AGAVE FLOWER VISITATION BY PALLID BATS, *ANTROZOUS PALLIDUS*, IN THE BIG BEND REGION OF TEXAS

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ABSTRACT

Pallid bats, *Antrozous pallidus,* though primarily insectivorous gleaning predators, are known to consume nectar of the cardón cactus, *Pachycereus pringlei*, in the Sonoran Desert. It is unknown whether a similar nectar feeding behavior may be occurring in the Big Bend region of Texas, where several researchers have captured pallid bats covered in pollen. I collected pollen samples from 67 pallid bats in Brewster County, Texas between April and August 2018. Pollen-covered pallid bats were captured in every month sampled. The pollen collected in all samples was homogeneous and identified as *Agave* pollen. Two species of *Agave* occur in this region of Texas, *Agave havardiana* and *Agave lechuguilla*. A linear discriminant analysis classified 556 of 723 of the pollen grains analyzed as *A. lechuguilla.* Additional evidence from infrared video footage collected in August of 2018, indicates that pallid bats are becoming covered in *A. lechuguilla* pollen as a result of nectarivory.

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INTRODUCTION

Pallid bats, *Antrozous pallidus,* are found throughout western North America from British Columbia to central Mexico including large portions of southern and western Texas (Hall 1981; Ammerman et al. 2012). Pallid bats are most abundant in low elevation desert and xeric habitats but also are present in lower abundance in mixed conifer forest habitats at elevations up to 2,440 m (Hermanson and O'Shea 1983). In desert habitats, pallid bats are most commonly encountered near rocky outcroppings with a nearby source of water (Hermanson and O'Shea 1983). Diurnal roosting sites are characteristically located in the crevices of exposed cliff faces (Miller and Jensen 2013; Schorr and Siemers 2013). Historically pallid bats were thought to remain relatively close to diurnal roosts, venturing <3 km in nightly foraging bouts (Bell 1982); however, recent advances in radio telemetry technology indicate that this might not always be the case (Ball 2002; Baker et al. 2008). Pallid bats tracked in the Great Basin Desert in central Nevada were found to regularly commute much greater distances of 6.5 and 8.5 km to foraging grounds (Ball 2002). The foraging behavior of pallid bats typically entails gleaning large arthropods (>17mm) from the ground or low foliage (Bell 1982; Ammerman et al. 2012). Unlike many other vespertilionid species, pallid bats do not use echolocation exclusively to locate their prey but instead can locate prey from noises generated by prey movement such as wing beats or foot falls (Fuzessery et al. 1993). Large prey items are carried to a night roost, where hard or indigestible body segments are removed before the prey is consumed (O'Shea and Vaughn

1977; Lenhart et al. 2010).

Pallid bats have been documented preying on a wide variety of arthropods and occasionally also preying on small vertebrates (Lenhart et al. 2010). Lenhart et al. (2010) identified prey items of pallid bats by collecting discarded body parts from underneath a colonial night roost in the Indio Mountains of West Texas with the most frequent taxa being orthopterans (44.1%), coleopterans (26.8%), and solifugids (16.2%); other identified taxa represented 4% or less of the total. Analysis of the stomach contents of 9 individuals collected in Big Bend National Park found orthopterans, lepidopterans, and neuropterans (antlions) to be the most frequently consumed insect orders; however, several other orders were also identified, and 38% of insect fragments were not identified to order (Easterla and Whitaker 1972). Significant variation in the diet of pallid bats has been documented in response to seasonal changes in prey abundance (Johnston and Fenton 2001) and also in response to extreme drought (Kuzdak 2017). During 2011, a year of extreme drought in Big Bend National Park, pallid bats consumed 2.33 times more diversity than their diet in a typical wet year (Kuzdak 2017).

Dietary variation also occurs as a result of individual dietary selectivity, so much so that the diet of an individual often is not reflective of the average population diet (Johnston and Fenton 2001). Scorpions, though infrequently consumed, are known to be prey items of pallid bats (O'Shea and Vaughn 1977), and recently, pallid bats have been shown to be immune to the sting of Arizona bark scorpions, *Centruroides sculpturatus*, which has an extremely painful sting that occasionally causes death in humans (Hopp et al. 2017).

Although considered gleaning insectivores, pallid bats have been observed on the fruit and flowers of some species of plants including organ pipe cactus, *Stenocereus thurberi*; Mexican giant cardón, *Pachycereus pringlei*; and Havard's agave, *Agave havardiana* (Howell 1980; Kuban 1989; Frick et al. 2009). Howell (1980) observed a night roost of pallid bats in which 70 individuals were smeared with the pulp and seeds from the fruit of the organ pipe cactus. Analysis of fecal material collected at the site was made up of 25% fruit pulp and seeds and 75% moth scales. Without observing the behavior firsthand, Howell (1980) hypothesized that pallid bats must have incidentally consumed fruit and seeds while preying on moths that were concentrated around the fruits.

Recent studies indicate that certain populations of pallid bats supplement their typical prey items with plant nectar and fruit when it is available (Howell 1980; Kuban 1989; Herrera et al. 1993; Frick et al. 2009; Aliperti et al. 2017). This seasonal change in diet has been observed in localities where the pallid bat is sympatric with a species of chiropterophilous mutualist plant (Frick et al. 2009). In the Sonoran Desert, the night-blooming flowers of the cardón cactus are frequently visited by a nectarivorous bat species (*Leptonycteris yerbabuenae*) that utilizes this nectar as a primary food source (Frick et al. 2009). Pallid bats in this area also have been found to visit cardón flowers and consume substantial quantities of nectar based on stable isotope analysis (Frick et al. 2014). During the blooming season of the cardón cactus, the diet of pallid bats switches dramatically from C_3 (arthropod) food sources to predominantly crassulacean acid metabolism (CAM) plant food sources, such as cactus and agave. Carbon stable isotope data from breath, blood, and

wing tissue samples indicates that 60-92% of their diet is made up of nectar in the spring (Frick et al. 2014).

Stable carbon isotope data from tissue samples collected from across the range of the pallid bat suggests that a similar behavior may be occurring in other locations where pallid bats are sympatric with bat-adapted cacti and agave (Herrera et al. 1993). This behavior might be occurring in a population of pallid bats in the Chihuahuan Desert of western Texas. Several researchers have documented or observed pallid bats being captured covered in pollen in Big Bend National Park, which is located in the Chihuahuan Desert of western Texas (Barbour and Davis 1969; Kuban 1989; Tuttle in litt., Ammerman pers. obs.). Barbour and Davis (1969) reported that 20 out of 22 pallid bats captured in the Chisos Mountains were heavily covered in pollen and speculated that the pollen was likely *Agave* pollen. During a study of the pollination biology of Havard's century plant, *Agave havardiana*, Kuban (1989) observed one incidence of a pallid bat crawling on the flowering panicles of *A. havardiana.* Additionally, while mist netting around flowering *A. havardiana*, Kuban (1989) captured three pallid bats that were heavily covered in pollen. In a project report to Big Bend National Park, Merlin Tuttle reported capturing pallid bats covered in pollen at a site in the desert lowlands and speculated that the source of the pollen might be *Agave lechuguilla*, which were observed blooming abundantly in the vicinity of the capture site.

It is possible that in the Chihuahuan Desert, pallid bats visit *Agave* or other flowering plants in order to consume insects and that, while gleaning insects from around the flowers, they contact the anthers and become covered in pollen. It is also possible that the

frequency of pollen-covered pallid bats captured and the high concentration of the pollen coverage indicates that some form of facultative nectarivory could be occurring in this region. To date, the identity of the pollen observed on captured pallid bats in Big Bend National park has not been studied or described in the literature. Regardless of the mechanism by which pallid bats become covered in pollen, the *Agave* native to this region are logical candidates for the source of this pollen.

Agave is a genus of succulent plants in the family Agavaceae that has a geographic range from the southwestern United States to northern South America with greatest diversity occurring in Mexico (Gentry 1982; Munguía-Rosas et al. 2009; Verhoek and Hess 2019). Plants in the genus *Agave* produce large quantities of nocturnal nectar and pollen and have been the subject of numerous studies investigating the selective influence of bat pollinators and bat-adapted floral characteristics (Munguía-Rosas et al. 2009). The evolution of *Agave* floral characteristics is thought to be influenced by bat pollination, especially by members of the subfamily Glossophaginae (Howell and Roth 1981; Gentry 1982; Kuban 1989; Flores-Abreu et al. 2019). These characteristics include nocturnal flowering, nocturnal nectar production, light-colored yellow or green-white flowers, flowers with large floral tubes, nocturnal pollen production, nocturnal initiation of stigma receptivity, tall inflorescence stalks, and the reproductive strategy of semelparity (Howell and Roth 1981; Kuban 1989; Slauson 2000; Rocha et al. 2005). Two species in the genus *Agave* occur in the Big Bend region of Texas: *Agave havardiana* and *Agave lechuguilla* (Gentry 1982). There also are thought to be rare hybrid populations of *Agave havardiana* and *Agave lechuguilla* which have been referred to as *Agave glomeruliflora* and *Agave chisosensis* (Gentry 1982).

Agave havardiana is a large paniculate agave in the subgenus *Agave* that grows on rocky, grassland slopes at elevations that range from 1,200 m to 2,000 m (Gentry 1982). There are records of *A. havardiana* occurring in many of the mountains of the Trans-Pecos region of Texas, with records occurring as far north as the Guadalupe Mountains and as far south as mountains in the Mexican states of Coahuila and Chihuahua (Gentry 1982). *Agave havardiana* are semelparous meaning that plants devote a massive amount of resources to one intense season of sexual reproduction and, thereafter, the plant withers and dies (Kuban 1989). Prior to flowering, rosettes grow for 8-20 years accumulating resources for reproduction (Gentry 1982). Each plant produces between 2,200 and 3,100 flowers that are clustered at the end of panicles that branch from a stalk that can grow in excess of 5m tall (Kuban 1989).

Agave havardiana flowers produce substantial amounts of nectar both diurnally and nocturnally (Kuban 1989). These nectar rewards attract insect pollinators and vertebrate pollinators including birds and a migratory species of nectarivorous bat, *Leptonycteris nivalis* (Kuban 1989). Kuban (1989) found that the most effective pollinators of *Agave havardiana* were Mexican long-nosed bats (*L. nivalis*), white-winged doves (*Zenaida asiatica*), and Scott's orioles (*Icterus parisorum*). Despite finding frequent pollination by diurnal birds, Kuban (1989) also observed that bat-adapted floral traits were conserved in *Agave havardiana* flowers (Kuban 1989). Flowers of *Agave havardiana* open at night and anthers dehisce nocturnally and release pollen during the second night (Kuban 1989). After each flower opens, nectar production gradually increases and peaks on the second night, the same period that the greatest quantity of pollen is available to floral visitors (Kuban 1989).

