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# Impacts of Manipulating Photoperiod on Circadian Rhythms of Agave Photosynthesis

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Honors Thesis

# IMPACTS OF MANIPULATING PHOTOPERIOD ON CIRCADIAN RHYTHMS OF AGAVE PHOTOSYNTHESIS

by
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ABSTRACT


# IMPACTS OF MANIPULATING PHOTOPERIOD ON CIRCADIAN RHYTHMS OF *AGAVE* PHOTOSYNTHESIS


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Bachelor of Science


*Agave* uses photoperiod, among other environmental conditions, to time its circadian rhythm of photosynthesis. Two species of *Agave* (*A. americana* and *A. murpheyi*) were tested under two extreme photoperiod conditions (constant light and constant dark) against a normal photoperiod control (12 hours light, 12 hours dark) to identify the impact of abnormal photoperiod on circadian rhythms of *Agave* photosynthesis. Under constant light conditions, normal oscillations in $CO_2$ gas exchange became unpredictable in duration and amplitude in both species and became very infrequent in *A. murpheyi.* Under constant dark conditions, net $CO_2$ uptake stabilized at a negative value and oscillations ceased in both species. Under a normal photoperiod, net $CO_2$ uptake mostly occurred during the second half of the day (phase IV) rather than at night. High nighttime temperatures (22 °C) prevented nighttime $CO_2$ uptake. This is likely an adaptation to prevent evapotranspiration through open stomates.

# ACKNOWLEDGEMENTS

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## Introduction

Hotter, dryer weather is predicted in coming years in the southwestern United States[1]. This climatic shift will alter agricultural yields in the region, requiring new methods and alternative crops. Traditional $C_3$ and $C_4$ crops are less water-use efficient than many crassulacean acid metabolism (CAM) plants. While $C_3$ and $C_4$ plants open their stomata and fix $CO_2$ during the daytime, CAM plants do the opposite, opening their stomata to receive and fix $CO_2$ mainly during the night. The $CO_2$ received during the night is stored in organic acids (such as malic acid) until daytime, when the organic acids are broken down to release the $CO_2$ to fuel the Calvin cycle. At nightfall, the Calvin cycle halts, and the cycle repeats itself. The stomata open and $CO_2$ is received and stored for the upcoming day. This inverted cycle allows CAM plants to significantly reduce both photorespiration and water loss, especially in hot, dry climates[2]. Therefore, CAM plants have been suggested as high-yield crops in arid, marginal lands[3].

*Agave* is one of the most promising CAM crops due to its high yield, drought-tolerance, and many uses[3,4]. Although *Agave* was cultivated by indigenous peoples in the arid U.S. Southwest[5], modern large-scale methods for *Agave* cultivation in the arid U.S. Southwest with minimal irrigation have not been well developed. To develop *Agave* as an efficient means of generating biomass in marginal lands, it is important to understand how novel environmental conditions alter its physiology. Farther from the equator, the ratio of light to dark becomes uneven during the winter and summer months. As new locations for *Agave* cultivation are proposed, it will be important to understand the effect of altered periods of light and dark on its photosynthetic rhythms. How will *Agave*

behave in these new environments? Specifically, how does differing photoperiod alter the CAM cycle of photosynthesis in *Agave*?

As an obligate CAM plant, *Agave* maintains the inverted photosynthetic cycle as previously described[6]. This cycle is maintained and regulated by both external factors, such as temperature[7], water, and light[8,9], and internal circadian rhythms in gene transcripts, proteins, and metabolites[10]. We hope to extend knowledge of how light, temperature, and water affect the photosynthetic rate of *Agave americana* and *Agave murpheyi*. This project has two parts. First, to investigate the effect of extreme light conditions on the diel cycle of photosynthesis, and second, to quantify the effect of water, temperature, and light on photosynthetic rate. Models have been created to generate environmental productivity indices (EPI) from environmental conditions for *A. fourcroydes*[8] and *A. tequilana*[11]. We hope to generate a similar preliminary model for *A. murpheyi and A. americana* that predicts photosynthetic rate.

We suspect that photoperiod has an important regulating effect on the diel cycle of photosynthesis in both *A. murpheyi* and *A. americana.* Under constant light conditions, we expect both species to maintain circadian oscillations of photosynthesis, but with an elongated period. Under constant dark conditions, we expect both species to decrease photosynthetic rate and eventually reach a steady, dormant state with no photosynthesis. Under conditions with a normal 12-hour photoperiod, we expect both species to maintain a pattern of CAM photosynthesis in which net $CO_2$ uptake primarily occurs during the dark period and net $CO_2$ uptake during the light period is minimal. If our hypotheses are correct, then we can conclude that photoperiod is necessary for resetting and adjusting the internal circadian clock in *A. murpheyi* and *A. americana* to match the photoperiod. If no

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oscillations in photosynthetic rate are observed in either constant light or dark conditions immediately after being placed under such conditions, we can conclude that the circadian clock that maintains the diel cycle of photosynthesis does not function without a steady photoperiod.

