PHENOLOGY OF LESSER LONG-NOSED BATS AND THEIR FOOD PLANTS

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Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to the Graduate College.

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ABSTRACT

Lesser long-nosed bats (*Leptonycteris yerbabuenae*) are important pollinators and seasonal residents along the U.S-Mexico border. Because they feed on flowering and fruiting plants, they are vulnerable to phenological shifts in those species. To evaluate synchrony between bats and their key food plants, we characterized seasonal abundance of bats and flowering and fruiting phenology of food plants at roosts in 2010, 2011, and 2021. Although phenology of bats and their food plants was generally consistent across years, we observed modest changes in aspects of flowering and fruiting of saguaro (*Carnegiea gigantea*) and organ pipe cacti (*Stenocereus thurberi*). At maternity roosts, bat abundance was synchronized more tightly with flowering phenology of saguaros in 2021 than in 2010-11. At post-maternity transient roosts, bat abundance was synchronized less tightly with flowering phenology of agaves (*Agave palmeri*) in 2021 than in 2010-11. We complemented these analyses by improving methods for non-invasive genetic sampling of this species. Specifically, we developed a two-step amplification approach to analyze microsatellite loci and identify individual bats via DNA extracted from fecal samples. As a proof of concept, five highly polymorphic microsatellite loci distinguished 434 individuals reliably. The probability of two closely related individuals having the same genotype at all five loci was 0.003, and the overall probability of identity was 7.5E-09. Addition of a multiplex step added minimal cost, improved amplification success, and conserved DNA extracts. Repeated analyses showed genotyping error was <2%. We explore the benefits and limits of our approaches for population studies of lesser long-nosed bats and other species that provide key ecosystem services and are commonly of conservation concern.

CHAPTER 1: PHENOLOGY OF LESSER LONG-NOSED BATS AND THEIR FOOD PLANTS

ABSTRACT

Global warming has altered the phenology of many plant and animal populations, often at different rates. When the phenology of interacting species shifts asymmetrically, mismatches have the potential to affect the fitness of one or both species. The lesser long-nosed bat (*Leptonycteris yerbabuenae*) migrates seasonally to give birth and raise young at maternity roosts in areas along the U.S-Mexico border, feeding on nectar, pollen, and fruits of saguaro (*Carnegiea gigantea*) and organ pipe cacti (*Stenocereus thurberi*). Later in the season, females and their young move to transient roosts at higher elevations where they feed on nectar and pollen from flowering Palmer's agave (*Agave palmeri*) before returning to Mexico. To evaluate the synchrony of the bats' phenology with that of their key food plants, we estimated abundance of bats and the proportion of food plants in flower and fruit in 2010, 2011, and 2021 at roosts near the U.S.-Mexico border. Bats arrived at maternity roosts prior to our first surveys in early May, their abundance peaked in June and July, and they abandoned roosts by August or September. Bats arrived at transient roosts in early July, their abundance peaked in late August, and they abandoned roosts in September or October. Bat abundance was associated positively with flowering and fruiting of saguaro and flowering of organ pipe cacti at maternity roosts and with flowering of agave at transient roosts. Peak abundance and seasonal variation in abundance of bats was similar in 2010-11 and 2021, although bats occupied transient roosts longer in 2021, remaining three to four weeks after agave had finished flowering. Timing of flowering and fruiting in food plants was generally similar between 2010-11 and 2021. However, more saguaros flowered and fruited longer in 2021 than in 2010-11, and organ pipe flowered and

fruited earlier and longer in 2021 than in 2010-11. At maternity roosts, seasonal patterns of bat abundance were synchronized more tightly with saguaro flowering in 2021 than in 2010-11. In contrast, seasonal patterns of bat abundance at transient roosts were synchronized less tightly with agave flowering in 2021 than in 2010-11. Our descriptions of seasonal and decadal variation in abundance of lesser long-nosed bats and phenology of saguaro, organ pipe, and agave help land managers better understand the life history of lesser long-nosed bats near the U.S-Mexico border and provide a foundation for continued monitoring.

INTRODUCTION

For many species, the timing of life-history events coincides with the seasonal availability of key resources, some of which are determined by the phenology of other species (Boyce 1979). There is widespread evidence that climate change has altered the timing of an array of seasonal events that are fundamental to relationships among plants and animals (Parmesan and Yohe 2003, Root et al. 2003, Inouye 2022). Variation in magnitude and direction of these phenological shifts has the potential to desynchronize important seasonal interactions between species, which has consequences for reproduction, survival, and population persistence (Thackeray et al. 2016, Renner and Zohner 2018, Walker et al. 2019).

The consequences of phenological mismatches on species interactions have been documented across the globe (Kharouba et al. 2018). For example, hatchling snow geese (*Anser caerulescens*) decreased in size as the interval between hatching date and availability of forage plants increased in northern Canada (Doiron et al. 2015). Similarly, first-year survival of roe deer (*Capreolus capreolus*) calves decreased as the interval between birth and availability of forage plants increased in eastern France (Plard et al. 2014). Finally, reproductive success of caribou (*Rangifer tarandus*) decreased as the interval between their annual migration and the availability

of forage plants increased in western Greenland (Post and Forchhammer 2008). Interactions between plants and pollinators might be especially sensitive to phenological mismatches because factors such as earlier warming in spring can cause seasonal life-history events, such as flowering, to occur earlier (Inouye 2022). Moreover, pollinators might respond differently to climate change because they sometimes rely on different seasonal cues than plants or have different sensitivities to the same cues (Hegland et al. 2009, Miller-Rushing et al. 2010, Kudo and Ida 2013).

Bats are the most important mammalian pollinators (Ratto et al. 2018). Populations of most species of bats are either in decline because of anthropogenic factors or have not been studied in detail (Frick et al. 2020). Further, bat species that are pollinators are trending towards extinction at a greater rate than non-pollinating species (Regan et al. 2015). In Mexico and the southern US, nectivorous bat species, the plants that they feed on, and their potential interactions are predicted to decrease as a result of climate change and land use (Gómez-Ruiz and Lacher 2019, Zamora-Gutierrez et al. 2021). Because pollination is central to the functioning of ecosystems and directly affects human welfare (Ratto et al. 2018), understanding how interactions between plants and pollinators are changing is a high priority in conservation (Frick et al. 2020).

The lesser long-nosed bat (*Leptonycteris yerbabuenae*) is a highly mobile species that has coevolved with the flowering plants on which it relies for food (Fleming et al. 2001). In southern Mexico, some lesser long-nosed bats are year-round residents that forage on a variety of flowering and fruiting plants (Valiente-Banuet et al. 1996). However, a portion of the population migrates north to breed in Baja California and Sonora, Mexico, and southern Arizona, exploiting seasonally abundant blooms of saguaro (*Carnegiea gigantea*) and organ pipe cacti (*Stenocereus*

thurberi) (Fleming et al. 1993). Pregnant females arrive at maternity roosts to give birth and forage on nectar and pollen from these cacti, which flower from mid-May to mid-July. As cacti finish flowering, bats feed on their ripened fruit (Cole and Wilson 2006). From late July through September, bats abandon maternity roosts and move to post-breeding transient roosts located at higher elevations throughout southeastern Arizona. They transition to a diet of nectar and pollen from Palmer's agave (*Agave palmeri*), on which they feed until they return to their winter range in Mexico (Fleming et al. 1993, Burke et al. 2021).

Because lesser long-nosed bats are capable of commuting long distances to forage (Ober et al. 2005, Goldshtein et al. 2020), they contribute to reproduction and gene flow of their food plants, which have ecological, economic, and cultural significance in the U.S. and Mexico (Fleming et al. 2009, López-Hoffman et al. 2010, Tremlett et al. 2020). Disruptions of the mutualistic relationship between lesser long-nosed bats and their key food plants, whether from anticipated declines in the abundance and geographic distribution of saguaro and agave (Albuquerque et al. 2018, Gómez-Ruiz and Lacher 2019) or a potential phenological mismatch between migrating bats and flowering plants (Rafferty et al. 2015), would have consequences for pollination and other ecosystem services (Kunz et al. 2011, Drezner 2014, Gómez-Ruiz and Lacher 2019).

To explore phenology of bats and their food plants, we characterized abundance of bats at maternity and transient roosts, and phenology of their food plants, near the U.S.-Mexico border between Arizona and Sonora. To evaluate potential changes in bat abundance and bat-plant interactions during the past decade, we combined our data with those collected throughout the same region in 2010 and 2011 (Cerro 2012). Our aims include describing how abundance of lesser long-nosed bats relates to the seasonal availability of food resources and evaluating

whether these relationships have changed during the last decade.

METHODS

Study area

We studied lesser long-nosed bats and their primary food plants at roost sites in northern Mexico and southern Arizona (Fig. 1.1). Elevation of maternity roosts ranged from 183 m to 620 m, with local vegetation dominated by desert scrub (McClaran and Brady 1994). Elevation of transient roosts ranged from 744 m to 1715 m, with vegetation dominated by desert grassland-scrub, juniper-pinyon woodland, and oak woodland (McClaran and Brady 1994). During the period when bats are resident, temperatures vary from 16 – 43 °C near maternity roosts and 9 – 35 °C near transient roosts (Fick and Hijmans 2017). The Sonoran Desert region receives sporadic rainfall from summer monsoons (Sheppard et al. 2002).

Phenology of bats and their food plants

We surveyed bats and their food plants at three maternity roosts and four transient roosts in 2010, four maternity roosts and six transient roosts in 2011, and three maternity roosts and six transient roosts in 2021 (Table 1.1). We surveyed three of the same maternity roosts and five of the same transient roosts in 2010-11 and in 2021.

We surveyed bats at each maternity roost an average of 7.1 times per year (SD = 1.9) from early May to August or September, and each transient roost an average of 7.1 times per year (SD = 2.4) from July to September or October. During these periods, we surveyed bats and their food plants at each roost every 7-14 days and concluded surveys when the estimated number of bats remaining at a roost had declined to <5% of the seasonal maximum.

To characterize seasonal abundance of bats, we recorded emergences using video cameras (Sony Handycam DCR-SR45 in 2010-11 and Axis Communications P1364 in 2021) and

infrared illumination. We placed cameras and illumination perpendicular to the flight pattern of emerging bats and deployed multiple cameras at roosts where bats used more than one entrance. We began recording approximately 20 min after sunset or at the first sign of bat activity and stopped recording when the net number of bats exiting the roost (no. exiting – no. returning) was ≤ 0 for 10 minutes.

We estimated abundance of bats emerging during each survey by reviewing recordings in slow motion and counting the number of bats that exited roosts. For most surveys (87%), we counted bats during every third minute of recorded video using a one-in-three systematic sample. For the remaining surveys (13%), we counted bats throughout the entire recording; we included at least one complete count for each roost in each year. For maternity roosts, we characterized peak abundance as using the highest estimate between 15 May and 15 June, and for transient roosts, we used the highest estimate from all surveys. For roosts surveyed in both 2010 and 2011, we used the mean of the two values for peak abundance we established in each year.

To characterize phenology of food plants, we established and surveyed one to three areas (1-2 km^2) located within 24 km of each roost, the average maximum distance lesser long-nosed bats forage from roosts (Sahley et al. 1993, Horner et al. 1998, Ober et al. 2005). If an area was located within 24 km of multiple roosts, we used the phenology data from that area for each roost. Within 10 days of each bat survey, we surveyed an average of 126 (SD = 173) reproductive plants of each focal species near each roost, and recorded the number that were flowering, fruiting, or senesced. For maternity roosts, we surveyed both organ pipe and saguaro cacti, which are iteroparous, and for transient roosts, we surveyed Palmer's agave, which is semelparous. For Palmer's agave, we recorded the number of branches on each inflorescence that were budding, flowering, or senesced. We repeated surveys in the same areas throughout the

season but did not necessarily survey the same plants. We characterized plant phenology three ways, by estimating 1) the week when the proportion of plants in flower or fruit peaked, 2) the peak proportion of plants in flower or fruit, and 3) the number of weeks during which the proportion of plants flowering or fruiting exceeded 0.5, which we used to characterize the duration of flowering and fruiting periods. We did not characterize plant phenology near the roost in Sonora because there was little vegetation within 24 km.

Statistical analysis

We characterized phenology of bats and their food plants by modeling species' traits as a function of year and survey date (Julian day), which we sometimes report as week of the year to simplify interpretation (e.g., 1 = week including January 1). Because we surveyed roosts multiple times in each survey season, we used mixed-effects models for analysis with roost classified as a random effect, which is appropriate for longitudinal data (Diggle 2001). In each analysis, we included a quadratic term for the seasonal component because we expected seasonality to induce curvilinear patterns in responses (Brown 1998). However, we only retained quadratic terms in models for inference when they explained appreciable variation in the response ($P < 0.1$). We standardized Julian day and year to a *Z*-score (mean = 0, SD = 1).

We modeled bat abundance (log transformed) as a function of Julian day, Julian day^2, year, and Julian day × year interactions using multiple regression. We modeled both the proportion of plants flowering and fruiting as a function of Julian day, Julian day^2, year, and Julian day × year interactions using logistic regression. For agave, we also modeled the proportion of branches budding, flowering, or senesced as a function of Julian day, Julian day^2, year, and Julian day × year interactions using logistic regression. To test whether phenology of

bat abundance or flowering and fruiting of plants changed between 2010-11 and 2022, we evaluated Julian day × year and Julian day^2 × year interactions.

To characterize the relationship between bats and phenology of their food plants, we modeled bat abundance (log transformed) as a function of the proportion of plants flowering or fruiting (logit transformed) and year using multiple regression. We then added flowering × year or fruiting × year interactions to the same model to test whether the relationship between bats and phenology of their food plants changed between 2010-11 and 2022.

RESULTS

Phenology of bats

Bats were present at maternity roosts during our first surveys in early May (Fig. 1.2). Their abundance was highest during June and July, and they abandoned roosts between late July and September (Fig. 1.2). Bats began to arrive at transient roosts in early July. Their abundance peaked in late August, and most roosts were abandoned between September and October (Fig. 1.2).

At maternity roosts, bat abundance varied seasonally in a curvilinear way that was consistent across years (P = 0.67 for Julian day × year interaction, Table 1.2). Peak abundance of bats at maternity roosts decreased slightly between 2010-11 and 2021 (Appendix 1). Specifically, for the three maternity roosts we surveyed in 2010-11 and in 2021, peak abundance decreased by an average of 7.0% from 2010-11 to 2021 (mean difference per roost = 6292 bats, SD = 31100).

