2016-06-01

Exploring the Possibility of Photosynthetic Plasticity in *Agave sensu lato*

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A thesis submitted to the faculty of
Brigham Young University
In partial fulfillment of the requirements for the degree of

Master of Science

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June 2016

ABSTRACT

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in *Agave sensu lato*

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Crassulacean acid metabolism (CAM) provides desert plants with distinct advantages over the C_3 and C_4 photosynthetic pathways in harsh climates where water is scarce. CAM is, however more metabolically costly than C_3 or C_4 photosynthesis, and some plants, such as *Mesembryanthemum crystallinum,* facultatively utilize CAM when water is abundant, and water conservation unnecessary. In such situations, these plants behave akin to a C_3 plant when photosynthesizing. CAM is divided into four phases, with each phase displaying unique metabolic processes. Certain changes, including changes in the timing of CO_2 fixation, stable carbon isotope ratios, and tissue malic acid content accumulation patterns can indicate that a plant has shifted from CAM to C_3 photosynthesis. Such shifts have been observed to be regulated primarily by water availability and ontogenic development. While facultative CAM is well documented in species like *Mesembryanthemum crystallinum*, and it has not been studied extensively in *Agave* with the exception of *Agave deserti,* and *Agave angustifolia.* A better understanding of this phenomenon would apply to the agricultural growth of this genus. This study aimed to trigger C_3 to CAM shifts in *Agave sensu lato* species, in order to expand upon the findings of previous studies, and better understand the prevalence of facultative CAM expression in the genus. Gas exchange and stable carbon isotope measurements were taken from 2-month-old, 10-month-old, and mature agaves grown in controlled ocnditions. Tissue acid content measurements were taken from mature plants.

Despite the *Agave sensu lato* species in this study being subjected to moisture applications ranging from dry to saturated, we were unable to observe any distinct shifts from CAM to C_3 photosynthesis in any of the species tested for both seedlings and mature plants. Diel net CO_2 fixation rates also increased with age, and water applications for seedlings, and decreased with heavy irrigation in mature plants. Stable carbon isotope ratios revealed that some carbon in the plant tissues was fixed by rubisco, and that some species (*Polianthes tuberosa, Prochnyanthes mexicana*) had carbon isotope ratios of a C_3 plant, but these ratios did not change with different irrigation treatments. Malic acid accumulation remained typical of CAM plants for the species tested as well, with one exception in *Polianthes tuberosa*. As such, we conclude that the *Agave sensu stricto* species tested in this study are obligate CAM plants, and that they perform poorly mature individuals are over-watered. Additionally, the *Agave sensu lato* species *P. mexicana, and P. tuberosa* appear to be C_3 plants given the results of this study.

Keywords: *Agave, Agave chrysantha, Agave deserti, Agave ellemeetiana, Agave marmorata, Agave mckelveyana, Agave palmeri, Agave parryi, Agave salmiana, Agave schotti, Agave striata, Agave tequilana, Agave toumeyana, Agave utahensis, Prochnyanthes mexicana, Polianthes tuberosa,* agave, succulent, photosynthesis, Crassulacean acid metabolism, CAM, hydroponic, automated irrigation

ACKNOWLEDGEMENTS

I would first like to thank my advisor, Dr. Ryan Stewart. His caring and earnest dedication to helping me grow mentally, and spiritually. Allowing me to carry out this research project, and present this thesis, the final capstone of this project. Secondly, I would also like to thank Dr. Antonio Villanueva, without whom, this project would never be finished. I would also like to thank Dr. Klaus Winter, Dr. Joseph Holtum, and Dr. Anne Borland, whose advice was invaluable in the design, and implementation of this experiment. Finally, I would like to thank my family, who have continued to support and help me through any situation with caring advice, encouragement, and custom-made materials.

I would like to thank Blake Anderson, Claire Poore, Héctor G. Ortiz, Justin Boyer, Matthew D. Evans, Megan McGhie, Neil Reed, Nicolas Mena, Stephanie Boudreaux, Stephen Florence, and Tysum Ruchti, who helped in obtaining measurements, and running lab equipment for the project.

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INTRODUCTION

Crassulacean acid metabolism (CAM) provides distinct advantages to plants over the C_3 and C_4 photosynthetic pathways in harsh climates where water is scarce. While the C_3 and C_4 pathways are more widely observed in plants (Nobel, 1994; Nobel, 2010), the unique CO_2-fixation patterns of CAM allows plants to avoid daytime transpirational water loss (Nobel, 1994; Nobel, 2010). By combining reduced water loss rates with physiological adaptations, such as succulent leaves and thick leaf cuticles, desert CAM plants, such as those in the *Agave* genus, have the ability to thrive in extremely arid environments where most C_3 and C_4 plants would struggle (Stewart, 2015). The use of CAM does incur some metabolic costs, which typically results in slower growth rates than plants using the C_3 and C_4 pathways (Nobel, 1994; Stewart, 2015). Consequently, in cases where soil moisture is abundant and extreme water conservation unnecessary, nocturnal uptake of CO_2 and low-to-zero daytime stomatal conductance would be disadvantageous. To overcome this disadvantage, some species, including *Mesembryanthemum crystallinum* and *Clusia pratensis,* are capable of facultatively utilizing CAM (Winter *et al.*, 2008; Winter *et al.*, 1978). Under wet conditions, these plants forgo the water-conserving mechanisms of CAM in favor of the less-costly C_3 photosynthetic pathway, and will shift to daytime CO_2 uptake. This shift in photosynthetic pathways allows a facultative CAM plant to take full advantage of abundant yet ephemeral watering events.

Shifts in CAM expression are often characterized by changes in CO_2 fixation and organic-acid-accumulation patterns during the four temporal phases of CAM (Winter *et al.*, 2008; Winter and Holtum, 2007). Phase I of CAM, which generally occurs at night, is characterized by increasing organic acid levels, high phosphoenolpyruvate carboxylase (PEPcase) activity, and decreasing carbohydrate levels (Lüttge, 2004; Osmond, 1978; Ting,

1985). During phase I, plant cells use PEPcase to fix CO_2 into malic acid within plant cell vacuoles. As the plant prepares to close its stomata to withstand high daytime temperatures during the early morning hours, a transition from high PEPcase activity to high ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity in the early morning characterizes phase II. Phase III is characterized by high Rubisco activity, decreasing organic acid levels, and increasing carbohydrate concentrations in photosynthetically active tissues during the day. In phase III, CAM plants break down stored malic acid into CO_2 to be used by Rubisco in the Calvin cycle in order to produce sugar. During phase IV, Rubisco activity ramps down and PEPcase activity increases during the evening, as the plant prepares to open its stomata for nocturnal CO_2 fixation.

Supposing a CAM plant were to transition to daytime atmospheric CO_2 uptake via open stomata, lower malic acid accumulation and CO_2 fixation rates would likely occur during phase I (Hartsock and Nobel, 1976; Winter *et al.*, 2014). Additionally, open stomata with active CO_2 fixation by Rubisco during phase III could occur. Such modifications to the phases of CAM would likely result in more CO_2 being fixed by Rubisco, as well as increased gas-exchange rates during phase III, with decreased gas-exchange rates during phase I (Hartsock and Nobel, 1976).

The unique ways in which Rubisco and PEPcase interact with stable C isotopes allow for identifying which enzyme is the most influential in fixing CO_2 from the atmosphere into carbohydrates (Coleman, 2012; Fry, 2007; Sternberg *et al.*, 1984). For example, Rubisco discriminates against the heavier ^{13}C isotope. Thus, a plant primarily using Rubisco will have a much lower concentration of ^{13}C isotopes than obligate C_4 and CAM plants (Coleman, 2012; Fry, 2007; Sternberg *et al.*, 1984). Conversely, PEPcase does not discriminate between ^{12}C and ^{13}C. As a result, using PEPcase to fix C will result in a less negative C isotope ratio in tissues

than if Rubisco was used to fix C (Coleman, 2012; Fry, 2007; Sternberg *et al.*, 1984). Consequently, CAM plants using PEPcase to fix carbon during the night and not exposing Rubisco to the atmosphere can be expected to have $^{12}C/^{13}C$ ratios closer to those of C_4 plants, which also use PEPcase to fix atmospheric C. Conversely, CO_2 fixed by Rubisco can be expected to have lower ^{13}C concentrations (-15 to -25‰) (Coleman, 2012).

Facultative CAM expression is also regulated by age in addition to environmental factors, such as soil water availability, humidity, and soil salinity (Winter *et al.*, 2008, 2011; Winter and Holtum, 2007, 2011). Seedlings of several desert CAM species, including *Opuntia ficus-indica* (Winter *et al.*, 2008) and *Opuntia elatior* (Winter *et al*., 2011), which are CAM obligates that exclusively utilize CAM as adults, have been shown to primarily use the C_3 photosynthetic pathway as young seedlings (Olivares and Medina, 1990; Winter *et al.*, 2008, 2011). These seedlings eventually transition to CAM as they age or when exposed to dry conditions during their development (Winter *et al.*, 2008, 2011). Some hypothesize that by using the less -costly C_3 pathway in early stages of development, seedlings of CAM plants are able to grow to a critical size before employing CAM (Hartsock and Nobel, 1976; Winter *et al.*, 1978). At this critical size, the plant is hypothetically able to store enough water to survive droughts common to their desert habitats (Hartsock and Nobel, 1976).

While some *Agave* species, namely *Agave deserti* and *Agave sisalana*, have been observed to express facultative CAM (Hartsock and Nobel, 1976; Matiz *et al.*, 2013), its prevalence within *Agave sensu lato* is not well documented (Matiz *et al.*, 2013). Indeed, only Hartsock and Nobel (1976) and Matiz *et al.* (2013) have reported the reversible transition from primarily nocturnal carbon fixation to diurnal carbon fixation in well-watered *Agave* species (Hartsock and Nobel, 1976; Matiz *et al.*, 2013). However, *Agave angustifolia* was found to be

unyielding in its expression of CAM when exposed to varying environmental conditions (Winter *et al.*, 2014). Even so, the degree to which facultative CAM is expressed within the *Agave sensu lato* genus—which includes genera not phylogenetically distinguishable at the molecular level (*Manfreda*, *Polianthes*, and *Prochynyanthes*) from *Agave sensu stricto*—remains unclear(Gentry, 1982; Good-Avila *et al.*, 2006). Even less clear is how these species utilize CAM and C_3 photosynthesis as they develop from seedlings to mature plants. Such knowledge would be important in deepening our understanding of the physiological adaptations of agaves to changing environmental conditions, which would be a crucial piece of information about this group of plants, which have benefited society in many ways for the past several hundred years (Brugge, 1965; Escamilla-Treviño, 2012; Stewart, 2015).

An increased understanding of how these plants utilize CAM could be beneficial in determining how to more sustainably cultivate agave as a crop, which has been cultivated for hundreds of years (Davis *et al.*, 2016), and is also being considered for production in marginal lands as a bioenergy feedstock crop, both under well-watered and arid conditions (Davis *et al.*, 2011; Davis *et al.*, 2016; Nobel, 1994; Stewart, 2015). However, while agaves have been reported to have biomass yields rivaling those of highly productive C_4 crops (Nobel, 1994), and grow better than conventional crops in dry regions, such as Arizona (Davis *et al.*, 2016), such claims are thought to be contrary to the survival-focused nature of CAM (Lüttge, 2002). Should these plants be able to switch from the more metabolically conservative CAM pathway to the C_3 pathway, light would be shed on how agaves might be able to achieve such growth rates while maintaining their xerophytic nature.

This study aims to determine whether select species of *Agave sensu lato* express photosynthetic mode-shifting as soil-water availability changes. Both seedling-stage and mature

Agave sensu lato plants were exposed to varying soil moisture levels, ranging from dry to saturated. In doing this, differences in photosynthetic pathway usage can be characterized between age classes and species. We hypothesized that well-watered mature plants primarily uptake CO_2 during the day over nocturnal CO_2 uptake. We also hypothesized that seedlings will predominantly engage in daytime CO_2 uptake, but will increasingly utilize the CAM pathway as they age. Additionally, we believe that *Agave sensu lato* species, such as *Prochnyanthes mexicana* and *Polianthes tuberosa*, which we assume are obligate CAM species, will shift from CAM to C_3 photosynthesis if abundant water is available.

MATERIALS AND METHODS

Seedling Experiment

Growing Conditions: Agave sensu lato seedlings (Rarepalmseeds, Muenchen, Germany) selected from 15 species (Table 1) were established over the course of one month under laboratory conditions at 24°C on a shelf rack equipped with time-controlled light-emitting diodes (LEDs) (width = 61 cm, length = 5 cm) (Custom LED Strip 5700K, BML Horticulture, Austin, TX) (Fig. 1). The LEDs were maintained at a 16/8-h day/night cycle. Seedlings were established in conetainers (diameter = 3.8 cm, height = 14 cm, volume = 107 cm^3) (SC7, Stuewe and Sons Inc., Tangent, OR) (diameter = 3.8 cm, height = 14 cm, volume = 107 cm^3) filled with a soilless mix consisting of a 3:1:1 ratio of superior-grade pumice (Hess Pumice Products, Malad, ID), industrial quartz sand (Unimin Corporation, New Canaan, CT), and shredded coconut coir (Black Gold Just Coir, Sun Gro Horticulture, Seba Beach, Alberta, Canada) on 25 Oct. 2014. Each conetainer was filled with 0.5 g of a 180-day slow-release fertilizer (13:13:13 NPK) (Arysta Life Science America Inc. New York, NY). The seedlings were divided into two age classes (2 months, 10 months), and two soil-moisture treatments, high water (watered to field

capacity daily), and low water (watered to field capacity every 3 days). Due to the need to have sufficient amount of plant material for measurements, up to five conspecific seedlings were established in conetainers for the 2-month-old plants. One plant was established in each conetainer for the 10-month-old seedlings. The conetainers were then randomly arranged in container holders on the shelf rack described above (Fig. 1, 2). High- and low-water treatment conetainers filled with soilless media, and absent of plants, were subjected to the same moisture treatments to serve as controls to determine the amount of medium-based respiration.