## Materials and Methods

### Extreme Light Conditions

In this investigation, we aimed to determine the effect of extreme light conditions on the diel cycle of photosynthesis. Two species, *A. murpheyi* and *A. americana,* two extreme light conditions, constant light (LL) and constant dark (DD), and a control light condition with a 12-hour photoperiod (LD), were investigated. Two replicates were used for each treatment combination, as well as the control treatment, resulting in a total of 12 plants. Three chambers were created inside the lab to house plants in each light condition, which were designated LL treatment, DD treatment, and LD control. Photosynthetic photon flux density or PPFD (Table 1) and spectral data (Fig. 1) were measured within each chamber using a LI-COR LI-180 spectrometer. Measurements were taken on the left-hand side of the chamber, in the middle, and on the right, both at bucket height (approximately 3 inches from the base of the plant and 10 inches from the chamber floor) and crown height (approximately 12 inches from the base of the plant and 19 inches from the chamber floor) resulting in a total of 6 measurements per chamber, which were then averaged together.
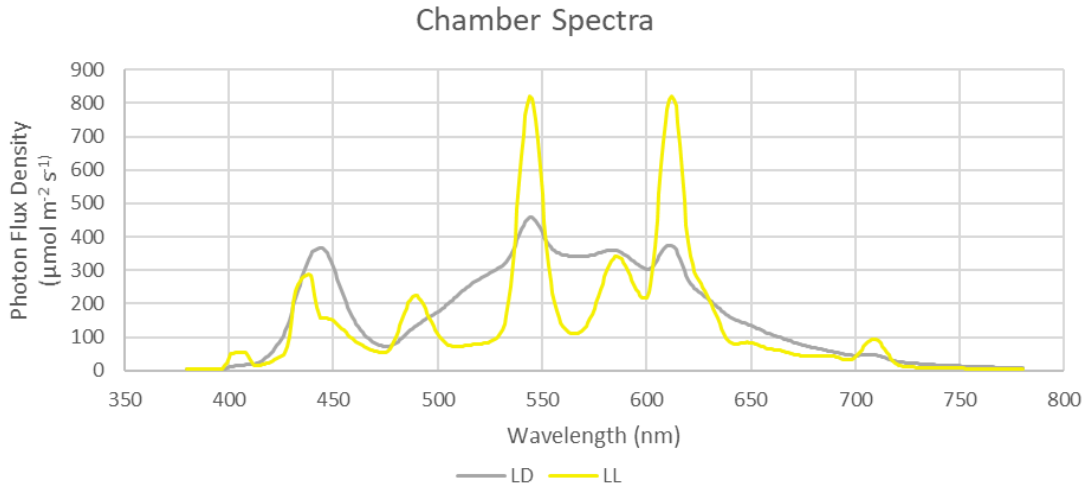
**Figure** 1. Average spectral data for the LD control and LL treatment chambers.

## DD Treatment

The DD treatment chamber was positioned directly above the LL chamber and was covered by a reflective cloth. To minimize the heating effect of the lights from the LL chamber and to provide fresh air to maintain constant levels of $CO_2$ in the chamber, two fans were installed in opposite corners of the DD treatment chamber. Nonetheless, lights from the LL chamber heated the DD chamber to a constant air temperature of 27 °C.

## LL Treatment

The LL chamber was surrounded on three sides by thin reflective material. The front of the chamber was partially covered by another sheet of reflective material, but gaps were left to allow for air flow. Eight 48-inch Sylvania 32-Watt neutral white fluorescent bulbs with a color temperature of 3500 Kelvin were placed on the ceiling of the chamber. Four 12-inch Intertek LED bars were also hung in front of the chamber. This combination of lights supplied an average PPFD of $240 \pm 10$ µmol m$^{-2}$ s$^{-1}$ at the height of the pots, and $272 \pm 10$ µmol m$^{-2}$ s$^{-1}$ at the crown height of the plants.

## LD Treatment

The LD chamber was on a lab bench separated from the LL and DD treatment chambers. Three 48-inch and four 12-inch Intertek LED bars were suspended from the ceiling. These lights provided an average PPFD of $250 \pm 16$ µmol m$^{-2}$ s$^{-1}$ at the height of the pots and $339 \pm 17$ µmol m$^{-2}$ s$^{-1}$ at the crown height of the plants. Lights were set to turn on at 9:00 and off at 21:00 to provide a 24-hour cycle of 12 hours of light and 12 hours of darkness.

## Plants

Young *A. murpheyi* and *A. americana* were potted in sandy-loam soil. Plants were randomly assigned to either the LL, DD, or LD chambers. Plants were then adjusted to lab conditions by being placed in the LD control chamber for ten days. During adjustment, as well as treatment, plants were watered to saturation once a week. At the end of ten days, plants assigned to the LL or DD treatments were moved to their respective chambers. Immediately after entry into their respective chambers, photosynthetic rate over a period of at least 24 hours was estimated by measuring net $CO_2$ uptake in one plant from each treatment combination. The air temperature, PPFD, and dimensions of the chambers are shown in Table 1. The average spectra supplied by the lights in the LD control and LL treatment chambers are displayed in Figure 1.

**Table 1.** Air temperature, PPFD at the height of the pot and the height of the crown of the plant, and dimensions of the LD control, DD treatment, and LL treatment chambers. The LD control chamber is separated into two rows, one displaying conditions during the light hours, and the other displaying conditions during the dark hours.

| Chamber | Air Temperature | PPFD at pot height | PPFD at crown height | Dimensions (L x W x H) |
|---|---|---|---|---|
| **LD Control (Light Hours)** | 24.6 | $250 \pm 16$ | $339 \pm 17$ | 92″ x 30″ x 30″ |
| **LD Control (Dark Hours)** | 21.8 | Less than 0.1 | Less than 0.1 | 92″ x 30″ x 30″ |
| **DD Treatment** | 27.2 | Less than 0.1 | Less than 0.1 | 48″ x 24″ x 24″ |
| **LL Treatment** | 24.2 | $240 \pm 10$ | $272 \pm 10$ | 48″ x 24″ x 29″ |

