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Running Head: Nighttime water loss in *Helianthus*

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*Helianthus* nighttime conductance and transpiration respond to soil water but not nutrient availability

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Abstract

We investigated the response of Helianthus sp. nighttime conductance ($g_{\text{night}}$) and transpiration ($E_{\text{night}}$) to soil nutrient and water limitations in nine greenhouse studies. The studies primarily used wild Helianthus annuus L., but also included a commercial and early domesticate of H. annuus, and three additional wild species (H. petiolaris Nutt., H. deserticola Heiser, and H. anomalus Blake). Well watered plants of all species showed substantial $g_{\text{night}}$ (0.023-0.225 mol m$^{-2}$ s$^{-1}$) and $E_{\text{night}}$ (0.29-2.46 mmol m$^{-2}$ s$^{-1}$) measured as instantaneous gas exchange. Based on the potential for transpiration to increase mass flow of mobile nutrients to roots, we hypothesized that $g_{\text{night}}$ and $E_{\text{night}}$ would increase under limiting soil nutrients, but found no evidence of responses in all six studies testing this. Based on known daytime responses to water limitation, we hypothesized that $g_{\text{night}}$ and $E_{\text{night}}$ would decrease when soil water availability was limited, and results from all four studies testing this supported our hypothesis. We also established that stomatal conductance at night was on average five times greater than cuticular conductance. Additionally, $g_{\text{night}}$ and $E_{\text{night}}$ varied nocturnally and across plant reproductive stages while remaining relatively constant as leaves aged. Our results further the ability to predict conditions under which nighttime water loss will be biologically significant and demonstrate that for Helianthus, $g_{\text{night}}$ can be regulated.
Introduction

It is widely accepted that plants regulate stomatal aperture both to minimize water loss for a given amount of carbon assimilated and to minimize xylem cavitation (Cowan, 1977; Sperry, 2000). C₃ and C₄ plants fix carbon during the day and lose water from leaves as an unavoidable cost of getting CO₂ to the site of carboxylation. Although these plants are generally expected to close their stomata at night to conserve water when carbon gain is not occurring, significant nighttime leaf conductance ($g_{\text{night}}$) and transpiration ($E_{\text{night}}$) have been observed in many C₃ species across a wide range of habitats (see Musselman and Minnick, 2000; Caird et al., submitted-a for reviews). Reported rates for $g_{\text{night}}$ typically range from 0.01 to 0.25 mol m⁻² s⁻¹ and can represent greater than 50% of daytime conductance ($g_{\text{day}}$). Nighttime transpiration depends on both $g_{\text{night}}$ and leaf-to-air vapor pressure deficit (VPDₐ), but is usually 5-15% of daytime transpiration ($E_{\text{day}}$). To date most studies document the magnitude of $g_{\text{night}}$ and $E_{\text{night}}$ and several have correlated these traits with environmental or physiological variables (Benyon et al., 1999; Oren et al., 2001; Kavanagh et al., 2007). However, there have been few manipulative experiments that individually test the effect of environmental factors on the regulation of stomata at night.

Several researchers have speculated that nighttime water loss could enhance nutrient uptake by increasing mass flow of soluble nutrients to plant roots (Snyder et al., 2003; Daley and Phillips, 2006; Caird et al., submitted-a). The Barber-Cushman model predicts that increasing water flux to the rhizoplane minimizes or eliminates the formation of a nitrate depletion zone around plant roots when conditions are appropriate for $E_{\text{night}}$ (Barber and Cushman, 1981; Barber, 1995). Empirically, McDonald et al. (2002) demonstrated a benefit of increased transpiration on nitrate delivery and uptake by *Populus* plants. Although the Tanner and Beevers (2001) study is sometimes cited as contrary evidence, it dealt only with effects of transpiration on long distance nitrogen transport within the xylem, not with mass flow delivery to roots. Thus, increased nutrient acquisition may represent a benefit that counters the cost of water loss at night.

If nighttime water loss increases nutrient acquisition then plants may benefit from the ability to regulate $g_{\text{night}}$ in response to nutrient conditions. The effects of nitrate availability on $g_{\text{day}}$ and $E_{\text{day}}$ have been investigated and are variable (Chapin, 1990; Fredeen et al., 1991; Ciompi et al., 1996; Cechin and Fumis, 2004). Potential regulatory pathways are still being debated (Dodd et al., 2003; Sakakibara et al., 2006). Two recent field studies with nutrient addition...
treatments found that \( g_{\text{night}} \) declined in response to nutrient additions (Ludwig et al., 2006; Scholz et al., 2007). However, the experimental designs of these studies did not permit direct effects due to reduced plant demand for nutrient acquisition regulating \( g_{\text{night}} \) to be separated from indirect effects of plant size or water status. More studies are needed that experimentally manipulate soil nutrient availability and test its effect on \( g_{\text{night}} \) and \( E_{\text{night}} \), independent of confounding variation in soil and plant water potential.

During the day stomatal conductance is regulated with respect to changing soil water potential and atmospheric demand, to minimize use of available water during CO\(_2\) uptake and maintain soil to leaf hydraulic continuity (Sperry et al., 2002). To further optimize use of limited soil water, regulation may also occur at night, reducing \( g_{\text{night}} \) and consequently \( E_{\text{night}} \). This expectation held true for droughted wheat plants, where \( g_{\text{night}} \) decreased as compared to well-watered controls (Rawson and Clarke, 1988). However, variable results have been obtained from studies that manipulated soil water potential with salt addition (Donovan et al., 1999) or through irrigation in the field (Donovan et al., 2003). At this time generalization about the effect of soil water availability on \( g_{\text{night}} \) and \( E_{\text{night}} \) is not possible and further examination in controlled experiments is needed.

The magnitude of \( g_{\text{night}} \) and \( E_{\text{night}} \) may also vary across temporally, as leaves age or across plant reproductive stages (e.g. pre-reproductive, reproductive). Field studies have shown that small juvenile plants have higher \( g_{\text{day}} \) and \( E_{\text{day}} \) and lower water use efficiency than larger adults (Donovan and Ehleringer, 1991, 1992; Casper, 2005). Leaf age has been shown to cause a decline in \( g_{\text{day}} \) in sunflowers (Cechin and Fumis, 2004). Similar to these daytime responses, Grulke et al. (2004) found higher \( g_{\text{night}} \) in large saplings than in mature trees and Blom-Zandstra et al. (1995) found \( g_{\text{night}} \) of rose leaves declined as leaves aged from three to six weeks. However, in both of these cases direct effects of reproductive stage and leaf age cannot be differentiated from additional variables such as plant size and age. Controlled studies are needed to accurately assess the role of plant reproductive stage and leaf age on \( g_{\text{night}} \).