Experimental Design: Agave sensu lato seedlings selected from 15 species (Table 1) were arranged in a randomized block design consisting of four groups blocked by time. Experimental units were defined as an individual conetainer with established plant material. Each block contained two conetainers with plant material of the same species and age class combination, if available (Table 1). Each experimental unit was randomly assigned to one of two water treatments (low or high). In total, there were 192 seedlings comprised of 24 species-age class combinations, with two of the same species-age class combinations, if available, represented in each of the four blocks being subjected to either the low- or high-water treatments.

Gas Exchange Measurements: Gas-exchange rates of plants of each species (including blank controls), age class, and treatment were measured once per block in each of the four blocks separated by time from Jul. 2015 to Oct. 2015 (Fig. 1). Gas-exchange rates were measured over 24 h for each experimental unit in a closed system by placing seedlings in one of four acrylic chambers (height = 7.62, diameter = 13.3 cm, volume = 159 cm^3) (Fig. 3). Each chamber was placed under a polyvinyl chloride (PVC) frame enclosed with a two-layer, light-excluding cloth made of black-out fabric and heat-reflective fabric, and subjected to a 16/8-h day/night cycle using four LEDs (BML Horticulture, Austin, TX) during the experiment. While measuring CO_2

uptake, air from the acrylic chamber was pumped through an infrared CO_2 analyzer (LI-6251, LI-COR, Lincoln, NE), and then back to the chamber over a 15-minute period in a closed system. In this state, CO_2 depletion was recorded using a datalogger (CR800, Campbell Scientific, Logan, UT). Depletion rate data were used to calculate seedling CO_2 fixation. While not being measured, air from outside the building was pumped in via a compressor to the lab, where it went through the acrylic chamber from the lab air supply valve and out into the surrounding atmosphere at 1 L min^{-1} in order to restore depleted CO_2 or vent excess CO_2. This established a new baseline for the next sample period. Net plant gas exchange was calculated using the following formula:

$$(FS = (((P \times V)/(8.514 \times T) * dC/dT))/A \quad (1)$$

where F is CO_2 fixation (μmol CO2 m^{-2} s^{-1}), P is atmospheric pressure (kPa), V is volume of the chamber in L, T = temperature (°C), dC is change in CO_2 content (ppm) after correction using the change in CO_2 content (ppm) from the matching blank, dT is change in time (s), and A is surface area (m^2) of the tissue in the chamber.

Surface area: Plant surface area was measured using three-dimensional (3D) imaging and modeling software (123d Catch, Autodesk, San Rafael, CA) installed on Apple iPad Pro tablets (Apple Inc., Cupertino, CA) Three-dimensional images of plants were scanned using this software and converted into object files. Object files were then processed with 3D-modeling software (Maya, Autodesk, San Rafael, CA) where the object files were cropped to only include the leaves and crown of the plants, and scaled to actual size using a solid reference cylinder (diameter = 2.54 cm, height = 5.08 cm). After being cropped and scaled, a standard algorithm for calculating surface areas was used in Maya to calculate the surface area of the object file. These data were used to estimate the surface area of each plant. This novel method produced

surface area and volume estimates, which correlated well with volume measurements acquired through liquid displacement measurements (data not published). The method allows for the estimation of surface area for plants with irregular shapes such as agave, and could likely be applied to other plants with succulent leaves.

Stable Carbon Isotope Ratios: All seedlings were dried in an oven at 60°C for 48 h. Each entire seedling was then crushed and homogenized using a ceramic mortar and pestle and 0.75-1.00 mg of tissue was placed in a 3.5 by 5 mm tin capsule and sent to the University of Utah SIRFER lab for stable carbon isotope analysis (Hall *et al.*, 2015).

Mature Plant Experiment

Growing Conditions: Five species of approximately 2-year-old *Agave sensu lato* plants from the southwestern U.S. and northern Mexico, including *Agave deserti* (accession number 19953, Huntington Botanical Garden, Los Angeles, CA), *Agave palmeri* (Mountain States Wholesale Nursery, Glendale, AZ), *Agave parryi* (accession numbers 21326, 21301, and 21291, Huntington Botanical Garden, Los Angeles, CA), *Agave utahensis* (Great Basin Natives, Holden, UT), and *Polianthes tuberosa* (Gardino Nursery Corp, Delray Beach, FL), were established for 3 months under greenhouse conditions at temperatures ranging from 24-29°C and a day/night cycle of 16/8 h, and were grown under supplemental lighting. Plants in the greenhouse were maintained using an automatic drip-irrigation system controlled by a datalogger (CR1000, Campbell Scientific, Logan, UT) as described by Bergsten and Stewart (2014).

Experimental Design: A total of 6 individuals from each of the species described above (30 individuals in all), were randomly arranged in a complete block design. Each pot containing a plant represented 1 experimental unit. Each treatment combination was represented in one of two blocks on a greenhouse bench (Fig. 4).

Agave plants were subjected to three soil moisture treatments (12 (drought), 33 (field capacity), and 100% (saturated (hydroponics)) volumetric water content (VWC)), based on a soil moisture curve that identified 33% as soil field capacity and 12% to be the midpoint between field capacity and permanent wilting point (unpublished data). Moisture treatments were applied on 27 Jan. 2015 with the automatic irrigation system. Plants in the 12 and 33% VWC treatments were established in 2.8-L (diameter = 23 cm, width = 17.78 cm) resin containers (Fiskars Corporation, Helsinki, Finland). Containers were filled with a soil-less mix as that reported for the seedling experiment on 25 Oct. 2014. Ten g of a 180-day slow-release fertilizer (13:13:13 NPK) (Arysta Life Science America) was mixed into each container. Plants subjected to the hydroponics treatment were established in containers (height = 24 cm, diameter = 24 cm, volume = 7.6 L) containing modified Steinberg nutrient solution (Nichols *et al.*, 2012; Steinberg, 1953) on 27 Jan. 2015. Conetainers without plants, but filled with soil-less medium and subjected to either 12 or 33% VWC treatments, served as controls to determine the amount of medium-based respiration.

Gas Exchange Measurements: Diel, whole-plant-level, gas-exchange measurements were collected for each experimental unit over a 24-hour period using a steady-state photosynthesis system (LI-6400XT, LI-COR, Lincoln, NE). Plants were placed in an acrylic cuvette (height = 22.9 cm, diameter = 25.4 cm, volume = 11.6 l) for gas-exchange measurements (Fig. 6). The cuvette was equipped with two circulatory fans (length = 40 mm, height = 40 mm) (MagLev KDE1204PFV3, Sunon Electric Machine Industry Co. Ltd., Kaohsiung, Taiwan) in order to ensure air within the chamber was well mixed. The cuvette was placed under a PVC frame covered by light-excluding cloth made of the same materials used in the seedling

experiment. Inside the PVC frame, the plants were subjected to a 16/8-h day/night cycle using four LEDs (BML Horticulture).

Measurements were taken continuously by monitoring the concentration of CO_2 in the air entering the cuvette as well as that of the air exiting the cuvette. Carbon dioxide concentrations were automatically maintained by the steady-state photosynthesis system at a constant concentration of 450 ppm CO_2 in order to establish a stable baseline for comparisons between the sample chamber containing the plant, and the reference chamber within the instrument. This was accomplished through the photosynthesis system, which automatically controlled the amount of CO_2 added to the incoming air, which had been purged of CO_2, using air from a compressed-CO_2 gas cylinder. As CO_2 was added to or removed from the chamber through respiration or fixation from the plants or soil-less media, the amount of CO_2 in the incoming air would then be automatically adjusted by the photosynthesis system mixer, in order to compensate for the addition or loss of CO_2 within the chamber containing the plant. After sampling, CO_2 fixation was calculated by comparing this adjusted concentration in the sample chamber containing the plant against the target of 450 ppm CO_2 in the reference chamber within the photosynthesis system. By doing this, we were able to calculate how much CO_2 was being added to or removed from the chamber in a given period of time. Net plant gas exchange was calculated using the following formula:

$$(F = (P_{st} - P_{bt})/A) \quad (2)$$

where F is whole-plant CO_2 fixation (μmol CO2 m^{-2} s^{-1}), P_{st} is plant sample CO_2 fixation at a given time, P_{bt} is blank CO_2 fixation (μmol CO2 m^{-2} s^{-1}) at the same time, and A is surface area of the plant (m^2).

Plant Surface Area: Plant surface area was measured using the same protocol and equipment as described for the seedlings.

Stable Carbon Isotope Ratios: Tissue samples for stable C isotope measurements were extracted from mature plants. The samples were oven-dried at 60°C for 24 h. After drying, samples were crushed using a ceramic mortar and pestle, and 0.75-1.00 mg of tissue was placed in a tin capsule (diameter = 3.5 mm, height = 5 mm). The ground tissues were sent to the University of Utah SIRFER lab for stable carbon isotope analysis (Hall *et al.*, 2015).

Acid Content: Tissue samples were extracted from leaves of mature plants 2 h before the end of the day cycle (~8:00 pm), and 2 h before the end of the night cycle (~4:00 am). Each tissue sample was extracted by cutting an entire leaf from the mid-section of the plant. A 1-cm cork borer was then used to extract four 1-cm diameter leaf discs from the middle of the cut leaf. The samples were then placed in aluminum foil and frozen in a -45°C freezer (Model #34, ScienceTemp, Adrian, MI) until they were tested for acid content. Samples were stored for several weeks at a time. Storage time did not have a significant effect on analysis results (data not published).

To measure acid content, tissues were flash-frozen in liquid N and crushed with a ceramic mortar and pestle. Two hundred mg of tissue were then submerged in a hot 80% methanol solution for 40 min. One mL of extract solution was then added to 2 mL of distilled H_2O followed by three drops of phenolphthalein indicator in a 5-mL Erlenmeyer flask. The solution was then titrated to a pH of 6.5 using 0.005M NaOH. The amount of malic acid was then calculated based on the amount of NaOH used (Winter *et al.*, 2011).

Statistical Analysis

The GLIMMIX procedure in SAS/STAT 14.1 software (SAS Institute Inc., 2015) was used to fit a normal linear model with repeated measures to the data on each of the response variables from both the seedlings and the mature plant experiments. For the mature plant experiment, the repeated measurements for a specific response variable consisted of 96 observations given by measuring responses every 15 minutes from 12:00 am to 11:45 pm on the same experimental unit. The model for the mean response included terms for the block effect; main effects; and interactions of species, moisture treatment, and CAM phase.

For the seedling experiment, the repeated measurements for a specific response variable consisted of 24 observations given by measuring that response every hour on the same experimental unit. The model for the mean response in this case included terms for the block effect; main effects; and interactions of age class, species, water treatment, and CAM phase. A first-order, autoregressive, moving-average covariance structure (ARMA(1,1)) was used as the model for correlations among repeated measurements on the same experimental unit in both the seedling and the mature plant experiments. The ARMA(1,1) covariance structure was selected among other covariance structures such as the first-order autoregressive (AR(1)), compound symmetry (CS), Toeplitz (TOEP), and first-order antedependence (ANTE(1)) through the comparison of values for some information criteria such as AIC (Akaike, 1974), AICC (Hurvich and Tsai, 1989), and BIC (Schwarz, 1978).

RESULTS

Seedling Experiment

Gas Exchange Measurements: Average CO_2 uptake did not differ at the species level ($P = 0.43$) (Table 2). Diel CO_2 fixation patterns did not differ for each of the four CAM phases

when comparing between treatments within species ($P = 0.98$). For all species, except for *A. ellemeetiana*, and *P. mexicana*, 71% of diel net CO_2 fixation occurred during CAM phase I, with phases II, III and IV accounting for 3, 11, and 15% of diel net CO_2 fixation (Fig. 7). In the case of *A. ellemeetiana,* respiration of CO_2 occurred during phases I and III, with phase I respiration 2.5 times greater than that of phase III. Additionally, for *A. ellemeetiana,* 54% of diel net CO_2 fixation occurred during phase II, with the remaining 46% occurring during phase IV (Fig. 8). Respiration of CO_2 for *P. mexicana,* also occurred mostly during phase I (Fig. 9). Additionally, for *P. mexicana,* phases I, II, and IV accounted for 15, 18, and 64% of diel net CO_2 fixation (Fig. 9). Though fixation patterns over a 24-h period were similar between age classes, the overall amount of CO_2 fixed by 10-month-old plants across all species and treatments was 2.5 times greater per unit surface area than the amount of CO_2 fixed by 2-month-old plants ($P = 0.0002$) (Fig. 10).

Stable Carbon Isotope Ratios: Soil-moisture treatments had no effect on C-isotope ratios of seedlings at the species level ($P = 0.90$). However, $^{12}C/^{13}C$ ratios of 2-month-old seedlings were, on average, 6% lower than that of 10-month-old seedlings across species represented in both age classes ($P = 0.0003$) (Figs. 11, 12 and Tables 1, 3). Although not significant, *P. mexicana* had the most negative $^{12}C/^{13}C$ ratio (-25.4‰) (Fig. 11, Table 3).

Mature Plant Experiment

Gas Exchange Measurements: Across species, soil moisture did not influence diel CO_2 fixation patterns for the four phases of CAM ($P = 0.59$). With the exception of *P. tuberosa*, net CO_2 fixation of all species and treatments occurred during phases I and II, which accounted for 64% and 36% of diel net CO_2 fixation, respectively (Tables 4, 5, 6, 7). Net CO_2 respiration for all species, except *P. tuberosa*, occurred during phases III and IV (Tables 4, 5, 6, 7), which

accounted for 47% and 53% of diel net respiration, respectively (Fig. 13). *Polianthes tuberosa* plants in the 12% moisture treatment respired across all four phases, with phases I, II, III, and IV, accounting for 31%, 14%, 21%, and 34% of diel net respiration (Fig. 14). For the 33% treatment of *P. tuberosa*, phases I and IV accounted for 17% and 83% of diel net respiration, while phases II and III accounted for 24% and 76% of diel net CO_2 fixation (Fig. 15). Carbon dioxide uptake during phase IV in the 100% soil-moisture treatment of *P. tuberosa* accounted for 100% of diel net CO_2 fixation, with net CO_2 respiration occurring during phases I, II, and III (Fig. 16).