Agave havardiana produce glucose and fructose rich nectar with an average of 4.3% sucrose (Kuban 1989). Hexose (glucose and fructose) rich – sucrose poor nectar is associated with flowers pollinated by bats (Freeman and Reid 1983; Baker et al. 1998). However, recent studies have shown that bat pollinators, Saussure's long-nosed bats (*Leptonycteris curasoae*) and long-tongued bats (*Glossophaga soricina*), show no preference for nectar with different sugar composition (Rodríguez-Peña et al. 2007).

Agave lechuguilla is a small agave in the subgenus *Littaea* that grows small rosettes of narrow leaves. It is most often found in limestone derived soils at elevations below 1500 m; however, its full elevational range is between 950 m and 2,300 m (Gentry 1982; Freeman and Reid 1985). *Agave lechuguilla* grows throughout the Chihuahuan Desert, from central Mexico to southern New Mexico, an unusually large range for an *Agave* species (Gentry 1982; Freeman and Reid 1985). It grows in dense clonal patches of rhizomatically-derived offsets (Gentry 1982; Freeman and Reid 1985). Despite this frequent clonal reproduction, *A. lechuguilla* plants also flower and produce viable seeds (Freeman 1973; Freeman and Reid 1985). In a typical year, *A. lechuguilla* blooms between May and June; however, during dry years, blooming continues until as late as October (Freeman and Reid 1985).

The flowering stalk of *A. lechuguilla* is spicate and generally shorter (1.5-2.5 m) and smaller than the stalks produced by *A. havardiana* (Gentry 1982; Kuban 1989; Fig. 1). Agave species with spicate and racemose inflorescence structure are associated with insect pollination; however, other spicate species of agave are known to attract bat pollinators including the southern long-nosed bat, *Leptonycteris curasoae*, the Mexican long-tongued bat, *Choeronycteris mexicana*, and a long-tongued bat species, *Glossophaga* sp. (Rocha et al.

Agave lechuguilla *Agave havardiana*

Fig. 1 — Comparison of inflorescence structure of *Agave lechuguilla* (left) and *Agave havardiana* (right). *Agave lechuguilla* produces spicate floral stalk, whereas that of *A. havardiana* is paniculate with branching clusters of flowers. The flowering stalk of *A. lechuguilla* is generally shorter, between 1.5m and 2.5m, than the flowering stalk of *A. havardiana* which are generally between 2m and 4m tall but can grow above 5 m (Gentry 1982; Kuban 1989; photos courtesy of Loren Ammerman).

2005). *Agave lechuguilla* is primarily pollinated by nocturnal hawk moths and large diurnal bees but is also visited by a variety of nectar robbers including hummingbirds that are not considered effective pollinators (Silva-Montellano and Eguiarte 2003). Silva-Montellano and Eguiarte (2003) conducted a latitudinal study of the pollinators of *A. lechuguilla* and expected to observe some visitation by nectarivorous bats; however, no bat visitation was observed over the course of that study. The floral traits of *A. lechuguilla* are similar to that of bat-pollinated *Agave* species like *A. havardiana,* in that many of the major floral events occur or initiate nocturnally (Kuban 1989: Freeman and Reid 1985). *Agave lechuguilla* flowers open in the late afternoon, and flowering begins at the bottom of the stalk and progresses upward (Freeman and Reid 1985). Once flowers open, filaments rapidly elongate during the first 24 hours and then anthers dehisce, making pollen available during the second flowering night (Freeman and Reid 1985). Nectar is produced nocturnally during the second and third flowering night and is composed primarily of fructose and glucose with 10% sucrose (Freeman et al. 1983). Flower styles also elongate after flowers open, and stigmas become receptive when the styles reach maximum length approximately 66 hours after flowers open (Freeman and Reid 1985).

These observations of pallid bats and studies of *Agave* floral characteristics suggest the possibility of a relationship between *Agave* species and the pallid bat similar to that of the pallid bat and cardón cactus in which nectar is an important component of the bat's diet. The primary objective of this study was to identify and document the species of pollen found on pallid bats. This objective was divided into two parts, first to determine if the pollen was *Agave* pollen and second to identify the pollen grains as *A. havardiana* or *A.*

lechuguilla. The concentration and distribution of pollen densities could be an important indicator of the mechanism by which these bats become covered in pollen. I hypothesized that pollen on the ventral surface of the body would be more common than the head based on observations reported by Kuban (1989). In addition, I hypothesized that pollen density would vary significantly by month with higher pollen densities during the peak of *Agave* flowering and that females would have higher pollen densities than males because of the energy requirements of pregnancy and lactation.

METHODS

Sampling Strategy — I conducted mist-netting surveys (Kunz et al. 2009) in Brewster County, Texas between April and August of 2018. Mist nets were erected over uncluttered, pools of water or blocking flyways near open water. Each sampling night, mist nets were opened at sunset and closed after 2 to 3 hours of netting effort, depending on successful capture of pallid bats. Due to seasonal fluctuations in the amount of water present in the pools at the sample sites, the number of mist nets set up at each site varied from one to four. Mist nets were monitored a minimum of every 15 minutes. A maximum of 15 pallid bats were sampled per night. This was deemed to be the greatest number of bats that could be sampled in one night, without holding bats for extended periods of time. Upon removing a pallid bat from the net, it was placed in a clean paper cup with a ventilated lid.

I recorded time of capture, morphometric measurements, age class, sex, and reproductive condition. When present, fecal samples were collected from the cups after the bats were released, thus fecal samples were not collected for every bat captured. All available fecal samples were collected and stored in microcentrifuge tubes and frozen at -20°C (Brice et al. 1989). These can be used in future studies to screen for pollen, for stable isotope testing, or diet analysis (Voigt et al. 2009). For captured non-target species, which were placed in clean cloth bags, I recorded age, sex, and reproductive condition before release.

All captured animals were handled and trapped following the guidelines from the National Park Service (NPS 2018) and the American Society of Mammalogists (Sikes et al. 2016). Prior to mist netting and sample collection, this study received approval from the

Angelo State University Institutional Animal Care and Use Committee (APPENDIX I) and the National Park Service (permit number BIBE-2018-SCI-0028). During all bat handling and mist netting we followed protocols for preventing the spread of the fungus *Pseudogymnoascus destructans*, causal agent of White-nose syndrome. Precautions included changing nitrile gloves between handling each bat, discarding paper cups after each use, and decontamination of cloth bags and nets after each survey night by submerging bags and nets in 10% bleach for 20 minutes (White-nosesyndrome.org 2016).

Study sites — Two sites in Big Bend National Park, Ernst Tinaja and Glenn Springs, were sampled at least once each month during April, May, June, July, and August. These sites were selected based upon the past frequency of capture of pollen-covered pallid bats (Ammerman pers. obs.). Ernst Tinaja (29.25609°N, -103.01184°W) is located in a limestone canyon on the western edge of the Dead Horse Mountains at an elevation of 680 m. The tinaja is a reliable water source for bats and typically holds water year-round. Glenn Springs (29.17416°N, -103.15778°W) is a series of shallow spring fed pools located approximately 11 km southeast of the Chisos mountains at an elevation of 774 m. Ernst Tinaja and Glenn Springs are low elevation sites (< 1000 m) and the floral communities of the areas surrounding both sites are classified as mixed desert scrub (Plumb 1991; Fenstermacher et al. 2008). This floral assembly is dominated by creosote (*Larrea tridentate*), Big Bend Prickly Pear (*Opuntia aggeria*), Graham dog cholla (*Opuntia grahamii*), mariola (*Parthenium incanum*), bristly nama (*Nama hispidum*), viscid acacia (*Acacia neovernicosa*), and in some areas, dense patches of *Agave lechuguilla*.

In August, additional mist netting and sample collection occurred at a third location, on a private ranch in Brewster County, Texas 30 km north of Big Bend National Park. Mist netting occurred over 4 nights at 4 different sites. Mist nets were erected over cattle tanks and a shallow pond. At this location pallid bats also were hand netted at a night roost, located in the rafters of a house porch.

Pollen sampling — Pollen density samples were taken from three body regions on each pallid bat: head, ventral surface of the wings, and ventral surface of the abdomen. To estimate pollen density, a 16mm by 20mm piece of tape was pressed to each body region and then attached to a labeled glass slide. The slide was placed in a slide box to prevent contamination between slides.

Pollen samples used for identifying pollen species were collected following the methods described by Kearns and Inouye (1993) and Jones (2012). For this technique forceps were used to dab a small cube of sticky glycerin over areas with visible concentrations of pollen or along the forearm to the wrist and in the ears of the bat when pollen was not immediately visible. After sample collection, each cube was stored individually in a labeled microcentrifuge tube and frozen at -20℃. Later in the lab, the cubes were melted onto microscope slides using a hotplate set at 90°C. A cover slip was placed on the melted sample and, once the slide cooled to room temperature, sealed around the edges with clear nail polish (Voigt et al. 2009).

The gel cubes used for sample collection were made in the lab prior to field collection following a recipe from Kearns and Inouye (1993). In a 50 ml beaker, 10.8 ml of deionized water and 3 grams of gelatin were heated and stirred on a hotplate until the

gelatin dissolved. The solution was removed from the hotplate and 9.2 ml of glycerin was added. I added the basic fuchsin powder gradually, almost one grain at a time, and with each addition, I stirred until the grains were completely dissolved and the solution was a dark, but still translucent, fuchsia. I poured the solution into a plastic tray and allowed it to cool and become firm. Once firm, the gel was cut the gel into cubes that had edge lengths of 2.5 mm and stored in individual microcentrifuge tubes at 4°C.

Pollen reference collection — Between April and August 2018 I collected pollen from identified plants and compiled a reference library of pollen samples (Powell 1998; Powell and Weedin 2004; Dodson and DeWitt 2012). This included pollen samples from *A. havardiana, A. lechuguilla*, and other plants that were observed blooming during the study period. Creating pollen libraries from the same geographic region and during a limited blooming season is an accepted method for identifying pollen when atlases and dichotomous keys are not available (Dafni et al. 2005). A voucher specimen of each plant species was collected, pressed, and dried for species verification (National Park Service Permit BIBE-2018-SCI-0030). The prepared specimens were deposited in the herbarium of the Angelo State Natural History Collection (APPENDIX II).