Most measures of plant water loss include loss across both the cuticular and stomatal pathways operating in parallel. Because cuticular conductance \( (g_{\text{cuticular}}) \) is very small compared to daytime conductance through open stomata \( (g_{\text{stomata}}) \), its contribution to \( g_{\text{day}} \) has traditionally been ignored. However, when considering much lower magnitude \( g_{\text{night}} \) and \( E_{\text{night}} \), cuticular losses may represent a substantial portion of the total measurement. Estimates of \( g_{\text{cuticular}} \),
ranging from 0.004-0.016 mol m\(^{-2}\) s\(^{-1}\), have been derived from gas exchange measurements of intact leaves where stomatal closure has been induced by either leaf wilting (water stress) or exogenous ABA application (Rawson and Clarke, 1988; Kerstiens, 1995; Boyer et al., 1997; Burghardt and Riederer, 2003; Nobel, 2005). These estimates include water loss through the cuticle and maximally closed stomata, and thus represent a functional definition of \(g_{\text{cuticular}}\). New techniques are available for estimating conductance and permeability of the cuticle separate from the stomatal pores, and they highlight the potential for variability in cuticular permeability (Schreiber et al., 2001; Santrueck et al., 2004; Kersteins 2006). However, it is still useful to measure water loss occurring through the cuticle plus stomata at maximal closure, because this represents a baseline that is not subject to short-term stomatal regulation.

We examined \(g_{\text{night}}\) and \(E_{\text{night}}\) in controlled greenhouse studies using wild \(Helianthus annuus\) L., \(H. annuus\) domesticates (commercial cultivar and Hopi domesticate), and a group of closely related wild species (\(H. anomalus\) Blake, \(H. deserticola\) Heiser and \(H. petiolaris\) Nutt.). Substantial \(g_{\text{night}}\) (0.08 to 0.10 mol m\(^{-2}\) s\(^{-1}\)) has been reported for \(H. annuus\) and \(H. anomalus\) in their native habitats (Snyder et al., 2003; Ludwig et al., 2006). The inclusion of several species allowed us to assess whether results for regulation of \(g_{\text{night}}\) and \(E_{\text{night}}\) can be generalized across closely related species. As large annuals, the \(Helianthus\) species were easily grown in the greenhouse allowing experimental manipulation of soil treatments under controlled environmental conditions. This allowed for robust tests of environmentally stimulated regulation and nighttime water loss at different phases of maturity.

Our objective was to investigate issues of regulation and variation in \(g_{\text{night}}\) and \(E_{\text{night}}\). Specifically, we addressed three questions: Are \(g_{\text{night}}\) and \(E_{\text{night}}\) regulated in response to soil nutrient and water availability? Under optimal soil conditions, do \(g_{\text{night}}\) and \(E_{\text{night}}\) vary nocturnally (within a night) and across leaf lifespan and plant reproductive stage? Finally, is \(g_{\text{night}}\) substantially larger than \(g_{\text{cuticular}}\) when the latter is defined functionally as conductance though the cuticle and maximally closed stomata?

**Results**

In all nine greenhouse studies (summarized in Tables 1, S1) the four species of wild \(Helianthus\) plus domesticated \(H. annuus\) and \(H. annuus\) Hopi all showed substantial loss of water at night. For sufficiently-watered plants, \(g_{\text{night}}\) averaged 0.057 mol m\(^{-2}\) s\(^{-1}\) (range: 0.023 to 0.225)
and $E_{\text{night}}$ averaged 0.69 mmol m$^{-2}$ s$^{-1}$ (range: 0.29 to 2.46). In comparison, averaged across these same plants $g_{\text{day}}$ was 0.54 mol m$^{-2}$ s$^{-1}$ and $E_{\text{day}}$ was 7.59 mmol m$^{-2}$ s$^{-1}$. VPD$_{1}$ for the gas exchange measurements averaged 1.17 kPa at night and 1.53 kPa during the day.

**Response of $g_{\text{night}}$ and $E_{\text{night}}$ to soil nutrient and water manipulation**

Six studies applied a soil nutrient treatment, four of which only manipulated soil nitrate (Table 1). There was no effect of nutrient limitation on $g_{\text{night}}$ and $E_{\text{night}}$ in any of these studies of *Helianthus* species (Fig. 1, Table S1, P>0.05 for all). The nutrient limitation was substantial enough to significantly reduce vegetative shoot biomass in all six studies (Table 2) and reproductive biomass in the studies where plant growth continued into the reproductive stage (Fall 2003-1 micro- and macronutrient manipulation, P<0.05 for all species except *H. deserticola*; Fall 2004-1, Spring 2005, Summer 2005 nitrogen manipulation, P<0.001; data not presented). Leaf total nitrogen content was also measured in four of the six nutrient manipulation studies. The limited nitrate treatment imposed as a modified Hoagland solution resulted in lower leaf nitrogen content (Table 2). Leaf nitrogen was measured in only one study involving total macro- and micronutrient manipulation and here the limited treatment resulted in significantly lower leaf nitrogen concentrations for *H. annuus* but not for *H. anomalus* or *H. petiolaris*.

In one of the nutrient limitation studies, Fall 2004-1, differences between wild *Helianthus* species were tested. A significant species effect was found ($g_{\text{night}}$, F=3.083,51, P<0.05; $E_{\text{night}}$, F=3.033,51, P<0.05), but a means separation test with Tukey’s HSD showed differences to be minimal and only significant between *H. deserticola* with the highest mean $g_{\text{night}}$ and $E_{\text{night}}$, and *H. petiolaris* with the lowest (P<0.05).

Four studies applied soil water treatments (Table 1): sufficient (maintained near field capacity) and limited. In all cases, plants with limited water showed substantially reduced $g_{\text{night}}$, $E_{\text{night}}$ (P<0.001), $g_{\text{day}}$, $E_{\text{day}}$ and photosynthesis (P<0.05 to 0.001) (Fig. 2, Table S1). In the Fall 2004-2 study, $g_{\text{night}}$ and $E_{\text{night}}$ were assessed in both wild *H. annuus* and *H. annuus* Hopi, but there was no interaction between accession and response to soil water limitation for these traits (P>0.05). During Fall 2005-2 xylem pressure potentials were measured at three points though the night and were consistently and substantially lower in the water limited *H. annuus* ($F_{1,14}$=30.82, P<0.001) (Fig. 3).
Variation in $g_{\text{night}}$ and $E_{\text{night}}$ nocturnally, across leaf lifespan and plant reproductive stages

A 24-hour time course was measured for $H. \text{annuus}$ in Fall 2005-2. $g_{\text{day}}$, $E_{\text{day}}$ and photosynthesis showed typical patterns, increasing rapidly in the morning and declining during the afternoon. $g_{\text{night}}$ and $E_{\text{night}}$, though low compared to daytime rates, increased through the night in the sufficiently-watered plants despite a small increase in atmospheric vapor pressure deficit ($\text{VPD}_a$) though the night (Fig. 3) (time effect for $g_{\text{night}}$ and $E_{\text{night}}$ respectively: $F_{2,11}=31.2$, $P<0.001$; $F_{2,11}=32.37$, $P<0.001$). In addition to instantaneous gas exchange measures, gravimetric measures were used to estimate total $E_{\text{night}}$ and total $E_{\text{day}}$ during the same time period. $E_{\text{night}}$ of sufficiently-watered plants was 0.86 (SE=0.10) for instantaneous gas exchange and 0.22 (SE=0.01) mmol m$^{-2}$ s$^{-1}$ for gravimetric measures. These rates were 5.7% and 6.5%, respectively, of the daytime rates measured by the same methods. Measures of $E_{\text{night}}$ and $E_{\text{day}}$ made with instantaneous and gravimetric methods were correlated ($E_{\text{night}} r^2=0.78$, $P<0.001$ and $E_{\text{day}} r^2=0.87$, $P<0.001$; Spearman rank correlations). During this same night and day period, average $\text{VPD}_a$ in the greenhouse was 0.6 kPa (SE=0.02) and 1.5 kPa (SE=0.12), respectively.