Though not statistically significant, CO_2 fixation was 1.9 times greater for plants across species in the 12% moisture treatment than of those in the 33% moisture treatment (Table 4, 5). Additionally, across species in the 33% moisture treatment, CO_2 fixation was 58% greater than of those in the 100% moisture treatment (Fig. 17).

Stable Carbon Isotope Ratios: The carbon-isotope ratio (-25‰) of *P. tuberosa* was 68% lower than all other species ($P < 0.0001$) (Fig. 18). Carbon-isotope ratios of *A. deserti, A. palmeri, A. parryi,* and *A. utahensis* fell within the range of -16.2 and 17.4‰ (Fig. 18). Soil-moisture treatments did not lead to changes in stable carbon isotope ratios within species ($P = 0.18$) (Table 8).

Tissue Acid Content: Within species, soil-moisture level had no significant effect on either nocturnal or diurnal leaf tissue acid content ($P \leq 0.0001$) (Table 9). On average, across treatments and species, leaf tissue acid content was 74% greater at the end of the night than it was at the end of the day ($P < 0.0001$) (Fig. 19). At the species level, tissue acid content for *P. tuberosa* was 35% greater at the end of the day than at the end of the night for plants in the 33% moisture treatment ($P < 0.0001$) (Table 9). Conversely, *P. tuberosa* acid content was 1.5 times higher at the end of the night than at the end of the day for plants in the 100% moisture treatment

($P < 0.0001$) (Table 9). In addition, acid content of *P. tuberosa* plants at the end of the day in the 12% moisture treatment was not significantly different than of conspecific plants at the end of the day ($P = 0.15$) (Table 9). For *P. tuberosa*, averaged across all treatments, leaf tissue acid content was 25% greater at the end of the night than at the end of the day (Table 9).

DISCUSSION

Despite subjecting young seedlings, 10-month-old seedlings, and mature *Agave sensu lato* plants to varying constant levels of soil moisture, we were unable to observe any overall deviations in diel CO_2 fixation patterns within species across treatments (Tables 2, 4, 5, 6, 7) from what has been observed in obligate CAM plants (Ting, 1985; Winter *et al.*, 2014). As such, we were unable to induce or observe any shifts from primarily nocturnal to primarily diurnal CO_2 uptake in any of the species tested, including *Agave deserti*, which was observed to be a facultative CAM plant by Hartsock and Nobel (1976). Our results corroborate with those reported by Winter *et al.* (2014), who found that *Agave angustifolia* was an obligate CAM plant irrespective of soil-moisture conditions.

Moreover, in the 33% moisture treatments, more CO_2 was fixed during the early morning than at night. However, possibly due to limitations in our sample sizes, the differences in CO_2 fixation between phase I of the 33% treatment and phase I of the 12% and 100% treatments were not statistically significant (Table 4, 5, 6, 7).

While most of the *Agave sensu stricto* species followed a gas exchange pattern typical of a constitutive CAM plant (Ting, 1985), some differed slightly in their uptake patterns. *Agave ellemeetiana* and *P. mexicana* seedlings in both high- and low-moisture treatments had relatively high uptake rates during phases II and IV of the CAM cycle (morning and evening), with respiration occurring during phases I and III (night and mid-day) (Table 2). This differed from

the CO_2 fixation pattern observed in the majority of *Agave s.s.* seedlings. In these species, net CO_2 fixation was primarily carried out in phase I (night), and to a lesser degree, in phase II (early morning) of CAM. Respiration occurred in phases III (mid-day) and IV (evening) of the CAM cycle for these species. Additionally, for mature *P. tuberosa* plants, CO_2 fixation was highest during phases II and III of CAM, and lowest in phases I and IV (Table 8). Interestingly, *A. ellemeetiana, P. mexicana,* and *P. tuberosa* come from regions in which rain is more abundant than the xeric habitats of most *Agave s.s.* species (Thiede, 2001). It may be that the gas-exchange patterns of these species have evolved to adapt to more mesic environments.

Stable C-isotope readings of *Agave s.s.* species revealed that Rubisco had some role in fixing CO_2 during the ontogenetic development of each of the plants we tested (Coleman, 2012; Fry, 2007). We found that C-isotope ratios for members of *Agave s.s.* became less negative as age classes became older (Tables 3, 8). This may indicate that the plants fixed CO_2 into structural sugars using Rubisco early in their ontogenic development (Coleman, 2012; Fry, 2007; Winter *et al.*, 2011; Winter and Holtum, 2007). As they aged, the structures created using sugars that originated from CO_2 molecules fixed by Rubisco became less abundant compared to the structures made with sugars formed by PEPcase. (Coleman, 2012; Fry, 2007). This is consistent with the findings of other studies that have observed that obligate CAM plants may use C_3 photosynthesis when young, but transition into CAM as they age and develop (Winter *et al.*, 2008, 2011).

Prochnyanthes mexicana and *P. tuberosa*, which are both considered part of *Agave sensu lato,* had stable C-isotope ratios consistent with C_3 plants (Tables 3, 8). This suggests a significant role of Rubisco in fixing CO_2 in these species (Coleman, 2012; Fry, 2007; Sternberg *et al.*, 1984).

Our measurements of acid content in leaf tissues corroborated our observations made from gas-exchange data (Table 4, 5, 6, 7). Members of *Agave s.s.* had significantly greater leaf tissue acid content at the end of the night (Table 9), reflective of typical CAM biology (Lüttge, 2004; Osmond, 1978; Ting, 1985). However, as indicated, *P. tuberosa*, which is not a member of *Agave s.s.*, deviated from this pattern (Table 9).

Differences between the results of our study and those of Hartsock and Nobel (1976) concerning *A. deserti* may be due to differences in gas-exchange measurement methods. In our study, gas exchange was measured on a whole-plant basis using a portable photosynthesis system. Hartsock and Nobel (1976) measured gas-exchange patterns of *A. deserti* plants by sealing a small chamber with a 2 cm by 5 cm opening around individual leaves (Hartsock and Nobel, 1976). According to Matiz *et al.* (2013), young tissues of agave plants, particularly young leaves, have been shown to utilize C_3 photosynthesis at first, and switch to CAM as they age. Since the chamber used by Hartsock and Nobel (1976) was inserted onto individual leaves near the middle of the rosette, we believe that the CAM to C_3 shifts recorded in well-watered *A. deserti* plants were potentially the result of young leaf tissues being measured. However, when measured on a whole-plant basis, as was done in our study and by Winter and Holtum (2008, 2014), the overall gas-exchange patterns of agave are those of an obligate CAM plant.

CONCLUSION

Any increases in yield by agaves grown under cultivated conditions with consistent irrigation appear not to be due to plants shifting from primarily nocturnal to primarily diurnal CO_2 uptake. We found that in addition to high soil-moisture content not inducing any significant shifts in gas exchange patterns (Table 2, 7), it also greatly reduces diel net CO_2 uptake in mature plants (Fig. 16). It may be possible, however, that Rubisco is playing some role in CO_2 fixation

during the transitional period between nocturnal CO_2 fixation with PEPcase and the diurnal processing of stored CO_2 using Rubisco during phase II. Net CO_2 uptake in *Agave s.s.* species was consistently observed during CAM phase II, and was notably higher in the 33% moisture treatment for mature plants (Table 5). This phase often occurs early in the morning, either just before or during sunrise. At this point of the day, air and soil surface temperatures of the arid environments where agaves naturally grow can be much cooler than later in the day. These cool temperatures likely facilitate lower transpiration rates, allowing the agaves to maintain gas exchange with relatively low water loss compared to daytime conditions. Further study would be needed to examine the potential role of Rubisco in phase II CO_2 fixation. It would appear that agave are able to maintain respectable growth rates without switching from the metabolically costly CAM pathway to the less costly C_3 photosynthesis pathway (Davis *et al.*, 2016; Nobel, 1994). Additional study is needed in order to identify the mechanisms that agave employ to accomplish this.

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4-13-H	3-19-H	1-14-H	Empty	Empty	2-9-L	2-12-H	3-16-L	3-17-L	1-23-L	1-10-L	Empty	1-10-H	Seedling Rack 3
1-12-H	4-6-H	Empty	4-18-H	Empty	4-12-H	Empty	2-3-L	4-21-H	2-25-L	3-9-H	Empty	4-3-L	
3-16-H	4-15-H	2-6-L	Empty	Empty	3-6-H	4-17-H	4-10-H	4-11-L	3-14-L	2-17-H	1-21-L	Empty	
Empty	3-18-H	4-26-L	1-16-H	1-19-H	1-22-H	2-20-L	4-11-H	1-26-L	Empty	1-9-H	1-27-H	1-14-L	
1-15-L	Empty	4-13-L	1-12-L	Empty	Empty	2-21-H	3-22-L	Empty	1-7-H	2-11-H	1-19-L	3-20-L	
3-13-H	Empty	2-13-H	3-6-L	Empty	1-4-H	3-15-L	4-14-H	4-6-L	2-16-L	4-5-L	4-i-L	4-2-L	
2-5-L	Empty	3-26-H	3-2-H	1-9-L	4-8-H	Empty	1-6-H	4-24-H	3-25-L	3-14-H	Empty	2-8-H	
2-27-L	3-11-L	4-25-L	2-26-L	Empty	2-18-L	3-12-H	1-11-H	3-4-L	3-15-H	3-20-H	2-19-L	3-13-L	Seedling Rack 2
Empty	1-2-L	4-4-H	Empty	Empty	1-8-L	3-12-L	3-23-H	2-17-L	4-5-H	3-3-H	2-15-L	2-21-L	
4-27-H	Empty	1-23-H	4-16-H	3-17-H	Empty	4-14-L	4-9-H	Empty	Empty	2-23-L	2-11-L	4-9-L	
3-27-H	4-10-L	Empty	Empty	2-6-H	3-7-H	Empty	2-19-H	Empty	1-7-L	2-5-H	3-18-L	2-3-H	
3-8-H	2-10-L	1-13-L	Empty	3-11-H	4-4-L	1-15-H	Empty	Empty	4-12-L	1-17-L	3-3-L	1-24-L	
Empty	2-18-H	Empty	3-8-L	2-4-H	4-2-H	1-18-H	Empty	2-24-H	1-22-L	2-2-L	Empty	Empty	
1-13-H	1-3-H	2-13-L	Empty	Empty	4-20-L	1-3-L	1-11-L	2-15-H	2-24-L	2-2-H	2-4-L	4-22-L	
4-7-L	Empty	4-7-H	3-9-L	4-16-L	Empty	4-23-L	1-27-L	3-24-H	Empty	3-27-L	1-5-L	2-9-H	Seedling Rack 1
4-25-H	3-21-H	Empty	2-22-L	Empty	Empty	1-4-L	4-18-L	2-16-H	3-4-H	3-21-L	Empty	Empty	
4-19-L	2-12-L	4-19-H	2-8-L	4-20-H	1-2-H	1-5-H	3-5-H	Empty	3-23-L	2-7-H	1-16-L	1-25-H	
4-24-L	4-26-H	4-22-H	Empty	Empty	3-10-L	Empty	2-10-H	2-20-H	1-18-L	2-25-H	1-24-H	2-26-H	
3-7-L	1-21-H	2-14-H	3-5-L	1-26-H	2-7-L	4-27-L	Empty	3-22-H	1-6-L	3-26-L	Empty	1-25-L	
1-8-H	4-23-L	3-24-L	3-10-H	Empty	1-17-H	3-25-H	1-20-L	Empty	Empty	2-27-H	1-20-H	4-15-L	
4-21-L	2-14-L	Empty	3-2-L	2-23-H	4-17-L	4-3-H	Empty	Empty	3-19-L	Empty	2-22-H	Empty	

Fig. 1. Block, Species and Treatment assignments for positions on the cone-tainer rack. The first number of each entry refers to the block assigned to the plant in that position. The second number refers to the # assigned to the species / age class combination in that position. Please refer to Table 1 for the species / age class combinations assigned to each number. The letter at the end of the entry refers to the moisture treatment. 'L' indicates a low water treatment in which plants were watered to field capacity every three days, and 'H' indicates a high water treatment in which plants were watered to field capacity daily.

Fig. 2. Pictured is are the seedlings used in this study under LED lights on a shelf rack planted in cone-tainers randomly assigned to positions in a cone-tainer rack.

Fig. 3. Pictured are the acrylic seedling gas exchange chambers with seedlings being measured contained within.

32) A. Palmeri 33%	31) A. Palmeri 12%	30) P. Tuberosa 100%	29) A. Deserti 100%	Block 2
28) P. Tuberosa 33%	27) A. Utahensis 12%	26) P. Tuberosa 12%	25) A. Utahensis 33%	
24) A. Parryi 100%	23) A. Deserti 12%	22) A. Palmeri 100%	21) A. Parryi 12%	
20) Blank 33%	19) A. Parryi 33%	18) A. Utahensis 100%	17) A. Deserti 33%	
16) Blank 12%	15) A. Palmeri 33%	14) P. Tuberosa 100%	13) A. Deserti 12%	Block 1
12) A. Deserti 33%	11) A. Utahensis 100%	10) P. Tuberosa 12%	9) A. Utahensis 33%	
8) A. Deserti 100%	7) A. Utahensis 12%	6) A. Parryi 33%	5) A. Parryi 100%	
4) P. Tuberosa 33%	3) A. Palmeri 12%	2) A. Parryi 12%	1) A. Palmeri 100%	

Fig. 4. This diagram indicates the species, treatment, and block assignments for plants and soilless media blanks placed on the greenhouse bench irrigation system.

