = 0.059, R^2 = 0.21 \), Figure 6). Accessions with smaller stomata had smaller \( \tau_{95A} \) (\( p < 0.001, R^2 = 0.51 \), Figure 6), smaller \( \tau_{95g_s} \) (\( p < 0.001, R^2 = 0.56 \), Figure 6), more negative \( g_{undershoot} \).
The majority of variation in average \( A \) under fluctuating PPFD was associated with steady-state \( A_{2000} \) (Figures 7A–C). In contrast, much of the variation in average \( iWUE \) under fluctuating PPFD was associated with the non-steady-state loss of \( iWUE \) following decreases in PPFD in addition to steady-state \( iWUE_{2000} \) (Figures 7D–F). This points to non-steady-state \( iWUE \) as an important contributor to overall \( iWUE \) under fluctuating PPFD, with decreases in PPFD being more impactful than increases in PPFD. This could in large part be attributed to the undershoot of steady-state by \( iWUE \) following decreases in PPFD (i.e., \( iWUE_{undershoot} \)), which showed significant variation among accessions and so could be a promising target for improvement.

The response of \( iWUE \) to a decrease in PPFD was biphasic, with an abrupt loss of \( iWUE \) driven by \( g \), followed by a gradual return to steady-state driven by \( g \) (Figure 1). Loss of \( iWUE \) was mitigated in accessions in which the undershoot of steady-state \( iWUE \) was less pronounced (i.e., greater, i.e., less negative, \( iWUE_{undershoot} \)) and \( iWUE \) returned to steady-state more rapidly (i.e., smaller \( t_{95,iWUE} \), Figure 6). The finding that \( t_{95,iWUE} \) and \( iWUE_{undershoot} \) were associated with corresponding traits of \( A \) rather than \( g \), is novel, and suggests that breeding for improved non-steady-state \( A \) traits, i.e., high \( t_{95,iWUE} \) and less negative \( A_{undershoot} \), could mitigate loss of \( iWUE \) under fluctuating PPFD (Figure 6).

Non-steady-state \( A \) following decreases in PPFD is influenced by different physiological processes. Undershoot of steady-state by \( A \) following decreases in PPFD may be attributed to kinetics of protective energy-dissipating mechanisms, collectively termed non-photochemical quenching, and photorespiration (Kaiser et al., 2015, 2018), and has been observed in \( C_3 \) dicots such as Arabidopsis, French bean (McAusland et al., 2016) and tobacco (Kromdijk et al., 2016). This could explain the rapid and pronounced undershoot of steady-state \( A \) following decreases in PPFD in accessions such as PI329656 (Supplementary Figure 2G). Large pools of metabolites involved in \( C_4 \) photosynthesis could buffer energy supply and sustain a higher \( A \) for some time following a decrease in PPFD (Stitt and Zhu, 2014). For instance, a large pool of active malate decreases in PPFD, causing a substantial loss of \( iWUE \) under fluctuating PPFD.

DISCUSSION

Improving \( iWUE \) under fluctuating PPFD is important to sustain or further increase crop yields (Leakey et al., 2019). This study shows significant variation among sorghum accessions in steady-state and non-steady-state \( A \) and \( g \), and stomatal density and size, reveals how variation in these traits drives variation in \( iWUE \), and discusses how tradeoffs between these should shape strategies for decreasing crop water use.

Decreases in PPFD Substantially Impair \( iWUE \) Under Fluctuating PPFD, With Loss of Non-Steady-State \( iWUE \) Associated With \( A \) Rather Than \( g \)

Almost all variation in average \( A \) under fluctuating PPFD was associated with steady-state \( A_{2000} \) (Figures 7A–C). In contrast, much of the variation in average \( iWUE \) under fluctuating PPFD was associated with the non-steady-state loss of \( iWUE \) following decreases in PPFD in addition to steady-state \( iWUE_{2000} \) (Figures 7D–F). This points to non-steady-state \( iWUE \) as an important contributor to overall \( iWUE \) under fluctuating PPFD, with decreases in PPFD being more impactful than increases in PPFD. This could in large part be attributed to the undershoot of steady-state by \( iWUE \) following decreases in PPFD (i.e., \( iWUE_{undershoot} \)), which showed significant variation among accessions and so could be a promising target for improvement.