The pollen samples that were used to create the reference library were collected by plucking whole stamens from the flower and placing them in individual, labeled microcentrifuge tubes. An alternative method, used to collect pollen without damaging the flower, was to dab cotton swabs along a stamen where pollen was visible and then place the cotton swab in a labeled microcentrifuge tube. Pollen samples were frozen at -20℃ until they were converted into light microscopy slides. Pollen from stamen samples or

cotton swab samples was converted into light microscopy slides using the same basic fuchsin gel cubes that were used for collecting pollen samples from bats (Kearns and Inouye 1993). Gel cubes were used to collect pollen from the stamen or cotton swab by rubbing the cube along the stamen or cotton swab.

Analysis of pollen density — I estimated pollen density for each body region by manually counting the pollen grains in a field of view from 8 photographs of the tape samples at 40x magnification and used the mean as an estimate of pollen density. Photographs were taken using a compound light microscope (Eclipse E200LED MVR, Nikon Corporation, Tokyo, Japan) and microscope camera (Infinity 1 Y-TV55, Nikon Corporation, Tokyo, Japan). Each photograph covered a $7mm^2$ area of the slide. Each tape sample was divided into eight unique regions and a photograph was taken within each region centered on a randomly selected point. I used the count tool in NIS Elements Documentation v 4.20 (Nikon Corporation, Tokyo, Japan) to tally pollen grains in each photograph. These pollen density estimates were used to compare the pollen densities between male and female bats and to determine on which body region pollen was most concentrated. Pollen densities also were used to evaluate seasonal changes by comparing mean pollen density by month for bats captured within BBNP. The sites located outside of the park on the private ranch were excluded from this analysis because they were only sampled in August. Statistical analyses were conducted in R-programming (R Development Core Team 2018). Quantile-quantile plots were used to evaluate the normality of monthly pollen density data (Becker et al. 1988). A Fligner-Killeen test was used to evaluate data for homogeneity of group variances (Conover et al. 1981). I used a log transformation to correct for non-normality (Fox and

Weisberg 2011; R Development Core Team 2018). I compared monthly pollen densities using a Welch's analysis of variance (ANOVA) followed by post-hoc tests using pairwise Welch's t-test and Bonferroni corrections to the p-values (Welch 1951; Fox and Weisberg 2011; R Development Core Team 2018). A p-value of ≤ 0.05 was used as the threshold for significance.

Pollen densities were compared between male and female bats to determine whether pollen densities differed between sexes. Quantile-quantile plots were used to evaluate the normality of pollen density data by sex (Becker 1988; Fox and Weisberg 2011; R Development Core Team 2018). A Brown-Forsythe test was used to evaluate data for homogeneity of group variances (Fox and Weisberg 2011). I used a log transformation to correct for non-normality. Pollen densities of male and female bats were compared using an ANOVA test (Fox and Weisberg 2011; R Development Core Team 2018). A p-value of ≤0.05 was used as the threshold for significance.

Identification of pollen species — The first step of pollen identification was to compare the characteristics of unknown pollen grains from bat samples to known pollen samples from the reference library of pollen. In a second identification step, I verified my proposed identification from the first step, using pollen grain measurements and features from published descriptions, images, and general keys of pollen from the same species or from species with similar pollen in the same genus (Erdtman 1969; Kapp 1969; Solomon et al. 1973).

Distinguishing the pollen of closely related species, such as species in the same genus, is difficult (Kaya et al. 2013). In this study, I designed a method to distinguish the

pollen of *Agave havardiana* from *Agave lechuguilla*. Using pollen samples from my reference collection, I statistically compared the difference in five pollen grain features/measurements (Fig. 2; Fig. 3) in order to identify characteristics that might be useful for species identification. The measurements included length of polar axis (Fig. 2), length of equatorial axis (Fig. 2), mean of three exine width measurements (Fig. 2), mean of 10 lumina surface area (Fig.3), and maximum lumen surface area (Punt et al. 2007; Kaya et al. 2013; Fig. 3).

Measurements of pollen grains were taken with oil immersion objective (1000x magnification) using a compound light microscope (Eclipse E200LED MVR, Nikon Corporation, Tokyo, Japan), microscope camera (Infinity 1 Y-TV55, Nikon Corporation, Tokyo, Japan), and a measuring tool in NIS Elements Documentation v 4.20 (Nikon Corporation, Tokyo, Japan). Not all pollen grains were included in this analysis; pollen grains that were damaged or in polar rather than equatorial orientation were not used for measurements.

The exine is the outer portion of the pollen grain that stains noticeably darker when stained with basic fuchsin (Kapp 1969; Moore and Webb 1978). Exine width was observed to vary over the extent of each pollen grain. To account for this variability, I recorded the mean of exine measurements taken at three locations: one measurement at each pole and one at the midline. The outer layer of *Agave* pollen has a network pattern that is defined as a reticulate exine pattern (Fig. 3; Fig. 4). Lumina (lumen, singular), often described as windows, are a feature of reticulate pollen grains where the exine is thinner, lacking some exine layers and appearing a lighter color when stained (Kapp 1969; Moore and Webb 1978;

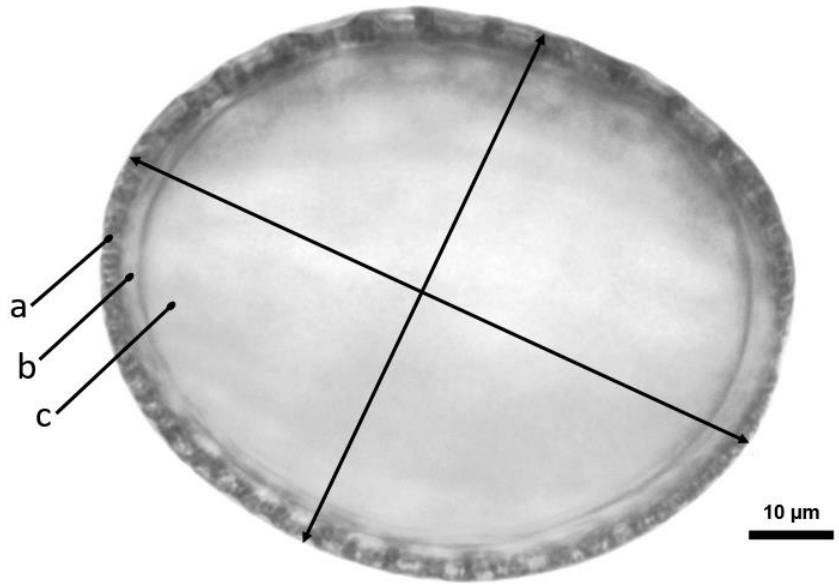

Fig. 2 — Labelled *Agave havardiana* pollen grain photograph. a.) exine b.) intine c.) cytoplasm. The polar axial measurement is the longer measurement from pole to pole and the equatorial axial measurement is perpendicular and midway between the two poles. *Agave havardiana* pollen grain at 1000x magnification (ASNHC herbarium accession number SAT 58750; APPENDIX IV).

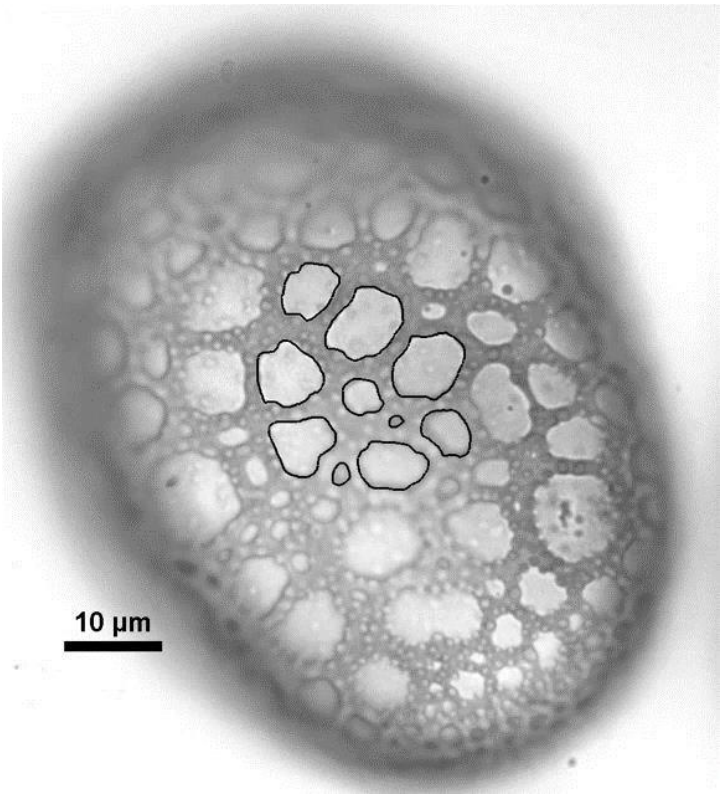

Fig. 3 — *Agave havardiana* pollen grain surface showing ten lumina surface areas outlined in a darker color using the area measurement tool in NIS Elements Documentation v 4.20 (Nikon Corporation, Tokyo, Japan). *Agave havardiana* pollen grain photographed at 1000x magnification (ASNHC herbarium accession number SAT 58750; APPENDIX IV).