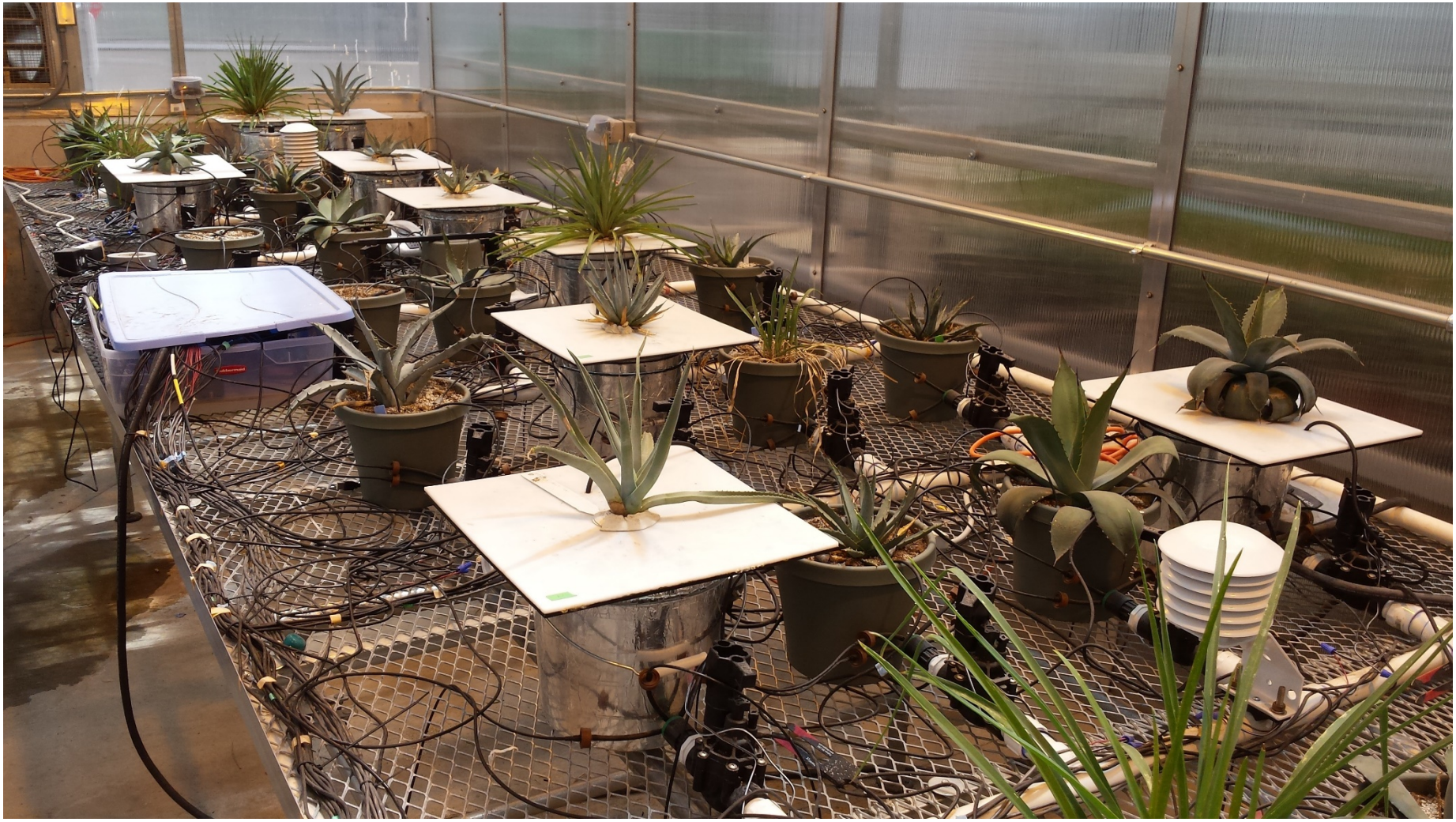

Fig. 5. Pictured is the irrigation system equipped greenhouse bench, with plants assigned to the positions listed in Fig. 4.

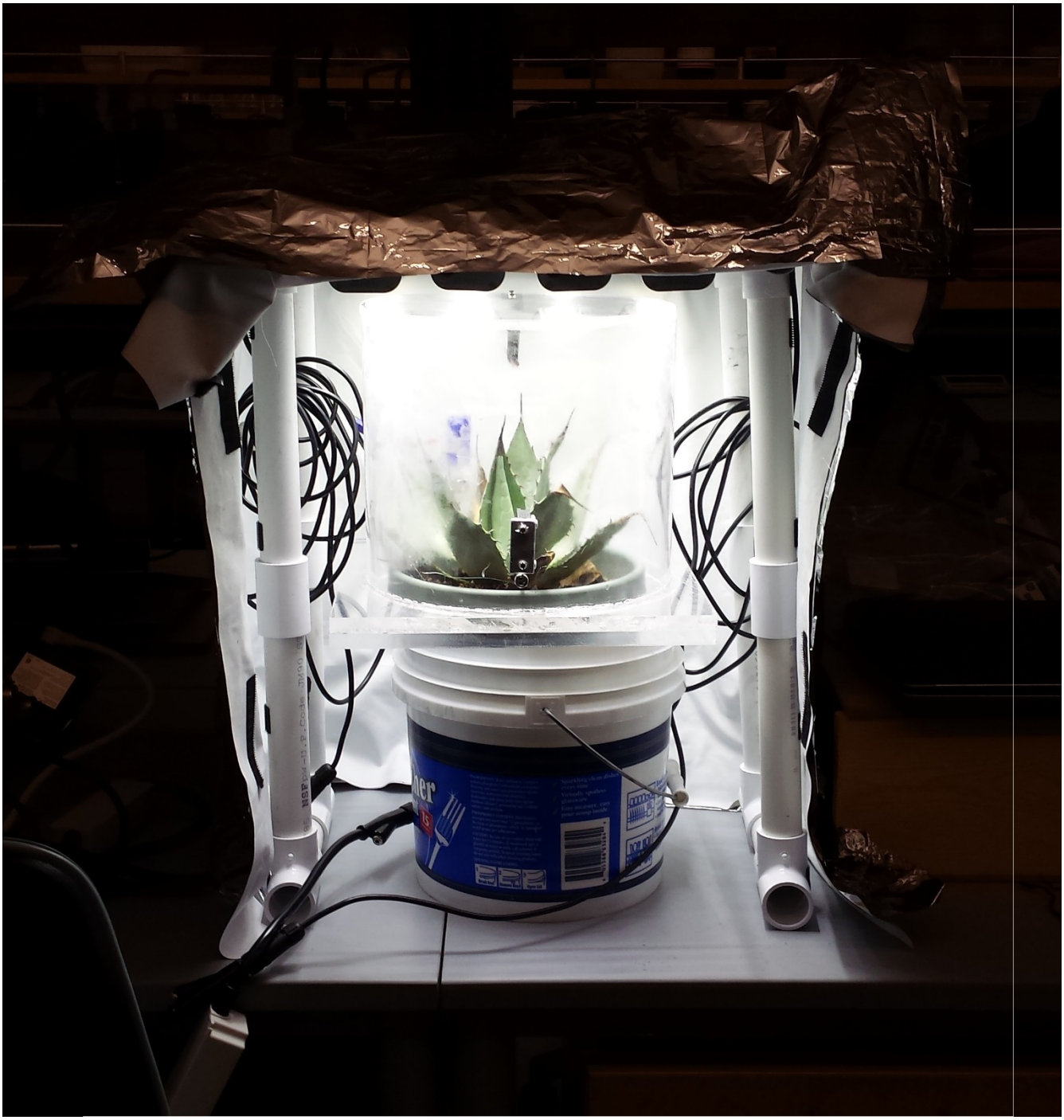

Fig. 6. Mature plant acrylic gas exchange chamber under a PvC frame equipped with LEDs and a light excluding shade cloth with plant contained within.

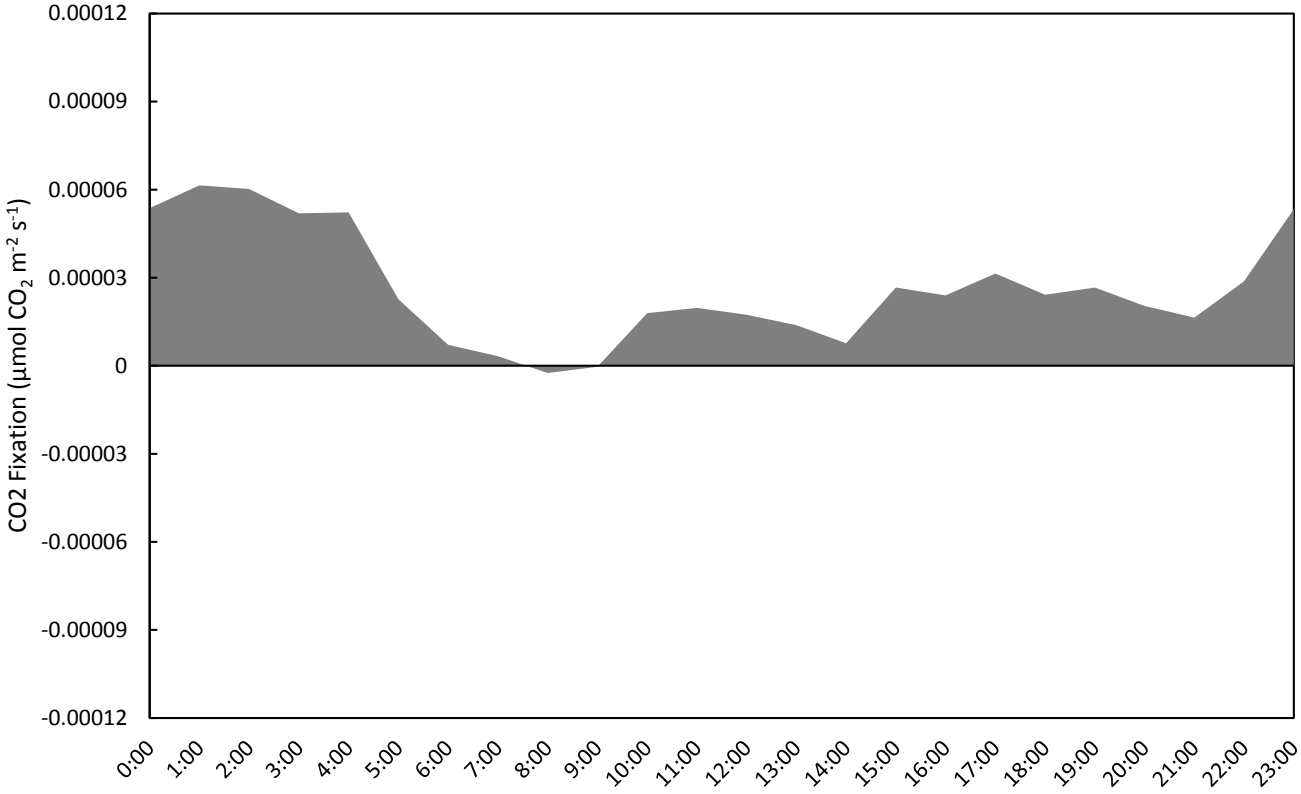

Fig. 7. Average net CO_2 fixation in µmol CO_2 m^{-2} s^{-1} for seedlings across age classes treatments, and species except for *Agave ellemeetiana* and *Prochnyanthes mexicana* per hour.

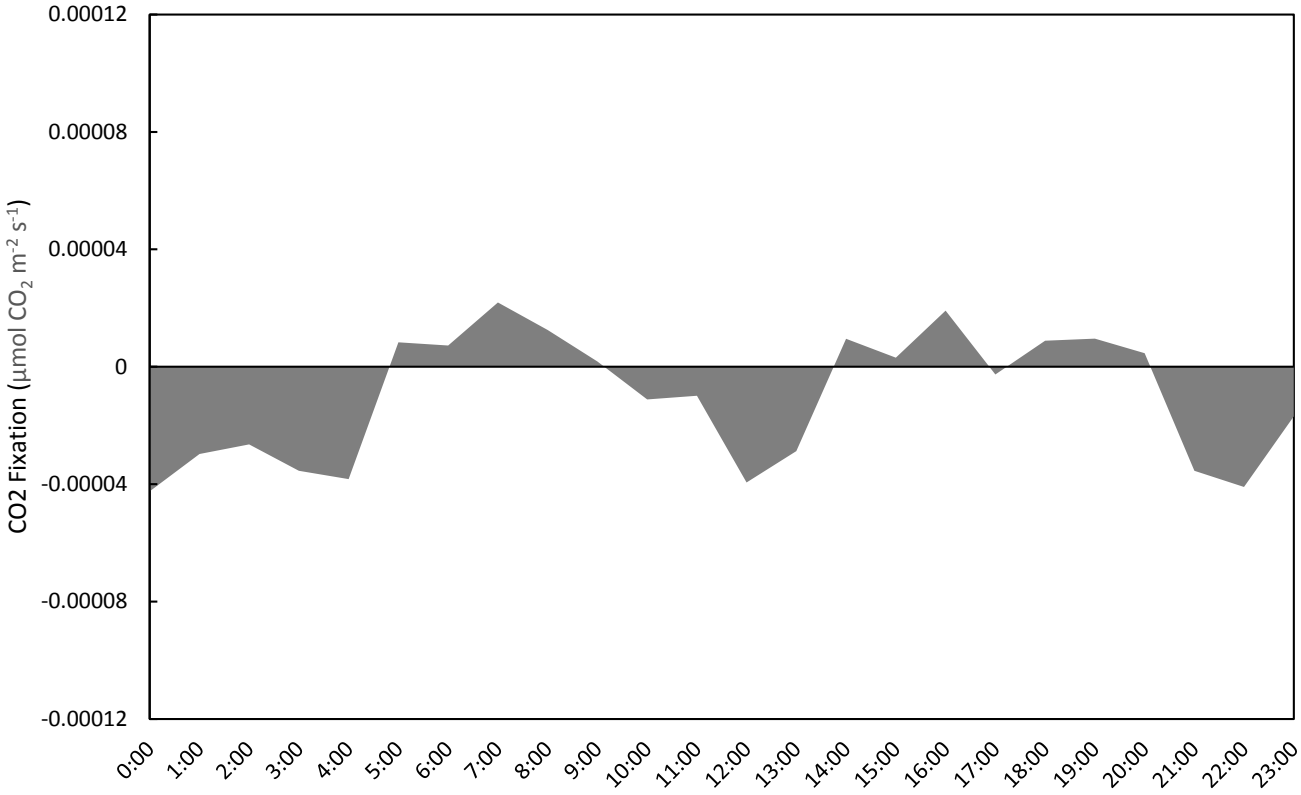

Fig. 8. Average CO_2 fixation in µmol CO_2 m^{-2} s^{-1} for *Agave ellemeetiana* across treatments per hour.