The response of \( iWUE \) to a decrease in PPFD was biphasic, with an abrupt loss of \( iWUE \) driven by \( g \), followed by a gradual return to steady-state driven by \( g \) (Figure 1). Loss of \( iWUE \) was mitigated in accessions in which the undershoot of steady-state \( iWUE \) was less pronounced (i.e., greater, i.e., less negative, \( iWUE_{undershoot} \)) and \( iWUE \) returned to steady-state more rapidly (i.e., smaller \( t_{95,iWUE} \), Figure 6). The finding that \( t_{95,iWUE} \) and \( iWUE_{undershoot} \) were associated with corresponding traits of \( A \) rather than \( g \), is novel, and suggests that breeding for improved non-steady-state \( A \) traits, i.e., high \( t_{95,iWUE} \) and less negative \( A_{undershoot} \), could mitigate loss of \( iWUE \) under fluctuating PPFD (Figure 6).
carries enough reductive power to reduce CO$_2$ for several seconds after a light to dark transition (Slattery et al., 2018). C$_4$ activity could be insufficient to achieve CO$_2$ saturation of Rubisco and eliminate photorespiration at low PPFD, leading to reduced steady-state $A$ (Kromdijk et al., 2010). If so, a leaf with CO$_2$-saturated Rubisco at high PPFD could maintain CO$_2$-saturation for some time after a decrease in PPFD, effectively maintaining photorespiration below its steady-state and therefore boosting $A$. This could explain the relatively gradual decline of $A$ following decreases in PPFD in accessions such as PI153852 (Figure 1A), enabling it to maintain $A$ above steady-state for several minutes following decreases in PPFD (Figure 3C). Here, we show that potential for improvement of non-steady-state $A$ in sorghum through breeding is supported by significant variation in $f_{95,A}$ and $A_{undershoot}$, which may result from variation in the processes above (Figure 2). This highlights the value of the methodology used in the present study to assess steady-state and non-steady-state gas-exchange traits, and demonstrates that sorghum is a relevant crop species to study diversity in these traits.
Interactions Between Non-Steady-State A and gₘ Impair iWUE Following Decreases in PPFD: This Could Be Resolved in Leaves With Smaller, More Sensitive Stomata

Many efforts to improve iWUE under fluctuating light have focused on faster stomatal closure (Lawson and Blatt, 2014; Bellasio et al., 2017; Lawson and Vialet-Chabrand, 2019). In a simplified model of leaf gas-exchange following a decrease in PPFD, where A has an instant step-change from one steady-state to the next, a faster decrease in gₘ would directly lead to a faster increase in iWUE, leading to overall improvement in iWUE under fluctuating PPFD. In the sorghum accessions studied here, this process was complicated by interactions between gₘ and A, which may reflect precise stomatal sensing of A (reviewed: Lawson and Matthews, 2020). In other words, accessions with rapid decreases in gₘ also had rapid decreases in A, negating much of the benefit to iWUE (Figures 1, 6). In fact, the undershoot of steady-state iWUE following decreases in PPFD (i.e., iWUEundershoot) was most negative in accessions with faster stomatal responses (i.e., smaller t₉₅ₘ, Figure 6). If the same coordination between non-steady state A and gₘ applies across species, this could explain the observation that faster stomatal closing speed did not translate to improved water saving across diverse plant species (Deans et al., 2019).

Because of this tradeoff, fast gₘ response to decreasing PPFD may be a difficult target for improvement of iWUE through breeding in sorghum, though it may be possible to bypass this tradeoff through transgenic means. The optimal leaf response following decreases in PPFD would be a slow decline in A with minimal undershoot of steady-state, paired with a rapid decline in gₘ. This might be achieved in leaves with enhanced stomatal sensitivity to A, in which even a slow decline in A following a decrease in PPFD could trigger a rapid stomatal response. In particular, stomatal aperture responds to light via two separate pathways: the photosynthesis-independent and guard-cell specific blue light pathway, and the photosynthesis-dependent red light pathway. The latter is thought to be the main mechanism coordinating stomatal behavior with photosynthesis (Matthews et al., 2020). Therefore, manipulation of components involved in red light sensing, such as the redox state of the chloroplastic plastoquinone pool (Głowacka et al., 2018), could be a good target for manipulation to increase stomatal sensitivity to changes in A and improve coordination of A and gₘ.