Repeated measures of $g_{\text{night}}$ and $E_{\text{night}}$ were also made on sufficiently-watered $H. \text{annuus}$ in the Fall 2004-1 study and showed similar trends to those documented in 2005 (Fig 3). $g_{\text{night}}$ and $E_{\text{night}}$ increased through the night (time effect respectively: $F_{2,29}=145.84$, $P<0.001$; $F_{2,29}=358.69$, $P<0.001$) despite increasing $\text{VPD}_a$, and these trends were not affected by nitrate treatment ($P>0.5$).

The effect of leaf aging on $g_{\text{night}}$ and $E_{\text{night}}$ was initially assessed in the Spring 2005 study. Repeated measures of $g_{\text{night}}$ and $E_{\text{night}}$ were made on the same leaves of $H. \text{annuus}$ across four weeks, starting when leaves were recently fully expanded. Start date for the four-week measurement sets were staggered across several weeks and used in the analysis to account for random environmental variation between nights. There was no decline in $g_{\text{night}}$ or $E_{\text{night}}$ due to leaf aging ($F_{1,321}=0.83$, $P>0.3$; $F_{1,321}=0.57$, $P>0.4$, respectively) (Table S1). In the Fall 2005-1 study, leaf age effects were assessed by further comparing a young fully mature and older fully mature leaf of the same plant using high nitrate treatment, 10-week old plants. Here again, $g_{\text{night}}$ and $E_{\text{night}}$ did not differ with leaf age ($t_{14}=1.21$, $P=0.2$; $t_{14}=1.22$, $P=0.2$, respectively).

The effect of plant reproductive stage on $g_{\text{night}}$ and $E_{\text{night}}$ was assessed in the Fall 2005-1 study. For $H. \text{annuus}$, plant reproductive stage affected $g_{\text{night}}$ and $E_{\text{night}}$ under both sufficient and
limited nitrate availability ($F_{2,46}=17.45, P<0.001; F_{2,46}=15.96, P<0.001$, respectively) (Fig. 4, Table S1). Pre-reproductive plants (5.5 weeks old) had higher $g_{\text{night}}$ and $E_{\text{night}}$ than did reproductive plants (10 or 15.5 week old). Plant reproductive stage also affected photosynthesis, which was higher in pre-reproductive plants ($F_{2,46}=6.69, P<0.01$), but not $g_{\text{day}}$ and $E_{\text{day}}$ ($P>0.05$).

The contribution of $g_{\text{cuticular}}$ to $g_{\text{night}}$

During the Fall 2004-1 and Spring 2005 studies $g_{\text{cuticular}}$, functionally defined as water loss through the cuticle with stomata at maximal closure, was measured on excised, wilted leaves. In Fall 2004-1 $g_{\text{night}}$ (total leaf conductance to water at night) was higher than $g_{\text{cuticular}}$ for all four wild *Helianthus* species (Fig. 5). In Spring 2005, $g_{\text{night}}$ was again higher than $g_{\text{cuticular}}$ (Fig. 5). In both studies $g_{\text{cuticular}}$ measured on leaves was higher than instrument error ($P<0.001$), which averaged $-7.5 \times 10^{-6}$ mol m$^{-2}$ s$^{-1}$ during $g_{\text{cuticular}}$ measurements. During Spring 2006, $g_{\text{cuticular}}$ was measured on intact leaves of plants infused with exogenous ABA into the xylem. $g_{\text{cuticular}}$ was lower than $g_{\text{night}}$ measured on intact leaves of control plants for both wild *H. annuus* and domesticated *H. annuus* (Fig. 5).

Looking across all three studies, $g_{\text{cuticular}}$ for wild *H. annuus* ranged from 0.013 to 0.023 mol m$^{-2}$ s$^{-1}$ and there was good agreement between measures made with the two different techniques (Fig. 5). Of the other three wild species only the estimate of $g_{\text{cuticular}}$ for *H. deserticola* was substantially larger than the range for *H. annuus*. Not considering *H. deserticola*, calculated $g_{\text{stomata}}$ for wild *Helianthus* was on average five times greater than $g_{\text{cuticular}}$.

Discussion

The *Helianthus* $g_{\text{night}}$ reported here for greenhouse grown plants (0.023-0.225 mol m$^{-2}$ s$^{-1}$) are within the range reported for two of these species in their native habitats (Snyder et al., 2003; Ludwig et al., 2007), and for C$_3$ and C$_4$ plants in general (Caird et al., submitted-a). The wild and domesticated *Helianthus* species in our studies had typical values for $g_{\text{day}}$, $E_{\text{day}}$, and photosynthesis (Table S1), and the $g_{\text{night}}$ values were relatively large and greater than explained by $g_{\text{cuticular}}$.

In the Fall 2005-2 study, gravimetric measures were compared to instantaneous measures of transpiration. The gravimetric measures were approximately four-fold lower, reflecting their integration over the entire night or day period, whereas instantaneous measures were timed to
capture maximal $E_{\text{night}}$ and $E_{\text{day}}$ rates. However, there was a strong correlation between the two measurement techniques. Additionally, the percentage total $E_{\text{night}}$ of total $E_{\text{day}}$ measured gravimetrically over the 24-hours gave an estimate of 6%, which agreed well with the 5% estimate from instantaneous gas exchange measures during the same day/night period. This added validity to our estimates based on instantaneous measures.

**Response of $g_{\text{night}}$ and $E_{\text{night}}$ to soil nutrient and water manipulation**

We hypothesized that regulation might occur for increased $g_{\text{night}}$ under limited nutrient conditions to increase bulk flow of soil solution to the roots and reduce the development of a nutrient depletion zone in the rhizosphere. Although the soil nutrient limitations were sufficient to limit shoot and reproductive biomass and generally to reduce leaf nitrogen concentration, they did not affect $g_{\text{night}}$ and $E_{\text{night}}$ in any of the wild *Helianthus* species or in domesticated *H. annuus*. Thus, for *Helianthus*, there is no evidence of nighttime stomatal regulation in response to soil nutrient limitations. Contrary to our *Helianthus* results, we have evidence that other species do respond to soil nutrient limitations imposed while controlling for plant water status; some with higher $g_{\text{night}}$ (*Distichlis spicata, Populus balsamifera ssp. trichocarpa*), and others with lower $g_{\text{night}}$ (*Arabidopsis thaliana*). M. Caird and A. Howard, Unpublished data). A broader range of species needs to be tested to support any generalizations. The variable response of $g_{\text{night}}$ to nutrient limitation may involve the same mechanisms that are currently being investigated for $g_{\text{day}}$ responses, such as ABA, pH, and cytokinin signals (Dodd et al., 2003; Sakakibara et al., 2006).