Net $CO_2$ Uptake

We used a portable real-time infrared gas analyzer and $CO_2$ gas exchange system (LI-6400XT Portable Photosynthesis System, LI-COR, Lincoln, NE, USA). A 2 cm x 3 cm cuvette (part no. 6400-02B) LED light source with a thick gasket (Li-Cor part no. 6400-37 thick gasket) was installed to enable proper sealing on the thick, curved *Agave* leaves during measurements. The measurements were made at a point on the leaf approximately two thirds the distance from the base. [12] The LI-6400XT was programmed to automatically record measurements using the AutoLog2 function (LICOR Biosciences, 2013). The equipment was set to record measurements every 30 seconds and averaging values in ten minutes intervals throughout night. In addition, the equipment was programmed to adjust changes in $CO_2$ reference and $CO_2$ samples, internal H2O reference and samples. The equipment used the function "match IRGAs" with a frequency of 30 minutes through the night, adjusting differential pressure changes between the IRGA and internal LI-6400XT computer.

Leaf Temperature

After four months of adjustment to the conditions within their respective chambers, the leaf temperature of each plant was measured using a handheld thermal camera, FLIR E5 Wifi (Teledyne FLIR LLC, Wilsonville, OR, USA). Images were taken every 4 hours for 24 hours, totaling 6 images per plant. This was repeated three times. To take images of the plants, they were temporarily removed from their chambers and placed on the floor of the lab. Images were taken within 1 minute of removal from their respective chamber at a height of approximately 12 in. above the crown height of the plant. The lights of the lab were turned on while plants that were under light conditions were imaged and turned off while plants that were under dark conditions were imaged. To minimize light while imaging plants that were under dark conditions, plants that were taken from the DD treatment chamber or the LD control chamber during the dark period were placed in a cardboard cylinder with a diameter of 24 in. and a height of 36 in.. After plants were placed in the cylinder, they were covered with a 30 in. square sheet of ¼ in. thick foil insulation, with a 3 in. diameter hole cut in the center. Images were then taken through this hole. Images were then analyzed using FLIR Tools software. Line measurements were taken along the center of four leaves of each plant (Fig. 2). The average of each line measurement was used to estimate the temperature of each leaf. The leaf temperature of each plant was estimated by taking the average of the four leaves' estimated temperatures. The average leaf temperatures of *A. murpheyi* and *A. americana* in each of the three chambers are shown in Figure 7
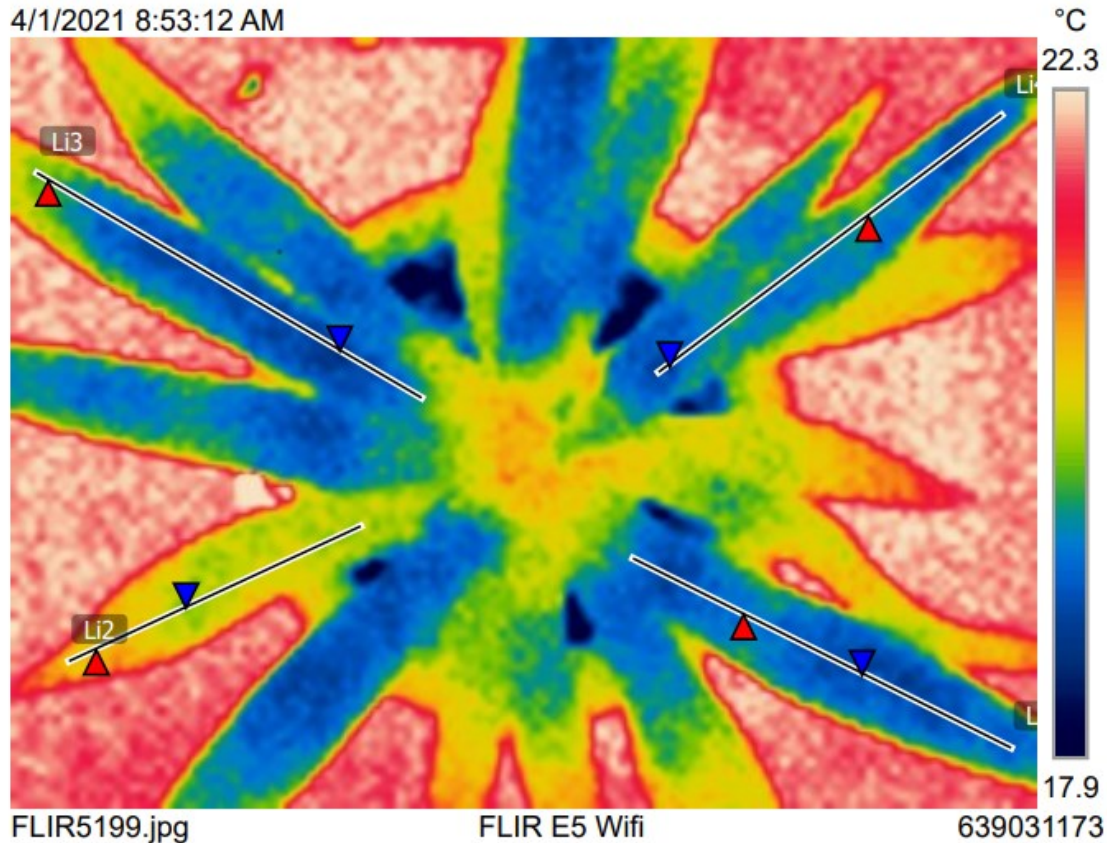
**Figure 2.** Example of a FLIR thermal image with line measurements taken on four leaves. This is an image of A. murpheyi taken within a cardboard cylinder with a foil insulation cover.