At transient roosts, bat abundance also varied seasonally in a curvilinear way that was consistent across years (P = 0.40 for Julian day × year interaction, Table 1.2). Peak abundance increased between 2010-11 and 2021 (Appendix 1). Specifically, for the five transient roosts we

surveyed in 2010-11 and in 2021, peak abundance increased by an average of 41.6% from 2010-11 to 2021 (mean difference per roost = 1479 bats, SD = 4096).

Phenology of food plants

Near maternity roosts, the timing of flowering and fruiting of the bat's two key food plants overlapped considerably (Figs. 1.3 and 1.4). Saguaro cacti were in flower during our first surveys in early May and continued to flower through late June. Organ pipe cacti flowered from early May to late August. Saguaro fruited from late May to late July and organ pipe cacti from mid-June to mid-September.

Phenological aspects of saguaro flowering and fruiting varied across years ($P < 0.0001$ for all Julian day × year interactions, Table 1.3). Although the proportion of saguaro in flower peaked at approximately the same time in 2010-11 and 2021 (Fig. 1.4), the peak was higher (0.77, SE = 0.08) in 2021 than in 2010-11 (0.56, SE = 0.11; Fig. 1.4). For all years, the proportion of plants in flower exceeded 0.5 for only one week (Fig. 1.4). The peak proportion of saguaro in fruit also was higher (0.84, SE = 0.04) in 2021 than in 2010-11 (0.60, SE = 0.06; Fig. 1.4). The proportion of saguaro in fruit exceeded 0.5 for four weeks in 2021 and only two weeks in 2010-11 (Fig. 1.4).

Phenological aspects of organ pipe flowering and fruiting also varied across years ($P < 0.0001$ for all Julian day × year interactions, Table 1.3). Organ pipe flowered earlier and longer in 2021 than in 2010-11. Specifically, the proportion of organ pipe in flower peaked during week 23 in 2021 and week 25 in 2010-11 (Fig. 1.4). Although the peak in the proportion of organ pipe in flower was similar in 2021 (0.57, SE = 0.04) and 2010-11 (0.51, SE = 0.01; Fig. 1.3), the proportion of plants in flower exceeded 0.5 for six weeks in 2021 and for only one week in 2010-11 (Fig. 1.3). Accordingly, the proportion of organ pipe in fruit peaked one week earlier in 2021

(week 29) than in 2010-11 (week 30; Fig. 1.4). The peak proportion of organ pipe in fruit was lower in 2021 (0.52, SE = 0.13) than in 2010-11 (0.78, estimated based on one survey). The proportion of organ pipe in fruit exceeded 0.5 for one week in 2021 and three weeks in 2010-11.

Near transient roosts, agave flowered from late June to late September, with modest variation in flowering phenology between survey years (Fig. 1.3). The proportion of agaves in flower peaked two weeks later in 2021 (week 28) than in 2010-11 (week 26), and the peak proportion of agave in flower was higher in 2021 (0.97, SE = 0.01) than in 2010-11 (0.82, SE = 0.03). The proportion of agaves in flower exceeded 0.5 for seven weeks in 2021 and eight weeks in 2010-11 (Fig. 1.3). The proportion of branches in flower peaked at similar times in 2021 and 2010-11 (week 27 and 28; Fig. 1.5), and the peak proportion of branches in flower was also similar in 2021 (0.17, SE = 0.06) and in 2010-11 (0.18, SE = 0.02). For all years combined, the proportion of branches in flower was highest in late July when the proportion of branches senesced and budding were approximately equal (Fig. 1.5).

Relationship between bats and their food plants

At maternity roosts, bat abundance was associated positively with flowering and fruiting of saguaro and flowering of organ pipe (Table 1.5, Figs. 1.6 and 1.7). There was some evidence that the association between bat abundance and the proportion of saguaros in flower changed slightly between 2010-11 and 2021 ($Z = 1.660$, $P = 0.104$ for flowering × year interaction, Fig. 1.8). When considering years separately, bat abundance was synchronized more tightly with saguaro flowering in 2021 (0.27 bats on the natural log scale/proportion of saguaros in flower on the logit scale, SE = 0.12) than in 2010-11 (0.03, SE = 0.5; Fig. 1.8). The association between bat abundance and the proportion of saguaros in fruit was similar between years ($Z = 0.08$, $P =$

0.90 for fruiting × year interaction), as was the association between bat abundance and the proportion of organ pipe in flower (Z = 1.63, P = 0.11).

At transient roosts, bat abundance was associated positively with flowering of agaves (Table 1.5, Fig. 1.6). The association between bat abundance and the proportion of agaves in flower changed somewhat between 2010-11 and 2021 (Z = -1.65, P = 0.101 for flowering × year interaction, Fig. 1.8). Excluding two surveys from 2021 where bats abandoned roosts unusually early, evidence for this decadal change becomes somewhat stronger (Z = -2.04, P = 0.04 for flowering × year interaction). Bat abundance was more tightly synchronized with agave flowering in 2010-11 (0.51 bats on the natural log scale/proportion of agaves in flower on the logit scale, SE = 0.11) than in 2021 (0.19, SE = 0.08, Fig. 1.8). Abundance of bats at transient roosts peaked approximately one month after the proportion of agaves in flower peaked. In 2021, thousands of bats remained at three roosts (mean = 4931, SD = 2873) after all nearby agave flowers had senesced, whereas in 2010-11, only one roost had a considerable number of bats (5178) after all nearby agave flowers had senesced (Figs. 1.2 and 1.3).

DISCUSSION

Identifying key food resources and how their seasonality influences the distribution of migratory species is important for understanding biogeography and for developing conservation strategies for rare and highly mobile species (Reynolds et al. 2018, Schuster et al. 2019). Although both biotic and abiotic resources are important distributional determinants for many animal species, the seasonal availability of food resources often explains why some species migrate tens to thousands of kilometers (Alerstam et al. 2003). Lesser long-nosed bats are important pollinators that migrate thousands of kilometers, providing pollination services that benefit both natural ecosystems and crop productivity (Trejo-Salazar et al. 2016, Tremlett et al.

2020). Globally, they represent the >80% of bat species that require focused research efforts (Frick et al. 2020), which is especially urgent as vertebrate pollinators are trending towards extinction at a greater rate than non-pollinators (Regan et al. 2015).

Maternity roosts

In southern Arizona and northern Mexico, the spatial and temporal aspects of migration and breeding in lesser long-nosed bats are linked closely to the timing of flowering and fruiting in their key food plants, saguaro cactus, organ pipe cactus, and agave. Here, we confirmed that spatial and temporal variation in abundance of bats is associated with seasonal availability of their primary food resources in Arizona near the U.S.-Mexico border. Although the seasonal distribution of lesser long-nosed bats has long suggested their residency is linked to the availability of food resources (Cockrum 1991, Fleming et al. 1993, Rojas-Martínez et al. 2009), this relationship has not yet been characterized formally, at least to our knowledge. We found that seasonal abundance of bats at maternity roosts near the U.S.-Mexico border coincided with the availability of flowers and fruits from their two primary food plants, saguaro cactus and organ pipe cactus. Therefore, lesser long-nosed bats time their migration and breeding so that pollen, nectar, and fruit from saguaro and organ pipe cacti are available to pregnant and lactating mothers (Fleming et al. 1993). Saguaro and organ pipe cacti provide a reliable source of pollen, nectar, and fruit used as food by many species (Wolf and Martinez del Rio 2000, Fleming et al. 2001, Drezner 2014), and their use by lesser long-nosed bats during breeding has been well documented (Hevly 1979, Horner et al. 1998, Fleming et al. 2001). Seasonal abundance of lesser long-nosed bats breeding at a roost in Sonora, Mexico was associated with the availability of food from nearby columnar cacti in previous work (Peñalba et al. 2006), consistent with our findings.

Timing of reproduction in mammals often coincides with resource availability in seasonal environments (Boyce 1979). Therefore, breeding seasons of some mammals, such as marsupial mice (*Antechinus stuartii*) and pygmy fruit bats (*Haplonycteris fischeri*), are synchronized tightly with seasonal peaks in their food resources (Bronson and Heideman 1994). Because gestation and lactation are energetically demanding periods, predictable food sources are necessary for successful recruitment (Gittleman and Thompson 1988).

Transient roosts

As the abundance of flowers and fruit from organ pipe and saguaro cacti decreased, abundance of bats at maternity roosts decreased as females and their young relocated to transient roosts where flowers from Palmer's agave were available. From late-June through September, Palmer's agave blooms provide nectar and pollen for multiple species in the Sonoran Desert (Slauson 2000, Scott 2004), including lesser long-nosed bats, who rely on it as a primary food source (Hevly 1979, Ober et al. 2005). Bats forage selectively in areas with disproportionally high agave densities (Ober et al. 2005) and visit agaves most frequently when flowering is near its peak (Ober and Steidl 2004).

In 2021 but not 2010-11, thousands of bats remained resident at some transient roosts 20 to 30 days after nearby agaves finished blooming (Figs. 1.2 and 1.3). We are unsure of the food source used by bats during this period. Lesser long-nosed bats roosting near cities have been observed foraging at hummingbird feeders late in the season, and their use of feeders has increased since at least 2007 (Fleming et al. 2021). The roosts where bats remained after agave finished blooming were all located within 20 km of cities (Benson, Sierra Vista, or Tucson), therefore, it is plausible that bats supplemented their diet with sugar water from hummingbird feeders as food from agave blooms diminished. Lesser long-nosed bats regularly commute one-

way distances of tens of kilometers to forage (Sahley et al. 1993, Ober et al. 2005), and have even been recorded traveling >100 km in one night from a roost to a foraging site in the Sonoran Desert (Goldshtein et al. 2020). Future studies could evaluate sugar source and content in the diet of these animals to clarify if feeders are their primary food resource at the end of the season.

A decadal perspective on phenology and species interactions

Monitoring species abundances and their interactions with key resources through time can help to identify responses to environmental change and likely stressors (Gibbs et al. 1998). Between 2010-11 and 2021, we observed only modest changes in the seasonal timing of food resources provided by plants important to lesser long-nosed bats. Near maternity roosts, the timing of seasonal peaks in flowering and fruiting of saguaro during 2010-11 and 2021 were consistent with observations from 1997 (Fleming et al. 2001). In 2010-11, the seasonal peak in flowering of organ pipe was consistent with observations from 1997, 2001, 2003, and 2004 (Fleming et al. 2001, Bustamante and Búrquez 2008). Near transient roosts, the timing of Palmer's agave flowering was similar in 2010-11 and 2021, which was consistent with observations from 1994-95 and 1997 (Slauson 2000, Scott 2004). Although our survey frequency limits us to detecting only coarse-scale changes in phenology, our observations provide a reliable foundation for future surveys that will allow biologists to determine whether the variation we observed is typical or indicative of systematic environmental change.

Bat abundance overall, and seasonal patterns in abundance at both maternity and transient roosts, also were similar between 2010-11 and 2021, suggesting that timing of breeding and migration did not change appreciably during the intervening decade. Although estimated peak abundance decreased notably from 2010-11 to 2021 at several roosts, it increased at others (Appendix 1). In 2018, the U.S. Fish and Wildlife Service's decision to delist the lesser long-

nosed bat was partly based on the opinion that populations in the United States were stable or increasing (US Fish and Wildlife Service 2018), which is consistent with our observations across this interval.

In southern Arizona, the timing of flowering in saguaro and organ pipe cacti responds to variation in temperature and precipitation (Bustamante and Búrquez 2008, Renzi et al. 2019). Therefore, climate change will likely alter the availability of food plants important to lesser long-nosed bats and other species (Fleming et al. 2001, Garfin et al. 2013, Drezner 2014). Although we found no marked changes in abundance and phenology of bats, their food plants, and bat-plant associations between 2010-11 and 2021, periodically characterizing the breeding and post-breeding abundance of bats and the phenology of their food plants will help identify potential mismatches and changes in distribution.

Directly or indirectly, climate change is altering the timing of migration in diverse terrestrial and marine animals (Davidson and Al 2020, Diehl 2020, Szesciorka et al. 2020). Because effects of climate change include shifts in plant phenology (Piao et al. 2019, Inouye 2022), many interactions between plants and animals have changed (Kharouba et al. 2018, Renner and Zohner 2018). Limited evidence suggests that climate change has already affected the phenology of some migratory bats that seem to have adjusted to changes in resource availability (Stepanian and Wainwright 2018). Bats with high wing aspect ratios and broad geographic ranges, like lesser long-nosed bats, may be more resilient to phenological shifts in their food plants than smaller species with limited range (Jones et al. 2003). A more immediate concern for the persistence of lesser long-nosed bats may be the predicted decline in distributions and diversity of chiropterophilous plants and bats in Mexico and the southwestern U.S. (Gómez-Ruiz and Lacher 2019, Zamora-Gutierrez et al. 2021). These predicted declines likely will be of

particular concern in central and southern Mexico, where plants with C3 metabolism are expected to be affected more by climate change than other types of plants(Zamora-Gutierrez et al. 2021). Although agave species are also predicted to decrease in distribution (Gómez-Ruiz and Lacher 2019), other plants with CAM metabolism, like columnar cacti, might increase in distribution (Zamora-Gutierrez et al. 2021). Maternity roosts in northern Mexico and southern Arizona, where lesser long-nosed bats primary food is columnar cacti, might play an increasingly important role for lesser long-nosed bat populations in the future.

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Table 1.1. Lesser long-nosed bat emergences by year in southern Arizona, USA, and northern Mexico, 2010, 2011, and 2021: number of emergences observed for each roost surveyed, the minimum, maximum, mean, and standard deviation.

Roost	Type	Year	*n*	Min.	Max.	Mean	SD
Bluebird Mine	Maternity	2010	5	2589	6117	4272	1415
		2011	7	4736	7133	5817	797
		2021	10	1456	9789	4820	2907
Copper Mountain Mine	Maternity	2010	8	18992	33354	28699	4532
		2011	7	15858	42882	31451	10284
		2021	10	610	86193	35699	26714
Pinacate Cave	Maternity	2010	6	2088	59772	38346	23345
		2021	6	0	23685	13735	8906
Old Mammon Mine	Maternity	2011	6	481	7450	2641	2909
Helena Mine	Transient	2011	6	906	7412	3269	2354
		2021	11	177	8937	4225	2985
Little Dragoon Mine	Transient	2010	8	26	4271	1457	1699
		2011	7	85	5508	2300	1842
		2021	11	33	4059	1460	1253
Montezuma Mine	Transient	2010	5	53	6234	1941	2751
		2011	5	12	2100	1002	994
Mustang Cave	Transient	2010	5	623	11529	5896	4611
		2011	5	70	7448	2342	2918
		2021	6	10	6035	1978	2358
Papago Springs Cave	Transient	2010	6	27	2916	1113	1285
		2011	8	74	2236	1049	823
		2021	8	330	7614	2276	2356
Pyeatt Cave	Transient	2011	9	66	13038	5400	4272
		2021	11	6970	18784	9400	5349
State of Texas Mine	Transient	2021	5	13	6794	3037	2506

Table 1.2. Estimates of fixed effects from a regression model for bat abundance (ln transformed) at maternity and transient roosts. The Julian day^2 × year interaction did not explain appreciable variation in abundance for either roost type ($P > 0.1$), so it was not retained.