Whether or not a species regulates $g_{\text{night}}$ in response to soil nutrients, a plant that is transpiring at night may have increased uptake of nutrients such as nitrate. McDonald et al. (2002) demonstrated that *Populus* plants transpiring continuously (day and night), instead of only during the day, took up more nitrogen. Given that there is genetic variation for $g_{\text{night}}$ and $E_{\text{night}}$ (*Arabidopsis thaliana*; M. Caird, Unpublished data), selection may favor high $g_{\text{night}}$ and $E_{\text{night}}$ in nutrient poor habitats if $E_{\text{night}}$ provides a nutrient uptake benefit. The four wild *Helianthus* species studied here are native to habitats differing in nutrient availability: *H. anomalus* and *H. deserticola* are endemic to nutrient poor desert dune habitats (Rosenthal et al., 2002; Brouillette et al., in press). We found that *H. deserticola* did have higher $g_{\text{night}}$ and $E_{\text{night}}$ than *H. petiolaris*, consistent with the direction predicted by selection for higher $g_{\text{night}}$ in lower
nutrient habitats, but the magnitude of difference was relatively small and appeared largely
driven by greater $g_{\text{cuticular}}$ in *H. deserticola*.

$g_{\text{night}}$ and $E_{\text{night}}$ did decline in response to water limitations that were generally sufficient
to decrease leaf predawn xylem pressure potential, $g_{\text{day}}$, $E_{\text{day}}$ and photosynthesis. Declines were
such that $g_{\text{night}}$ in the limited water treatments was generally within the range we recorded for
functionally defined $g_{\text{cuticular}}$. For three of the four studies, the water limitation was short term
and consisted of withholding water just prior to measurements on fully mature leaves, so that the
effect on $g_{\text{night}}$ could not be due to a long term change in leaf structure, stomatal density or size,
or cuticle. The decline in $g_{\text{night}}$ and $E_{\text{night}}$ due to water limitation demonstrates that guard cell
regulation of nighttime water loss is possible, analogous to daytime regulation of water loss in
response to soil drying. Our results agree with previous results showing lower $g_{\text{night}}$ associated
with decreased plant water status in *Hibiscus cannabinus* (Muchow et al., 1980), *Pseudostuga mezensii* (Running, 1976; Blake and Ferrell, 1977) and *Helianthus anomalus* (Ludwig et al.,
2006), and a water stress treatment resulting in decreased water loss at night in wheat plants
(Rawson and Clarke, 1988). The nighttime stomatal response to drought likely involves many of
the same mechanisms that are currently being investigated for daytime responses, such as ABA
and pH signals (Dodd, 2003; Davies et al., 2005; Li et al., 2006), although this remains to be
determined.

Variation in $g_{\text{night}}$ and $E_{\text{night}}$ nocturnally, across leaf lifespan and plant reproductive stages

Assessing temporal variation is necessary for interpreting the significance of
instantaneous leaf-level measures of $g_{\text{night}}$ and $E_{\text{night}}$. We complemented single instantaneous
measures on most recently fully expanded leaves of mature plants with studies that assessed
variation nocturnally, across leaf lifespan, and across plant reproductive stages. Beginning with
nocturnal variation (across a single night), repeated measures during the night in two studies both
showed a significant increase in $g_{\text{night}}$ and $E_{\text{night}}$. A similar gradual increase in $g_{\text{night}}$ has been
observed in several other species including *Arabidopsis thaliana*, desert shrubs, and trees
(Lasceve et al., 1997; Leymarie et al., 1998, 1999; Donovan et al., 2003; Bucci et al., 2004;
Dodd et al., 2004, 2005), and potential regulatory mechanisms are being investigated (Lasceve et
al., 1997; Gardner et al., 2006).
When \( g_{\text{night}} \) was measured across three nocturnal time-points for sufficiently-watered \( H. \) \textit{annuus} in Fall 2005-2, the increase in \( g_{\text{night}} \) was associated with an increase in VPD\(_a\), although of small magnitude (approximately 0.5 to 0.7 kPa, Fig. 3). Thus, over this range of VPD\(_a\), the correlation between \( g_{\text{night}} \) and VPD was not the negative relationship expected from daytime VPD\(_a\) responses (Franks and Farquhar, 1999). However, a larger range of VPD\(_a\) is needed to test nighttime VPD responses. Correlative data from other studies suggest that \( g_{\text{night}} \) does decline in response to increased VPD\(_a\), similar to \( g_{\text{day}} \), and responses may be species specific (Oren et al., 2001; Bucci et al., 2004; but see Barbour et al., 2004 for contrast). Bakker (1991) found a decline in \( g_{\text{night}} \) in response to experimentally manipulated VPD. However, more studies are needed that experimentally manipulate VPD\(_a\) and VPD\(_l\) and account for potentially confounding factors such as \( g_{\text{cuticular}} \), circadian rhythms and xylem pressure potential recovery.

We assessed variation in \( g_{\text{night}} \) and \( E_{\text{night}} \) across entire leaf lifetime. In contrast to Cechin and Fumis (2004), who found that \( g_{\text{day}} \) declined as sunflower leaves aged, and Blom-Zandstra et al. (1995) who found that \( g_{\text{night}} \) declined as rose leaves aged, we found no decline in \( H. \) \textit{annuus} \( g_{\text{night}} \) and \( E_{\text{night}} \) as recently matured (i.e. fully expanded) leaves aged over the following four weeks. Measures on the same plants indicated that leaf lifespan (number of days from 1 cm leaf blade length to 50% of leaf senesced) averaged 40 days (5.7 weeks). Thus our gas exchange measurements captured the majority of leaf lifespan. The lack of decline in nighttime gas exchange rates over leaf lifespan suggests that for \textit{Helianthus}, instantaneous \( g_{\text{night}} \) on a recently matured leaf may be used to scale up to instantaneous \( g_{\text{night}} \) for whole plant leaf area, provided that there is an open canopy structure.

To generalize across plant life stages we investigated variation in \( g_{\text{night}} \) and \( E_{\text{night}} \) across plant reproductive stages, controlling for leaf age. Pre-reproductive \( H. \) \textit{annuus} showed significantly higher \( g_{\text{night}} \) and \( E_{\text{night}} \) than individuals that were flowering or setting seeds. This trend was not mirrored in daytime rates. Our results are consistent with those of Grulke et al. (2004) who found \( g_{\text{night}} \) to be higher in large saplings compared to mature ponderosa pine.

Young plants, during rapid vegetative growth, expend a large portion of respiratory energy on nutrient uptake and this proportion generally declines as plants age (Marschner, 1995). Thus, although \textit{Helianthus} species appear unable to regulate nighttime water loss in response to soil nutrient conditions, an inherently higher \( E_{\text{night}} \) for younger plants may be beneficial if it reduces formation of a nutrient depletion zone around roots at night, as suggested by results with the
Barber-Cushman model. Nutrient depletion zones may be more pronounced around roots of young *H. annuus* due to a significantly lower root mass ratio in the pre-reproductive plants (F_{2,46}=6.89, P<0.01). Whether or not increased E_{night} represents a nutrient uptake benefit for pre-reproductive phase plants, it is possible that estimates of total water flux in mixed aged stands or integrated over the life of a crop are underestimated when based on a combination of E_{day} and E_{night} measured only on reproductive aged individuals.