Another factor influencing the speed of change in gₘ is stomatal size, with smaller stomata generally showing faster movement, possibly due to greater guard cell membrane surface area to volume ratio (Drake et al., 2013; Raven, 2014; Lawson and Vialet-Chabrand, 2019; Lawson and Matthews, 2020). Therefore, leaves with smaller stomata might allow greater sensitivity of gₘ to A by enabling mechanically faster stomatal closure. However,
stomatal size is usually negatively correlated with stomatal density (Hetherington and Woodward, 2003). Here, stomatal density and size were negatively correlated (Figure 6), and leaves with more numerous and smaller stomata had faster $g_s$ but also had reduced $iWUE_{2000}$, more negative $iWUE_{undershoot}$, and increased $g_s$ (Figures 5, 6). This points to a tradeoff between leaves with more numerous, smaller stomata which show high steady-state $g_s$ but rapid $g_s$ responses to PPFD, and leaves with fewer, larger stomata which show low steady-state $g_s$ but slow $g_s$ responses to PPFD. The finding that steady-state $iWUE_{2000}$ was mainly associated with $g_s$ rather than $A_{2000}$ is consistent with prior observations in sorghum (Geetika et al., 2019) and other C$_4$ grasses (Leakey et al., 2019), but the tradeoff with non-steady-state $g_s$ identified here (Figure 6) is not widely recognized.

Sorghum leaves with fewer and smaller stomata might achieve the best of both worlds with low steady-state $g_s$ but rapid stomatal responses to PPFD. In C$_4$ crops such as sorghum, where photosynthesis is typically CO$_2$-saturated even under subambient conditions, modestly reducing $g_s$ at steady-state may not impair $A$, compounding benefits to $iWUE$ (Leakey et al., 2019; Pignon and Long, 2020). Further, at a given SD, C$_4$ grass stomata are smaller than those of related C$_3$ grasses (Taylor et al., 2012). Transgenic approaches may hold potential to break the relationship between stomatal density and size (reviewed: Leakey et al., 2019).

Increases in PPFD were much less disruptive to $iWUE$ than decreases in PPFD (Figure 3). The fact that $iWUE$ was slightly above steady-state following increases in PPFD suggests that increase in $A$ was faster than $g_s$, and a model (Bellasio et al., 2017) applied to photosynthesis induction data in maize (Chen et al., 2013). By comparison, in many other C$_3$ and C$_4$ dicots and monocots, the return of $iWUE$ to steady-state following an increase in PPFD was much slower (e.g., >30 min), reflecting a pronounced desynchronization between $A$ and $g_s$ (McAusland et al., 2016). In our study, the response of $g_s$ to an increase in PPFD occurred within seconds, whereas in C$_3$ species there may be a lag of up to several minutes before stomata begin to open (Lawson and Blatt, 2014). Together these findings suggest exceptional coordination between $A$ and $g_s$ in sorghum.

### Relative to Other Species, iWUE in Sorghum Is High Both at Steady-State and Non-Steady-State

The range of natural variation among sorghum accessions tested here in $iWUE_{2000}$ of 166–215 μmol mol$^{-1}$ (Figure 2) is similar to published variation in sorghum RILs (100–140 μmol mol$^{-1}$; Kapanigowda et al., 2014) and accessions (143–176 μmol mol$^{-1}$; Xin et al., 2009), but less variable compared to measurements in closely related NADP-ME C$_4$ grasses such as maize (80–140 μmol mol$^{-1}$; Yabiku and Ueno, 2017), sugarcane (100–180 μmol mol$^{-1}$; Viswanathan et al., 2014) and elephant grass (100–160 μmol mol$^{-1}$; Sollenberger et al., 2014), whereas the widest range of variation tends to be found in C$_3$ species such as soybean (40–115 μmol mol$^{-1}$; Tomeo and Rosenthal, 2017), wheat (25–65 μmol mol$^{-1}$; Jahan et al., 2014), and rice (50–80 μmol mol$^{-1}$; Giuliani et al., 2013).