Roost Type	Factor	Estimate	SE	*Z*	*P*
Maternity	Intercept	8.361	0.424	19.708	<0.001
	Year	-0.117	0.427	-0.273	0.790
	Julian day	-1.683	0.441	-3.817	<0.001
	Julian day^2	-0.585	0.250	-2.337	0.023
	Julian day × Year	-0.100	0.218	-0.458	0.649
Transient	Intercept	7.223	0.353	20.456	<0.001
	Year	0.484	0.351	1.378	0.187
	Julian day	2.491	0.356	6.997	<0.001
	Julian day^2	-2.557	0.292	-8.758	<0.001
	Julian day × Year	0.193	0.227	0.850	0.397

Table 1.3. Estimates of fixed effects from a logistic regression model for the proportion of plants flowering and fruiting for three species.

Species	Characteristic	Factor	Estimate	*SE*	*Z*	*P*
Saguaro	Flowering	Intercept	-11.515	0.491	-23.470	<0.001
		Year	-3.305	0.506	-6.532	<0.001
		Julian day	-11.674	0.050	-231.879	<0.001
		Julian day^2	-3.116	0.016	-200.242	<0.001
		Julian day × Year	-3.866	0.053	-72.379	<0.001
		Julian day^2 × Year	-1.249	0.016	-75.894	<0.001
	Fruiting	Intercept	-3.706	0.125	-29.610	<0.001
		Year	1.933	0.129	14.970	<0.001
		Julian day	-12.424	0.074	-167.110	<0.001
		Julian day^2	-8.229	0.037	-221.660	<0.001
		Julian day × Year	2.235	0.080	28.060	<0.001
		Julian day^2 × Year	1.097	0.039	27.830	<0.001
Organ pipe	Flowering	Intercept	-1.695	0.064	-26.524	<0.001
		Year	0.637	0.065	9.769	<0.001
		Julian day	-2.559	0.011	-223.772	<0.001
		Julian day^2	-1.102	0.006	-190.966	<0.001
		Julian day × Year	1.436	0.010	139.810	<0.001
		Julian day^2 × Year	0.714	0.005	130.908	<0.001
	Fruiting	Intercept	0.450	0.054	8.311	<0.001
		Year	-0.346	0.055	-6.258	<0.001
		Julian day	0.074	0.009	7.864	<0.001
		Julian day^2	-2.203	0.011	-197.499	<0.001
		Julian day × Year	0.238	0.008	28.612	<0.001
		Julian day^2 × Year	1.115	0.010	111.843	<0.001
Palmer's agave	Flowering	Intercept	1.671	0.110	15.143	<0.001
		Year	0.485	0.112	4.326	<0.001
		Julian day	0.620	0.011	58.473	<0.001
		Julian day^2	-3.581	0.015	-242.854	<0.001

Julian day × Year	0.108	0.011	9.994	<0.001
Julian day^2 × Year	-0.816	0.015	-53.379	<0.001

Table 1.4. Estimates of fixed effects from a logistic regression model for the proportion of Palmer's agave branches that were budding, flowering, and senesced by day of year and survey year.

Characteristic	Factor	Estimate	*SE*	*Z*	*p*
Budding	Intercept	-0.410	0.107	-3.829	<0.001
	Year	-0.046	0.109	-0.420	0.674
	Julian day	-2.937	0.024	-123.835	<0.001
	Julian day^2	-0.129	0.038	-3.393	<0.001
	Julian day × Year	0.117	0.023	5.142	<0.001
	Julian day^2 × Year	0.090	0.038	2.378	0.017
Flowering	Intercept	-1.663	0.074	-22.408	<0.001
	Year	-0.007	0.075	-0.093	0.926
	Julian day	-0.318	0.028	-11.526	<0.001
	Julian day^2	-1.697	0.048	-35.664	<0.001
	Julian day × Year	-0.073	0.025	-2.974	0.003
Senesced	Intercept	-0.266	0.131	-2.038	0.042
	Year	0.051	0.133	0.382	0.703
	Julian day	3.014	0.025	120.244	<0.001
	Julian day^2	-0.181	0.034	-5.251	<0.001
	Julian day × Year	-0.138	0.024	-5.691	<0.001
	Julian day^2 × Year	0.151	0.034	4.387	<0.001

Table 1.5. Estimates of fixed effects from a regression model for the relationship between bat abundance, characteristics of food plants, and year.

Species	Factor	Estimate	SE	*Z*	*P*
Saguaro	Intercept	9.789	0.460	21.326	<0.001
	Year	0.175	0.410	0.426	0.685
	Flowering	0.134	0.062	2.153	0.037
	Fruiting	0.121	0.053	2.273	0.028
Organ pipe	Intercept	9.670	0.506	19.116	<0.001
	Year	-0.194	0.417	-0.465	0.668
	Flowering	0.416	0.170	2.449	0.020
	Fruiting	0.012	0.093	0.128	0.899
Palmer's agave	Intercept	6.666	0.314	21.221	<0.001
	Year	0.569	0.318	1.787	<0.001
	Flowering	0.398	0.077	5.192	0.098

Figure 1.1. Locations of lesser long-nosed bat roost sites surveyed in southern Arizona and northern Mexico in 2010, 2011, and 2021. Some roost sites were not surveyed every year. We characterized food plants within 24 km of roosts, as indicated by the circles, except for Pinacate Cave. Maternity roosts: 1) Bluebird Mine, 2) Copper Mountain Mine, 3) Old Mammon Mine, 4) Pinacate Cave. Transient roosts: 5) Helena Mine, 6) Little Dragoon Mine, 7) Montezuma Mine, 8) Mustang Cave, 9) Papago Springs Cave, 10) Pyeatt Cave, 11) State of Texas Mine.

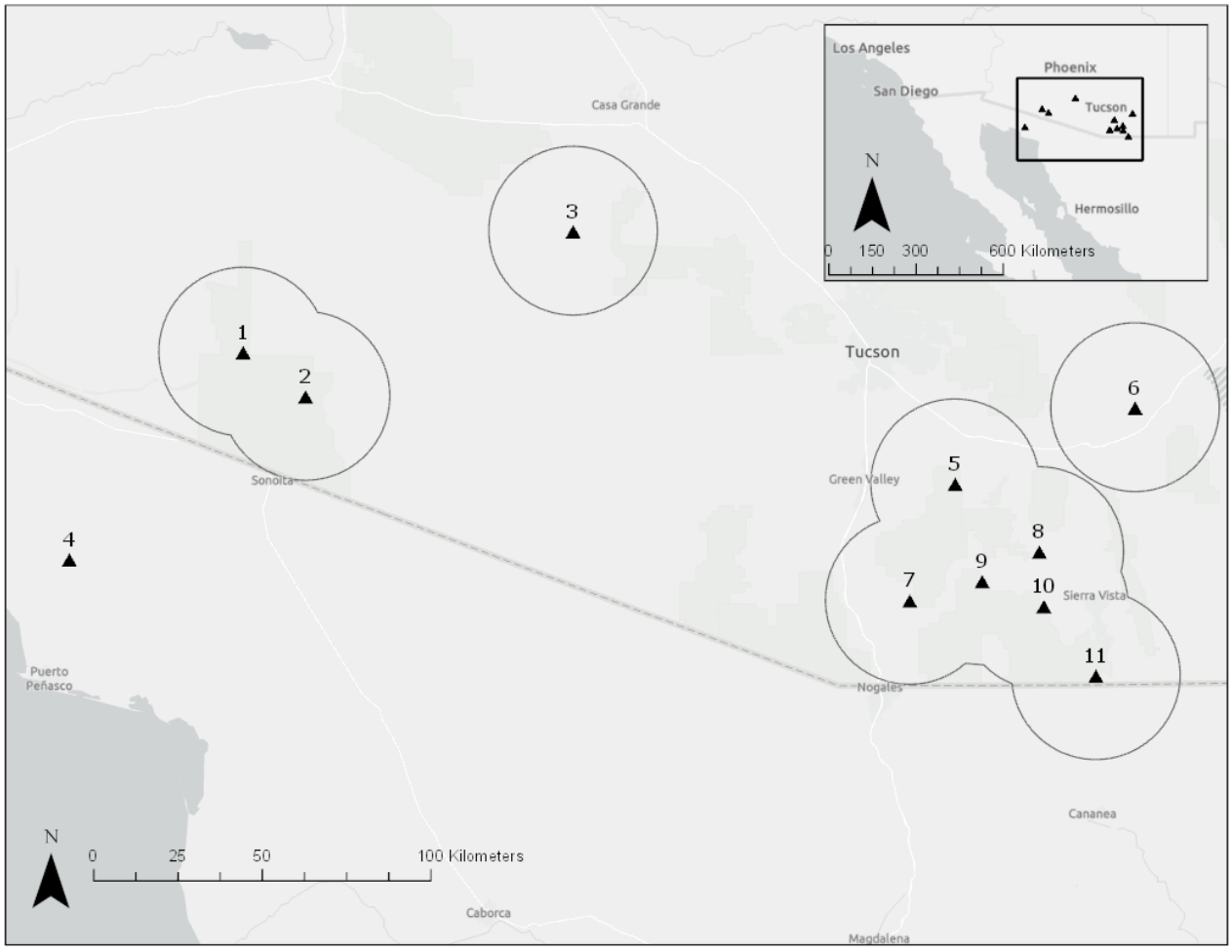


Figure 1.2. Abundance of lesser long-nosed bats across the spring and summer in southern Arizona and northern Mexico, 2010, 2011, and 2021. Squares symbolize bat abundance at maternity roosts, and circles symbolize bat abundance at transient roosts. Statistics for regression lines are provided in Table 1.2. In non-leap years, 1 May is Julian date 121.

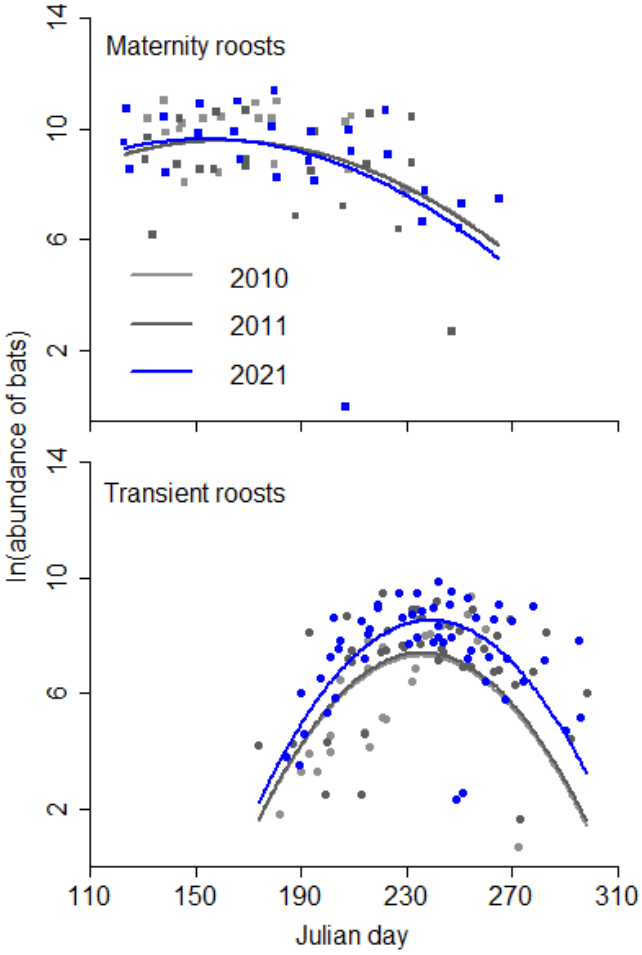


Figure 1.3. Proportion of saguaro, organ pipe, and agave plants flowering within 24 km of lesser long-nosed bat roosts across spring and summer in southern Arizona, 2010-11, 2021.

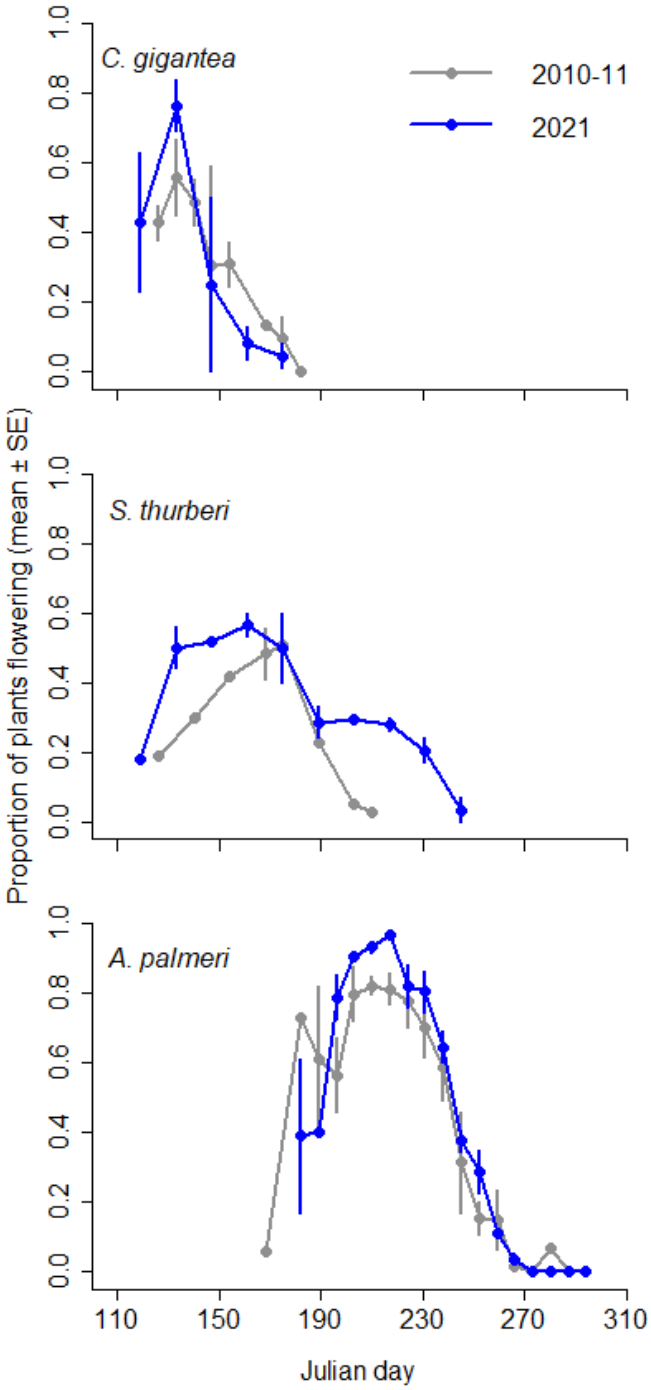


Figure 1.4. Proportion of saguaro and organ pipe plants fruiting within 24 km of lesser long-nosed bat maternity roosts across spring and summer in southern Arizona, 2010-11, 2021.