## Results

### Photosynthetic Rate

The net $CO_2$ uptake of plants in the LD control, LL treatment, and DD treatment chambers are displayed in Figure 3, Figure 4, and Figure 5, respectively.

### LD Control

In the control LD chamber, both *A. murpheyi* and *A. americana* exhibited two distinct peaks in net $CO_2$ uptake. The most pronounced peak occurred in phase IV, which started earlier than expected (around 14:00-21:00 in *A. murpheyi* and around 16:00-21:00 in *A. americana*). Both species also exhibited a wide sloping peak throughout the night in

phase I (22:00-8:00). The average net $CO_2$ uptake within phase I and phase IV is shown in Table 2. Variation between replicate measurements was greater in phase I than in phase IV for both *A. murpheyi and A. americana.* Average net $CO_2$ assimilation was greater, and variation between replicates was less in *A. murpheyi* than in *A. americana* in both phases. A majority of net $CO_2$ uptake occurred in phase IV, during the light period. This is unusual for *Agave,* which is generally considered an obligate CAM plant[6]. A pattern of net $CO_2$ uptake with peaks in phases I and IV has been observed in *A. americana*[13]. and *A. tequilana*[11]. under lab conditions. However, in such measurements, most net $CO_2$ uptake occurred in phase I, during the dark period, rather than in phase IV. The reversed pattern of $CO_2$ photosynthesis in our experiment may have been caused by warm temperatures during the dark period in our LD control chamber. In the experiments by Nobel et al.[11] and Niechayev et al.[13], daytime temperature was 30 °C and 27 °C respectively and nighttime temperature for both experiments was 15 °C. This is a difference of 12 to 15 °C. In contrast, the difference between daytime and nighttime temperatures in our experiment was only 3 °C. Daytime temperature was around 25 °C and nighttime temperature was around 22 °C. Nighttime net $CO_2$ uptake is known to decrease when nighttime temperatures are warm (25 °C or higher) in *A. americana*[7] and *A. Lechuguilla.*[14]
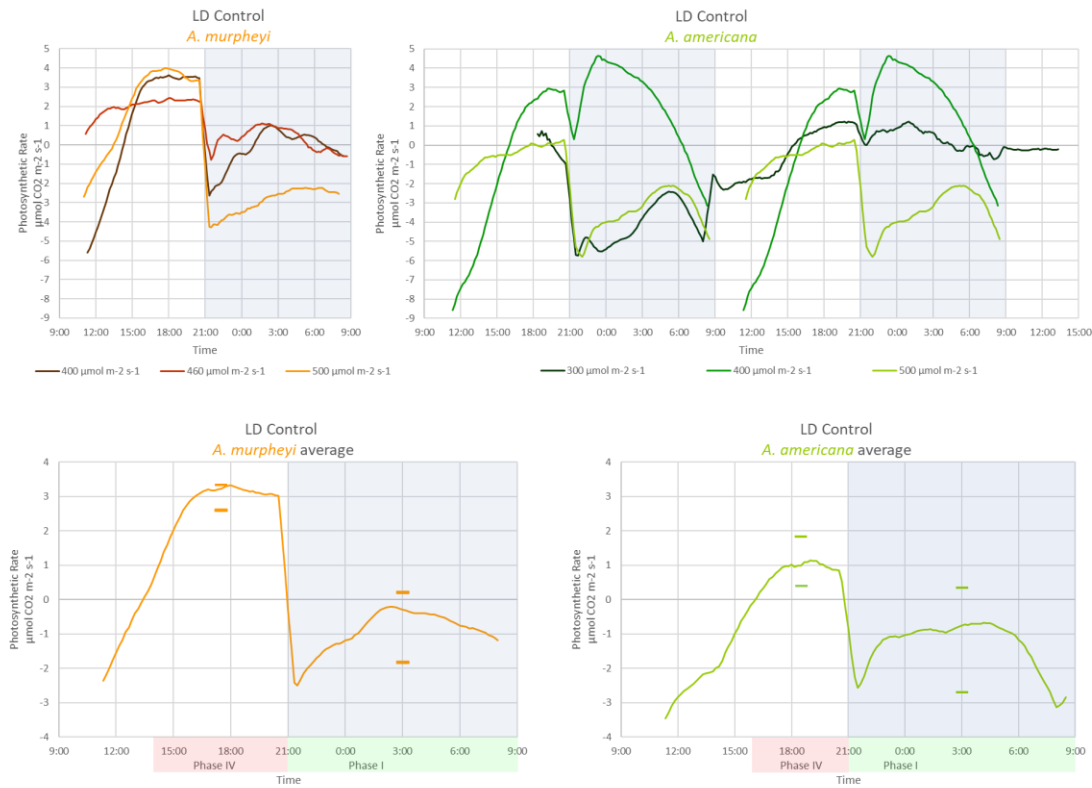
**Figure 3.** Net $CO_2$ uptake of A. murpheyi and A. americana under LD control conditions. Periods of dark are shaded blue, and periods of light are left white. Photosynthetic rate is estimated by net $CO_2$ uptake in µmol $CO_2$ m-2 s-1.
**Upper Graphs**: The moving average (n=5) of three separate measurements for each species. These measurements were each taken with a slightly different PAR within the LICOR chamber. For A. americana, the measurement taken at 300 µmol m-2 s-1 PAR (dark green) lasted for nearly two complete LD cycles. On the other hand, measurements taken at 400 and 500 µmol m-2 s-1 (green and light green) lasted only one LD cycle. Therefore, measurements taken at 400 and 500 µmol m-2 s-1 (green and light green) are shown twice, once in the first LD cycle and a second time in the second LD cycle to show how they can align with either LD cycle of the measurement taken at 300 µmol m-2 s-1.
**Lower Graphs**: The moving average (n=5) of all replicates of A. murpheyi and A. americana at time points where at least three measurements were taken. Error bars indicate the SEM of the photosynthetic rate within either phase I (green) or phase IV (red). The SEM was calculated by first averaging the photosynthetic rate within each phase for each individual measurement. Then, the average and SEM of these averages was calculated for each phase (Table 1).