Fig. 9. Average CO_2 fixation in µmol CO_2 m^{-2} s^{-1} for *Prochnyanthes mexicana* across treatments per hour.

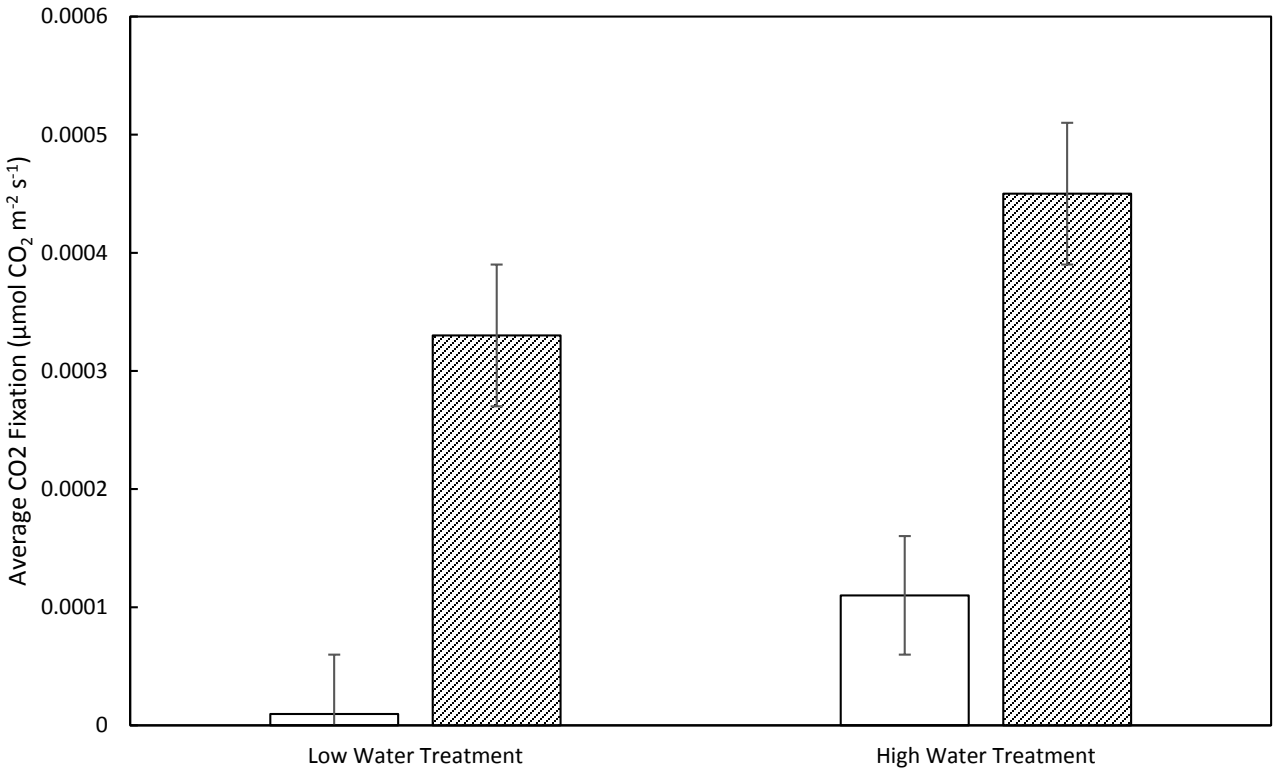

Fig. 10. Net seedling CO_2 fixation across species by treatment, and age classes. Two-month-old seedlings are represented by non-patterned bars, while 10-month-old seedlings are represented by patterned bars. Low water treatment plants were watered every 3 days, while high water treatment plants were watered daily. Refer to Table 1 for a list of seedling species and age class combinations.

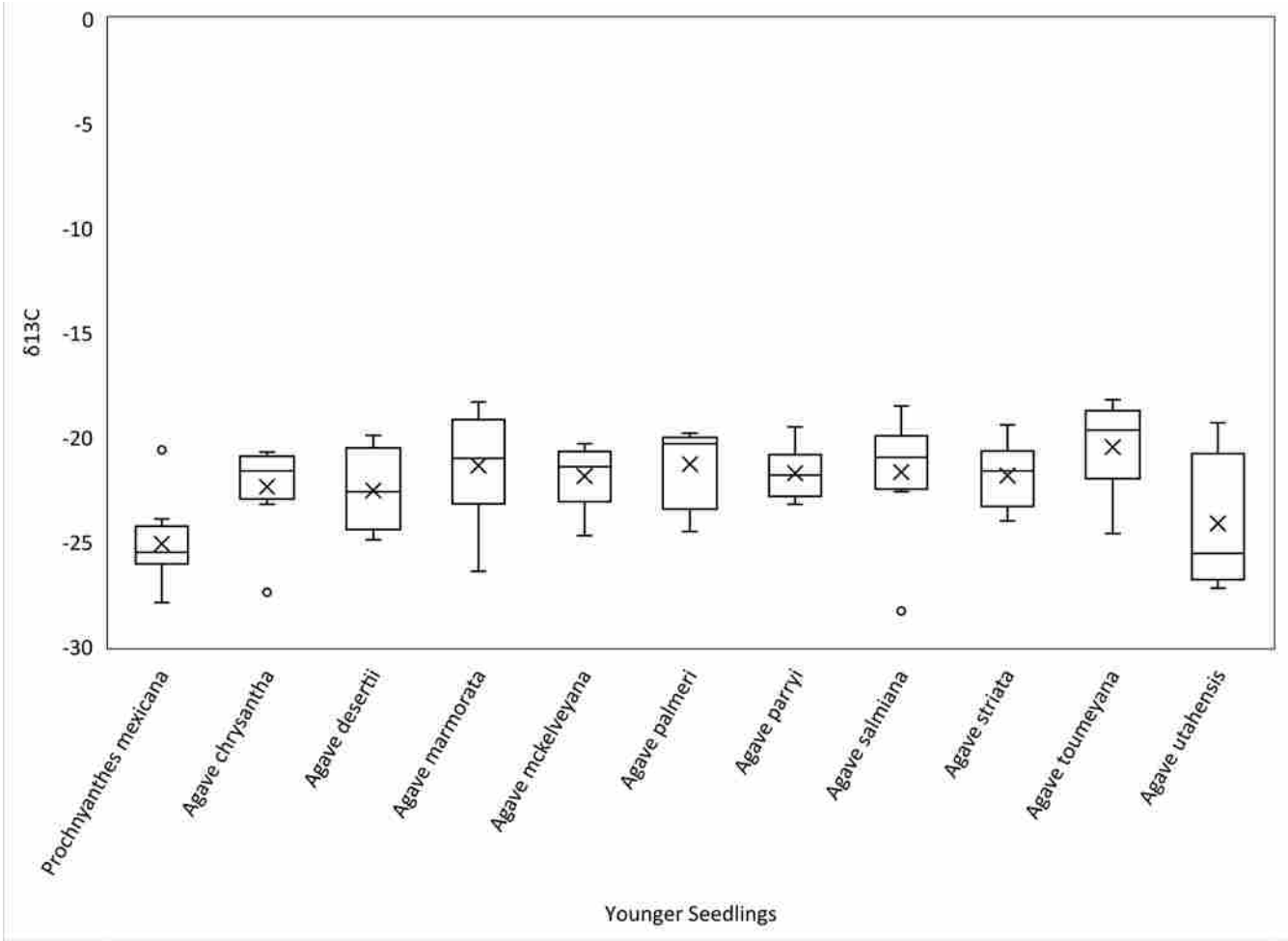

Fig. 11. 2-month-old age class seedling carbon 12 and 13 isotope ratios in parts per million by species across treatments.

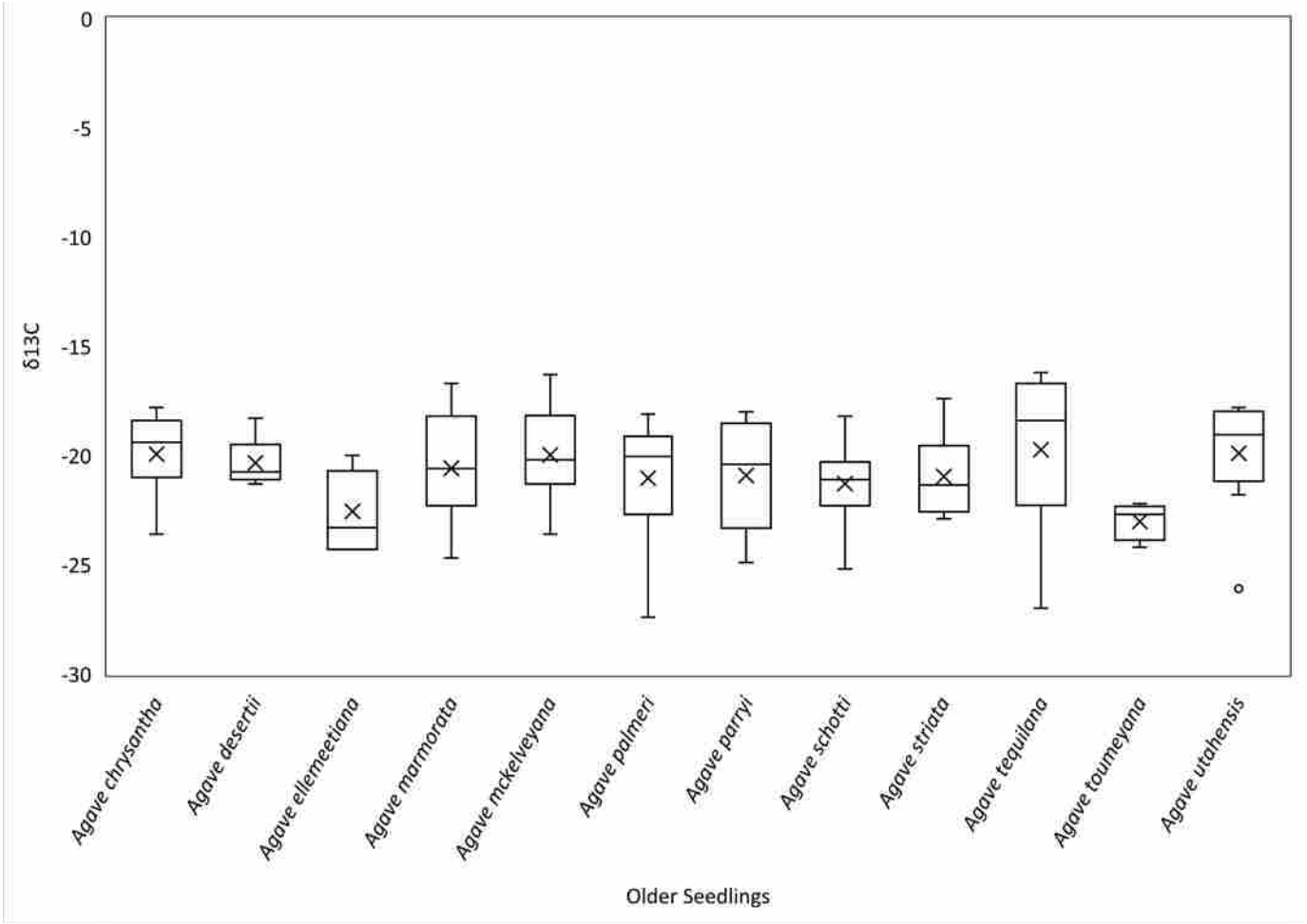

Fig. 12. 10-month-old age class seedling carbon 12 and 13 isotope ratios in parts per million by species across treatments.

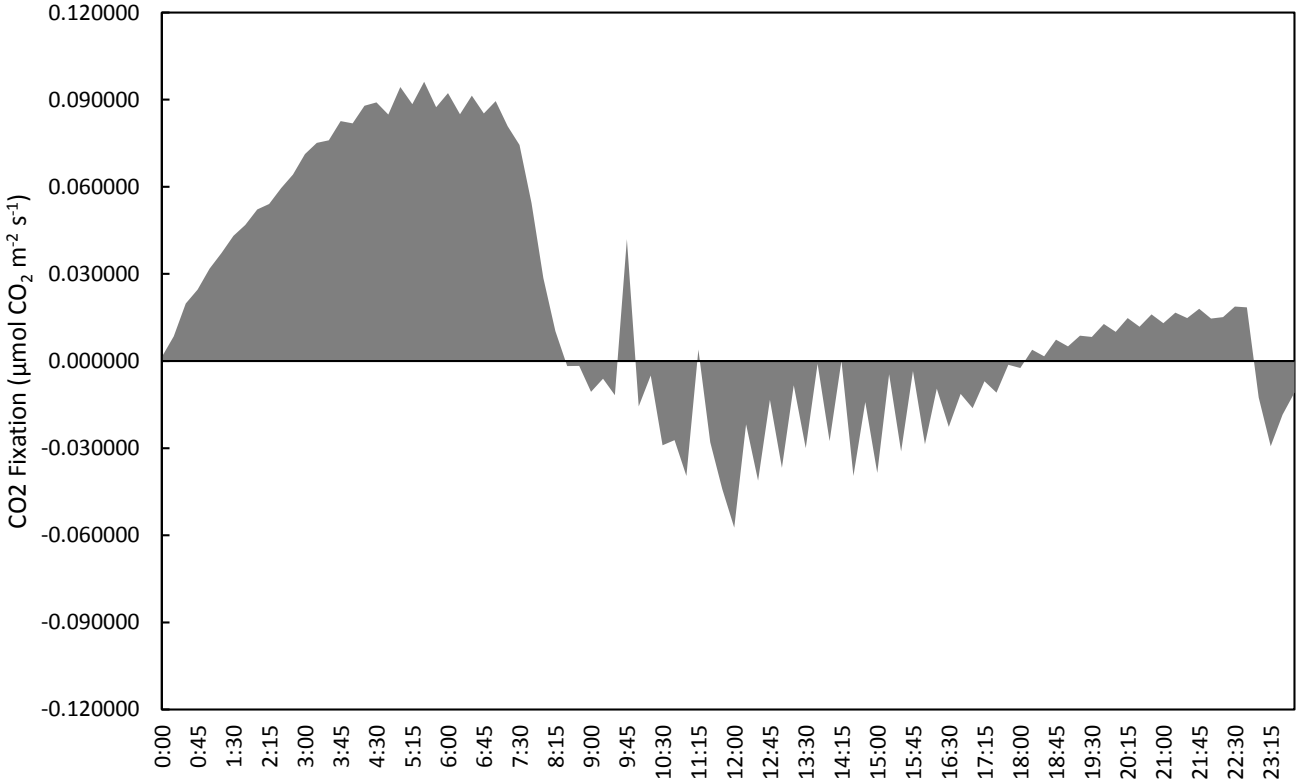

Fig. 13. Average net CO_2 fixation in µmol CO_2 m^{-2} s^{-1} for mature species except for *Polianthes tuberosa* (*Agave deserti, Agave palmeri, Agave parryi, and Agave utahensis*) across treatments per hour.