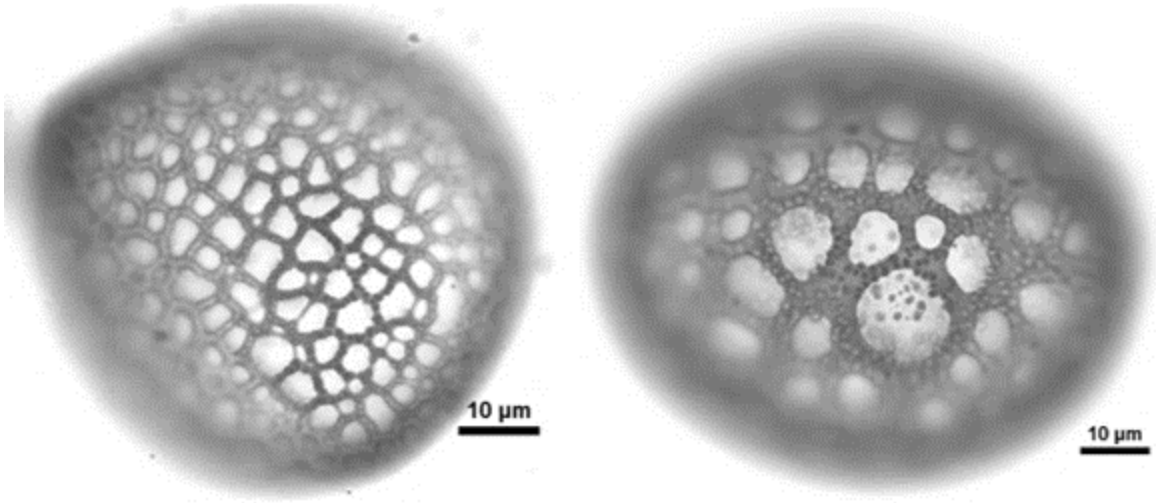

Fig. 4 — Comparison of the reticulate surface of *Agave lechuguilla* pollen (left) and *Agave havardiana* pollen (right).

Punt et al. 2007). *Agave* pollen are heterobrochate meaning that the lumina are variable in size rather than being uniform (Punt et al. 2007). Variability in the size of the lumina could be a distinguishing feature between *Agave* species (Erdtman 1969; Kapp 1969; Punt et al. 2007). After measuring a group of 10 lumina using a freehand tracing tool in in NIS Elements Documentation v 4.20 (Nikon Corporation, Tokyo, Japan), I calculated the mean lumen surface area for each pollen grain. The 10 lumina that I selected for measurement were near the midpoint of the pollen grain and were adjacent to each other. I determined the midpoint based upon the intersection of the polar and equatorial axial measurements. The lumen with the largest surface area measured in this group was recorded as the maximum lumen surface area and this was included as the fifth pollen grain feature used for identification of species.

Statistical analysis of pollen identification — I used a linear discriminant analysis (LDA) in the MASS package of R-programming to distinguish the pollen of *A. havardiana* and *A. lechuguilla* using the previously described five pollen grain measurements (Venables and Ripley 2002; R Development Core Team 2018). A linear discriminant analysis (LDA) is a supervised algorithm that evaluates the ratio of variance between classes to the variance within classes and it can be used as a classification tool where there is overlap between classes. I created a training dataset of pollen grain measurements from known pollen grains collected from plant specimens of *A. havardiana* and *A. lechuguilla*. The LDA uses the training dataset to generate a coefficient (β_x) for each of the pollen measurements ($f(x) = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_5$). The resulting function combines the data from all five pollen grain measurements into a score that classifies the pollen as *A. havardiana* or *A. lechuguilla*.

I used a test dataset of measurements from 100 reference collection pollen grains, 50 from *A. havardiana* and 50 from *A. lechuguilla*, to determine the accuracy of the LDA function by finding the proportion of the pollen grains that were correctly identified. The pollen grains were sourced from reference collection plants from five *A. lechuguilla* specimens and five *A. havardiana* specimens (APPENDIX II).

I collected the same five measurements from the unknown *Agave* pollen grains originating from gel cube swabs of bats: polar axis, equatorial axis, mean exine width (n=3), mean lumen surface area (n=10), and maximum lumen surface area. I used the LDA function to determine the probable species identification of each pollen grain as either *A. havardiana* or *A. lechuguilla*. Ideally, a sample of 20 pollen grains per pallid bat sample were measured for classification using the LDA analysis. However, many bat samples contained damaged pollen grains, pollen grains in an unmeasurable orientation, or very few (< 20) pollen grains. All of the pollen grains that were measured were included in the LDA analysis, but in some instances only one pollen grain was included per bat sample.

Video observations— As part of a wildlife documentary project directed by Skip Hobbie, nightly video recordings were taken of five captive pallid bats on a private ranch in Brewster County, Texas between 9 August and 14 August 2018. The flight tent was 3 x 3 meters with a ceiling that vaulted to approximately 3.5 meters in the center. The flight tent was set up in a large garage that was sheltered from direct light, extreme heat, and inclement weather. The center of the tent was made into a small landscape simulation made of dirt, rocks, and plants from the surrounding habitat. Pallid bats were captured in mist nets over water on 9 August 2018 and placed in the flight tent for recorded

observations. After six nights of filming, all bats were released at the site of capture. The health of each bat was monitored by tracking the body weight and body condition of each bat prior to filming. A RED Epic Dragon camera (RED Digital Cinema, Irvine, California) capable of recording in near infrared light was used for video observations. Infrared lights produce light wavelengths between 850 and 940 nm, which does not negatively impact bat behavior (Mistry and McCracken 1990). Video was recorded at 120 frames per second at a resolution of 4K (4096 x 2160) pixels per frame. On 12 August 2018 a blooming inflorescence of *A. lechuguilla* was cut and placed in the flight tent. Each night between 12 August and 14 August 2018 observations were made of the body position of the bat relative to floral organs during interactions with the *A. lechuguilla* flower inflorescence.

RESULTS

Sampling strategy and study sites — I conducted 18 nights of mist netting between 27 April 2018 and 13 August 2018 at sites within BBNP and sites located on a private ranch in Brewster Co., Texas. A total of 297 bats were captured of 12 different species (APPENDIX III) and 77 pallid bats were captured from both the BBNP sites and the private ranch sites (APPENDIX IV). The majority of the mist netting nights (13/18 nights) occurred within BBNP at Ernst Tinaja and Glenn Springs. Though 18 nights of mist-netting occurred, pallid bats were only captured successfully on 12 of these nights. In April and May pallid bats only were successfully captured at one site, Glenn Springs. In June, July, and August pallid bats were captured and sampled at both Glenn Springs and Ernst Tinaja (Fig. 5). Additional sample collection occurred at a private ranch located in Brewster Co., Texas between 9 August 2018 and 13 August 2018. During this period, pollen samples were collected from a total of 10 pallid bats (Fig. 5). This included pollen samples that were collected from three pallid bats that were hand netted at a night roost on 12 August 2018.

Analysis of pollen density — Tape pollen samples were collected from 67 pallid bats and fuchsin gel samples were collected from 60 pallid bats (APPENDIX IV). Wing samples were found to have significantly higher pollen densities than samples collected from the head and body (Welch's ANOVA $F_{2,\ 107.1}$= 7.95, P<0.001; Fig. 6). Mean pollen densities from wing tape samples ($\bar{x}$ = 4.52 grains/mm^2) were five times higher than head ($\bar{x}$ = 0.80 grains/mm^2) and body ($\bar{x}$ = 0.79 grains/mm^2) samples (Welch's t-test, p_{adj}<0.001). After determining that wing samples contained more pollen than samples from other body

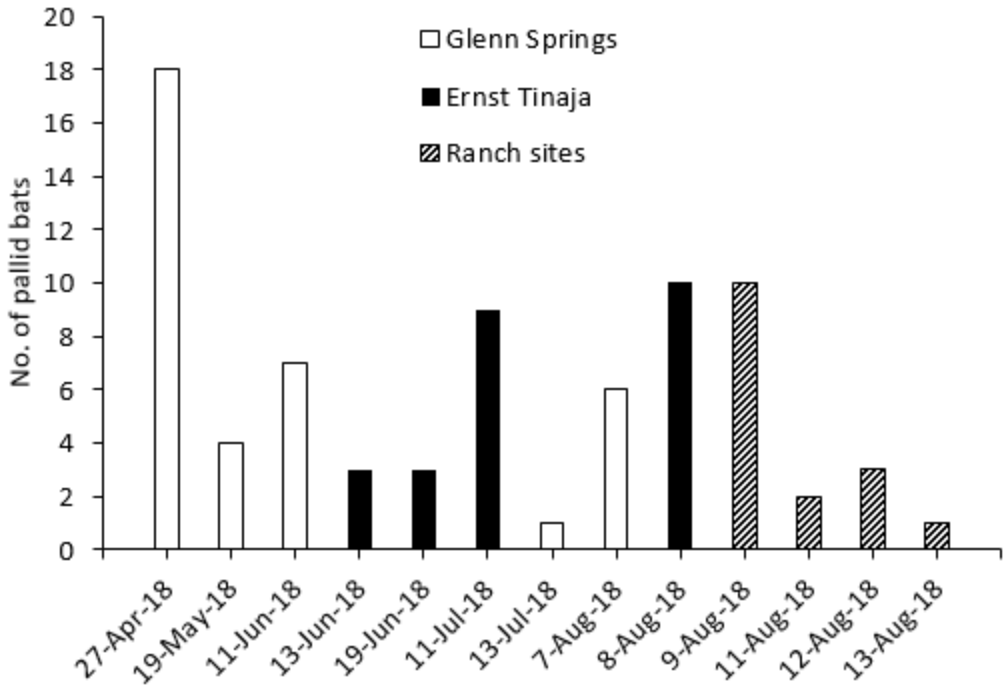

Fig. 5 — Sampling site and date for 77 pallid bats captured in Brewster Co., Texas between 27 April 2018 and 13 August 2018. On 12 August 2018, 3 pallid bats were hand netted at a night roost instead of being captured over a water source.

regions, I used wing samples as the primary measurement of pollen density on the bats for all additional comparisons.

All of the 67 pallid bats sampled were found to have detectable levels of pollen on their wings; however, 11 pallid bat wing samples had low mean pollen densities of < 0.15 pollen grains/mm^2 (Fig. 7). The remaining 56 pallid bats had substantial pollen densities of greater than 0.15 pollen grains/mm^2. Thirteen pallid bats had wing samples with high pollen densities of > 14.3 pollen grains/mm^2. In several instances so much pollen was collected on the tape that it would not adhere to the glass slide.

Comparison of mean pollen densities from the 56 pallid bats captured within BBNP indicated that the pollen densities varied between sampling months (Welch's ANOVA, $F_{4,17.19} = 8.1568$, $P<0.001$; Fig. 8). Pallid bats captured in June (n=13) had significantly lower pollen densities than bats captured in July and April (Welch's t-test, $P_{adj}<0.05$). However, mean pollen densities were not significantly different between the months of April, May, July, and August (Welch's t-test, $P_{adj} > 0.05$). No difference was found in the pollen densities between female (n=48) and male (n=19) pallid bats (ANOVA, $F_{5,342}=1.05$, $P=0.3098$; Fig. 9).