**The contribution of g_{cuticular} to g_{night}**

Measures of g_{night} and E_{night} include cuticular and stomatal pathways in parallel, yet only water loss through stomata, at an aperture greater than maximal possible closure, may be subject to guard cell regulation. For all wild *Helianthus* species except for *H. deserticola*, g_{stomata} was five times greater than g_{cuticular}, suggesting that most nighttime water loss can be regulated. With the exception of the extremely high g_{cuticular} for *H. deserticola*, which deserves further investigation, the remaining g_{cuticular} for *Helianthus* were in the upper range of those reported in the literature using comparable techniques (Rawson and Clarke, 1988; Kerstiens, 1995; Boyer et al., 1997; Burghardt and Riederer, 2003; Nobel, 2005). More characterizations are needed of inter- and intra-specific variation in g_{cuticular}, including the extent to which growth conditions and atmospheric humidity can change g_{cuticular} components (Schreiber et al., 2000; Kerstiens et al., 2006; Kock et al., 2006).

**Variation among studies in magnitude of g_{night}**

Although our tests of g_{night} responses to nutrients and water occurred within each study, and cross study comparisons were not preplanned, the study differences in maximum g_{night} deserve some comments. For wild *H. annuus* in the nutrient and water manipulation studies, g_{night} of sufficiently-watered plants ranged from 0.04 to 0.12 mol m^{-2} s^{-1} (Figs. 1-2, Table S1). Because studies were conducted in different seasons and years, some of the variation may have been due to differences in the growth environment, and to VPD_{l} differences during the nights and days of gas exchange measurements. However, the study with the lowest g_{night} (Fall 2005-1) did not stand out as having the highest VPD_{l} on the night or accompanying day of gas exchange measurements, or an unusual VPD_{a} across the growth interval of the study. It is possible that using study means obscures a specific time interval where VPD_{a} affected leaf development and
maximum $g_{\text{night}}$, but there are many other potential contributing factors. We recommend more exploration of growth environment (temperature, humidity, CO$_2$ levels, light quantity and quality, plant nutritional status, growth medium, etc.) on leaf structure, stomatal density and size, cuticular properties, and maximum $g_{\text{night}}$ (Hetherington and Woodward, 2003; Bergman et al., 2006; Kock et al., 2006). Additionally, the effects of VPD$_a$ and VPD$_l$ prior to and during the gas exchange measurements deserve more attention (Franks and Farquhar, 1999; Schreiber et al., 2001).

Across multiple studies we demonstrate substantial $g_{\text{night}}$ and $E_{\text{night}}$ in Helianthus wild species and domesticates. For Helianthus, nighttime water loss occurs largely through stomata, and is regulated in response to plant water stress but not soil nutrient availability. Additionally, Helianthus $g_{\text{night}}$ varies nocturnally and across plant reproductive stages, but does not vary for individual leaves as they age. More research is needed to test the commonality of these findings in plants of various life histories and native to diverse habitats. Building generalities for variation and regulation of $g_{\text{night}}$ and $E_{\text{night}}$ is necessary for predicting the conditions under which nighttime water loss will be biologically significant.

Materials and Methods

The objectives were addressed in nine greenhouse studies carried out at the Biological Sciences Plant Growth Facility at the University of Georgia, Athens, GA (Table 1). The studies included four wild annual sunflower species (Helianthus annuus L., H. anomalus Blake, H. deserticola Heiser and H. petiolaris Nutt.), commercial H. annuus cv. Gray Stripe (referred to as H. annuus domesticate) and the Hopi domesticate of H. annuus (referred to as H. annuus Hopi).

Achenes of the four wild sunflower species were collected in Juab County, UT, USA, except for the H. annuus from Keith Country, NE, USA, used in the Fall 2003-2 and Fall 2004-2 studies, and the H. petiolaris collected in Washington County, UT, USA. The achenes of H. annuus domesticate used in Fall 2003-2 and Spring 2006 studies were obtained from Carolina Biological (Carolina Biological Supply Co., Burlington, NC USA). The achenes of H. annuus Hopi (PI 432504 NPGS Accession) used in Fall 2004-2 study were originally collected from Shungopovi Village, Hopi Indian Reservation, Navajo County, AZ, USA.

The wild Helianthus species and the H. annuus Hopi achenes were germinated in Petri dishes and transferred to pots after the seedlings developed root hairs. The H. annuus
domesticate achenes were sown directly into the study pots. The study pots (20-25 cm diameter) contained a mix of sand and Turface (fritted clay, Profile Products LLC, Buffalo Grove, IL, USA), except for the Fall 2003-1 and Fall 2003-2 studies that used all sand. All plants were grown in a greenhouse with natural daylight supplemented to 12 to 14 hours with metal-halide lamps. Temperatures were generally set to be at or above 26ºC (day) and 16ºC (night). For the six studies that had greenhouse weather available for the growth interval (Fall 2004-1, Fall 2004-b, Spring 2005, Summer 2005, Fall 2005-1 Spring 2006), the average night VPDa and day VPDa across studies (n=6) was 0.88 (SE=0.11) and 1.57 (SE=0.10) kPa, respectively.

**Nutrient and water treatments**

Nutrient treatments manipulated either total macro- and micronutrients (slow-release fertilizer, Osmocote Plus, Scotts-Sierra Horticultural Products Corp., Marysville OH, USA) or manipulated just nitrogen (available only as nitrate). The latter was achieved with thrice weekly applications of a modified Hoagland solution containing 140 or 7 µg mL⁻¹ nitrogen as nitrate. The sufficient and limited nitrate Hoagland solutions contained equal amounts potassium (176 µg mL⁻¹ K) and phosphorus (31 µg mL⁻¹ P). Additional macronutrients were calcium (50 µg mL⁻¹ in high; 10 µg mL⁻¹ in low), sulfur (8 µg mL⁻¹ in high; 120 µg mL⁻¹ in low) and magnesium (55 µg mL⁻¹ in high; 6 µg mL⁻¹ in low). Micronutrients included: Cl (0.443 µg mL⁻¹), B (0.068 µg mL⁻¹), Mn (0.027 µg mL⁻¹), Zn (0.033 µg mL⁻¹), Cu (0.008 µg mL⁻¹), Mo (0.012 µg mL⁻¹) and Fe (0.698 µg mL⁻¹ as FeEDTA). In the three studies without a nutrient treatment, the plants either received the high nitrate Hoagland solution or weekly application of 20:10:20 NPK soluble fertilizer (Peter’s Peat-Lite Special, Scotts-Sierra Horticultural Products Corp.).