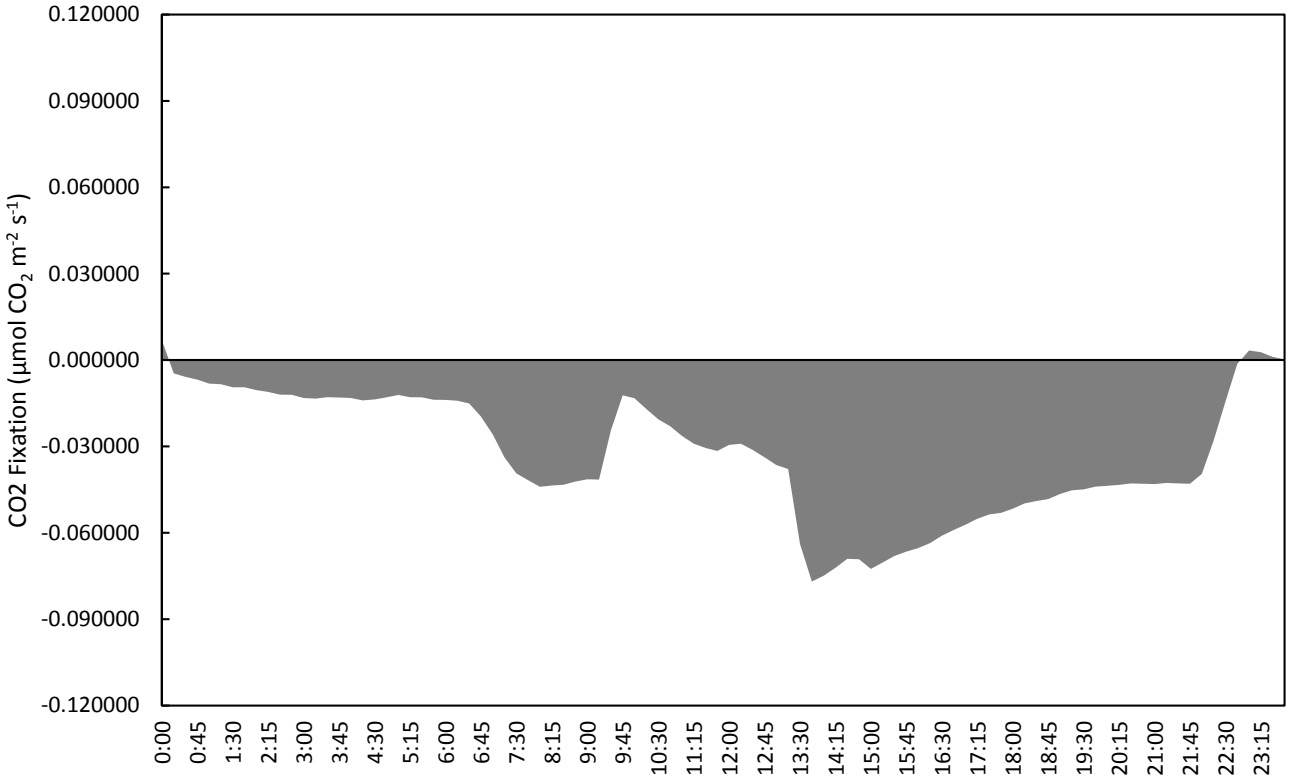

Fig. 14. Average net CO_2 fixation for *Polianthes tuberosa* in the 12% moisture treatment per hour. Moisture treatment percentages refer to the % content of water in the growing medium.

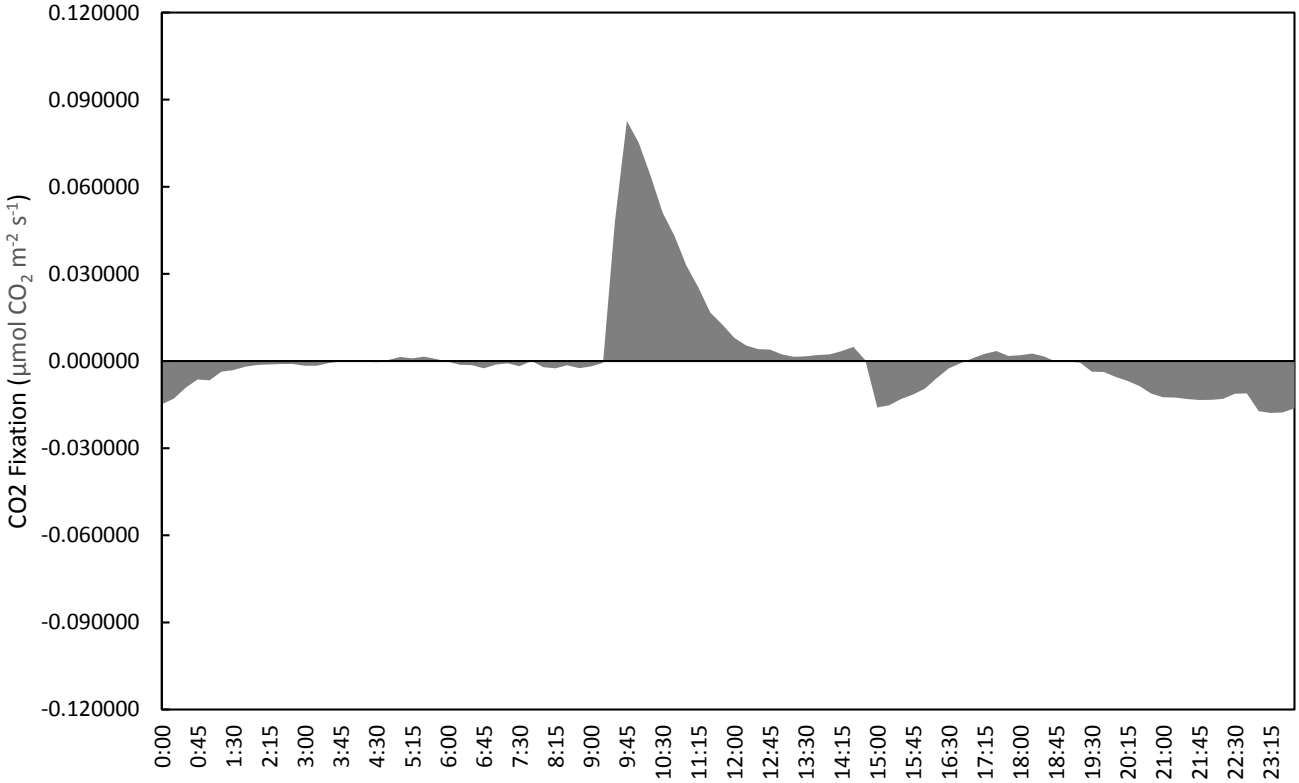

Fig. 15. Average net CO_2 fixation for *Polianthes tuberosa* in the 33% moisture treatment per hour. Moisture treatment percentages refer to the % content of water in the growing medium.

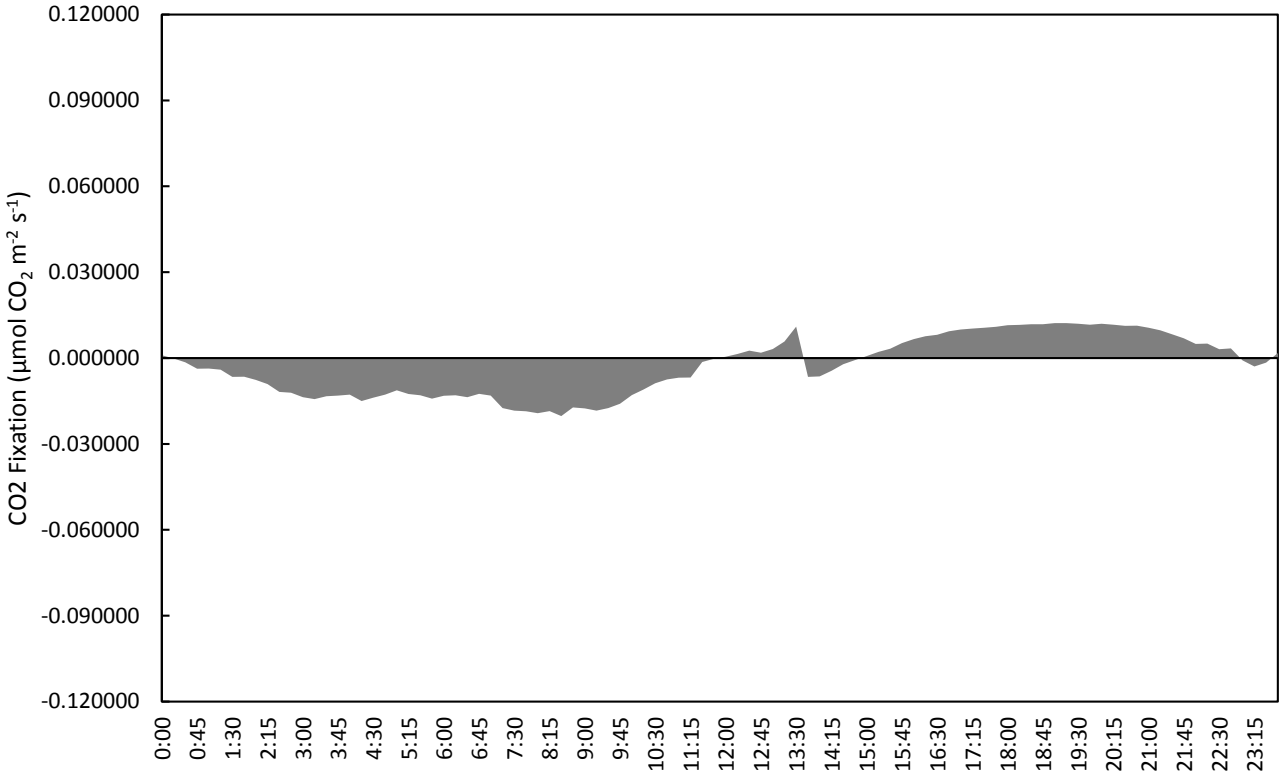

Fig. 16. Average net CO_2 fixation for *Polianthes tuberosa* in the 100% moisture treatment per hour. Moisture treatment percentages refer to the % content of water in the growing medium.

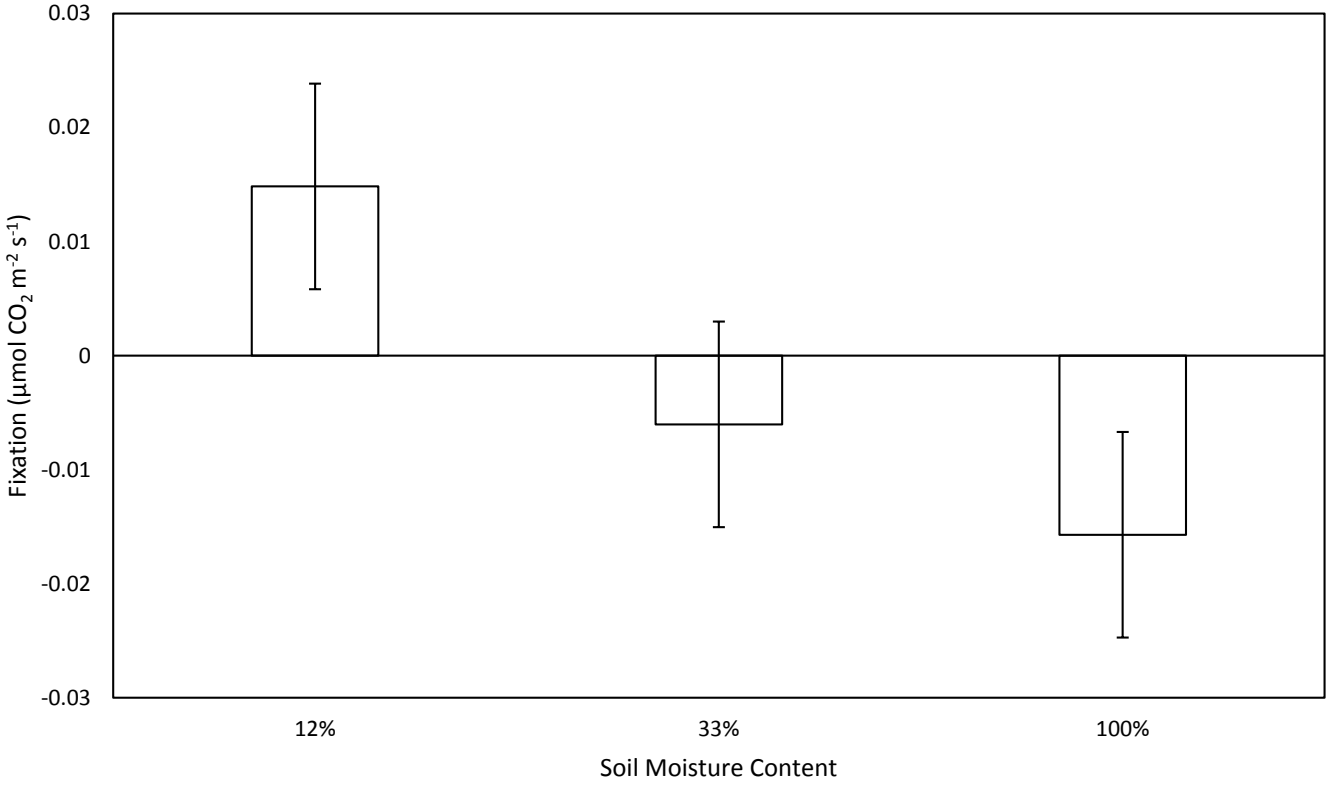

Fig. 17. Average diel CO_2 fixation for each treatment across mature species (*Agave deserti, Agave palmeri, Agave parryi, and Polianthes tuberosa*) in µmol CO_2 m^{-2} s^{-1}. Moisture treatments indicate the % composition of moisture in the plant's growing medium.