Pollen identification — The morphology of pollen grains from fuchsin gel cubes and tape samples was clearly homogeneous for all observed pollen grains (Fig. 10). Fuchsin gel cube samples were collected for 60 of the 77 pallid bats captured and all of the pollen from these samples was identified as *Agave* spp. pollen. Of the 60 fuchsin gel samples collected from pallid bats, 8 samples did not have pollen grains present in the sample. Pollen grains from 52 pallid bat samples were measured and analyzed using the LDA.

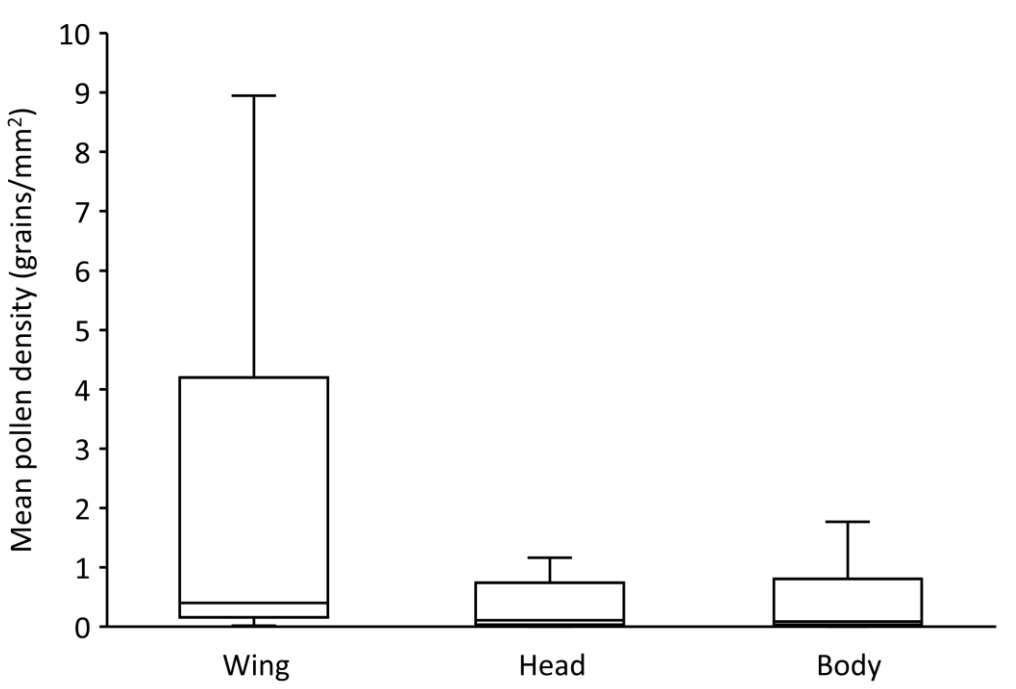

Fig. 6 — Comparison of the mean pollen density from wing, head, and body tape samples collected from 67 pallid bats collected in Brewster Co., Texas between April and August 2018.

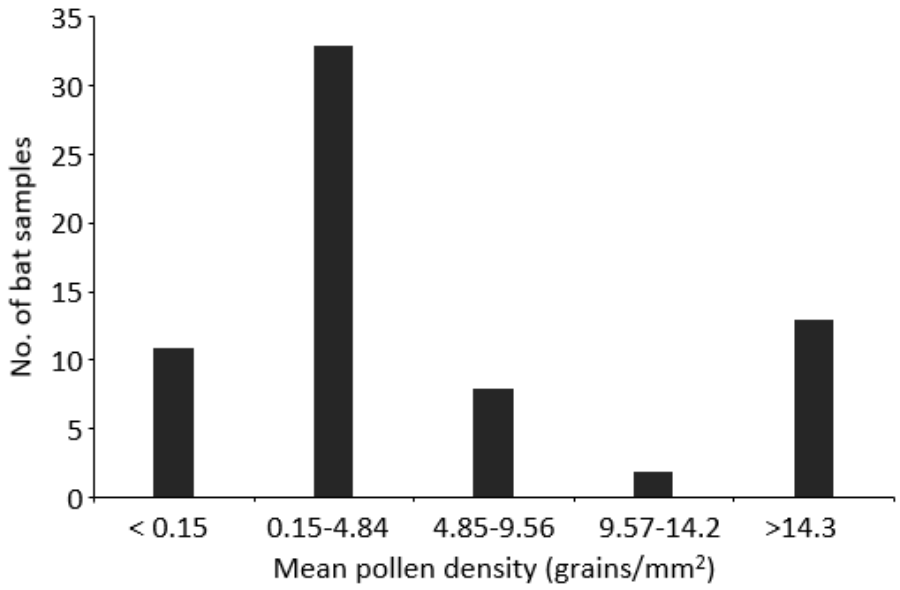

Fig. 7 — Pollen density on the wings of pallid bats categorized in five groups of increasing mean pollen density. Wing samples were collected from 67 pallid bats captured in Brewster Co., Texas between April and August 2018.

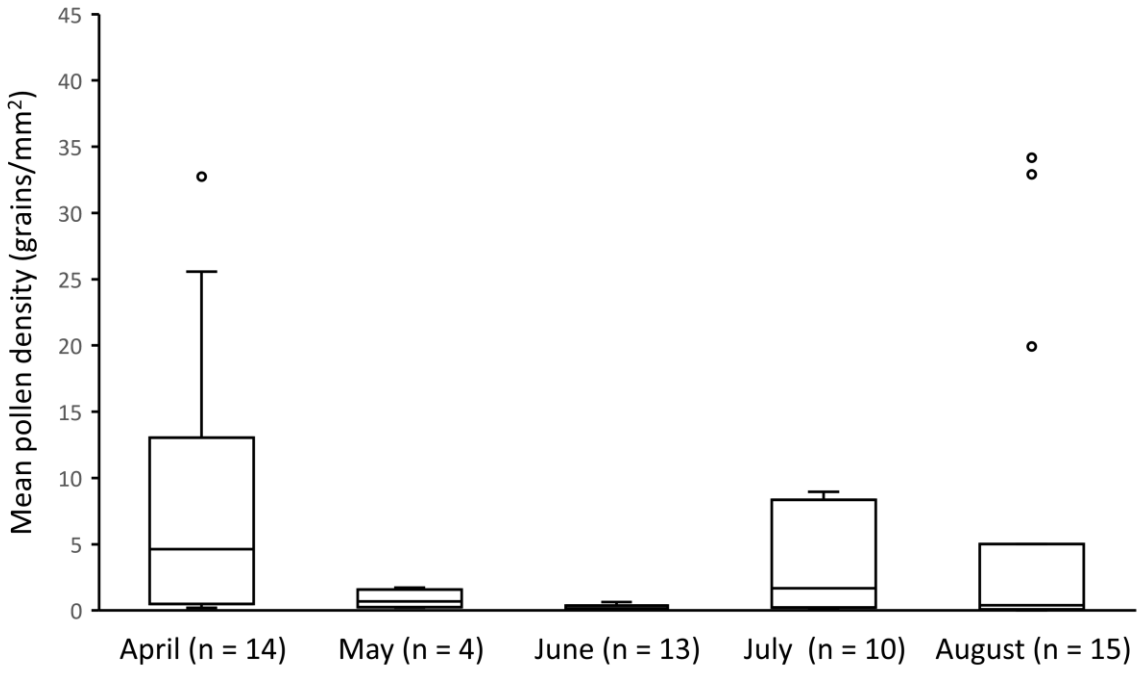

Fig. 8 — Comparison of monthly differences in mean pollen density from the wing samples of 56 pallid bats that were captured in Big Bend National Park between April and August 2018. This includes combined data from Ernst Tinaja and Glenn Springs, however, excludes data from Ranch sites which were only sampled in August.

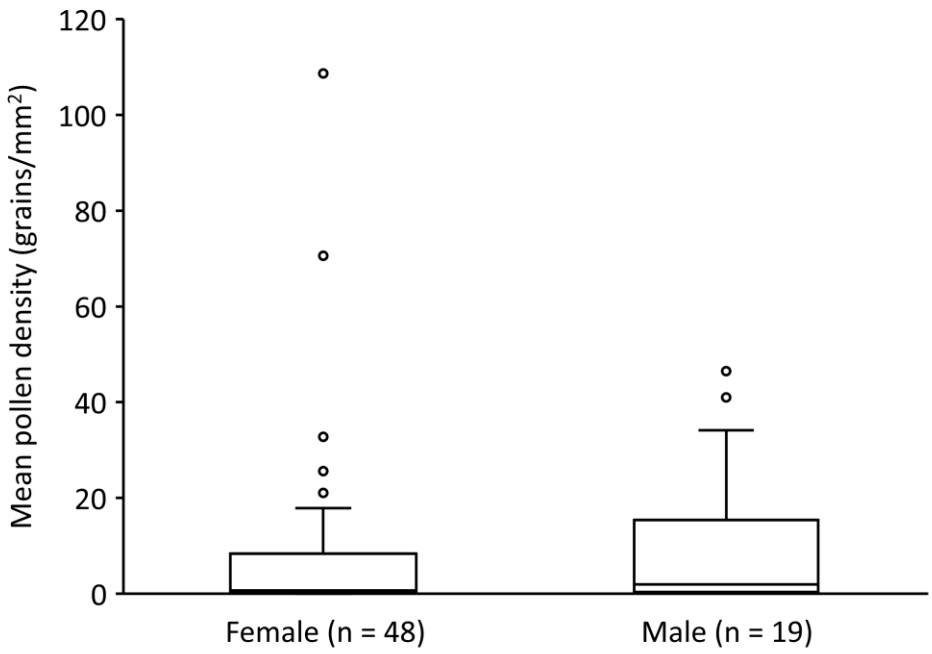

Fig. 9 — Comparison of mean pollen density on the wings of male and female pallid bats. Samples were collected from 67 pallid bats captured in Brewster Co., Texas between April and August 2018.