**Table 2.** Average photosynthetic rate, estimated by net $CO_2$ (µmol $CO_2$ m-2 s-1) within phase I and phase IV for A. murpheyi and A. americana. First, net $CO_2$ uptake within each phase was estimated for each replicate measurement by calculating the average net $CO_2$ uptake within the corresponding time range. For phase I, the average net $CO_2$ uptake was calculated between 22:00 and 24:00 for both species. For phase IV, the average net $CO_2$ uptake was calculated between 14:30 and 20:30 for A. murpheyi, and between 16:30 and 20:30 for A. americana. The average and SEM of the replicates for each phase and species are displayed below.

| Species | Phase I | Phase IV |
|---|---|---|
| *A. americana* | -1.18 ± 1.52 | 1.12 ± 0.70 |
| *A. murpheyi* | -0.81 ± 1.02 | 2.96 ± 0.36 |

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After being placed in the LL treatment chamber, net $CO_2$ assimilation decreased in *A. murpheyi* and remained stable in *A. americana* until 9:00 when the lights would have turned on in the LD control chamber. Then, net $CO_2$ assimilation increased in both species, until peaking sharply at 21:00, where the lights would have turned back off. After this peak in $CO_2$ assimilation at 21:00, $CO_2$ assimilation again decreased in *A. murpheyi* and remained stable in *A. americana.* The peaks around 21:00 are possibly remnants of the phase IV peak observed in the LD control measurements. Even after 36 hours of constant conditions, including intense light (1000 μmol $m^{-2}$ $s^{-1}$ within the LICOR chamber), both plants timed these peaks precisely to within 20 minutes of when the lights would have turned off. Therefore, *A. murpheyi and A. americana* must rely somewhat on internal clocks to time their rhythms of net $CO_2$ uptake.

Sharp increases and decreases in $CO_2$ assimilation before 0:00 are likely due to the plants still adjusting to the environment of the LICOR chamber. Noise at the beginning of the first two measurements in the LL chamber was more intense than in other measurements both in the LL treatment and in other treatments. This is likely because in these first two measurements, the light intensity inside the LICOR chamber was 1000, compared to 300-500 in other measurements.

### *A. americana*

After one week of being in the LL chamber, net $CO_2$ gas exchange of *A. americana* was measured again. In all three measurements, oscillations between around 0 and 3 μmol $CO_2$ $m^{-2}$ $s^{--}$ were most observed, lasting from 3-18 hours or more. Excluding one outlier, the average maximum peak height of these oscillations was

$3.6 \pm 0.4$ μmol $CO_2$ m$^{-2}$ s$^{-1}$. One peak with a maximum of 10.8 μmol $CO_2$ m$^{-2}$ s$^{-1}$ was also observed. Oscillations had no predictable repeating pattern in either duration or amplitude. Interestingly, net $CO_2$ uptake rarely dropped below 0 μmol $CO_2$ m$^{-2}$ s$^{-1}$. Rather, average net $CO_2$ uptake was $2.3 \pm 0.1$ μmol $CO_2$ m$^{-2}$ s$^{-1}$, which is higher than plants in both the LD and DD chambers.

*A. murpheyi*

After three weeks of adjustment to the LL chamber, gas exchange of *A. murpheyi* was measured again. Partial peaks were observed at the beginning and end of the measurement, but no full oscillations were observed. Maximum net $CO_2$ uptake reached 3.5 μmol $CO_2$ m$^{-2}$ s$^{-1}$ in the first peak and 1 μmol $CO_2$ m$^{-2}$ s$^{-1}$ in the second peak. However, between these two peaks, net $CO_2$ gas exchange dropped to $0.0 \pm 0.004$ μmol $CO_2$ m$^{-2}$ s$^{-1}$ for a period of 30 hours.