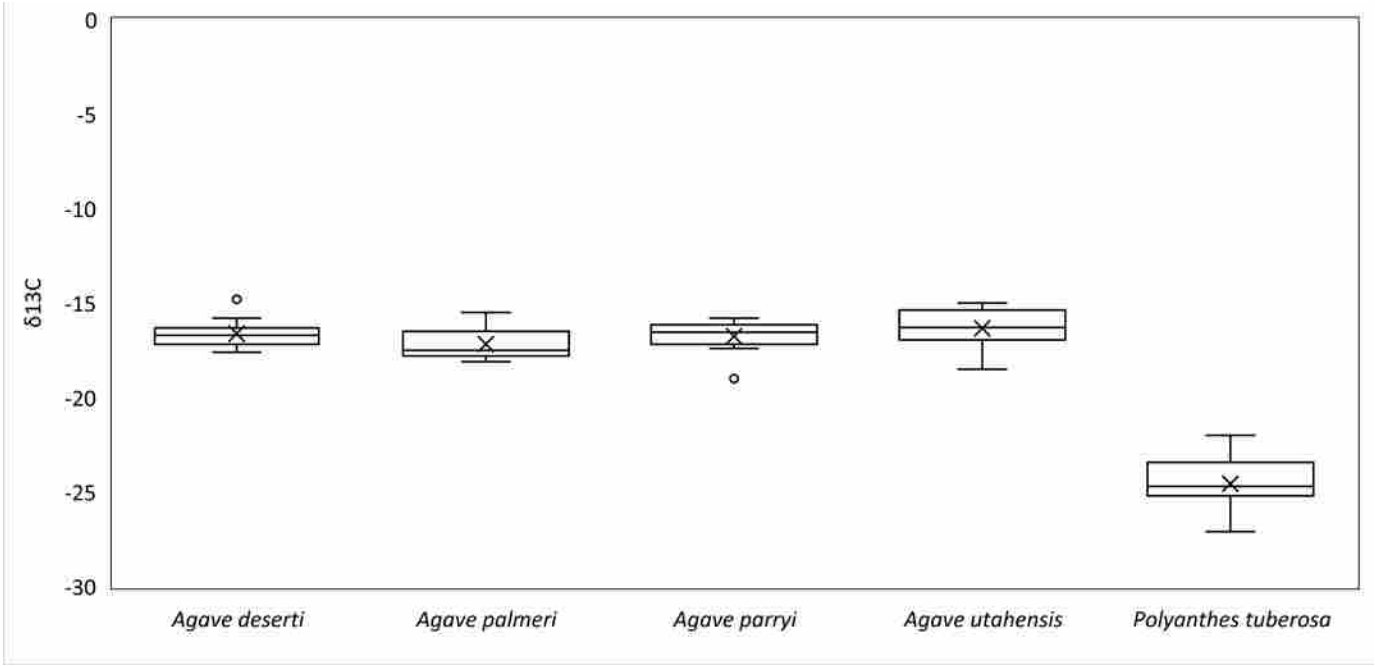

Fig. 18. Average carbon 12 and 13 isotope ratios for mature plant species across treatments in parts per million.

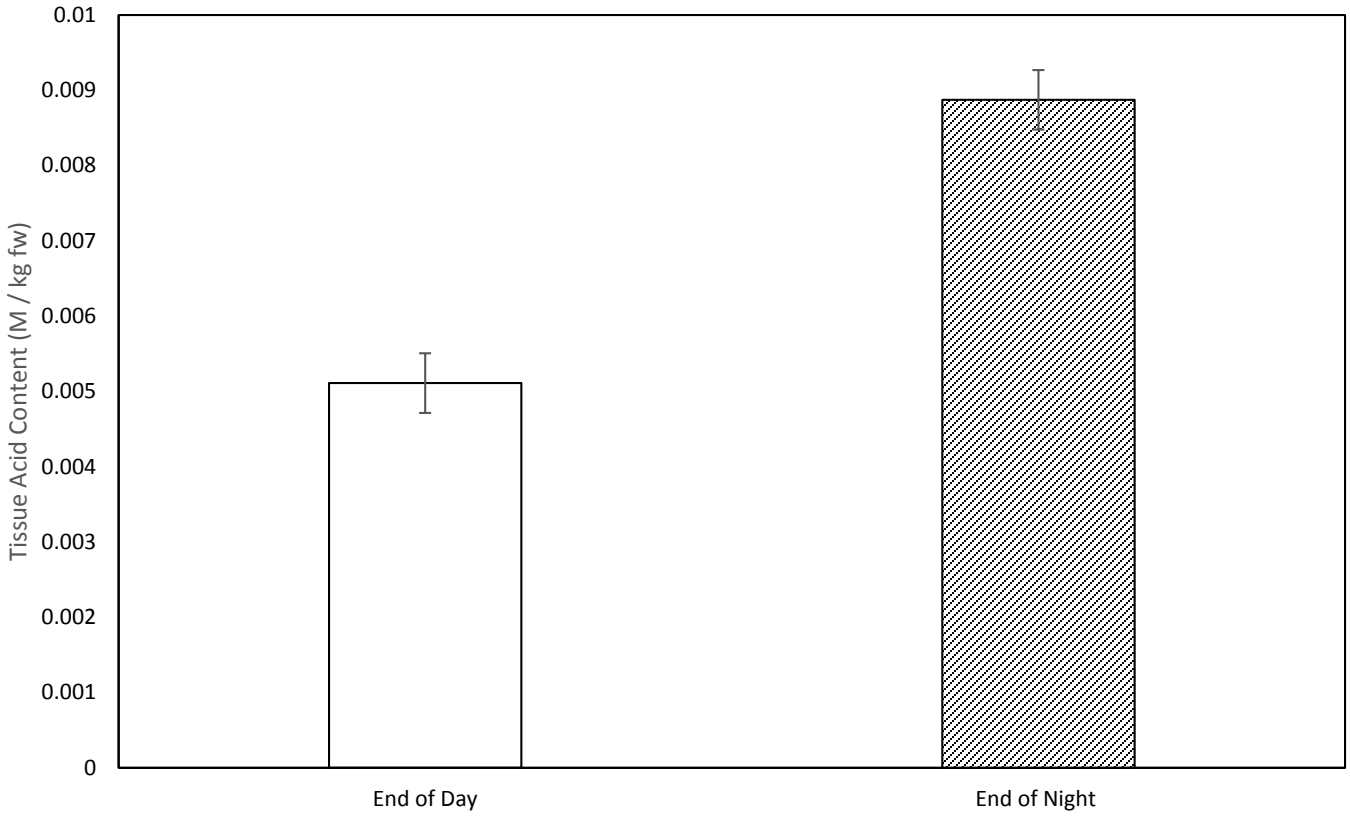

Fig. 19. Mature *Agave sensu lato* tissue acid content in Moles per kg of fresh weight at the end of the day and at the end of the night.

Table 1. List of Agave species used in the seedling experiment. The key number is unique to each species and age class combination. These numbers were used to identify species / age class combinations in Fig. 1.

Species	Key #	Age Class
Agave ellemeetiana	1	2-month-old
Agave mckelveyana	2	2-month-old
Agave salmiana	3	2-month-old
Agave chrysantha	4	2-month-old
Procnyanthes mexicana	5	2-month-old
Agave palmeri	7	2-month-old
Agave marmorata	8	2-month-old
Agave striata	9	2-month-old
Agave toumeyana	10	2-month-old
Agave parryi	11	2-month-old
Agave utahensis	12	2-month-old
Agave deserti	13	2-month-old
Blank	14	2-month-old
Agave deserti	15	10-month-old
Agave parryi	16	10-month-old
Agave palmeri	17	10-month-old
Agave tequilana	18	10-month-old
Agave ellemeetiana	19	10-month-old
Agave toumeyana	20	10-month-old
Agave marmorata	21	10-month-old
Agave utahensis	22	10-month-old
Agave schotti	23	10-month-old
Agave striata	24	10-month-old
Agave chrysantha	25	10-month-old
Agave mckelveyana	26	10-month-old
Blank	27	10-month-old

Table 2. Average fixation rates per species, age class and phase across all moisture treatments in μmol CO_2 m^{-2} s^{-1}. Capital letters shared between entries designate no significant difference for that comparison. Lower case letters shared between entries designate no significant difference for that comparison. Plants marked to have pairs of species age groups were represented in both the 2-month-old and 10-month-old age classes. Species marked to have no age pairs were only represented in one of the age classes.

			Overall Estimates (μmol CO_2 m^{-2} s^{-1})				
	Species	Age Class	Phase 1	Phase 2	Phase 3	Phase 4	Average
Species / Age Group Pairs	*A. chrysantha*	2-months	0.000007045 ± 0.000018 hijklmno	0.000003832 ± 0.000022 hijklmno	-0.0000000236 ± 0.000023 ijklmno	0.000011 ± 0.000022 fghijklmno	0.000006016 ± 0.000018 DEF
		10-months	0.000122 ± 0.000019 a	0.000068 ± 0.000024 bcdefgh	0.000089 ± 0.000025 abcd	0.000074 ± 0.000024 abcdefg	0.000100 ± 0.000019 A
	A. deserti	2-months	0.000015 ± 0.000018 fghijklmno	-0.00000819 ± 0.000022 lkmno	0.000003375 ± 0.000023 hijklmno	0.000009576 ± 0.000022 hijklmno	0.000008395 ± 0.000018 DEF
		10-months	0.000098 ± 0.000019 abc	0.000043 ± 0.000024 bcdefghijkl	0.000098 ± 0.000019 bcdefghij	0.000075 ± 0.000024 abcdef	0.000079 ± 0.000019 AB
	A. marmorata	2-months	0.000016 ± 0.000018 fghijklmno	0.000006684 ± 0.000022 hijklmno	0.000008102 ± 0.000023 hijklmno	0.000011 ± 0.000022 fghijklmno	0.000012 ± 0.000018 DEF
		10-months	0.000019 ± 0.000019 efghijklmno	0.000004272 ± 0.000024 hijklmno	0.000002312 ± 0.000025 hijklmno	0.0000009726 ± 0.000024 jklmnopq	0.000011 ± 0.000019 DEF
	A. mckelveyana	2-months	0.000021 ± 0.000018 efghijklmno	0.000009694 ± 0.000022 hijklmno	0.000027 ± 0.000023 defghijklmno	0.000026 ± 0.000022 defghijklmno	0.000021 ± 0.000018 DEF
		10-months	0.000071 ± 0.000018 abcdefg	-0.00001 ± 0.000022 lkmno	0.000027 ± 0.000023 efghijklmno	0.000045 ± 0.000022 bcdefghijkl	0.000046 ± 0.000018 BCD
	A. palmeri	2-months	0.000015 ± 0.000018 fghijklmno	0.000021 ± 0.000022 efghijklmno	0.000018 ± 0.000023 efghijklmno	0.000008598 ± 0.000022 hijklmno	0.000015 ± 0.000018 DEF
		10-months	0.000068 ± 0.000018 abcdefgh	0.0000005016 ± 0.000022 ijklmno	0.000032 ± 0.000023 defghijklmn	0.000045 ± 0.000022 bcdefghijkl	0.000047 ± 0.000018 BCD
	A. parryi	2-months	0.000011 ± 0.000018 fghijklmno	-0.000000918 ± 0.000022 ijklmno	0.000002265 ± 0.000023 ijklmno	0.000008365 ± 0.000022 hijklmno	0.000007187 ± 0.000018 DEF
		10-months	0.000041 ± 0.000018 cdefhijkl	-0.00001 ± 0.000022 klmno	0.000023 ± 0.000023 defghijklmno	0.000027 ± 0.000022 defghijklmno	0.000027 ± 0.000018 CDEF
	A. striata	2-months	0.000007552 ± 0.000018 hijklmno	0.000005279 ± 0.000022 hijklmno	0.000013 ± 0.000023 fghijklmno	0.000012 ± 0.000022 fghijklmno	0.00000873 ± 0.000018 DEF
		10-months	0.000030 ± 0.000018 defghijklmn	-0.00001 ± 0.000022 lmno	0.000019 ± 0.000023 efghijklmno	0.000017 ± 0.000022 fghijklmno	0.000018 ± 0.000018 DEF
	A. toumeyana	2-months	0.000022 ± 0.000018 efghijklmno	0.000008502 ± 0.000022 hijklmno	0.000018 ± 0.000023 efghijklmno	0.000009984 ± 0.000022 ghijklmno	0.000017 ± 0.000018 DEF
		10-months	0.000039 ± 0.000018 cdefghijkl	-0.00003 ± 0.000022 no	-0.00000187 ± 0.000023 ijklmno	0.000002285 ± 0.000022 ijklmno	0.000050 ± 0.000018 DEF
	A. utahensis	2-months	0.000021 ± 0.000018 efghijklmno	0.000030 ± 0.000022 defghijklmn	0.000043± 0.000023 bcdefghijkl	0.000009643 ± 0.000022 hijklmno	0.000025 ± 0.000018 CDEF
		10-months	0.000057 ± 0.000018 bcdefghij	-0.00000241 ± 0.000022 jklmno	0.000011 ± 0.000023 fghijklmno	0.000024 ± 0.000022 defghijklmno	0.000034 ± 0.000018 BCDE
No Age Pairs	*A. salmiana*	2-months	0.000008268 ± 0.000022 hijklmno	-0.0000264 ± 0.000022 jklmno	0.000001851 ± 0.000023 ijklmno	0.000015 ± 0.000022 fghijklmno	0.00000643 ± 0.000018 DEF
	P. mexicana	2-months	-0.00002 ± 0.000018 lmno	-0.00000366 ± 0.000022 jklmno	-0.00002 ± 0.000023 lmno	0.0000004595 ± 0.000022 ijklmno	-0.00001 ± 0.000018 EF
	A. schotti	10-months	0.000099 ± 0.000019 ab	0.000036 ± 0.000024 defghijklm	0.000039 ± 0.000025 defghijklm	0.000064 ± 0.000024 abcdefghi	0.000072 ± 0.000019 ABC
	A. tequilana	10-months	0.000076 ± 0.000018 abcde	-0.00000701 ± 0.000022 klmno	0.000027 ± 0.000023 defghijklmno	0.000050 ± 0.000022 bcdefghijk	0.000050 ± 0.000018 ABCD
	A. ellemeetiana	10-months	-0.00002 ± 0.000018 mno	-0.00001 ± 0.000022 lkmno	-0.00004 ± 0.000023 o	-0.00000777 ± 0.000022 klmno	-0.00002 ± 0.000018 F