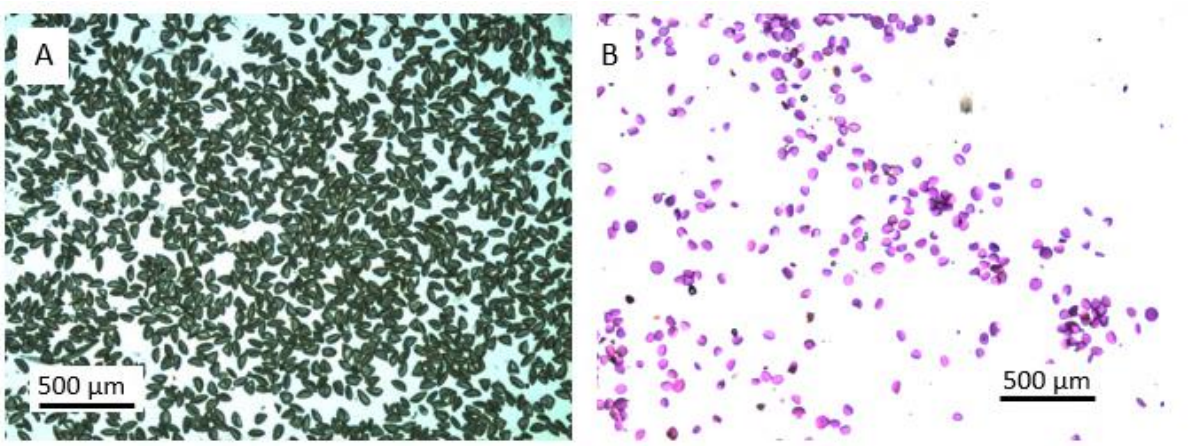

Fig. 10 — Homogeneous pollen morphology was observed in both tape samples and fuchsin gel cube samples. A.) Pollen (100 x magnification) from a wing sample collected using clear tape, from a pallid bat captured in August 2018 in Brewster Co., Texas. B.) Pollen (100 x magnification) collected using a fuchsin gel cube from a pallid bat captured in July 2018 in Brewster Co., Texas.

A dataset of measurements from reference collection pollen grains was used to train the LDA and establish classes for *A. lechuguilla* and *A. havardiana*. The LDA training dataset was composed of five measurements for each pollen grain and included 77 *A. lechuguilla* pollen grains from 5 reference collection plants and 100 *A. havardiana* pollen grains from 5 reference collection plants (Table 1; APPENDIX IV). The LDA generated a coefficient for each of the five pollen grain measurements (Table 2).

The LDA correctly classified 100% of the test dataset (*n*=100) of pollen from reference collections of *A. lechuguilla* and *A. havardiana*. The classification probability for 78% (39 of 50) of the *A. lechuguilla* test pollen grains was ≥ 0.9, whereas only 60% (30 of 50) of the *A. havardiana* test pollen grains had classification probabilities ≥ 0.9 (Fig. 8). Pollen grains with classification probabilities < 0.9 were labeled as intermediate pollen grains, 22% (11/50) of *A. lechuguilla* pollen grains were intermediate and 40% (20/50) of the *A. havardiana* pollen grains were intermediate.

A total of 723 unknown *Agave* pollen grains were analyzed from 52 pallid bat samples (Fig. 12). The result of this analysis indicated that 556 of the pollen grains were *A. lechuguilla* with a classification probability of ≥ 0.9 (Fig. 12). Only three pollen grains were assigned to the *A. havardiana* group with classification probabilities ≥ 0.9, and these were sourced from three different bat samples. Of the 723 pollen grains analyzed, 701 pollen grains had a probability < 0.5 of being *A. havardiana*.

Video observations — During the periods where the *A. lechuguilla* inflorescence was available to the captive pallid bats in the flight tent, the pallid bats were observed to

Table 1— Mean and standard deviation for measurements of reference collection pollen grains of *A. havardiana* and *A. lechuguilla*. A total of 77 pollen grains were measured from 5 plant specimens of *A. lechuguilla*, and 100 pollen grains were measured from 5 plant specimens of *A. havardiana*.

Species	Length of polar axis (µm)	Length of equatorial axis (µm)	Mean lumen surface area (μm^2)	Maximum lumen surface area (μm^2)	Mean exine width (µm)
A. havardiana (*n*=100)	86.88 $\pm$ 8.9	70.35 $\pm$ 7.89	26.98 $\pm$ 11.40	49.81 $\pm$ 25.43	2.89 $\pm$ 0.31
A. lechuguilla (*n*=77)	72.82 $\pm$ 8.37	57.29 $\pm$ 10.74	7.279 $\pm$ 3.21	13.7 $\pm$ 21.55	2.31 $\pm$ 0.69

Table 2 —Coefficients (β_x) produced by the linear discriminant analysis to distinguish pollen grains of *A. havardiana* and *A. lechuguilla*.

Measurement	Coefficient (β_x)
Length of polar axis	-0.01257674
Length of equatorial axis	-0.03752386
Mean lumen surface area	-0.10697274
Maximum lumen surface area	0.01425318
Mean exine width	-0.50358096

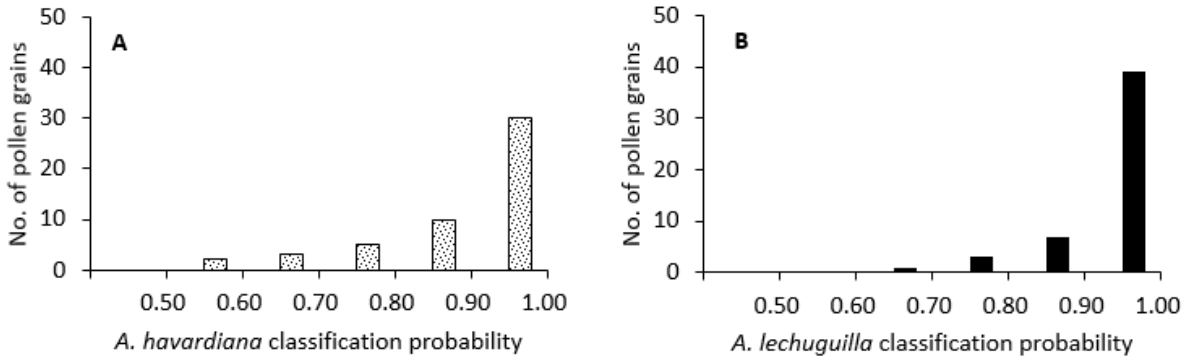

Fig. 11 — Linear discriminant analysis (LDA) classification of 100 pollen grains from reference collections of *Agave havardiana* and *Agave lechuguilla.* Pollen samples were collected from pallid bats captured in Brewster Co., Texas between April and August 2018. A.) All 50 *A. havardiana* pollen grains were correctly classified as *A. havardiana* and 30 of 50 had high classification probabilities ≥ 0.9 B.) All 50 *A. lechuguilla* pollen grains were correctly classified as *A. lechuguilla* and 39 of 50 had high classification probabilities ≥ 0.9.

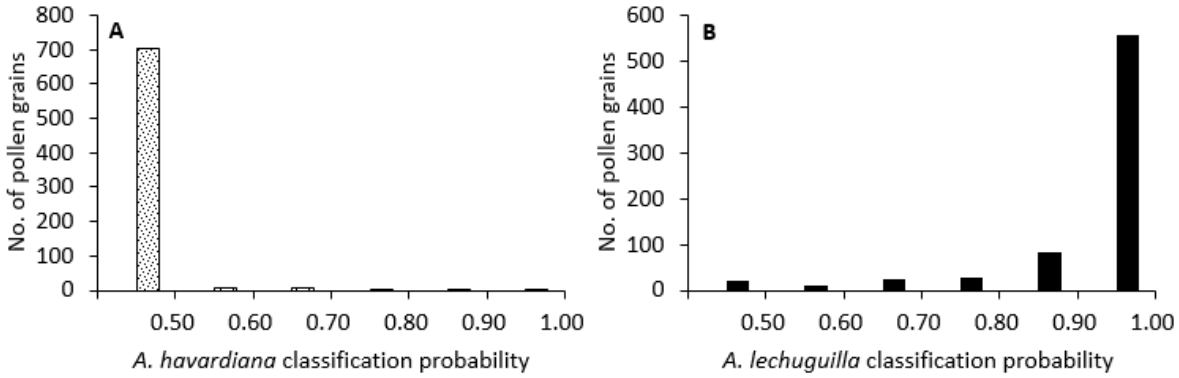

Fig. 12 — Linear discriminant analysis classification of unknown pollen grains from pallid bat samples. Analysis included 723 unknown *Agave* pollen grains from 52 bats captured in Brewster Co., Texas between April and August 2018. A.) Probability that unknown pollen grains are *Agave havardiana* B.) Probability that unknown pollen grains are *Agave lechuguilla.*

frequently land and cling to the flower. Pallid bats were recorded repeatedly (at least 12 separate occasions) landing on an *Agave lechuguilla* inflorescence and licking the base of the flower style where nectaries are located (Freeman and Reid 1985). The bats most often landed on the area of the flower where nectar was being produced and anthers had dehisced earlier in the night. In three recorded instances, bats "missed" and did not land on the flowers that were producing nectar. When this occurred, bats adjusted their position by crawling up or down the inflorescence until they reached the region of flowers that were producing nectar.

Nectar production was observed to occur nocturnally and only produced in flowers with newly dehisced anthers when large quantities of pollen was available. *A. lechuguilla* flowers have shallow floral tubes and do not hold large volumes of nectar; however, beads of nectar were observed clinging by surface tension between the base of the style and the edge of the lower sepals. Nectar from one flower was measured with a pipette and found to be approximately 100 µl in volume.

DISCUSSION

Overall, I have found compelling evidence that pallid bats are visiting *Agave* flowers and consuming *Agave* nectar. The majority (54 of 67) of the pallid bats sampled had substantial pollen densities on their wings. Both fuchsin gel samples and tape samples contained remarkably homogeneous pollen and no aberrant pollen morphologies were observed (Fig. 10). Thus, the pollen found on pallid bats was exclusively *Agave* pollen. The LDA function identified most (556 of 723, classification probability ≥ 0.9) of the pollen grains as *Agave lechuguilla* (Fig. 12). Opportunistic video observations of captive pallid bats provided further confirmation that pallid bats interact with *A. lechuguilla*. When considered together, data from pollen analyses and video observations clearly indicates that pallid bats are becoming covered in pollen as a result of nectarivorous consumption of *Agave* nectar.

The distribution of the pollen on the body of pallid bats was concentrated on the ventral surface of the wings, though it was also present in lower abundance on the heads and bodies of bats. This pollen density difference might indicate that tape samples were better at collecting pollen from unfurred wing membranes. However, the clinging posture of the pallid bats that was observed in the video footage provides a convincing reason that pollen densities might be greater on the wings than other body regions. In the video observations, pallid bats were observed grasping flower styles and stamen with thumbs and feet while hugging the flower stalk with wing membranes. The wing membranes were observed folding around pollen-covered anthers and any shifting or moving by the bats increased wing membrane contact with anthers. The posture of the pallid bats and the

position of the wings could explain why pollen is concentrated on ventral body surfaces, particularly wing surfaces.