The soil water treatments consisted of supplying plants with ample water to maintain soils near field capacity (sufficient), and limiting the soil water availability (limited) either just prior to gas exchange measures or as a sustained treatment throughout the study. The limitation of soil water availability prior to gas exchange measures consisted of withholding water until visual wilting and depression of daytime gas exchange rates were achieved. The sustained water limitation in the Fall 2003-2 study consisted of watering every four to five days, beginning two weeks after germination. For the Fall 2005-2 study leaf predawn xylem pressure potentials were sampled to accompany gas exchange measurements using a pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA).
Gas exchange procedures

Leaf level measurements of daytime and nighttime gas exchange were made with a portable photosynthesis system (LI-6400, LI-COR Inc., Lincoln, NE, USA). Measurements were made on a young fully expanded leaf of each plant, except when testing leaf age effects in the Spring 2005 study. The chamber light level was set to be 0 or 2000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) during the night and day, respectively. To view equipment and plants at night we used green safety headlamps with intensity not detectable by an LI-190 sensor (0 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) photosynthetic photon flux density (PPFD); LiCor, Lincoln, Nebraska, USA) to avoid promoting stomatal opening. During the Fall 2004 study and part of the Spring 2005 study, leaves of some species were too small for the standard chamber and an Arabidopsis (6400-15, LiCor Inc.) chamber was used. This chamber lacks an internal light source and daytime measurements were therefore only taken on sunny days when photosynthetically active radiation exceeded 1500 \( \mu \text{mol m}^{-2} \text{s}^{-1} \).

For both chambers air temperature was set to ambient and \( \text{CO}_2 \) was supplied at 400 \( \mu \text{mol mol}^{-1} \). Flow was set to 125 to 200 \( \mu \text{mol s}^{-1} \) at night and 700 \( \mu \text{mol s}^{-1} \) during the day. Chamber fan speed was set to high. To partially compensate for removal of the boundary layer due to the chamber mixing fan, chamber relative humidity was manually manipulated to a target 5-10% above ambient (assessed with open chamber). The standard chamber directly measures leaf temperature and before every set of measurements the leaf thermocouple was checked to ensure it was reading accurately to within 0.1 \( ^\circ \text{C} \). Sample and reference IRGAs were matched prior to every plant for nighttime measurements. Measurements were also made with an empty chamber or with dry paper in the chamber every four to six leaf measures to assess instrument error. Averaged by study, estimates of instrument error obtained with the standard or Arabidopsis chamber at night yielded values for \( g \) from 0.001 to 0.016 \( \text{mol m}^{-2} \text{s}^{-1} \), which was always substantially lower than plant measures. Plant measures were logged when readings were stable and typically within 1-2 minutes of clamping onto the leaf.

Whenever possible leaves were chosen that would fill the leaf chamber (6 \( \text{cm}^2 \) for standard chamber; 0.8 \( \text{cm}^2 \) for Arabidopsis chamber). When leaves that did not fill the chamber were used, all leaves in the measurement set (including those that filled the chamber) were marked before removal from the chamber to indicate placement of the chamber gaskets. The following day gas exchange leaves were cut to remove all area that was not inside the chamber and scanned (Winfolia, Regent Instruments Inc., Quebec, Canada) to determine area. Leaves that did not fill
the chamber were used in the Arabidopsis chamber in Fall 2004-1 (minimum area 0.45 cm\(^2\)) and in the standard chamber in Fall 2005-1 (minimum area 4.5 cm\(^2\)).

Daytime measurements were typically made between 9 am and 2 pm and nighttime measurements were typically made between 1 am and the beginning of astronomical twilight (sun 12\(^0\) below the horizon). Measures made at three times spaced through the night confirmed that this period captured maximum g\(_{\text{night}}\) but was well before a predawn stomatal opening would occur.

In the Fall 2005-2 study nighttime water loss was measured both instantaneously using the LI-6400 as well as gravimetrically. Gravimetric measures of transpiration made over a 24-hour time span were achieved by sealing the pot and root system in a bag, bagging all flower heads and weighing at the beginning and end of the day and night periods. To obtain water loss per area, all leaves were harvested the following day and total leaf area was measured using a LI-3100 leaf area meter (LiCor, Lincoln, Nebraska, USA).

**Assessment of cuticular water loss**

Cuticular conductance (g\(_{\text{cuticular}}\)) was defined functionally as conductance through the cuticle and stomata at maximum closure induced by either leaf wilting (water stress) or exogenous ABA application. As such it includes both water loss through the cuticle and water loss through stomata at minimum aperture. The conductance provided by the LI-6400 (g\(_{\text{night}}\) or g\(_{\text{day}}\) in this study) is a total of both g\(_{\text{cuticular}}\) and g\(_{\text{stomata}}\) in parallel. Stomatal conductance at night was calculated as g\(_{\text{night}}\) minus g\(_{\text{cuticular}}\) (Nobel, 2005).

Cuticular water loss for excised, wilted leaves, was estimated both by weighing (Rawson and Clarke, 1988) and by gas exchange measurements with the LI-6400. For weighing, excised leaves (cut end of petiole sealed with wax) were allowed to dry and wilt in the dark at ambient room temperature and VPD. Weights were taken approximately every 15 minutes and, after initial rapid loss of water during which time stomata presumably closed, the linear relationship of water loss and time was used to estimate E\(_{\text{cuticular}}\). During this period of linear water loss, g\(_{\text{cuticular}}\) and E\(_{\text{cuticular}}\) were also measured with the LI-6400 set to match ambient temperature and VPD. In the Fall 2004-1 study, E\(_{\text{cuticular}}\) from these methods, including all four species of wild *Helianthus*, were highly correlated (r\(^2\) = 0.939, P<0.0001, n=24). Thus, only the instantaneous LI-6400 measurements of g\(_{\text{cuticular}}\) and E\(_{\text{cuticular}}\) are reported.
Cuticular conductance and transpiration were also measured on leaves for which stomatal closure had been induced by exogenous ABA application. ABA was fed into the xylem sap of sufficiently-watered plants (Borel et al., 2001). Funnels were sealed around the stems of treatment plants and filled at predawn with degassed ABA solution (1.6 mol m\(^{-3}\) synthetic (±)-ABA, 0.4 mol m\(^{-3}\) Ca(NO\(_3\))\(_2\) and 2.0 mol m\(^{-3}\) KH\(_2\)PO\(_4\)). Stems were drilled radially below the surface of the solution. Funnels were covered with silver foil and the solution was topped off as needed during the following day and night to ensure the drill hole was always below the surface of the solution. Plants were watered amply throughout the period of experimentation and gas exchange measures were made on the first leaf above the infusion point.

**Leaf tissue analysis**

In most of the nutrient treatment studies, leaves used for gas exchange were collected after measurement, dried, ground, and analyzed for nitrogen content (Carbo Era NA 1500 CN analyzer, Milan, Italy). When a factorial design of water and nutrient treatments was present only the plants in the high water treatment were analyzed for leaf nitrogen. In the Fall 2004-1 study, gas exchange measurements were made on two dates per plant and these two leaves were combined for analysis of nitrogen content.

**Biomass measures**

Plants were generally harvested after reaching reproductive maturity and when plants began to show shoot senescence. Plants in the Fall 2003-2, Fall 2005-2 and younger age classes in Fall 2005-1 studies were harvested before or shortly after the appearance of first flower. Plant shoots were divided into vegetative and reproductive components, dried at 60°C, and weighed.