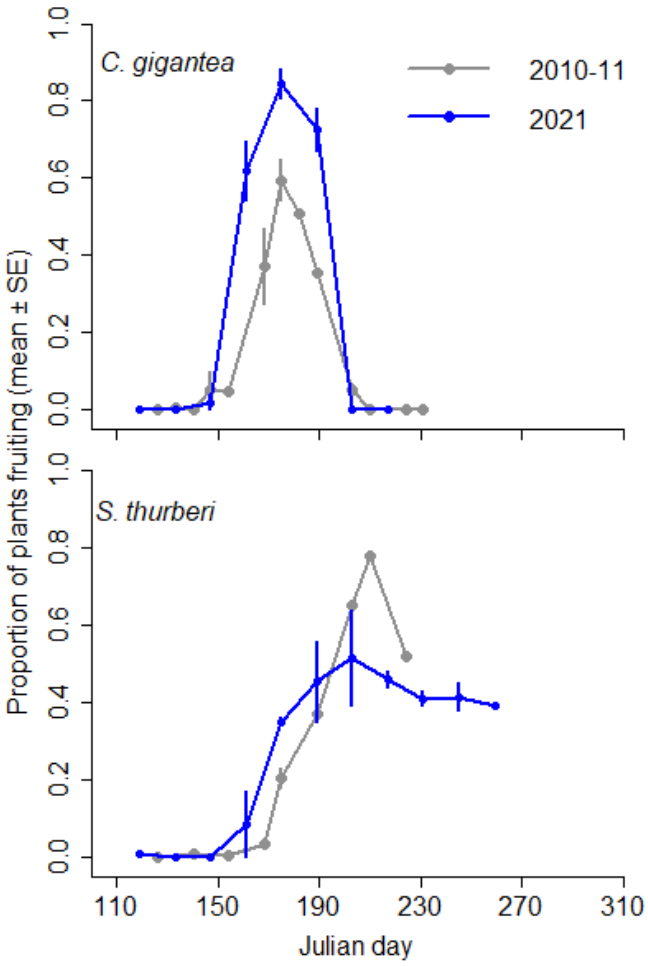


Figure 1.5. Predicted proportion of agave branches budding, flowering, and senesced near lesser long-nosed bat transient roosts in southern Arizona, 2010, 2011, and 2021. Statistics for regression lines are provided in Table 1.4.

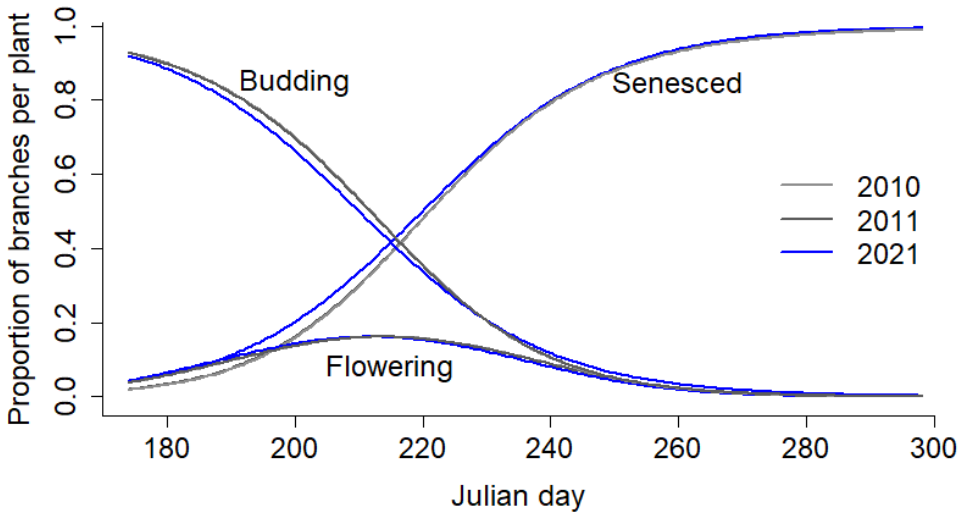


Figure 1.6. Abundance of lesser long-nosed bats and the proportion of food plants flowering at maternity and transient roosts in southern Arizona, 2010, 2011, and 2021. Predicted effects of flowering is represented with solid black lines and 95% confidence intervals with dashed lines. Statistics for regression lines are provided in Table 1.5.

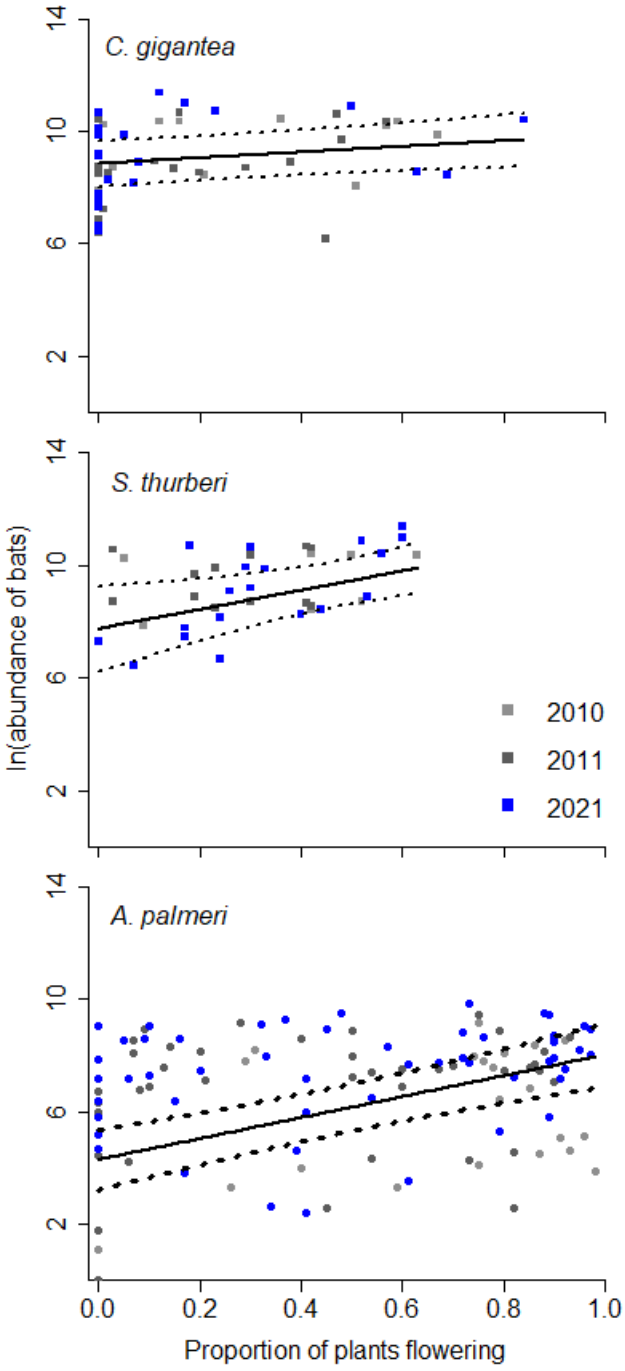


Figure 1.7. Abundance of bats and the proportion of saguaro plants fruiting at maternity roosts in southern Arizona, 2010, 2011, and 2021. The predicted effect of saguaro fruiting on abundance of bats is represented with a solid black line and 95% confidence intervals with dashed lines. Statistics for regression lines are provided in Table 1.5.

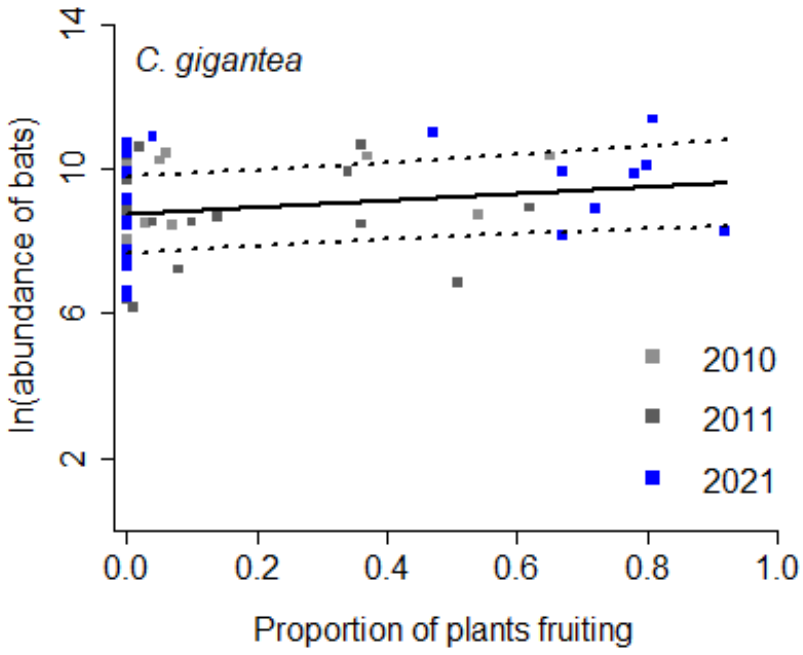
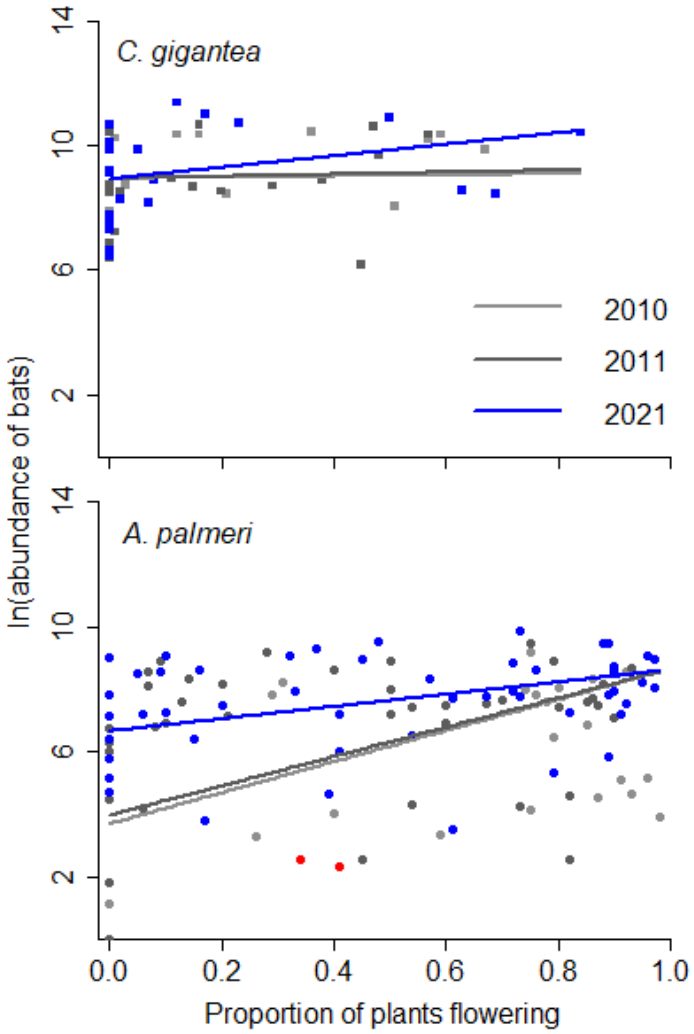


Figure 1.8. Differential effects of saguaro and agave flowering on abundance of lesser long-nosed bats across years at maternity and transient roosts in southern Arizona, 2010, 2011, and 2021. We modeled the effect of agave flowering on bat abundance with and without two near-zero values when bats abandoned a roost unusually early, which are depicted in red. Statistics for regression lines are provided in Table 1.5.



Appendix 1. Estimated maximum abundance of lesser long-nosed bats in southern Arizona, USA, and northern Mexico, 2010-11 and 2021.

Roost	Roost Type	2010-11	2021	% Change
Bluebird Mine	Maternity	5861	5075	-13.4
Copper Mountain Mine	Maternity	36795	58482	58.9
Pinacate Cave	Maternity	59772	19995	-66.5
Helena Mine	Transient	7412	8937	20.6
Little Dragoons Mine	Transient	4890	4059	-17.0
Mustang Cave	Transient	11529	7448	-35.4
Papago Springs Cave	Transient	2576	7614	195.6
Pyeatt Cave	Transient	13038	18784	44.1

CHAPTER 2: A NON-INVASIVE APPROACH FOR MICROSATELLITE GENOTYPING OF LESSER LONG-NOSED BATS (*LEPTONYCTERIS YERBABUENAE*)

ABSTRACT

Characterizing demography and movements of highly mobile, nocturnal animals is always challenging, but especially when disturbance to animals, disease risks, or field-safety concerns preclude handling or close observation. Non-invasive genetic samples from feces have been shown to be a cost- and time-efficient alternative for population-level studies, especially when microsatellite data are used to generate genetic tags ("molecular fingerprints") to identify and track individuals uniquely. However, because DNA from feces of animals that forage on nectar and fruit, including many bats, often is low in quality and quantity. Therefore, it is not clear whether fecal DNA can be used to provide individual identification to inform population-scale insights for this group, which are essential to ecosystem function. We developed and optimized a non-invasive approach based on microsatellite analysis of DNA from feces to identify individual lesser long-nosed bats (*Leptonycteris yerbabuenae*), which pollinate columnar cacti and agave across much of Mexico and into the southwestern U.S. We collected feces from roosts near the U.S-Mexico border, extracted total genomic DNA from air-dried feces that we suspended in an inexpensive buffer, and developed a two-step amplification approach to characterize five highly polymorphic microsatellite loci. Addition of a multiplex step improved amplification success and conserved DNA extracts while adding minimal cost. In an initial screening of 434 samples, focal loci distinguished individuals reliably; the probability that two closely related individuals could randomly have the same genotype at all five loci was 0.003. Repeated analyses showed that our analyses had a genotyping error rate <2%. We explore the benefits and limits of our approach for population studies of lesser long-nosed bats and similar

nectivorous and frugivorous species that provide key ecosystem services and are often of conservation concern.