Pallid bats with detectable pollen densities were captured in every month sampled between April to August of 2018 (Fig. 8). Monthly comparisons of mean pollen density data from bats captured at BBNP sites show that mean pollen densities were not significantly different between the months of April, May, July, and August. Finding pallid bats with roughly equivalent pollen densities throughout the sampling period, except for June, was surprising. I expected to observe higher pollen densities coinciding with a single peak in *Agave* blooming followed by declining pollen densities reflecting the gradual end in the flowering season. It could be that 2018 was an unusual flowering season for *A. lechuguilla*. Freeman and Reid (1985) observed that *Agave lechuguilla* bloom over a longer season and in lower abundance following a dry winter. The National Weather Service recorded 4.57 cm of precipitation from October 2017 to April 2018 at weather stations in BBNP, whereas the mean precipitation is 11.56 cm for the October to April period based on 1981-2010 precipitation records (National Weather Service 2019). Thus, an unusually dry preceding winter may have resulted in a longer flowering season for *A. lechuguilla*.

Though measurable pollen densities were observed for every month sampled, pallid bats from June had significantly lower wing pollen densities than bats captured in April and July (Fig. 8). The reason for this difference was unclear. It could indicate that there were fewer blooming *A. lechuguilla* in June and more blooming in April and July. However, past studies of the reproductive ecology of *A. lechuguilla* (Freeman and Reid 1985) and records

of museum specimen collections of flowering *A. lechuguilla* from Gentry (1982) indicate that June should be within the flowering period for this species in BBNP.

The pollen of *A. lechuguilla* and *A. havardiana* were surprisingly similar considering the differences in size and appearance of the plants (Fig. 1; Fig. 4). Despite differences in mean measurement, there was also a high degree of variability within feature measurements and overlap between the two species, so that no single feature could be used as a reliable diagnostic feature (Table 1). This is a common problem in pollen species identification; in many plant groups pollen morphology is extremely similar and difficult to distinguish within family and genus (Erdtman 1969; Kaya et al. 2013). Automated pollen classification tools like linear discriminant analyses (LDA) are an increasingly common tool used to distinguish species with similar pollen (del Pozo-Baños et al. 2015). The LDA function correctly classified 100% of the reference collection pollen grains that were used to test it; however, the degree of certainty, represented by the classification probability, was low for 22% (11/50) of the *Agave lechuguilla* classifications and 40% (20/50) of the *Agave havardiana* classifications. Pollen grains that had low classification probability were pollen grains that had feature measurements that are intermediate between the two species. In order to account for intermediate pollen grains, I used a threshold of classification probability ≥ 0.9. The LDA function classified 556 of 723 pollen grains as *Agave lechuguilla* and 3 of 723 pollen grains as *Agave havardiana* (Fig. 12). The small number of pollen grains identified as *A. havardiana* does not provide enough evidence to determine with certainty that pallid bats are also visiting *A. havardiana*.

The location of the study sites could have influenced the species of pollen found on pallid bats. It is possible that *A. lechuguilla* was the only *Agave* species within the 8.5 km pallid bat commuting distance of the capture sites (Ball 2002). The primary sites sampled in this study were Glenn Springs and Ernst Tinaja which are low elevation sites (774m and 680m respectively). *Agave lechuguilla* occurs at low elevations and is known to be abundant in the vicinity of both sites; however, *A. havardiana* only occurs at elevations above 1200m. Of the two BBNP sites, Glenn Springs is nearer to the foothills of the Chisos mountains and closer to locations where *A. havardiana* is known to occur. Specifically, *A. havardiana* are known to occur in Juniper Canyon; however, Juniper Canyon is approximately 10km away from Glenn Springs.

Although the results of this study only indicate that pallid bats visit *A. lechuguilla* it seems probable that pallid bats in high elevation habitats also would utilize *A. havardiana* as a nectar source. Past researchers working at high elevation sites in BBNP have captured pollen-covered pallid bats and these bats could have been visiting either *Agave* species (Barbour and Davis 1969; Kuban 1989). Kuban (1989) observed a pallid bat crawling on the flowers of *A. havardiana* and captured three pollen-covered pallid bats in mist nets erected around *A. havardiana* flowers. Further study in habitats where *A. havardiana* is abundant is necessary to confirm whether pallid bats also engage in nectarivory with this *Agave* species.

Finding *A. lechuguilla* as the primary *Agave* species visited by pallid bats was unexpected because nectar feeding bats in the subfamily Glossophaginae have not been observed feeding from this *Agave* species (Silva-Montellano and Eguiarte 2003). However, Easterla (1972) speculated that *A. lechuguilla* was a food source of *L. nivalis* after multiple

captures of *L. nivalis* at low elevation sites in BBNP including Ernst Tinaja. Relative to the nectar production of large paniculate agaves like *A. havardiana* where flowers can have more than 700 µl of nectar (Kuban 1989), *A. lechuguilla* flowers, which hold droplets of 100 µl, provide significantly smaller nectar reward. Though *A. lechuguilla* produce less nectar per plant than *A. havardiana*, they typically are much more abundant on the landscape (pers. observ.; Gentry 1982) and could be a substantial nectar source collectively.

Both *A. lechuguilla* and pallid bats are commonly found throughout Chihuahuan desert habitats. This extensive overlap in geographic ranges suggests that pallid bat– *Agave lechuguilla* nectarivory is unlikely to be isolated to the Big Bend region of Texas and could be a widespread phenomenon across Chihuahuan desert habitats. However, Silva-Montellano and Eguiarte (2003) conducted 54 hours of nocturnal observations of blooming *A. lechuguilla* at 11 different sites and observed no bat visitation, which indicates the opposite: that this behavior is rare. This suggests that the nature of pallid bat – *A. lechuguilla* interaction may not be uniform over the large geographic area where the two species are sympatric. Pallid bats might visit some areas of blooming *A. lechuguilla* more frequently, whereas other patches are visited infrequently or not at all. Frick et al. (2009) found pallid bat visitation varied between sites and that pallid bat visitation was higher at sites located in the vicinity of permanent sources of freshwater. The pollen-covered pallid bats sampled in this study were all captured over fresh water, except for 3 bats hand netted at a night roost. Future efforts to document wild pallid bat – *A. lechuguilla* interactions might be more successful if conducted in habitats near a water source.

Evidence from video observations and studies of the flowering phenology of *A. lechuguilla* indicates that pallid bats are potentially effective pollinators of *A. lechuguilla* (Freeman and Reid 1985). Pallid bats have been established as effective pollinators in other systems; Frick et al. (2013) found that pallid bats deposited more pollen per visit on the stigmas of cardón cacti than the specialized nectarivorous bat species, *Leptonycteris yerbabuenae*. In video observations, I recorded three instances where pallid bats initially landed on the *A. lechuguilla* stalk just above or just below the portion of the stalk where flowers were producing nectar. After "missing" and not finding nectar, the bats crawled up or down the stalk toward the area where flowers were producing nectar. In the process of finding the nectar producing flowers, bats contacted many flowers in various stages of bloom (Freeman and Reid 1985). The flowers with receptive stigmas are typically located below the flowers where nectar is produced (Freeman and Reid 1985). Thus, instances where bats "missed" and landed on lower flowers were potential pollination events. Further study is necessary to investigate whether pallid bats make contact with receptive stigmas and the resultant seed sets as a result of pallid bat visitation.

If pallid bats effectively and consistently pollinate *Agave lechuguilla*, they could exert some selective pressure on the *A. lechuguilla* floral features. Silva-Montellano and Eguiarte (2003) observed no bat visitation in their study of *A. lechuguilla* pollinators but also found that throughout its latitudinal range *A. lechuguilla* flowers had anthers that dehisced nocturnally and produced nectar nocturnally. Pallid bats as effective bat pollinators of *A. lechuguilla* could explain why some bat-adapted floral characteristics are conserved in *A. lechuguilla*.

In general, *Agave* diversity declines as distance to the equator increases and the number of nectarivorous bat species also decreases (Gentry 1982; Munguía-Rosas et al. 2009). In the northern extent of their geographic range, species of nectar feeding bats in subfamily Glossophaginae are seasonal residents that migrate north and arrive during the blooming season of a plant food source that produces copious nectar resources (Munguía-Rosas et al. 2009). However, the arrival of these nectarivorous bat pollinators is not always synchronized with the peak bloom of their nectar food source (Easterla 1972; Kuban; Scott 2004). Easterla (1972) observed one year where *L. nivalis* was apparently absent from its Chisos Mountain roost, and Kuban (1989) noted that in two of the four years studied *L. nivalis* arrived after the peak *A. havardiana* bloom. Scott (2004) observed no bat visitation by *L. yerbabuenae* or *C. mexicana* for 60% of the blooming period of *Agave palmeri*, Palmer's agave, in Arizona and New Mexico. In response to the decreased selection from bat pollinator activity, *Agaves*, like *A. havardiana*, are thought to progressively adopt generalist pollination strategies, often involving characteristics that facilitate diurnal pollination by insects and birds (Kuban 1989; Slauson 2000). Despite the unpredictability of the arrival phyllostomid bat pollinators, both *A. havardiana* and *A. palmeri* produce nocturnal nectar and pollen (Kuban 1989; Slauson 2000). Silva-Montellano and Eguiarte (2003) proposed that conservation of these traits could be the result of phylogenetic constraints. However, if pallid bats are engaging in facultative nectarivory with *Agave* species across their range they could act as resident bat pollinators of *Agave* in northern latitudes. Pallid bats as *Agave* pollinators could be selecting for the conservation of floral

characteristics, like nocturnal nectar production and nocturnal anther dehiscence, at the edge of and outside of the migratory range of nectarivorous phyllostomid bat pollinators.

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APPENDIX I — Angelo State University Institutional Animal Care and Use Committee (IACUC) approval letter for project 18-204.

ANGELO STATE UNIVERSITY
College of Graduate Studies & Research
Institutional Animal Care & Use Committee

04/17/18

Loren K. Ammerman, Ph.D.
Professor of Biology
Curator of Tissues, ASNHC
Angelo State University
ASU Station #10890
San Angelo, TX 76909

Dear Dr. Ammerman:

Your proposed project titled, "An investigation of Agave flower visitation by Antrozous pallidus in the Big Bend Region of Texas" was reviewed by Angelo State University's Institutional Animal Care and Use Committee (IACUC) in accordance with the regulations set forth in the Animal Welfare Act and P.L. 99-158.