Table 3. Seedling carbon 12 and carbon 13 isotope ratios in parts per million by species and age class across treatments. Letters shared between entries designate no significant difference for that comparison. Plants marked to have pairs of species age groups were represented in both the 2-month-old and 10-month-old age classes. Species marked to have no age pairs were only represented in one of the age classes.

	Species	Age Class	Carbon Signature
Species / Age Group Pairs	*A. chrysantha*	2-month-old	-22.1694 ± 0.747 DEFG
		10-month-old	-19.9427 ± 0.8059 ABC
	A. deserti	2-month-old	-22.1469 ± 0.8059 CDEFG
		10-month-old	-20.3375 ± 0.7449 ABCD
	A. marmorata	2-month-old	-21.35 ± 0.7449 ABCDEF
		10-month-old	-20.4844 ± 0.8059 ABCD
	A. mckelveyana	2-month-old	-21.85 ± 0.7440 BCDEF
		10-month-old	-19.975 ± 0.7449 ABC
	A. palmeri	2-month-old	-21.275 ± 0.7449 ABCDE
		10-month-old	-21.0375 ± 0.7449 ABCDEF
	A. parryi	2-month-old	-21.7125 ± 0.7449 ABCDEF
		10-month-old	-20.9125 ± 0.7449 ABCDEF
	A. striata	2-month-old	-21.825 ± 0.7449 BCDEF
		10-month-old	-20.9625 ± 0.7449 ABCDEF
	A. toumeyana	2-month-old	-20.4625 ± 0.7449 ABCD
		10-month-old	-23.025 ± 0.7449 FG
	A. utahensis	2-month-old	-24.1125 ± 0.7449 GH
		10-month-old	-19.9 ± 0.7449 AB
No Age Pairs	*A. salmiana*	2-month-old	-21.6625 ± 0.7449 ABCDEF
	P. mexicana	2-month-old	-25.3756 ± 0.747 H
	A. schotti	10-month-old	-21.121 ± 0.8059 ABCDEF
	A. tequilana	10-month-old	-19.7375 ± 0.7449 A
	A. ellemeetiana	10-month-old	-22.746 ± 0.8059 EFG

Table 4. Average fixation rates by species, age class and phase for 12% moisture treatment applications in μmol CO_2 m^{-2} s^{-1}. Capital letters shared between entries designate no significant difference for that comparison. Lower case letters shared between entries designate no significant difference for that comparison. The % moisture indicates the % content of water in the plant's growing medium.

	12% Moisture (μmol CO_2 m^{-2} s^{-1})				
Species	Average	Phase 1	Phase 2	Phase 3	Phase 4
Agave deserti	0.004287 ± 0.04692 ABC	0.03856 ± 0.04697 abcdef	0.1239 ± 0.04992 ab	-0.115 ± 0.05081 g	-0.03382 ± 0.04992 bcdefg
Agave palmeri	0.08756 ± 0.04692 A	0.09035 ± 0.04697 ab	0.09433 ± 0.04992 ab	0.08532 ± 0.05081 abc	0.08025 ± 0.04992 abc
Agave parryi	-0.00038 ± 0.03853 ABC	0.000713 ± 0.04697 abcdefg	0.000613 ± 0.04992 abcdefg	-0.00424 ± 0.05081 abcdefg	0.08025 ± 0.04992 abcdefg
Agave utahensis	0.05574 ± 0.03853 AB	0.06172 ± 0.03857 abcd	0.05589 ± 0.04096 abcde	0.04771 ± 0.04169 abcdef	0.05767 ± 0.04096 abcde
Polianthes tuberosa	-0.07303 ± 0.04692 C	-0.6837 ± 0.04697 efg	-0.07366 ± 0.04992 gf	-0.07662 ± 0.05081 gf	-0.07347 ± 0.04992 gf

Table 5. Average fixation rates by species, age class and phase for 33% moisture treatment applications in μmol CO_2 m^{-2} s^{-1}. Capital letters shared between entries designate no significant difference for that comparison. Lower case letters shared between entries designate no significant difference for that comparison. The % moisture indicates the % content of water in the plant's growing medium.

	33% Moisture (μmol CO_2 m^{-2} s^{-1})				
Species	Average	Phase 1	Phase 2	Phase 3	Phase 4
Agave deserti	-0.1350 ± 0.04692 ABC	-0.01312 ± 0.04697 bcdefg	-0.00975 ± 0.04992 bcdefg	-0.01676 ± 0.05801 bcdefg	-0.01436 ± 0.04992 bcdefg
Agave palmeri	-0.01800 ± 0.04692 ABC	-0.01732 ± 0.04697 bcdefg	-0.01310 ± 0.04992 bcdefg	-0.02101 ± 0.05081 bcdefg	-0.02055 ± 0.04992 bcdefg
Agave parryi	-0.01017 ± 0.04692 ABC	-0.00887 ± 0.04697 bcdefg	-0.00715 ± 0.04992 bcdefg	-0.01341 ± 0.05081 bcdefg	-0.01126 ± 0.04992 bcdefg
Agave utahensis	-0.03388 ± 0.06748 ABC	-0.00626 ± 0.06754 abcdefg	-0.04960 ± 0.07165 bcdefg	-0.03615 ± 0.07289 bcdefg	-0.04351 ± 0.07165 bcdefg
Polianthes tuberosa	-0.00303 ± 0.04692 ABC	-0.00210 ± 0.04697 abcdefg	0.000969 ± 0.04992 abcdefg	-0.00646 ± 0.05081 bcdefg	-0.00452 ± 0.04992 abcdefg

Table 6. Average fixation rates by species, age class and phase for 100% moisture treatment applications in μmol CO_2 m^{-2} s^{-1}. Capital letters shared between entries designate no significant difference for that comparison. Lower case letters shared between entries designate no significant difference for that comparison. The % moisture indicates the % content of water in the plant's growing medium.

	100% Moisture (μmol CO_2 m^{-2} s^{-1})				
Species	Average	Phase 1	Phase 2	Phase 3	Phase 4
Agave deserti	-0.02160 ± 0.04692 ABC	-0.02055 ± 0.04697 bcdefg	-0.02069 ± 0.04992 bcdefg	-0.02305 ± 0.05081 bcdefg	-0.02211 ± 0.04697 bcdefg
Agave palmeri	-0.06032 ± 0.04692 BC	-0.05347 ± 0.04697 cdefg	-0.06813 ± 0.04992 efg	-0.06350 ± 0.05081 defg	-0.05618 ± 0.04992 cdefg
Agave parryi	0.002906 ± 0.04692 ABC	0.003171 ± 0.04697 abcdefg	0.002089 ± 0.04992 abcdefg	0.003038 ± 0.05081 abcdefg	0.003327 ± 0.04992 abcdefg
Agave utahensis	0.050509 ± 0.04692 AB	0.1024 ± 0.04697 ab	0.1568 ± 0.04992 a	-0.07127 ± 0.05081 efg	0.01443 ± 0.04992 abcdefg
Polianthes tuberosa	-0.00173 ± 0.04692 ABC	-0.00168 ± 0.04697 abcdefg	-0.00287 ± 0.04992 abcdefg	-0.00215 ± 0.05081 abcdefg	-0.00023 ± 0.04992 abcdefg

Table 7. Average fixation rates by species, age class and phase for mature plants across moisture treatment applications in μmol CO_2 m^{-2} s^{-1}. Capital letters shared between entries designate no significant difference for that comparison. Lower case letters shared between entries designate no significant difference for that comparison.

	Average of All Treatments (μmol CO_2 m^{-2} s^{-1})				
Species	Average	Phase 1	Phase 2	Phase 3	Phase 4
Agave deserti	-0.01027 ± 0.02709 A	0.001631 ± 0.02712 ab	0.03114 ± 0.02882 a	-0.05042 ± 0.02934 b	-0.02343 ± 0.02882 ab
Agave palmeri	0.003083 ± 0.02709 A	0.006521 ± 0.02712 ab	0.004367 ± 0.02882 ab	0.000269 ± 0.02934 ab	0.001173 ± 0.02882 ab
Agave parryi	-0.00255 ± 0.02709 A	-0.00166 ± 0.02712 ab	-0.00148 ± 0.02882 ab	-0.00487 ± 0.02934 ab	-0.00218 ± 0.02882 ab
Agave utahensis	0.02415 ± 0.03007 A	0.05263 ± 0.03010 a	0.05436 ± 0.03198 a	-0.01990 ± 0.03255 ab	0.009529 ± 0.03198 ab
Polianthes tuberosa	-0.02593 ± 0.02709 A	-0.02405 ± 0.02712 ab	-0.02519 ± 0.02882 ab	-0.02841 ± 0.02934 ab	-0.026-9 ± 0.02882 ab

Table 8. Mature plant carbon 12 and carbon 13 isotope ratios by species across treatments for mature plants. Letters shared between entries designate no significant difference for that comparison.

Species	12%	33%	100%	Average
Agave deserti	-16.7 ± 0.5012 abc	-16.025 ± 0.5012 ab	-17.125 ± 0.05012 abc	-16.4278 ± 0.2894 A
Agave palmeri	-17.425 ± 0.5012 bc	-16.45 ± 0.4012 abc	-17.625 ± 0.5012 c	-16.6167 ± 0.2894 A
Agave parryi	-17.325 ± 0.5012 bc	-16.4 ± 0.5012 ac	-16.525 ± 0.5012 abc	-16.7500 ± 0.2894 A
Agave utahensis	-15.8333 ± 0.4092 a	-16.35 ± 0.7088 abc	-17.1 ± 0.5012 abc	-17.1667 ± 0.3199 A
Polianthes tuberosa	-25.875 ± 0.5012 e	-24.8 ± 0.5012 e	-23.025 ± 0.5012 d	-24.5667 ± 0.2894 B

Table 9. Mature plant malic acid content in moles per Kg of fresh weight by species and time of sampling across treatments per CAM phase and averaged across CAM phases. Letters shared between entries designate no significant difference for that comparison.

Species	Sampling Time	Tissue malic acid content (Moles / Kg FW) 12% Treatment	33% Treatment	100% Treatment	Average
Agave deserti	End of Day	0.004844 ± 0.001496 efghi	0.002572 ± 0.001496 i	0.007306 ± 0.001496 cdefg	0.004907 ± 0.000864 C
	End of Night	0.009544 ± 0.001504 bcd	0.00701 ± 0.001504 cdefgh	0.009162 ± 0.001504 cde	0.008572 ± 0.000868 AB
Agave palmeri	End of Day	0.005386 ± 0.001496 defghi	0.004629 ± 0.001496 fghi	0.003065 ± 0.001496 i	0.00436 ± 0.000864 C
	End of Night	0.01382 ± 0.001504 ab	0.007244 ± 0.001504 cdefgh	0.006305 ± 0.001504 defghi	0.009122 ± 0.000868 A
Agave parryi	End of Day	0.004275 ± 0.001496 ghi	0.003965 ± 0.001496 ghi	0.006893 ± 0.001496 cdefghi	0.005044 ± 0.000864 C
	End of Night	0.005363 ± 0.001504 defghi	0.006618 ± 0.001504 cdefghi	0.01397 ± 0.001504 a	0.008649 ± 0.000868 AB
Agave utahensis	End of Day	0.004074 ± 0.001222 ghi	0.005353 ± 0.002116 defghi	0.005577 ± 0.001496 defghi	0.005002 ± 0.000955 C
	End of Night	0.006024 ± 0.001228 defghi	0.01569 ± 0.002127 a	0.008918 ± 0.001504 cdef	0.01021 ± 0.00096 A
Polianthes tuberosa	End of Day	0.006938 ± 0.001496 cdefgh	0.008021 ± 0.001496 cdefg	0.003773 ± 0.001496 ghi	0.006244 ± 0.000864 BC
	End of Night	0.006637 ± 0.001504 cdefghi	0.005922 ± 0.001504 defghi	0.01089 ± 0.001504 abc	0.007817 ± 0.000868 AB