INTRODUCTION

Monitoring demography of animal populations reliably is central to identifying population-level changes in time to take management action (O'Shea and Bogan 2003). When disturbance to animals, disease risks, or field-safety concerns preclude handling or close observation, estimating abundance or other demographic attributes can be challenging (Jones et al. 1996, Powell and Proulx 2003, Cook et al. 2021)., but especially for species that are nocturnal, cryptic, rare, challenging to capture, or that inhabit environments that preclude efficient survey methods (e.g., Eggert et al. 2003, Zylstra et al. 2010, Blanc et al. 2013, Roffler et al. 2019).

Non-invasive genetic sampling can be a cost-efficient and practical approach for studying animals without having to capture, handle, injure, or otherwise disturb them (Waits and Paetkau 2005, Woodruff et al. 2016, Ferreira et al. 2018, Pfeiler et al. 2020). For example, DNA extracted from shed feathers, hair, or feces can be analyzed to confirm species identifications, which can contribute to information on taxonomy, phenology, or monitoring for ecological and evolutionary studies that span diverse scales (e.g., Rees et al. 2014, Walker et al. 2016, Arnold et al. 2017, Pawlowski et al. 2021). Similarly, molecular analyses of DNA from environmental samples can be used to determine a unique "molecular fingerprint" for individual animals, providing an important tool for population genetics, demography, or individual-scale analyses (Adams et al. 2019, Sigsgaard et al. 2020, Andres et al. 2021).

A traditional approach for molecular fingerprinting is to analyze microsatellite loci that are polymorphic among individuals (see Waits and Paetkau 2005). Often referred to as single-

sequence repeats or single-sequence tandem repeats, microsatellites are short motifs of DNA sequences, typically a few nucleotides in length, that repeat multiple times in a particular region of the genome (Vieira et al. 2016). Differences in the number of repeats at a given locus result in different allele sizes among individuals (Hardy et al. 2003). By measuring allele sizes for multiple loci, a distinctive individual fingerprint can be inferred from a DNA sample.

For species that roost gregariously, large samples of materials such as feces can be gathered for non-invasive microsatellite analyses with less effort and disturbance to animals than the capture-based methods used to generate DNA samples from wing punches, muscle tissues, blood, or buccal epithelium (Oyler-McCance et al. 2018). By sampling roosting repeatedly, approaches such as mark-recapture models based on fingerprints from fecal DNA can be used to estimate abundance, distribution, movements, and other demographic parameters with more precision and larger sample sizes than traditional capturing techniques (Williams et al. 2002, Woodruff et al. 2016, Ferreira et al. 2018, Oyler-McCance et al. 2018, Pfeiler et al. 2020). However, these approaches require that DNA in feces is of sufficient quantity and quality for amplification and characterization with a low error rate (see Taberlet et al. 1999). DNA from feces of nectivores and frugivores can be low in quantity because (1) relatively few epithelial cells are excreted with the fecal material relative to organisms with fibrous or bulky feces, (2) presence of inhibitors, (3) occurrence of DNA from bacteria and other microbes, and (4) rapid degradation of DNA after excretion, especially in warm climates (see Piggott et al. 2004, Boston et al. 2009a, Marrero et al. 2009, Baldwin et al. 2010, Arnold et al. 2017). Because nectar-feeding and frugivorous bats typically are mutualists of the plants with which they affiliate, they are important in shaping plant reproduction and demography (Ramírez-Francél et al. 2022). Therefore, estimating abundance of these animals, monitoring changes in their abundances over

time, and understanding their movements at a landscape scale are important for understanding plant ecology and ecosystem function (Ratto et al. 2018).

The lesser long-nosed bat (*Leptonycteris yerbabuenae*) is a nectivore and frugivore that is an important pollinator of iconic columnar cacti and agave across its range in Mexico and the southwestern U.S. (Fleming et al. 2001, US Fish and Wildlife Service 2016*a*). The species is resident in the U.S.-Mexico border region from April to September and roost in caves and abandoned mines (Fleming et al. 1993). Upon arriving in region, lesser long-nosed bats inhabit maternity roosts in areas near flowering columnar cacti, where pregnant mothers give birth to pups (Fleming et al. 1998). Abundance at maternity roosts can reach a seasonal maximum of >50,000 individuals (Chapter 1). Later in the summer, mothers and young of the year move to transient roosts in areas near flowering agave (Cockrum 1991), where abundances can exceed 7000 individuals (Chapter 1). Although *L. yerbabuenae* was delisted from the Endangered Species Act in 2018 (US Fish and Wildlife Service 2018), the species is considered vulnerable to human activities such as infrastructure projects, human trafficking, and drug smuggling that reduce habitat or disturb roosts (US Fish and Wildlife Service 2016*a*). Additionally, changes to the distribution and phenology of food plants and the spread of white-nosed syndrome are of concern (US Fish and Wildlife Service 2016a, Frick et al. 2020). Therefore, estimating abundance and monitoring trends of lesser long-nosed bats in the region remains a conservation priority for this species.

Existing methods for estimating abundance of lesser long-nosed bats, such as counting bats from video recordings of emergences or capture-recapture methods that rely on marked individuals, are informative but also labor-intensive (Ober et al. 2005, Peñalba et al. 2006). High-quality video recordings require good weather and estimating abundance effectively

depends on consistent flight behaviors during emergences, with error increasing as the number of bats increases (O'Shea and Bogan 2003). Capturing and marking bats requires trained handlers, can occasionally injure bats, and may be limited due to concerns regarding disease transmission (Ellison 2008). Developing alternative methods to characterize demography of *L. yerbabuenae*, such as non-invasive genetic sampling of fecal samples for molecular analyses, can provide information from more sites, minimize disturbances, and improve long-term monitoring while also providing DNA samples that can be used for studies of diet, health, or microbiomes (Piggott and Taylor 2003, Waits and Paetkau 2005).

Although non-invasive genetic sampling has advantages, several challenges remain for studies that require fingerprints to distinguish individual animals. The first challenge is to develop an informative microsatellite panel for species of interest. Ramirez et al. (2011) identified and characterized 12 microsatellite loci for lesser long-nosed bats, including five simple dinucleotide repeats, one tetranucleotide repeat, and six complex repeats. Alleles for these loci range were reported to range from 125 to 300 nucleotides (often given as base pairs, bp), with 7-26 alleles per locus recorded in previous work (mean = 13.4 alleles/locus; Ramirez 2011). Successful use of this panel with DNA from feces has not yet been confirmed, as Ramirez et al. (2011) and Ramirez (2011) used wing punches and buccal cells from captured bats as DNA sources. Repeated analyses to estimate error rate have not been performed with this panel, but are needed especially because of the limited quality and quantity of fecal DNA (Taberlet et al. 1999, Waits and Paetkau 2005). The capacity to differentiate individuals with only a subset of the 12 loci, ideal as a cost-saving measure, also has not been explored.

A second challenge is to minimize costs. Although non-invasive methods often are considered less expensive than more traditional methods, the cost of laboratory reagents and

effort required for analyzing multiple microsatellite loci can be limiting in ecological applications (Ferreira et al. 2018), especially if amplification frequently fails and requires repeated attempts. Several studies have addressed similar limitations by using a multiplex-singleplex approach to amplification (Piggott et al. 2004, Hedmark and Ellegren 2006, Arandjelovic et al. 2009). This two-step approach involves an initial PCR with primers for several loci (the "multiplex" step, as there are multiple primer pairs present). The resulting product is used as template for singleplex PCRs to amplify each locus separately (the "singleplex" step, wherein only a single locus is amplified at a time). Microsatellite amplification with multiplex-singleplex PCR typically reduces genotyping errors such as allelic dropout and false alleles that can complicate individual identification and bias population estimates (Taberlet et al. 1999, Waits and Paetkau 2005). Because these errors increase in frequency with the number of loci analyzed, it is ideal to choose a minimal set of highly polymorphic loci that can still provide high discriminatory power for individual identification (Taberlet and Luikart 1999). To our knowledge, these approaches have not yet been applied in studies of lesser long-nosed bats nor in non-invasive sampling of bat DNA more broadly.

We identified and then optimized molecular approaches to reliably fingerprint individual lesser long-nosed bats from fecal samples. First, we screened 12 microsatellite loci previously described by Ramirez et al. (2011) to select a subset that amplified consistently from DNA isolated from feces, which we collected from maternity and transient roosts in southern Arizona. We then developed and optimized a multiplex-singleplex PCR approach to maximize amplification of target loci from fecal DNA that we isolated with a kit chosen because of its versatility and efficacy with fecal samples. We used these approaches to develop a cost-effective

approach for amplifying and scoring five focal loci, which were reliable in terms of a low error rate and highly discriminatory in terms of distinguishing individual bats.

METHODS

Studies based on DNA of bats typically use wing punches, blood, or buccal cells as source material (e.g., Ramirez 2011, Corthals et al. 2015), as these sample types generally provide high-quality genomic DNA (Worthington-Wilmer and Barratt 1996, Brooks et al. 2003). We began our study by obtaining DNA from buccal cells so that we could test and optimize methods that we then applied to DNA from fecal samples, which we anticipated to be of lower quality (Taberlet et al. 1999).

Collection of cells from buccal swabs

In August and September 2019, we live-trapped lesser long-nosed bats in mist nets near roost entrances at Organ Pipe National Monument (ORPI) and at hummingbird feeders at several private residences near Tucson and Hereford, AZ (Fig. 2.1). Our work was conducted with permission from the National Park Service (permit no. ORPI-2019-SCI-0015), the Arizona Game and Fish Department (permit no. SP650349), and the University of Arizona Institutional Animal Care and Use Committee (protocol 16-092).

We captured, recorded sex and age class (juvenile or adult), and gathered DNA from 5-70 bats in good condition on each of six trapping nights (Table 2.1) for a total of 184 bats over the trapping period (Table 2.2). Immediately after capture, an experienced handler used a sterile swab (Medical Wire & Equipment, UK) to rub the buccal mucosa (inner cheek) of each of the 184 bats for 5-10 seconds to obtain epithelial cells. Each bat was swabbed once, therefore, each swab represented a unique individual, providing a positive control for subsequent attempts to determine whether previously described microsatellite loci (Ramirez et al. 2011) can reliably

distinguish individuals of this species. The average time we handled each bat from capture to release was 88 minutes (SD = 61). On each sampling night, we also collected at least one negative control. Each negative control consisted of a sterile swab that was opened, waved in open air for 5-10 seconds, and then processed in the same manner as the buccal swabs. We placed each swab tip immediately into a 2 mL collection tube containing 500 µL of sterile phosphate-buffered saline (PBS) solution. Tubes were stored in a cooler and then transferred to a -20°C freezer within 12 hrs, where they were stored until DNA was extracted.

We used the DNEasy PowerSoil kit (Qiagen, USA) to extract total genomic DNA from each swab. We chose this kit because it has been used successfully to analyze DNA from buccal swabs of other vertebrates (e.g., Taylor et al. 2019), it is optimized for use with diverse environmental samples including vertebrate feces (Arnold et al. 2017, Parejo-Farnés et al. 2018), and because we aimed to use the same extraction method for both buccal and fecal samples. The PowerSoil kit also offers a value-added benefit of extracting high-quality microbial DNA, which could facilitate future studies of the bat microbiome or diet from these DNA samples (e.g., Avena et al. 2016, Alberdi et al. 2020, Aizpurua et al. 2021). We followed the manufacturer's protocol except that we incubated samples initially in lysis buffer for 10 min at 65°C (see Arnold et al. 2017). We eluted the extraction products to 100 µL and froze them immediately at -45°C.

Collection of fecal samples

We collected fecal samples non-invasively from transient (i.e., non-breeding) roosts of lesser long-nosed bats at ORPI and Coronado National Memorial (CORO) (Fig. 2.1). Our work was conducted with permission from the National Park Service (permits ORPI-2019-SCI-0015, CORO-2019-SCI-0005, and CORO-2020-SCI-0004). We entered roosts only after bats had exited for the evening, and we wore full personal protective equipment when in the roost (i.e.,

N95 masks, Tyvek suits and booties, and latex gloves). We disinfected all materials before leaving the roost area following US Fish and Wildlife Service's White-Nosed Syndrome Decontamination Protocol (US Fish and Wildlife Service 2016*b*).

We collected fecal samples at ORPI over two nights in August 2019 (Table 2.1). Our aim was to place plastic sheeting on the floor of the roost and collect fecal samples that did not overlap frequently on the sheet. For each sampling event, we placed a 1.4 m x 1.8 m polyethylene sheet (2-mm thick) inside the north entrance of the roost after bats had emerged. We left the sheet in place for 24 hours and retrieved it after bats had exited the roost the following evening. We stored sheets at -20°C.

Each of our sampling nights yielded hundreds of fecal samples that were well-spaced and typically did not overlap on the sheets (Fig. 2.2). Because > 40,000 bats were present in the ORPI roost during these surveys (Chapter 1), the probability of detecting the same individual more than once was low. Therefore, we shifted our focus to CORO, where the State of Texas roost is used by 2000-6000 bats annually (Chapter 1).

At CORO, we deployed a 2.7 m × 3.7 m polyethylene sheet (2-mm thick), but repeated the deployment and retrieval of sheets over three to five nights in succession (Table 2.1). We completed five multi-night sampling events in 2019 and three in 2020 (Table 2.1). Each sheet collected several thousand non-overlapping fecal samples.

In the laboratory, we carefully used a sterile swab (Puritan, USA) to collect individual fecal samples from the surface of sheets (Fig. 2.2). We wore N95 masks and worked in well-aerated spaces. We collected a range of differently colored fecal samples, including reds, browns, and yellows. Observation by compound microscopy revealed that the samples contained abundant pollen from *Agave* spp., readily visible at 100x magnification.

We placed each swab tip immediately into a 1.5 mL tube containing 500 µL of PBS solution. We stored samples at -20°C until they were processed for DNA extraction. Ultimately, we collected >15,500 fecal samples and selected 253 from CORO at random for analysis (Table 2.1).

Confirmation of DNA quality for amplification of microsatellite loci

We first determined whether DNA extracted with the PowerSoil kit was of sufficient quality for amplifying the microsatellite loci developed by Ramirez et al. (2011). This step was important because previous work used DNA extracted by the Qiagen Blood and Tissue kit (see Ramirez et al. 2011). We focused on buccal swabs, which we expected to have higher quality and quantity of DNA relative to fecal samples. Ramirez et al. (2011) reported high DNA concentrations from buccal swabs and wing punches, but because quantities were not presented, we could not compare our yields directly with those from previous work. Therefore, we used amplification by the polymerase chain reaction (PCR) as an indirect test of our DNA quantity and quality.