It appears that under these extended LL conditions of several weeks, *A. murpheyi* entered a state of partial dormancy. Since these *Agave* were never supplied with extra nutrients, this may be due to stress from nutrient starvation.
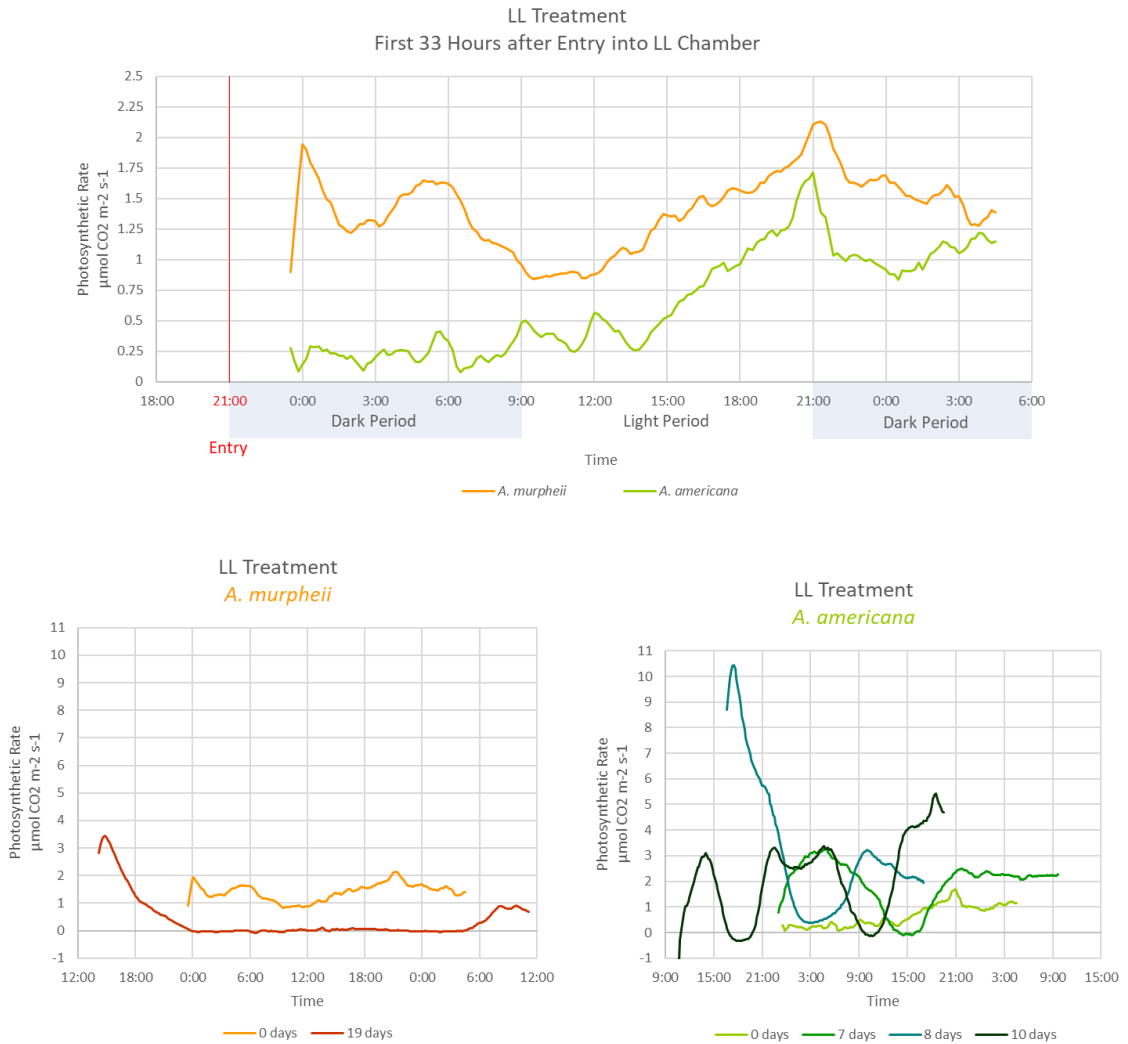
**Figure 4.** Net $CO_2$ uptake of A. murpheyi and A. americana under LL treatment conditions. The graphs are left white to indicate constant light conditions. All graphs display a moving average (n=5) of individual replicates. **Upper Graph**: The first 33 hours of net $CO_2$ uptake for A. murpheyi and A. americana after plants were moved from the LD control chamber into the LL treatment chamber. There are gaps between entry into the LL chamber and the start of measurements to allow leaves to adjust to the conditions within the LICOR measurement chamber. The times that the plants would have experienced a dark period in the LD control chamber are shaded in blue along the x-axis; the times that the plants would have experienced a light period are left white. The time that plants were moved from the LD control chamber to the LL treatment chamber is indicated in red at 21:00. **Lower Graphs**: Net $CO_2$ uptake for A. murpheyi and A. americana after an indicated number of days, (including initial measurements from the upper graph) measured for periods of approximately 25-40 hours. Notice the larger scale compared to the upper graph.

## DD Treatment

In the first 24 hours following placement in the DD chamber, both *A. murpheyi* and *A. americana* maintained a slow oscillation of $CO_2$ gas exchange. However, net $CO_2$

uptake was very low (-14 to -8 for *A. americana* and -10 to -6 for *A. murpheyi*). Over time, oscillations decreased in amplitude, and average net $CO_2$ increased, but remained negative. *A. murpheyi* lost its circadian rhythm more quickly and more completely than *A. americana,* but *A. murpheyi* maintained a higher net $CO_2$ uptake than *A. americana.* In *A. murpheyi,* oscillations completely disappeared and photosynthetic rate stabilized around -1 $\mu$mol $CO_2$ m$^{-2}$ s$^{-1}$. In *A. americana,* oscillations were extremely slow (more than 24 hours for half a cycle) and very slight.

**Figure 5.** Net $CO_2$ uptake of A. murpheyi and A. americana under DD treatment conditions. The graphs are shaded blue to indicate constant dark conditions. All graphs display a moving average (n=5) of individual replicates. **Upper Graph:** The first 25 hours of net $CO_2$ uptake for A. murpheyi and A. americana after plants were moved from the LD control chamber into the DD treatment chamber. There are gaps between entry into the DD chamber and the start of measurements to allow leaves to adjust to the conditions within the LICOR measurement chamber. The times that the plants would have experienced a dark period in the LD control chamber are shaded in blue along the x-axis; the times that the plants would have experienced a light period are left white. The time that plants were moved from the LD control chamber to the LL treatment chamber is indicated in red at 9:00. **Lower Graphs:** Net $CO_2$ uptake for A. murpheyi and A. americana after an indicated number of days, (including initial measurements from the upper graph) measured for periods of approximately 18-22 hours.