This protocol was approved for three years, effective 4-17-2018, and it expires three years from this date; however, an annual review and progress report form (www.angelo.edu/content/files/22583-iacuc-annual-review-progressreport) for this project is due on August 15 of each year. If the study will continue beyond three years, you must submit a request for continuation before the current protocol expires.

The protocol number for your approved project is 18-204. Please include this number in the subject line of in all future communications with the IACUC regarding the protocol.

Sincerely,

Steven T. Brewer, Ph.D.
Assistant Professor,
Co-Chair, Institutional Animal Care and Use Committee
Director, MS Program in Experimental Psychology
Psychology & Sociology
Angelo State University
Member, Texas Tech University System
ASU Station #10907
San Angelo, TX 76909-0907

ANGELO STATE UNIVERSITY
College of Graduate Studies & Research
Institutional Animal Care & Use Committee

6-14-19

Loren K. Ammerman, Ph.D.
Professor of Biology
Curator of Tissues, ASNHC
Angelo State University
ASU Station #10890
San Angelo, TX 76909

Dear Dr. Ammerman:

Your proposed amendment for protocol #18-204, "An investigation of Agave flower visitation by Antrozous pallidus in the Big Bend Region of Texas" was reviewed by Angelo State University's Institutional Animal Care and Use Committee (IACUC) in accordance with the regulations set forth in the Animal Welfare Act and P.L. 99-158.

This amendment was approved, effective 6-14-2019. Expiration of this protocol is three years from the original protocol approval date; an annual review and progress report form (www.angelo.edu/content/files/22583-iacuc-annual-review-progressreport) for this project is due no later than 04-17 of each year. If the study will continue beyond three years, you must submit a request for continuation before the current protocol expires.

Please remember to include the protocol number (18-204) in the subject line of all future communications with the IACUC regarding this protocol.

Sincerely,

X

Steven T. Brewer, Ph.D.
Assistant Professor

Director, MS Program in Experimental Psychology
Co-Chair, Institutional Animal Care and Use Committee
Department of Psychology & Sociology
Angelo State University
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ASU Station #10907
San Angelo, TX 76909-0907
Phone: 325-486-6124

APPENDIX II — Angelo State Natural History Collection Herbarium accession number, collection date, and location data for reference collections of *Agave havardiana* and *Agave lechuguilla* from Brewster County, Texas. A voucher specimen of each plant species was collected, pressed, and dried for species verification (National Park Service Permit BIBE-2018-SCI-0030).

Accession number	Collection date	Species	Coordinates (latitude, longitude)	Location description
SAT 58726	8-Jun-2018	*A. havardiana*	30.33098, -103.09299	Hwy 385 roadside between Fort Stockton and Marathon
SAT 58730	9-Jun-2018	*A. havardiana*	29.26901, -103.30159	Big Bend National Park, Chisos Mountain Basin, cabins
SAT 58731	9-Jun-2018	*A. havardiana*	29.27542, -103.30148	Big Bend National Park, Chisos Mountain Basin
SAT 58749	9-Jun-2018	*A. havardiana*	29.24685, -103.29495	Big Bend National Park, Chisos Mountains, Boot Springs area
SAT 58750	9-Jun-2018	*A. havardiana*	29.25263, -103.30236	Big Bend National Park, Chisos Mountains, Pinnacles area
SAT 57566	10-Jun-2018	*A. lechuguilla*	29.27363, -103.28413	Big Bend National Park, along Green Gulch Road
SAT 57569	14-Jul-2018	*A. lechuguilla*	29.20427, -102.99060	Big Bend National Park, Old Ore Road
SAT 57570	14-Jul-2018	*A. lechuguilla*	29.20379, -102.99072	Big Bend National Park, Old Ore Road
SAT 57571	14-Jul-2018	*A. lechuguilla*	29.20300, -102.98975	Big Bend National Park, Old Ore Road
SAT 57567	10-Jun-2018	*A. lechuguilla*	29.27452, -103.28413	Big Bend National Park, near Lost Mine trailhead

APPENDIX III — A total of 297 bats of 12 different species were captured over 18 nights of sample collection. Mist netting occurred between 27 April 2018 and 8 August 2018 at two sites within Big Bend National Park. Sample collection occurred at 5 sites on a private ranch between 9 August 2018 and 13 August 2018.

Species	Glenn Springs	Ernst Tinaja	Private ranch 1	Private ranch 2	Private ranch 3	Private ranch 4	Porch night roost
Mormoops megalophylla	49	1	—	—	—	—	—
Myotis ciliolabrum/californicus	2	6	1	—	1	—	—
Myotis thysanodes	2	—	—	—	—	—	—
Myotis velifer	11	—	1	7	—	—	—
Lasiurus cinereus	7	—	—	—	—	—	—
Eptesicus fuscus	1	—	—	—	—	—	—
Parastrellus hesperus	44	59	8	—	—	4	—
Corynorhinus townsendii	5	2	—	—	—	—	—
Antrozous pallidus	36	25	10	—	2	1	3
Nyctinomops femorosaccus	—	—	1	—	—	—	—
Tadarida brasiliensis	8	—	—	—	—	—	—

APPENDIX IV — Pallid bat captures including date, site, bat identification number, sex, and whether pollen samples were collected from that individual. Two types of pollen samples were collected, fuchsin gel cubes and tape samples.

Date	Site	Sex	Fuchsin gel sample	Clear tape sample
27-Apr-18	Glenn Springs	female	yes	yes
27-Apr-18	Glenn Springs	female	no	no
27-Apr-18	Glenn Springs	female	yes	yes
27-Apr-18	Glenn Springs	female	no	no
27-Apr-18	Glenn Springs	female	yes	yes
27-Apr-18	Glenn Springs	male	yes	yes
27-Apr-18	Glenn Springs	female	yes	yes
27-Apr-18	Glenn Springs	female	yes	yes
27-Apr-18	Glenn Springs	female	yes	yes
27-Apr-18	Glenn Springs	male	yes	yes
27-Apr-18	Glenn Springs	female	yes	yes
27-Apr-18	Glenn Springs	female	yes	yes
27-Apr-18	Glenn Springs	female	yes	yes
27-Apr-18	Glenn Springs	female	yes	yes
27-Apr-18	Glenn Springs	female	yes	yes
27-Apr-18	Glenn Springs	female	yes	yes
27-Apr-18	Glenn Springs	—	no	no
27-Apr-18	Glenn Springs	—	no	no
19-May-18	Glenn Springs	female	yes	yes
19-May-18	Glenn Springs	male	yes	yes
19-May-18	Glenn Springs	female	yes	yes
19-May-18	Glenn Springs	female	yes	yes
11-Jun-18	Glenn Springs	female	no	yes
11-Jun-18	Glenn Springs	female	no	yes
11-Jun-18	Glenn Springs	female	yes	yes
11-Jun-18	Glenn Springs	female	no	yes
11-Jun-18	Glenn Springs	female	yes	yes
11-Jun-18	Glenn Springs	female	yes	yes
11-Jun-18	Glenn Springs	female	yes	yes
13-Jun-18	Ernst Tinaja	female	yes	yes
13-Jun-18	Ernst Tinaja	male	yes	yes

APPENDIX IV — Continued

Date	Site	Sex	Fuchsin gel sample	Clear tape sample
13-Jun-18	Ernst Tinaja	female	yes	yes
19-Jun-18	Ernst Tinaja	male	yes	yes
19-Jun-18	Ernst Tinaja	female	yes	yes
19-Jun-18	Ernst Tinaja	male	yes	yes
11-Jul-18	Ernst Tinaja	female	yes	yes
11-Jul-18	Ernst Tinaja	male	yes	yes
11-Jul-18	Ernst Tinaja	male	yes	yes
11-Jul-18	Ernst Tinaja	female	yes	yes
11-Jul-18	Ernst Tinaja	female	yes	yes
11-Jul-18	Ernst Tinaja	male	yes	yes
11-Jul-18	Ernst Tinaja	female	yes	yes
11-Jul-18	Ernst Tinaja	male	yes	yes
11-Jul-18	Ernst Tinaja	female	yes	yes
13-Jul-18	Glenn Springs	female	yes	yes
7-Aug-18	Glenn Springs	female	yes	yes
7-Aug-18	Glenn Springs	female	yes	yes
7-Aug-18	Glenn Springs	female	yes	yes
7-Aug-18	Glenn Springs	male	yes	yes
7-Aug-18	Glenn Springs	female	yes	yes
7-Aug-18	Glenn Springs	—	no	no
8-Aug-18	Ernst Tinaja	male	yes	yes
8-Aug-18	Ernst Tinaja	female	yes	yes
8-Aug-18	Ernst Tinaja	male	yes	yes
8-Aug-18	Ernst Tinaja	female	yes	yes
8-Aug-18	Ernst Tinaja	female	yes	yes
8-Aug-18	Ernst Tinaja	female	yes	yes
8-Aug-18	Ernst Tinaja	female	yes	yes
8-Aug-18	Ernst Tinaja	female	yes	yes
8-Aug-18	Ernst Tinaja	male	yes	yes
8-Aug-18	Ernst Tinaja	female	yes	yes
9-Aug-18	Ranch site 1	female	yes	yes
9-Aug-18	Ranch site 1	female	yes	yes
9-Aug-18	Ranch site 1	male	yes	yes
9-Aug-18	Ranch site 1	male	yes	yes

APPENDIX IV — Continued

Date	Site	Sex	Fuchsin gel sample	Clear tape sample
9-Aug-18	Ranch site 1	female	no	no
9-Aug-18	Ranch site 1	male	no	no
9-Aug-18	Ranch site 1	female	no	no
9-Aug-18	Ranch site 1	female	no	no
9-Aug-18	Ranch site 1	female	no	no
9-Aug-18	Ranch site 1	female	no	no
11-Aug-18	Ranch site 3	male	yes	yes
11-Aug-18	Ranch site 3	male	yes	yes
12-Aug-18	Porch night roost	female	yes	yes
12-Aug-18	Porch night roost	female	yes	yes
12-Aug-18	Porch night roost	male	yes	yes
13-Aug-18	Ranch site 4	female	yes	yes