We first amplified three of the 12 microsatellite loci developed by Ramirez et al. (2011): *Leye*27, *Leye*CT2, and *Leye*GT2. We selected these loci because they were amplified by Ramirez et al. (2011) at one annealing temperature, and thus represented a straightforward test in which reactions for the three loci could be run at the same time on a single PCR machine.

In a set of initial trials, we used DNA from buccal swabs from eight bats (individuals 3, 4, 5, 6, 7, 8, 10, 11). For PCR, we used 10 µL reaction volumes containing AMP solution from the cost-effective Extract-N-Amp reagent system (Sigma-Aldrich, St. Louis, MO). Our initial reactions included 5.0 µL of AMP solution, 0.5 µL of each forward and reverse primer, 2.0 µL of sterile molecular-grade water, and 2.0 µL of template. Primers are listed in Ramirez et al. (2011).

Cycling reactions were run on a MJ Research PTC-200 thermocycler (Waltham, MA) following Ramirez et al. (2011): denaturation for 5 min at 94°C; 40 cycles consisting of 30 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C; and 7 min at 72°C. We used unlabeled (i.e., non-fluorescent primers) in initial trials, therefore we evaluated success by observing bands via gel electrophoresis. We observed faint bands from these initial PCRs, suggesting weak but positive amplification.

To ensure that weak amplification was not due to selection of specific loci that shared one annealing temperature, we ran subsequent PCRs as described above to amplify five additional loci: *Leye*3a, *Leye*11, *Leye*21, *Leye*17, and *Leye*23. For these reactions, we used annealing temperatures specified by Ramirez et al. (2011): 55°C, 53°C, 53°C, 58°C, and 58°C, respectively. Once again, we observed faint bands. Finally, we amplified the remainder of the 12 loci developed by Ramirez et al. (2011), assessing the success of PCRs at higher annealing temperatures employed (i.e., 61°C and 67°C; *Leye*4, *Leye*7, *Leye*67, and *Leye*71). We observed faint bands with these loci as well, suggesting that limited amplification was not only occurring at lower annealing temperatures.

Based on these results, we anticipated that a multiplex PCR could be useful as a prelude to singleplex reactions (Piggott et al. 2004). In a multiplex PCR, initial amplification of multiple loci in a single sample is used to generate template DNA used in subsequent singleplex (single locus) reactions. We next tested the multiplex-singleplex approach with DNA from buccal swabs.

Initial test of multiplex-singleplex approach

We focused first on 3 of 12 microsatellite loci developed by Ramirez et al. (2011): *Leye*11, *Leye*27, and *Leye*GT2. We chose these loci for three reasons. First, *Leye*11, *Leye*27, and

*Leye*GT2 were amplified by Ramirez et al. (2011) at relatively low and relatively high annealing temperatures (i.e., 53°C for *Leye*11, 67°C for *Leye*27 and *Leye*GT2) that spanned the range of annealing temperatures used for the full set of 12 loci (i.e., 53°C to 67°C). Thus, successful amplification of all three in a multiplex reaction, followed by successful singleplex reactions, would suggest that the DNA samples and multiplex process were robust to a range of annealing temperatures. Second, the three loci represent a diversity of repeat motifs. *Leye*11 is among the most complex repeat motifs reported by Ramirez et al. (2011), whereas *Leye*27 and *Leye*GT2 are simple dinucleotide repeats. Amplification of all three would suggest that the DNA samples and multiplex process were of sufficient quality to amplify both simple and complex motifs.

Finally, alleles from the three loci differ in their sizes (*Leye*11, 235-300 bp; *Leye*27, 125-165 bp; *Leye*GT2, 200-245 bp; Ramirez et al. 2011). Together, these represent the shortest and longest sizes among loci developed previously for lesser long-nosed bats (Ramirez et al. 2011), and their limited overlap in fragment sizes meant that they were likely to be visible via gel electrophoresis as three distinct bands/sample.

In the first multiplex reactions, we used 20 µL reaction volumes with 9.5 µL of DreamTaq Master Mix, 0.5 µL of each forward and reverse primer, 2.5 µL of sterile molecular-grade water, and 4.0 µL of DNA extract. We used DNA from buccal swabs of bats 8, 10, and 11. Cycling parameters followed Ramirez et al. (2011) with an annealing temperature of 56°C. We then used 3.0 µL of a 3:7 dilution of multiplex PCR product and sterile molecular-grade water as the template in singleplex PCRs that followed the recipe and cycling protocols for each locus as described by Ramirez et al. (2011). We found the three loci amplified successfully, as demonstrated by visible bands on gels following electrophoresis.

We then tested three multiplex reactions in which we amplified loci with compatible annealing temperatures: *Leye*3a, *Leye*17, *Leye*21, *Leye*23, with an annealing temperature ranging from 55°C to 58°C; *Leye*67, *Leye*71, and *Leye*CT2, with an annealing temperature ranging from 60°C to 61°C; and *Leye*4 and *Leye*7, with an annealing temperature of 67°C. These multiplex reactions generally yielded a product that supported successful singleplex reactions, confirming that the multiplex approach was suitable across a range of annealing temperatures and that up to four loci could be amplified successfully in a single multiplex-singleplex approach. We subsequently confirmed successful amplification via multiplexes that included up to seven loci, with similar results.

We then completed a series of optimization steps to ensure that we were using the appropriate amount of DNA from the multiplex reactions as template for our singleplex reactions. We found that the most consistent amplification in singleplex reactions occurred when we used a 3:7 dilution of the multiplex product in PCR-grade water as template. With this protocol established, we next selected loci for our main analyses.

Selection of loci

We selected six loci that satisfied four criteria: they amplified consistently from buccal samples; they were compatible in a single multiplex PCR, amplifying successfully at a single annealing temperature; they demonstrated considerable allelic variation (i.e., 7-26 per locus; Ramirez (2011)); and they showed limited departure from Hardy-Weinberg equilibrium (Taberlet and Luikart 1999, Ramirez 2011; Table 2.3). Based on these criteria, we selected *Leye*3A, *Leye*4, *Leye*17, *Leye*71, *Leye*CT2, and *Leye*GT2.

We tested our candidate loci with DNA from eight representative buccal samples and 18 representative fecal samples (Table 2.4). We shifted from gel electrophoresis to capillary

electrophoresis analysis on the Bioanalyzer 2100 platform with fluorescent primers (Applied Biosystems, dye set DS-33; ThermoFisher 2022; Table 2.4). We developed a four-color dye-fluorescence system with unique dye colors for loci *Leye*3A, *Leye*17, and *Leye*GT2, and a single dye color for the loci *Leye*CT2 and *Leye*71, which differed in their fragment lengths and thus could be read as distinct outputs (Fig. 2.3).

We pooled the singleplex reactions from each DNA sample into a single 10uL volume for fragment analysis on the Bioanalyzer 2100 platform at the University of Arizona Genetics Core. Because the Bioanalyzer is restricted to five outputs per run, we focused on *Leye*3A, *Leye*17, *Leye*71, *Leye*CT2, and *Leye*GT2 (Fig. 2.2). *Leye*4 was amplified by the multiplex but was not needed for singleplex reactions unless two samples were considered the same on the basis of the five-locus system, and thus needed the sixth locus to distinguish them.

Having selected our locus set, we then optimized our multiplex approach further, seeking high-quality chromatograms from singleplex reactions. We first optimized our methods for buccal samples, then evaluated that optimization for fecal samples.

Multiplex optimization for high-quality singleplex reactions

We used DNA from buccal swabs to test three multiplex approaches with our selected loci and dye system. For the first approach, we used 2× Qiagen Multiplex Master Mix. Our reaction volumes for these tests were 30 µL, including 12.5 µL of Master Mix, 0.6 µL of each forward and reverse primer, 0.8 µL of sterile molecular-grade water, 1.5 µL of 50 mM $MgCl_2$, and 8.0 µL of template DNA. We followed the manufacturer's recommendations for thermocycling conditions: denaturation for 5 min at 95°C; 40 cycles consisting of 30 sec at 94°C, 90 sec at 58°C, 60 sec at 72°C; and 30 min at 60°C. For the second, we used 2× Multiplex Master Mix plus an additive for GC-rich targets, again following the manufacturer's

recommendation for thermocycling conditions and the above recipe. For the third, we used DreamTaq Master Mix and followed the thermocycling conditions described by Ramirez et al. (2011). We used a 3:7 dilution of each multiplex product in PCR grade water as template for singleplex amplifications of individual loci.

We assessed electropherograms visually from the Bioanalyzer analyses and determined that 2 × Multiplex Master Mix without the additive, following the manufacturer's thermocycling conditions, performed best. We then applied our refined multiplex-singleplex approach to analyze DNA from buccal swabs of eight bats (bats 1-8), as well as DNA from six representative fecal samples (samples 74-79). We consistently generated high-quality electropherograms for loci *Leye*3A, *Leye*17, *Leye*71, *Leye*CT2, and *Leye*GT2 from buccal and fecal samples.

As a final step in developing our workflow for fecal samples, we compared our multiplex-singleplex approach with a traditional, single-PCR approach for each locus. This test was important because we sought to minimize costs: if the multiplex step was not needed, we could eliminate it.

Comparing standard PCR vs. a multiplex-singleplex approach for DNA from fecal samples

We analyzed 21 fecal samples twice: once using only a singleplex PCR and once using the multiplex-singleplex PCR we developed. We first used gel electrophoresis to compare the proportion of successful amplifications using DNA from six fecal samples, and later used the more sensitive Bioanalyzer to evaluate the proportion of successful amplifications from 15 fecal samples and a negative control. We found that the singleplex-multiplex method was robust for fecal samples, resulting in consistently detectable signals from the Bioanalyzer as described below. Therefore, we elected to use the multiplex-singleplex approach for DNA from fecal samples and finalized our approach for microsatellite analyses.

Final approach for microsatellite analyses

Following our optimization steps above, we analyzed DNA from 180 buccal samples and 253 fecal samples (Table 2.1) with the following steps. First, we used multiplex PCR to amplify loci *Leye*3A, *Leye*4, *Leye*17, *Leye*71, *Leye*CT2, and *Leye*GT2. We used 6.25 µL of 2× Multiplex Master Mix, 0.3 µL of each forward primer and reverse primer, 0.75 µL of $MgCl_2$, 0.4 µL of sterile molecular-grade water, and 4.0 µL of DNA extract. Cycling parameters were 5 min at 95°C; 40 cycles consisting of 30 sec at 94°C, 90 sec at 58°C, and 60 sec at 72°C; and 30 min at 60°C. Next, we amplified *Leye*3A, *Leye*17, *Leye*71, *Leye*CT2, and *Leye*GT2 separately in singleplex reactions that included 4.75 µL of DreamTaq Master Mix, 0.25 µL of a fluorescently labeled forward primer (Applied Biosystems), 0.25 µL of the relevant reverse primer, 3.25 µL of sterile molecular-grade water, and 1.5 µL of a 3:7 dilution of multiplex product in PCR grade water. Cycling for all singleplex reactions followed Ramirez et al. (2011). We pooled the resulting singleplex products for fragment analysis using a ratio that produced consistent signals across loci: 2.5 µL of *Leye3A* product, 2.0 µL of *Leye17* product, 1.5 µL of *Leye71* product, 1.0 µL of *LeyeCT2* product, and 4.0 µL of *LeyeGT2* product. As a proof of concept, we examined buccal DNA from 10 samples to evaluate whether *Leye*4, which was to be amplified in the multiplex, could be amplified and scored via a subsequent singleplex step, as for the other loci we considered.

Negative controls and assessment of error

In all analyses we ran negative controls in the form of swabs exposed only to air, extraction blanks (with water as template), and PCR blanks (with water as template). We assessed error rates for representative buccal and fecal samples by analyzing a total of 74 samples (34 buccal, 40 fecal) up to three times. We did not compare allele sizes from runs of

high quality against those of low quality (defined by very low or missing peaks, which occurred at times during our optimization steps), instead comparing only those runs in which our confidence was relatively high. In each case, we compared allele sizes to determine whether there was any change between runs. If a change was detected, we recorded the nature of the change (e.g., a minor shift of one nucleotide or a major change resulting in determination of a different allele size). We considered our five focal loci in a total of 182 individual comparisons based on buccal DNA, and 243 comparisons based on fecal DNA.

Fragment analysis and data analyses

We scored each sample for presence and size of alleles for each locus via the cloud-based Microsatellite Analysis software (Thermofisher Scientific). We used the GS500 (-35,-250,-340) LIZ size standard (B. Fransway, personal communication). We set a peak threshold of 50 Relative Fluorescent Units (RFU) for all loci, but typically accepted low peaks only after scrutinizing chromatograms, typically with validation from samples that were analyzed multiple times during our optimization process. We used the Microsatellite Analysis software for genotyping, with all peaks confirmed by visual inspection. Average peak sizes for representative data are reported below. We analyzed data from buccal swabs before analyzing data from fecal samples. We sorted allele lengths into discrete classes (i.e., 'binned' alleles) with TANDEM, which applies a least-squares minimization of rounding errors, estimates repeat sizes when the exact repeats are unclear (e.g., with complex repeats such as *Leye*4, *Leye*17 and *Leye*CT2), and uses the Nelder-Mead Downhill Simplex algorithm for parameter optimization (Matschiner and Salzburger 2009).

We used GenAlEx ver. 6.5 (Peakall and Smouse 2012) to estimate observed and expected heterozygosity, allele frequencies, and probability of identity. To explore the possible occurrence

of null alleles and scoring errors from stuttering, we used MICRO-CHECKER (van Oosterhout et al. 2004).

RESULTS AND DISCUSSION

For rare or elusive species, identifying or enumerating individuals to estimate population parameters reliably can be challenging. Non-invasive genetic sampling and microsatellite analysis can be used to identify animals without having to see, capture, or handle them (Taberlet and Luikart 1999). Subsequently, mark-recapture methods can be used to estimate population parameters (Miller et al. 2005, Miotto et al. 2014, Woodruff et al. 2016, Oyler-McCance et al. 2018). Although this strategy holds promise for many species, low quantities of DNA from fecal samples and the costs of laboratory materials can limit implementation.

We developed methods for collecting feces without disturbing bats, and designed and validated a protocol for analyzing microsatellite loci from buccal and fecal DNA samples. We confirmed that the PowerSoil DNA extraction kit is suitable for both sample types and optimized a novel multiplex-singleplex PCR to improve amplification of DNA from fecal samples and minimize genotyping error (Piggott et al. 2004). Because DNA from fecal material is often low in concentration and degraded, and PCR inhibitors are common in feces of frugivorous species (Waits and Paetkau 2005), maximizing amplification and minimizing genotyping error helps to prevent bias in population analyses (Taberlet et al. 1999).