For both steady-state and non-steady-state traits, variation in $iWUE$ was narrower than for $A$ and $g_s$ (Figures 2, 4). This resulted from coordination in $A$ and $g_s$, e.g., accessions with high $A_{2000}$ also had high $g_s$ and accessions with high $g_{s2000}$ also had high $g_{sundershoot}$ (Figure 6). The accessions studied here showed faster $g_s$ responses to changes in PPFD and higher $iWUE$ compared to diverse gymnosperms and C$_3$ dicots (Deans et al., 2019), C$_3$ monocots such as wheat and rice, and even closely related C$_4$ monocots such as maize and Miscanthus (Chen et al., 2013; McAusland et al., 2016). Understanding how sorghum maintains coordination between $A$ and $g_s$ to sustain high $iWUE$ may be valuable to design strategies for improvement in species where coordination is less tight.

In our study, $g_s$ declined over the course of the fluctuating PPFD timecourse, suggesting that stomatal opening during increases in PPFD was slower than stomatal closing during decreases in PPFD (Figure 3). Faster stomatal closure than opening may be a consequence of sorghum’s adaptation to dry, high-light environments, where water is more limiting than light and rapid stomatal closing can maximize $iWUE$ (Vico et al., 2011; McAusland et al., 2016). On the contrary, species adapted to shaded environments such as a forest understory, where light is more limiting than water, typically show faster stomatal opening than closing, which can maximize $A$ with little penalty to $iWUE$. These patterns suggest that dynamic stomatal traits are driven by ecological adaptation rather than evolutionary lineage (Deans et al., 2019). Increased steady-state $iWUE$ of C$_4$ photosynthesis may have been a driver for evolution of this pathway (Osborne and Sack, 2012). The fact that C$_4$ grasses such as sorghum display fast stomatal responses to fluctuating light (Grantz and Assmann, 1991; Knapp, 1993; McAusland et al., 2016) may be an additional evolved mechanism to improve non-steady-state $iWUE$.

While a drought treatment was not included, we show genetic variation that may be exploited to reduce crop water demand and avoid drought (Leakey et al., 2019). An important next step will be to determine whether the trait correlations identified here are also observed in water-limited plants. Plants that develop under water-limited conditions can produce fewer leaves with fewer and/or smaller stomata to mitigate water loss. In these smaller plants, reduced canopy density may limit the prevalence of light fluctuations and alter the microenvironment including VPD and temperature. However, leaves that develop with sufficient water supply but are water-limited afterward have fewer options to acclimate. With stomatal density and size already fixed during development, stomatal closure is the main mechanism available to reduce steady-state $g_s$. Leaves that permanently operate in a reduced range of stomatal apertures could have an altered relationship between steady-state traits (e.g., $g_s$) and non-steady-state traits (e.g., $g_{sundershoot}$). This could also affect the relative association of $A$ and $g_s$ traits with $iWUE$: at low apertures, stomatal control may be less precise (Kaiser and Kappen, 2001) and so more wasteful in terms of water loss.
The next step will be to determine the degree of plasticity in steady-state and non-steady-state $A$, $g_i$, and iWUE traits under different environments.

**CONCLUSION**

In this study, we show that a common measurement, the steady-state PPFD response curve, can be used to derive valuable insight into both steady-state and non-steady-state $A$, $g_i$, and iWUE (Figures 1, 2). The relevance of these non-steady-state traits could be seen when leaves were exposed to a fluctuating PPFD regime, as natural diversity in traits such as $I_{95A}$, $A_{undershoot}$, and iWUE$_{undershoot}$ correlated with the deviation of $A$ and iWUE from steady-state under fluctuating PPFD (Figures 3, 4, 6). Remarkably, the deviation of $A$ and iWUE from steady-state under fluctuating PPFD was substantial even under the relatively lengthy PPFD fluctuations, spaced 5.5 min apart, of the fluctuating PPFD response curves used here (Figure 7). In a crop canopy, where most light fluctuations are more rapid (<5 s) (Kaiser et al., 2018), the non-steady-state processes quantified here, especially the photosynthetic traits $I_{95A}$ and $A_{undershoot}$, would likely be even more important in driving overall iWUE. Variation among accessions in steady-state and non-steady-state traits may be exploited to reduce crop water demand and avoid drought, but our results emphasize that translating this into breeding strategies will require careful consideration of emerging tradeoffs due to co-variation between traits.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

**AUTHOR CONTRIBUTIONS**

CP designed and performed the research, data collection, analysis and interpretation, and wrote the manuscript. AL, SL, and JK performed the data analysis and interpretation and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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