In addition to changes in photosynthetic rate, we observed etiolation in

*A. americana* after one month under constant dark conditions (Fig. 6). The older leaves of

the plants began to die, and young leaves began to grow rapidly at the base of the stalk,

pushing the leaf to unfold at the base of the stalk while the tips remained wrapped around

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the top of the stalk. The rapidly growing bases of the new leaves remained white, producing no chlorophyll.

After 6 months in the DD treatment chamber, plants of both species began to die. All old leaves of *A. americana* died and new leaves were completely white and produced no chlorophyll (Fig. 6).



**Figure 6.** Etiolation in *A. americana* after 1 month (photos framed in black on the left), and after 6 months of constant dark conditions (photo framed in red on the right).

Temperature

After four months of adjustment to the LD control, LL treatment, and DD treatment, the leaf temperature of *A. murpheyi and A. americana* was warmer than the air temperature of the chamber when in the light, and cooler than the air temperature of the chamber when in the dark. This held true in all three chambers (LD, DD, and LL).

The leaf temperature of *A. americana* was slightly higher than that of *A. murpheyi* in the DD and LL treatments, but in the LD control, the leaf temperature of *A. murpheyi* was warmer than that of *A. americana.*
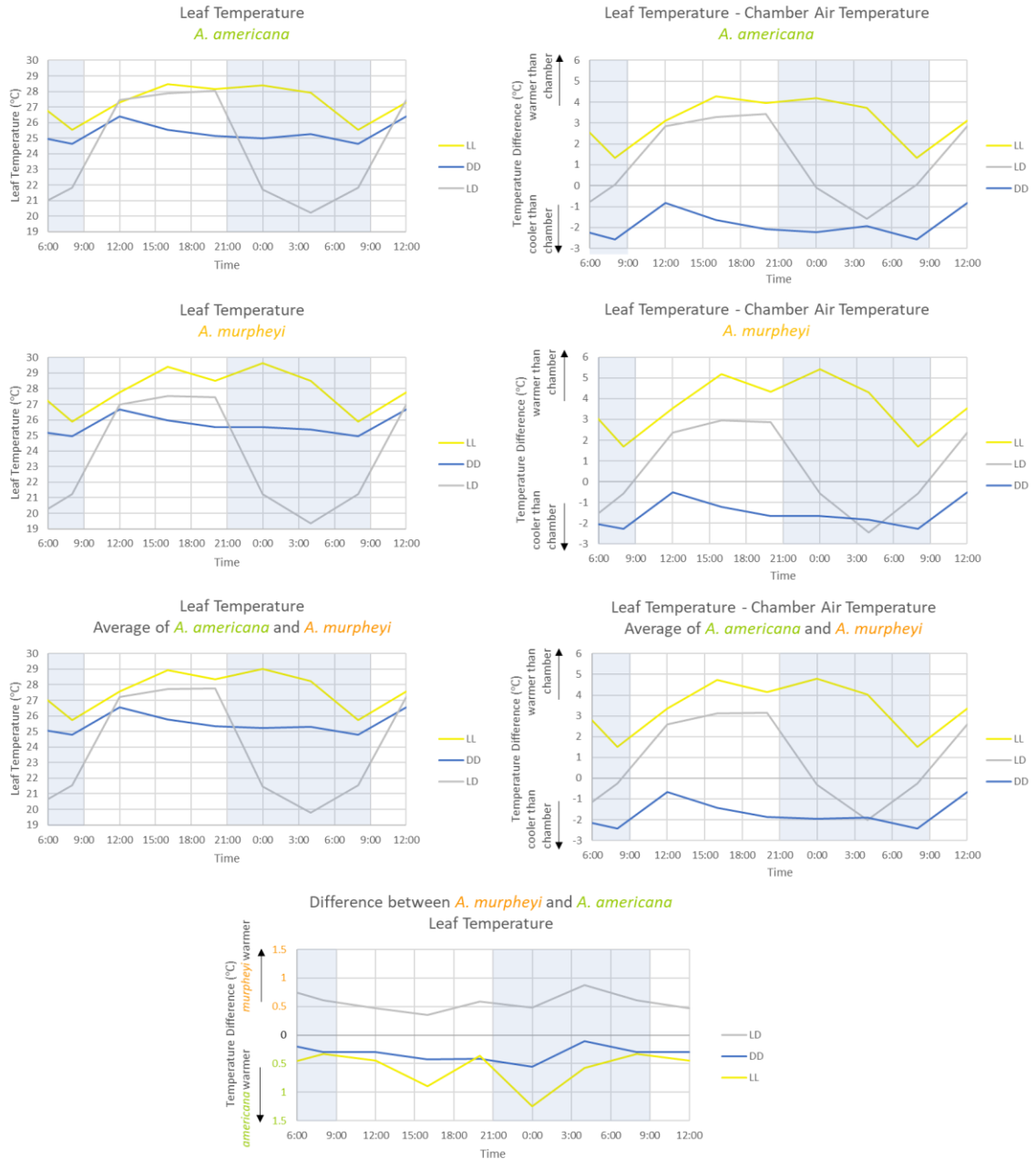
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**Figure 7.** Each point represents the average of three replicates. Each replicate is the average of four leaves on the same plant. **Left Graphs:** Average leaf temperatures of A. murpheyi and A. americana after 4 months of adjustment to the LD control, DD treatment, and LL treatment chambers. **Right Graphs:** Average difference in leaf temperature and chamber air temperature. Positive values signify leaf temperatures warmer than the surrounding air temperature of the corresponding chamber at the corresponding time. **Lower Graph:** Average difference in leaf temperature of A. murpheyi and A. americana.