Before analyzing fecal samples, we evaluated and optimized conditions for amplifying microsatellites with buccal samples, which typically contain higher quality DNA than fecal samples (Brooks et al. 2003, Broquet et al. 2007). In our initial analysis, all five loci amplified successfully from 76.1% of buccal samples (n = 137). We obtained data for only four loci from 14.4% of buccal samples, and data for only three loci from 9.4% of buccal samples. For fecal

samples, five loci amplified successfully from 61.3% of samples (n = 155). We obtained data for only four loci from 21.3% of fecal samples, three loci from 15.0%, and two loci from 2.4%. One sample failed completely.

These values are lower than those reported by Ramirez (2011), potentially a result of our use of the PowerSoil kit. In future work, we propose to test the Qiagen Blood and Tissue kit used previously for buccal swabs (Ramirez 2011, Ramirez et al. 2011) and to test its efficacy for fecal swabs. Our use of negative controls helped ensure that our success rate was not inflated artificially by contamination. Our success rate for genotyping fecal samples, defined as the percent of fecal samples for which all loci were amplified successfully, is comparable to similar studies of bats or other small mammals (Ferreira et al. 2018, Oyler-McCance et al. 2018, Ibouroi et al. 2021). Although each individual sample had a unique microsatellite genotype in our initial work, those that failed to amplify in all loci would be of limited use for mark-recapture analyses because confirming a repeated individual detection could be ambiguous when data for one or more loci are not obtained (Mills et al. 2000). As described below, our data show that even three or four loci have high discriminatory power given the number of bats we sampled, as revealed by the probability of identity. Thus, our approaches, which included at least three loci for >99% of buccal samples and 98.6% of fecal samples, might be especially useful in smaller roosts.

Multiplex approach

We confirmed that multiplex pre-amplification improved our ability to size microsatellite loci from fecal samples. Successful sizing requires that the fluorescent signals from amplified microsatellites meet a threshold, represented as peaks in a chromatogram. When we used only a singleplex approach, mean peak height for all loci was 173.0 RFU (95% C.I. = 153.1-192.9) for fecal samples, which did not differ meaningfully from spurious peaks detected in the negative

control (Wilcoxon test, $P = 0.20$). The difficulty in resolving peaks from samples relative to spurious peaks in negative controls indicates the singleplex samples were of insufficient quality for further consideration. In contrast, mean peak height for all loci was sevenfold higher overall when a multiplex step was included prior to the singleplex reaction (mean = 1177.6 RFU, 95% C. I. = 923.8-1431.4). We observed strong improvements in peak height for all loci with the multiplex approach relative to the singleplex alone (Table 2.5, Fig. 2.4). Enhancement of peak height ranged from 3.8-fold (*Leye*3A) to 19.9-fold (*Leye*GT2). Therefore, the multiplex step is an important element in generating useful microsatellite data from DNA extracted from feces of lesser long-nosed bats.

Low error rate detected via repeated analyses

In repeated analysis of 34 buccal samples, 181 of 182 (99.5%) of allele size calls were consistent for individual samples. We observed minor sizing differences in 11 of 182 cases (6%) that did not affect determination of allele size after binning. The allele size call that differed markedly was for locus *Leye*CT2. One individual accounted for 4 of 11 minor changes (36.3%) and the single marked change, suggesting relatively low DNA quality from that individual. In repeated analysis of 40 fecal samples, 240 of 243 (98.8%) allele-size calls were consistent for individual samples. Minor changes were observed in only two cases. The allele-size calls that differed markedly were for loci *Leye*17, *Leye*L71, and *Leye*CT2, all from different fecal samples. Overall, our assessment of error rates in allele-size calls indicates that data from both buccal and fecal swabs are reliable, with approximately 99% consistency in allele size determinations.

Evidence for few null alleles

Null alleles occur when one allele fails to amplify, and the locus is mistakenly scored as homozygous (van Oosterhout et al. 2004). We detected a low estimated frequency ($r < 0.1$) for

null alleles in all cases except for *Leye*CT2 (Chakraborty et al. 1992). For buccal samples, the occurrence of null alleles might reflect mutations in the primer-binding region (Selkoe and Toonen 2006) or minor population structuring in bats from the region we sampled (Ramirez 2011). For fecal samples, we anticipate that null alleles also might result from low DNA quantity (Selkoe and Toonen 2006). We did not detect evidence for large allele dropout or inconsistencies in our scoring that could be attributable to stutter peaks, which can be mistaken for "true" allele peaks. Negative controls from DNA extraction, multiplex PCR, and singleplex PCR steps confirmed that potential contamination with extraneous DNA did not affect our analysis.

Population-level data

The bats included in our study likely represented a subset of the same large population studied by Ramirez (2011), as observed heterozygosity (H_O) and expected heterozygosity (H_E) for the five loci we analyzed generally resembled the values reported previously (Table 2.3). However, we detected a larger number of alleles (Na) for each locus relative to the values reported by Ramirez (2011), even when only buccal samples are considered (Table 2.3). If we exclude several alleles that occurred at low frequency (< 0.5%), our observed number of alleles decreased an average of 5.6 alleles per locus (SD = 4.5), but numbers still exceeded those reported by Ramirez (2011) (Table 2.3). In contrast, another study of *L. yerbabuenae* (Arteaga et al. (2018) reported fewer alleles per locus than Ramirez et al. (2011), attributing fewer alleles to sampling from a more limited range of sites in the Baja California Peninsula. However, two loci developed by Ramirez et al. (2011) for *L. yerbabuenae* were used in studies of Mexican long-tongued bats (*Leptonycteris nivalis*) (Pourshoushtari and Ammerman 2021). Those authors reported a high number of alleles for *Leye*17 (Na = 17) and *LeyeL*71 (Na = 24), potentially highlighting differences in the sensitivity of equipment or thresholds for scoring peaks.

Alternatively, population abundance and dynamics of *L. nivalis* may be different from that of *L. yerbabuenae*, potentially reflecting differences between the polygynandrous mating system of *L. nivalis* and the polygynous mating system of *L. yerbabuenae* (Hensley and Wilkins 1988, Ceballos et al. 1997). Although several studies using microsatellites from bats have reported a similarly high or even higher number of alleles for other species (Boston et al. 2009*b*, Nagy et al. 2010, Oyler-McCance et al. 2018), the differences we observed from similar studies of the same species warrant further exploration, and highlights the challenge of comparing microsatellite-based studies among laboratories and in different timeframes (Shafer et al. 2015).

One challenge in comparisons with previous studies is that complex microsatellites can be difficult to bin *de novo* in traditional tools such as GENEMAPPER, which require reference data that rarely exist for non-model species (see Matschiner and Salzburger 2009). Manual binning by rounding to the expected periodicity given the repeat size (e.g., to allele sizes at intervals of four bp in the case of the tetranucleotide repeat of *Leye*3a) can combine alleles and underestimate allelic diversity (Matschiner and Salzburger 2009). Programs such as TANDEM can estimate periodicity of complex microsatellite repeats based on data without references and incorporate error profiles for each data set, with the added benefit of presenting histograms of allele-size frequency for visual inspection. In reviewing TANDEM output, we found in general that the number of alleles detected was higher in fecal samples than in buccal samples. If degraded DNA in fecal samples was responsible for erroneous allele-size calls, we would not have expected such a low error rate when the same samples were reanalyzed multiple times. We anticipate that studies aiming to detect individuals from fecal samples might benefit from leaving sheets in roosts for shorter periods of time to reduce the chances of mixing samples or contamination by aerosolized or dried excreta.

We note that the number of alleles was particularly elevated for *Leye*CT2 relative to the values reported by Ramirez (2011). *Leye*CT2 is a complex repeat that showed evidence of null alleles at a higher frequency than other loci, for which successful amplification was lowest, and incidence of errors was higher than for other loci (above). In future work, therefore, another locus might prove more useful than *Leye*CT2, such as the dinucleotide repeats *Leye*23 or *Leye*27 (Ramirez et al. 2011). Both *Leye*23 and *Leye*27 conform to expectations of Hardy-Weinberg equilibrium (Ramirez et al. 2011) and the annealing temperatures needed for amplifying them (58°C and 60°C) are compatible with our multiplex approach. Ramirez et al. (2011) reported a size range of 200-255 bp for *Leye*23 and 125-165 bp *Leye*27. Given the constraint in the number of dyes that can be analyzed simultaneously, we anticipate that replacing *Leye*CT2 with *Leye*27 could be useful in future work. More generally, improvements over time in algorithms, reagents, and analysis technologies may yield more precise data, a prediction that could be assessed if data sets are deposited publicly.

Utility of Leye4

We amplified *Leye*4 twice in 10 samples successfully. All amplifications produced high-quality reads in the expected size range. The repeated analysis for each sample yielded consistent results in all cases and each individual had a distinctive value for each allele. Therefore, we confirmed that the *Leye4* locus amplified reliably and could provide additional discriminatory power if needed.

Costs

At an estimated cost of $8.25 per sample ($1.65 per locus per individual), the approach for microsatellite analysis we developed provide a viable alternative to traditional techniques for identifying individual lesser long-nosed bats. The multiplex step added only 10% to the cost per

sample and allowed more samples to be processed with less materials going to waste, reducing total effort and cost. Additionally, genotyping error generally is reduced, and DNA extract is conserved when using multiplex products as a template for subsequent singleplex reactions (Piggott et al. 2004). The remaining DNA could be analyzed to gain additional information about demographics, microbiome, or diet of bats (e.g., Gaona et al. 2019, Lance et al. 2022). The costliest step in our workflow was DNA extraction, which accounted for ca. 75% of the material and analysis cost. We anticipate that less-expensive methods might be equally useful (Videvall et al. 2017), which can be explored in future studies.

Probability of identity and perspective on estimating abundance

The loci we considered have high power to discriminate individual lesser long-nosed bats (Table 2.7; Waits et al. 2001). Overall, the probability of identity given the five focal loci was 7.5E-09, and the cumulative probability that two unique but closely related individuals could have the same genotype at all five loci was 3.3E-03. Had any two samples matched at all five loci, or differed at only one locus, multiple amplifications and analyzing a sixth locus (*Leye*4) would reduce the chance of misidentification from genotyping error and confirm the two genotypes were the same individual (Taberlet et al. 1996). We note that the discriminatory power of three or for loci is also high (Table 2.7), such that partial data still have the capacity to distinguish individuals when resident population sizes are similar to those in many transient roosts (Chapter 1).

Because several thousand individual lesser long-nosed bats can use a single roost (Chapter 1), mark-recapture abundance estimates would require microsatellite signatures of more individuals than we considered. For example, Robson and Regier (1964) suggest two samples of 500 individuals are required to estimate abundance of a closed population of 5000 with a high

level of precision using Lincoln-Petersen methods. For multi-year open population mark-recapture designs, effective sample sizes are difficult to define and depend on the parameters of interest (Lindberg 2012). At present, little is known about roost fidelity or other factors influencing the number of bats using a given transient each year. If repeated sampling with larger within-year sample sizes were achieved, then additional population parameters could be estimated using a robust design that incorporates aspects of both open and closed mark-recapture designs (Pollock 1982).

Conclusions

Developing non-invasive methods to identify animals, characterize their movements, and estimate their abundances are fundamental steps in conservation and management. The workflow we developed for fecal samples collected passively provides relatively inexpensive and reliable data from five microsatellite loci that we found reliable for distinguishing individual lesser long-nosed bats; we did not record the same molecular fingerprint twice. Given the relatively high number of individuals inhabiting breeding roosts (Chapter 1), it might be appropriate to develop a complementary multiplex-singleplex system for additional or other loci (Ramirez et al. 2011). The approach we have used could be made more cost-effective by changing to a less expensive process for DNA extraction, providing the opportunity to process more samples without incurring high costs. More broadly, the methods we outline provide a workflow that, although focused on *L. yerbabuenae*, can be expanded to other species, provided microsatellite panels for those species are established. Our study provides a proof of concept for reliably characterizing individuals in a primarily nectarivorous species via non-invasive and low-cost DNA fingerprinting of feces, with a general workflow that is readily transferrable to other species of nectivores and frugivores that may be of conservation concern.

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Table 1.1. Summary of buccal and fecal samples collected for microsatellite analysis from lesser long-nosed bats. Collection dates for fecal samples represent the period when sheets were deployed in roosts. Fecal samples that were collected but not processed, and DNA samples that were obtained but not extracted, are stored at the University of Arizona for future work. ORPI is Organ Pipe National Monument and CORO is Coronado National Memorial.

Sample type	Location	Collection date	No. collected	No. extractions	No. analyzed
Buccal	ORPI	3-Aug-19	70	70	70
	Hereford, AZ	24-Aug-19	22	21	21
	Elgin, AZ	31-Aug-19	13	13	13
	Tucson, AZ	14-Sep-19	5	5	5
	Hereford, AZ	21-Sep-19	43	42	42
	Tucson, AZ	27-Sep-19	30	29	29
Fecal	ORPI	3-Aug-19	48	0	0
	ORPI	3-Aug-19	378	77	0
	ORPI	4-Aug-19	596	0	0
	ORPI	4-Aug-19	607	0	0
	CORO	26-Aug-19 to 2-Sep-19	208	207	0
	CORO	2-Sep-19 to 5-Sep-19	48	0	0
	CORO	5-Sep-19 to 7-Sep-19	2837	500	196
	CORO	7-Sep-19 to 9-Sep-19	2887	312	57
	CORO	9-Sep-19 to 12-Sep-19	2012	312	0
	CORO	17-Aug-20 to 19-Aug-20	>2000	0	0
	CORO	19-Aug-20 to 21-Aug-20	>2000	0	0
	CORO	21-Aug-20 to 24-Aug-20	>2000	0	0

Table 1.2. Sex and age class of lesser long-nosed bats from which buccal swabs were obtained at sites in southern Arizona, 2019.

		n Females		*n* Males		
Location	Collection date	Juvenile	Adult	Juvenile	Adult	Total
ORPI	3-Aug-19	32	23	15	0	70
Hereford, AZ	24-Aug-19	6	14	1	1	22
Elgin, AZ	31-Aug-19	9	2	2	0	13
Tucson, AZ	14-Sep-19	5	0	0	0	5
Hereford, AZ	21-Sep-19	27	1	15	0	43
Tucson, AZ	27-Sep-19	21	0	9	0	30

Table 1.3. Characteristics of 12 microsatellite loci as reported by Ramirez (2011) and the present study. H_O is observed heterozygosity, H_E is expected heterozygosity, and * indicates significant deviations from Hardy-Weinberg equilibrium. Values in brackets indicate the number of alleles observed only from buccal samples in the present study. Values in parentheses indicate the total number of alleles from the present study when those with a frequency of < 0.5% are excluded. We did not obtain data for *Leye*4 to estimate allele characteristics: the other five loci reliably distinguished individuals in our sample, such that *Leye*4 was amplified from only a few individuals as a proof that it could be used if needed.