**Experimental design and statistical analysis**

Experiments were either complete randomized block designs or completely randomized (Table 1). When gas exchange measurements were made across several days and nights, plants were grouped by block so that random effects due to night of measurement (e.g. VPD\(_a\)) were accounted for by the block effect. Measurements of different species or treatments made in one night and block were randomized to avoid confounding treatment results with effects of circadian rhythm or changing VPD though the night.
Most data were analyzed using a mixed-model ANOVA, with block treated as a random effect (PROC MIXED; SAS, 2004) or with a general linear model ANOVA when blocking was not present (Fall 2005-2 study; PROC GLM; SAS, 2004). In some cases, plant death, outliers, or difficulties with treatment application (e.g. ABA application, Spring 2006) resulted in an unbalanced design. When additional tests only involved two levels of a single variable, paired or independent t-tests were used as appropriate. The Fall 2004-1 and Fall 2005-2 studies included repeated gas exchange measures during a 24-hour period and these data were analyzed in a repeated-measurement mixed model in PROC MIXED with an unstructured covariance matrix. In all analyses variables were log transformed when necessary to approach model assumptions of normality of residuals and homogeneity of variance.

**Supplemental Material**

Table S1 provides a summary of means, and statistical results for results summarized in text for $g_{\text{night}}$, $E_{\text{night}}$, $g_{\text{day}}$, $E_{\text{day}}$, and photosynthesis.
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Table 1: Overview of nine studies including *Helianthus* species, water and nutrient treatments, and experimental design. The *H. annuus* was wild, except where designated: *H. annuus* dom. is a commercial domesticate and *H. annuus* Hopi is an early domesticate. See methods for composition of high and low nitrate modified Hoagland solution. For experimental design, RCBD is randomized complete block and CR is completely randomized.

| Study          | Species                  | Nutrient treatment | Water stress treatment | Additional tests                                | Experimental design                                                                 |
|----------------|--------------------------|--------------------|------------------------|----------------------------------------------|-------------------------------------------------------------------------------------|
| **Fall 2003-1**| *H. annuus*, *H. anomalus*, *H. deserticola*, *H. petiolaris* | Yes: 40g or 4g Osmocote | No                     | ---                                         | RCBD: 4 species * 2 NPK trts * 3 blocks * 3 replicates = 72 plants                  |
|                |                          |                    |                        |                                              | (gas exchange measures taken on each species separately)                           |
| **Fall 2003-2**| *H. annuus* dom.         | Yes: Hydrosol, then 10 or 1 g Osmocote | Yes: sustained         | ---                                         | RCBD: 2 NPK trts * 2 water trts * 3 blocks * 4 replicates = 48 plants              |
| **Fall 2004-1**| *H. annuus*, *H. anomalus*, *H. deserticola*, *H. petiolaris* | Yes: 140 or 7 µg mL\(^{-1}\) N (as nitrate) Hoagland | No                     | Species Cuticular - wilting Nocturnal time course | RCBD: 4 species * 2 nitrogen trts * 3 blocks * 3 replicates =72 plants.             |
| **Fall 2004-2**| *H. annuus*, *H. annuus* Hopi | No: 20:10:20 NPK soluble fertilizer | Yes: before measurements | Accession (wild vs. Hopi)                     | RCBD: 2 accessions * 2 water trts * 3 blocks * 3 replicates = 36 plants.          |
| **Spring 2005**| *H. annuus*               | Yes: 140 or 7 µg mL\(^{-1}\) N (as nitrate) Hoagland | No                     | Leaf age Cuticular - wilting                 | RCBD: 2 nitrogen trts * 3 blocks * 3 replicates = 18 plants.                      |
| **Summer 2005**| *H. annuus*               | Yes: 140 or 7 µg mL\(^{-1}\) N (as nitrate) Hoagland | Yes: before measurements | ---                                         | RCBD: 2 nitrogen trts * 2 water trts * 3 blocks * 3 replicates = 36 plants.       |
| **Fall 2005-1**| *H. annuus*               | Yes: 140 or 7 µg mL\(^{-1}\) N (as nitrate) Hoagland | No                     | Plant age Leaf age                           | RCBD: 3 ages * 2 nitrogen trts * 3 blocks * 3 replicates = 54 plants.             |
| **Fall 2005-2**| *H. annuus*               | No: 140 µg mL\(^{-1}\) N (as nitrate) Hoagland | Yes: before measurements | 24-hour time course Gravimetric E            | CR: 2 water trts * 13-14 replicates (10 for gas exchange & 3-4 for xylem pressure potential) = 27 plants |
| **Spring 2006**| *H. annuus*, *H. annuus* dom. | No: 140 µg mL\(^{-1}\) N (as nitrate) Hoagland | No                     | Cuticular - ABA                             | CR: 2 accessions * 2 trts (ABA or control) * 3-7 replicates = 19 plants          |
Table 2: Vegetative shoot biomass at harvest and total leaf nitrogen (N) content of gas exchange leaves for studies that included a nutrient limitation treatment. If no treatment is designated (i.e. “---”) then all plants in that study received sufficient levels of that resource. Values are lsmeans ± 1 SE. F-values and associated degrees of freedom (F df num, df denom.) are presented for each model effect (PROC MIXED ANOVA, block as random). F-values in bold indicate statistical significance (* P<0.05, ** P<0.01, *** P<0.001).

| Study and species | Nutrient treatment | Water treatment | shoot (g) | N (mg g⁻¹) for gas exchange leaf |
|-------------------|--------------------|----------------|----------|--------------------------------|
| **Fall 2003-1**   |                    |                |          |                                |
| *H. annuus*       | sufficient         | ---            | 7.3 ± 3.3| 5.54 ± 0.39                     |
|                   | limited            | ---            | 1.8 ± 3.3| 4.17 ± 0.41                     |
|                   | **Nutrient effect**|                | 29.57    | 26.57                           |
|                   |                    |                | 1, 14*** | 1, 6**                          |
| *H. anomalus*     | sufficient         | ---            | 14.5 ± 3.0| 4.30 ± 0.22                     |
|                   | limited            | ---            | 5.1 ± 3.0| 4.13 ± 0.22                     |
|                   | **Nutrient effect**|                | 10.46    | 1.24                           |
|                   |                    |                | 1, 14**  | 1, 6                           |
| *H. deserticola*  | sufficient         | ---            | 15.4 ± 3.3| Not assessed                    |
|                   | limited            | ---            | 3.3 ± 3.9| Not assessed                    |
|                   | **Nutrient effect**|                | 5.6      | Not assessed                    |
|                   |                    |                | 1, 8*    |                                |
| *H. petiolaris*   | sufficient         | ---            | 10.4 ± 2.9| 5.85 ± 0.36                     |
|                   | limited            | ---            | 4.7 ± 2.9| 4.89 ± 0.32                     |
|                   | **Nutrient effect**|                | 7.01     | 4.10                           |
|                   |                    |                | 1, 12*   | 1, 5                           |
| **Fall 2003-2**   |                    |                |          |                                |
| *H. annuus dom.*  | sufficient         | sufficient     | 8.0 ± 0.4| Not assessed                    |
|                   | limited            | sufficient     | 4.2 ± 0.4| Not assessed                    |
|                   | sufficient         | limited        | 2.8 ± 0.4| Not assessed                    |
|                   | limited            | limited        | 1.7 ± 0.4| Not assessed                    |
|                   | **Water effect**   |                | 78.33    | 30.69                           |
|                   |                    |                | 1, 42*** | 1, 42***                        |
|                   | **Nutrient effect**|                | 9.83     | 9.83                           |
|                   |                    |                | 1, 42**  | 1, 42**                         |
| **Fall 2004-1**   |                    |                |          |                                |
| *H. annuus*       | sufficient         | ---            | 4.5 -1.0, +1.2| 2.86 ± 0.23                     |
|                   | limited            | ---            | 1.6 -0.3, +0.4| 2.82 ± 0.223                    |
| *H. anomalus*     | sufficient         | ---            | 7.3 -1.6, +2.1| 3.70 ± 0.24                     |
|                   | limited            | ---            | 1.0 -0.3, +0.3| 3.44 ± 0.26                     |
| *H. deserticola*  | sufficient         | ---            | 7.8 -1.7, +2.2| 3.53 ± 0.24                     |
|                   | limited            | ---            | 2.9 -0.6, +0.8| 3.19 ± 0.23                     |
| *H. petiolaris*   | sufficient         | ---            | 7.9 -1.7, +2.1| 4.17 ± 0.23                     |
|                   | limited            | ---            | 1.2 -0.3, +0.4| 3.44 ± 0.26                     |
|                   | **Nitrate effect** |                | 70.56    | 6.78                            |
|                   |                    |                | 1, 56*** | 1, 56*                          |
|                   | **Species effect** |                | 2.45     | 10.16                           |
|                   |                    |                | 3, 56    | 3, 56***                        |
|                   | **Nitrate*species**|                | 2.38     | 1.21                            |
|                   |                    |                | 3, 56    | 3, 56                           |
| **Spring 2005**   |                    |                |          |                                |
| *H. annuus*       | sufficient         | ---            | 81.7 -5.5, +5.9| Not assessed                    |
|                   | limited            | ---            | 7.8 -0.5, +0.6| Not assessed                    |
|                   | **Nitrate effect** |                | 561.23   | Not assessed                    |
|                   |                    |                | 1, 13*** |                                |
### Summer 2005