## Discussion

### Photoperiod Affects Circadian Rhythm of Photosynthesis

Both *A. murpheyi* and *A. americana* were expected to maintain oscillations in photosynthetic rate under constant light conditions, but with an elongated period. In both species, a peak was observed almost exactly 24 hours after entry into the LL treatment chamber, suggesting that the circadian clock of both species is precise, and can maintain the timing of photosynthetic oscillations for a short period of time without external light input from photoperiod. However, after an extended amount of time, oscillations were no longer aligned with photoperiod in either species, suggesting that external light input from photoperiod is necessary to maintain a normal cycle of photosynthesis.

In *A. americana,* oscillations were observed after 10 days, but they had no consistent amplitude or period and were not predictable. Therefore, we can conclude that the circadian clock can still create oscillations in photosynthetic rate, although unpredictable, after over 10 days without external timing by the photoperiod. However, external light input during the day is essential for resetting the circadian clock that keeps the diel cycle of photosynthesis aligned with the photoperiod in *A. americana.*

In *A. murpheyi,* external light input is even more important in maintaining the diel cycle of photosynthesis. After 19 days under constant dark conditions, photosynthesis halted for a period of 30 hours. The circadian clock that maintains the diel cycle of photosynthesis stopped functioning and the plant only occasionally photosynthesized with no alignment with photoperiod. Therefore, external light input from photoperiod may be necessary for proper functioning of the circadian clock that maintains the diel cycle of photosynthesis in *A. murpheyi.*

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The diel cycle of photosynthesis was altered even further under constant dark conditions. As expected, oscillations in photosynthesis were reduced, and photosynthetic rate came to a halt in both species. Surprisingly, the net $CO_2$ uptake of both species was lowest in the 12 hours after being placed in the DD treatment chamber rather than after an extended time under constant dark conditions. Plants reacted more extremely to constant dark immediately after a normal photoperiod than after an extended time. Therefore, they may have adjusted to the constant dark and entered a dormant state. However, etiolation indicates that the plants were not completely dormant, and that they were attempting to find light. In addition, because plants died after 6 months of constant dark conditions, we can conclude that the *Agave* does not enter a sustainable dormant state under constant dark conditions.

Interestingly, in the control chamber, a majority of net $CO_2$ uptake occurred during the light period, in phase IV, rather than during the dark period, in phase I. These results are consistent with the conclusion that cool nighttime temperatures are essential for normal photosynthesis in *Agave* and that warm nighttime temperatures can reduce nocturnal $CO_2$ uptake[7]. The nighttime temperature in our control chamber remained high, at 21.8 °C, which was less than three degrees cooler than the daytime temperature of 24.6 °C.

## Limitations and Future Research

Variability in the pattern of photosynthesis can be high between individuals of *A. americana*[13]. More replicates will be needed for our conclusions to be robust.

Photosynthetic rate is affected by many variables other than photoperiod, including temperature[7], $CO_2$ concentrations, soil nutrients, light intensity and quality, and

soil water content[9]. Although we aimed to keep each chamber at the same constant temperature, the temperature was not the same in each chamber, and the temperature was different in the light and dark periods in the control chamber. Therefore, differences in temperature could be a confounding variable that affects photosynthetic rate in the plants. Air circulation differed between chambers as well because each chamber was enclosed differently. This could have led to differences in $CO_2$ concentrations, humidity, temperature, and evapotranspiration, all of which could be confounding variables that affect photosynthetic rate. The intensity and quality of light supplied in the LL treatment and LD control chambers were not the same, and different areas of the chamber received different intensities and spectra of light. Each plant may have been responding differently due to its own differing microclimate within the chamber. To eliminate potential confounding variables, this experiment should be repeated in a controlled growth chamber with constant temperatures, $CO_2$ concentrations, humidity, light intensity and quality, and air flow. A more controlled environment would better isolate photoperiod as the independent variable.

Plants were not always measured at the same time after watering. Although *Agave* is a drought-tolerant plant, the difference in soil water content between measurements could have affected photosynthetic rate as well. Plants also never received any additional nutrients throughout the experiment. After an extended time, plants may have been stressed due to nutrient deficiencies, which could have altered later results. In future experiments, measurements should be taken at the same time after watering, and nutrient solution should be added monthly. Biofertilization is also known to help *Agave* access

water and nutrients. Therefore, to ensure good access to water and nutrients, potting soil should be inoculated with mycorrhiza at the beginning of the experiment[15].

Measurements in *A. americana* and *A. murpheyi* were not taken at the same time after being moved into the treatment chambers. This makes it difficult to compare results between the two species. In future experiments, measurements should be taken at the same time after being moved into treatment chambers.

This experiment tests two extremes: constant light and constant dark. We suspect that the response of *Agave* to photoperiod length is a spectrum, and that there may be certain minimum and maximum thresholds of photoperiod length that are required to maintain a normal cycle of photosynthesis. Varying lengths of photoperiod length from near-constant light to near-constant dark should be tested to better characterize the effect of photoperiod on the photosynthetic cycle in *Agave*.

*Agave* is a diverse group of plants with differing responses to stimuli. To improve our understanding of the effect of photoperiod on the photosynthetic cycle in *Agave*, more species should be included in future experiments and compared to one another.

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