		From Ramirez (2011)			Present study		
Locus	Size Range	No. alleles	H_O	H_E	No. alleles	H_O	H_E
*Leye*3a	135-200	8	0.739	0.741	13 [9] (11)	0.819	0.833
*Leye*4	200-290	26	0.330	0.851	NA	NA	NA
*Leye*17	200-240	12	0.721	0.722	20 [18] (16)	0.722	0.800
*Leye*L71	130-180	8	0.513	0.766*	27 [23] (21)	0.803	0.902
*Leye*CT2	200-280	7	0.628	0.618	27 [26] (13)	0.630	0.796
*Leye*GT2	200-245	16	0.865	0.866	21 [15] (19)	0.827	0.930

Table 1.4. The proportion of samples for which successful amplification was observed at each locus during optimization. Initial tests used gel electrophoresis and visualization to confirm amplification and a subset of loci then were tested using the more sensitive capillary electrophoresis (i.e., Bioanalyzer). *Leye*4 and *Leye*7 were not included in initial tests.

	Gel		Bioanalyzer
Locus	Buccal (n = 8)	Fecal (n = 18)	Buccal (n = 6)
*Leye*3A	1.00	0.33	1.00
*Leye*4	NA	NA	NA
*Leye*17	0.75	0.00	1.00
*Leye*L71	1.00	0.89	1.00
*Leye*CT2	1.00	0.61	1.00
*Leye*GT2	1.00	0.28	1.00
*Leye*7	NA	NA	NA
*Leye*11	0.88	0.17	NA
*Leye*21	0.00	0.00	NA
*Leye*23	0.13	0.00	NA
*Leye*27	1.00	0.22	NA
*Leye*67	0.88	0.11	NA

Table 1.5. Results of amplification of six microsatellite loci via singleplex-only PCR (S) and the multiplex-singleplex PCR approach we developed for this study (MS). Values indicate the proportion of samples for which amplification was successful for each locus. Six fecal samples were analyzed by gel electrophoresis initially and 15 fecal samples were analyzed subsequently by capillary electrophoresis (Bioanalyzer).

	Gel		Bioanalyzer	
Locus	S	MS	S	MS
*Leye*3A	0.00	0.17	0.00	1.00
*Leye*4	0.00	0.17	0.00	1.00
*Leye*17	0.00	0.17	0.00	0.93
*Leye*L71	0.00	0.83	0.00	1.00
*Leye*CT2	0.00	0.50	0.00	0.80
*Leye*GT2	0.00	0.00	0.00	0.93

Table 1.6. The occurrence of null alleles at each microsatellite locus for buccal and fecal DNA samples. Estimated frequencies (*r*) of null alleles at each locus are calculated using the methods of Chakraborty et al. (1992), with values < 0.1 considered negligible to zero (Pourshoushtari and Ammerman 2021).

	Frequency	
Locus	Buccal	Fecal
*Leye*3a	0.0040	0.0034
*Leye*4	NA	NA
*Leye*17	0.0540	0.0000
*Leye*L71	0.0690	0.0363
*Leye*CT2	0.0560	0.1378
*Leye*GT2	0.0620	0.0098

Table 1.7. The probability of identity (PI) and probability of identity of closely related individuals (PI_{sibs}) for increasing combinations of loci based on analyses of *Leye*3A, *Leye*17, *Leye*71, *Leye*CT2, and *Leye*GT2 from buccal and fecal DNA samples.

	Probability	
Number of loci	PI	PI_{sibs}
1	4.7E-02	3.5E-01
1+2	8.1E-04	1.0E-01
1+2+3	1.2E-05	3.1E-02
1+2+3+4	6.8E-07	1.1E-02
1+2+3+4+5	7.5E-09	3.3E-03

Figure 1.1. Roost sites where lesser long-nosed bats were captured to obtain buccal swabs (circles) and from which their feces were collected (triangles) in southern Arizona, 2019 and 2020. 1: Copper Mountain Mine, ORPI; 2: Private residence; 3: Appleton-Whittell Research Ranch; 4: Private residence; 5: Private residence; 6: State of Texas Mine, CORO.

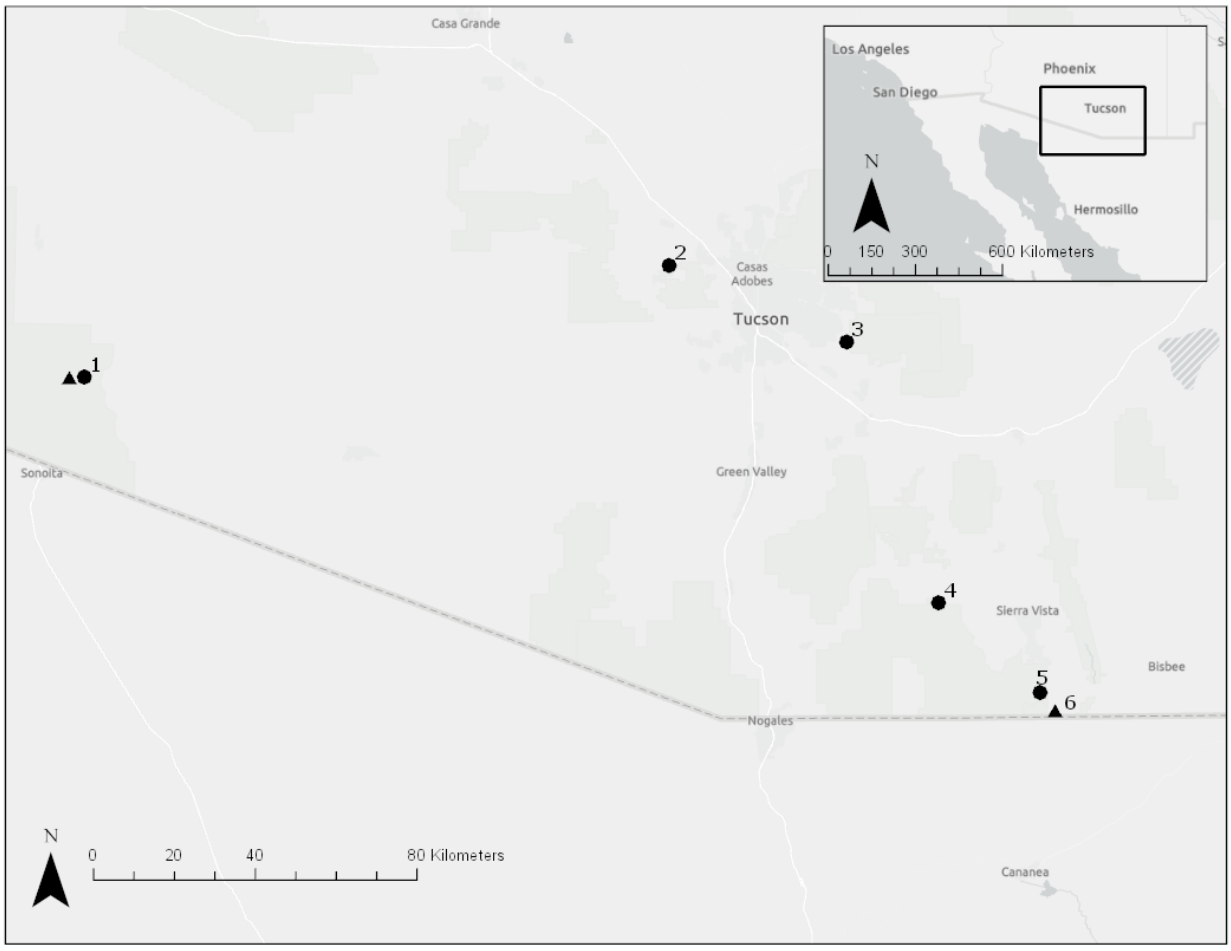

Figure 1.2. Feces of lesser long-nosed bats were widely spaced on plastic sheets placed in roosts (left), facilitating collection of individual samples with a sterile swab (right).

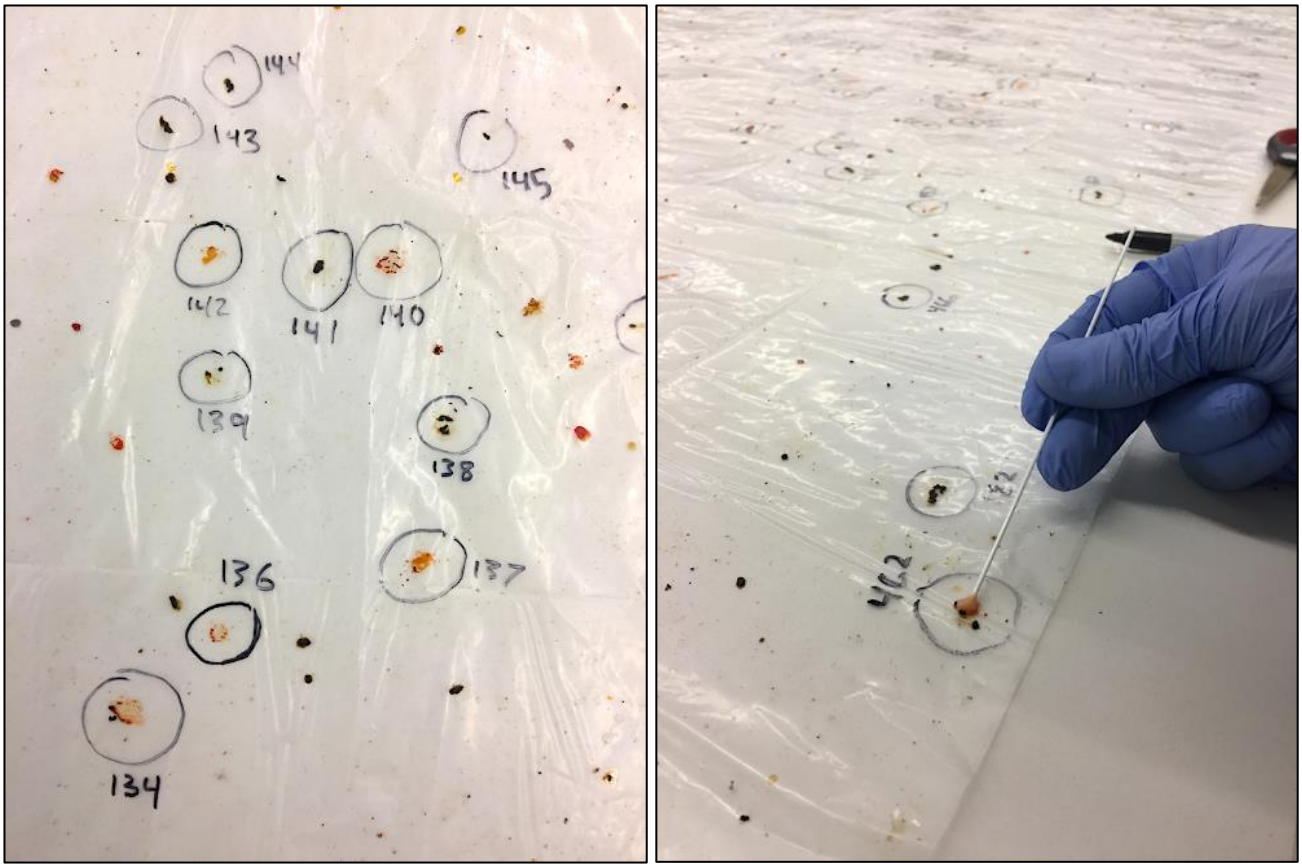

Figure 1.3. Size ranges (base pairs) and dyes for the panel of five microsatellite loci selected for the multiplex-singleplex approach. We used Applied Biosystems DS-33 dye set: blue = 6-FAM™, green = VIC™, yellow = NED™, red = PET™, and orange = LIZ™. The locus *Leye4* (size range 200 – 290 BP) was included in multiplex PCR but was not analyzed; we retained it to provide further information if any individuals matched at the five loci analyzed initially.

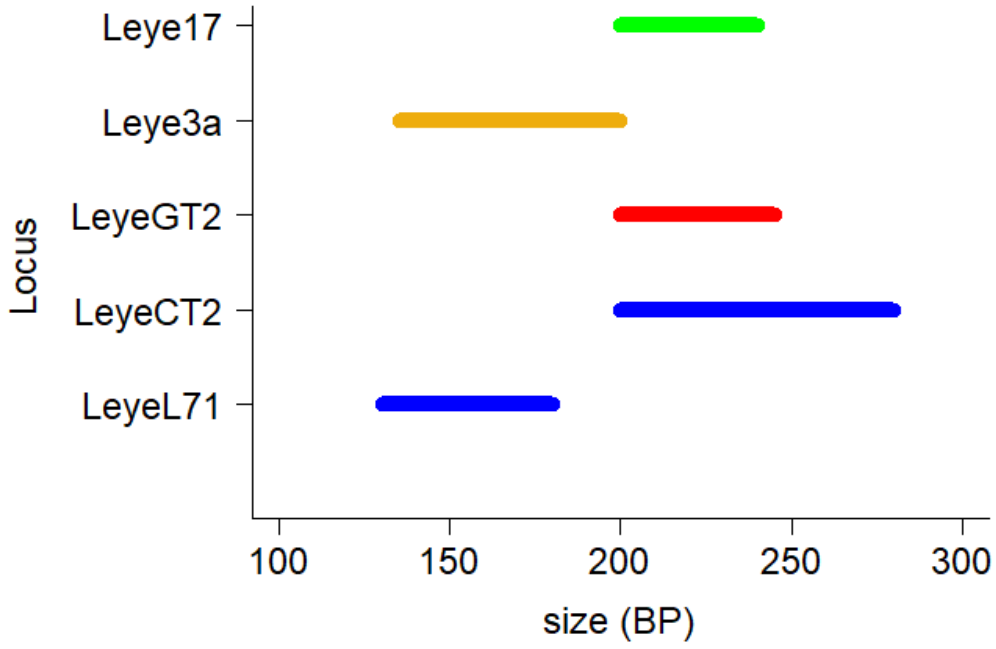

Figure 1.4. Comparison of Relative Fluorescent Units (RFU) on the natural log scale for five microsatellite loci from 15 fecal samples following amplification by the multiplex-singleplex approach vs. a singleplex-only approach. Boxes represent the first and third quartiles, the median is symbolized with a heavy horizontal line, and vertical lines symbolize values that are within 1.5 times the interquartile range.