**H. annuus**

| Condition       | Age     | Water effect | Nitrate effect | Water*nitrate |
|-----------------|---------|--------------|----------------|---------------|
| sufficient      | 15.5 wk | 114.0 ±10.5, +11.6 | 3.83 ± 0.10 | -             |
| sufficient      | 10 wk   | 71.6 ±6.3, +6.8   | 2.17 ± 0.10 | Not assessed  |
| limited         | 15.5 wk | 6.2 ±0.6, +0.6    | 3.83 ± 0.10 | -             |
| limited         | 10 wk   | 4.6 ±0.4, +0.4    | 2.17 ± 0.10 | Not assessed  |

### Fall 2005-1

**H. annuus**

| Condition       | Age     | Water effect | Nitrate effect | Water*nitrate |
|-----------------|---------|--------------|----------------|---------------|
| sufficient;     | 15.5 wk | 87.2 ±16.6, +20.5 | 2.75 ± 0.18 | -             |
| sufficient;     | 10 wk   | 10.2 ±2.0, +2.5  | 3.87 ± 0.18 | -             |
| sufficient;     | 5.5 wk  | 0.6 ± 0.1     | 5.17 ± 0.18 | -             |
| limited; 15.5   | wk age  | 7.6 ±1.4, +1.8 | 1.82 ± 0.18 | -             |
| limited; 10 wk  | wk age  | 1.3 ±0.2, +0.3 | 2.81 ± 0.18 | -             |
| limited; 5.5 wk | wk age  | 0.3 ± 0.1     | 4.27 ± 0.18 | -             |

**Nitrate effect**

|                |            | Water | Nitrate | Water*nitrate |
|----------------|-------------|-------|---------|---------------|
|                |             | 1.62  | 2065.79 | 130.65        |

**Plant age effect**

|                |            | Water | Nitrate | Water*nitrate |
|----------------|-------------|-------|---------|---------------|
|                |             | 1.62  | 2065.79 | 130.65        |

**Nitrate*plant age**

|                |            | Water | Nitrate | Water*nitrate |
|----------------|-------------|-------|---------|---------------|
|                |             | 1.62  | 2065.79 | 130.65        |
Figure 1: Effect of manipulating soil nutrient availability on nighttime leaf conductance ($g_{\text{night}}$) showing all of the tests for wild Helianthus annuus. In Fall 2003-1 availability of all macro- and micronutrients were manipulated, whereas only nitrogen, available as nitrate, was manipulated in the additional four studies. Bars are lsmeans ± 1 SE. See Table S1 for nutrient treatment comparisons for H. annuus domesticate, H. annuus Hopi, and other Helianthus species.

Figure 2: Effect of manipulation soil water availability on nighttime leaf conductance ($g_{\text{night}}$) during Fall 2003-2 (A), Fall 2004-2 (B), Summer 2005 (C) and Fall 2005-2 (D). In studies where both a water and nutrient treatment were applied (A, C) bars represent data from the high nutrient treatment only. Bars are lsmeans ± 1 SE. $g_{\text{cuticular}}$ for H. annuus and H. annuus dom., measured in Fall 2004-1, Spring 2005 and Spring 2006, ranged from 0.013 to 0.023 mol m$^{-2}$ s$^{-1}$.

Figure 3: Variation in Helianthus annuus nighttime leaf conductance ($g_{\text{night}}$) and transpiration ($E_{\text{night}}$) across a single night during Fall 2004-1 (A) and Fall 2005-2 (B) studies. Included are independent measurements of atmospheric VPD (VPD air). Fall 2005-2 included measurements of xylem pressure potential (C) made on separate, randomly chosen plants from each treatment level. Points represent means ± 1 SE, n=5-6 for $g_{\text{night}}$ and $E_{\text{night}}$, and n=3-4 for xylem pressure potential. $g_{\text{cuticular}}$ for H. annuus, measured in Fall 2004-1, Spring 2005 and Spring 2006, ranged from 0.013 to 0.023 mol m$^{-2}$ s$^{-1}$.

Figure 4: Effect of plant reproductive stage on nighttime leaf conductance ($g_{\text{night}}$) (A) and transpiration ($E_{\text{night}}$) (B) in Helianthus annuus during the Fall 2005-1 study. Measurements were made on most recently fully mature leaves produced concurrently. Five and a half week old plants were pre-reproductive while 10 and 15.5 week old plants were both reproductive. Bars are lsmeans ± 1 SE, n=8-9.

Figure 5: Instantaneous measures of nighttime leaf conductance ($g_{\text{night}}$) and cuticular conductance ($g_{\text{cuticular}}$), functionally defined as conductance though both the cuticle and stomata at maximal closure. Measures of $g_{\text{cuticular}}$ were made on excised, wilted leaves during the Fall 2004-1 study (A) and Spring 2005 study (B) and on intact leaves of plants infused with exogenous ABA during the Spring 2006 study (C). Bars are means ± 1 SE, n=5-6 bulked across nitrate treatment.
in Fall 2004-1, n=9 bulked across nitrate treatment in Spring 2005, and n=3-7 high nitrate treated plants during Spring 2006. Measures were made on different leaves of the same plant in Fall 2004-1 and Spring 2005 and made on separate control or ABA treatment plants during one night during Spring 2006. t-values and associated degrees of freedom are presented from a paired t-test in Fall 2004-1 and Spring 2005, and from an independent t-test in Summer 2006 (* P<0.05, ** P<0.01, *** P<0.001).
