Analytical Chemistry Analysis of Bat Guano: An Investigation Into Species-Specific Bat Detection Via Olfaction

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INTRODUCTION

With the increasing pressures on rare species that demand better information and data to guide and support recovery efforts, the use of dogs to seek out evidence of wildlife (e.g., scat), if not individual animals, has seen a dramatic increase over the past several years. The US Fish and Wildlife Service (USFWS) currently deploys or sanctions deployment of Wildlife Detection Dogs (WDDs) for a variety of species throughout the US, and has been involved in development of one WDD program, to include a species-specific certification standard. This was the Mojave Desert Tortoise K9 Program (DTK9) funded by the US Department of Defense as well as several military installations, and conducted in close collaboration with the USFWS Desert Tortoise Recovery Office in Nevada (Cablk and Harmon, 2011; Heaton et al., 2008; Nussear et al., 2008; Cablk et al., 2007; Cablk and Heaton, 2006). Ultimately an overarching WDD standard is needed to establish the basic elements that a WDD team demonstrates to gain approval for use in conservation efforts (e.g., conduct field surveys, among others) for USFWS listed species. Outside of DTK9 there are no published standards, best practices or other regulatory guidance to offer a measurement of safety or capability for species-specific WDDs currently in use, or for WDDs in general (Cablk and Harmon, 2011).

To date, most of the research investigating WDDs relates to dogs trained for scat detection (e.g., DeMatteo et al., 2014; Wasser et al., 2012; Smith et al, 2003). Outside of the DTK9 Program, there have been few studies published demonstrating the efficacy of WDD teams to locate live animals within an experimental framework in either a controlled or field setting. Kapfer et al (2012) compared results from human surveys with WDDs to locate box turtles. Mathews et al (2013) compared human surveys with WDDs to located bat casualties from wind-turbines, which was technically not a scat detection study, however the animals were deceased thus eliminating risk to the wildlife. Similar research was conducted for bird strikes by Paula et al (2011). In other studies where WDDs were used to survey for live animals, the dogs were not expected to, and were not able to directly interface with the survey species. Rather, the dogs alerted the handler to the presence of odor at a burrow, presumably at some level of concentration that the dog has been trained to or itself deems enough to indicate the presence of an animal. Studies such as Duggan et al (2011), using dogs to survey for Franklin's Ground Squirrel (*Poliocitellus franklinii*) and (Reindl-Thompson et al., 2006), using dogs to locate black-footed ferrets (*Mustela nigripes*), report problems with false positives and the inability to accurately validate dog responses. Risk to wildlife in these types of surveys would relate more toward negative impacts to species burrows and habitat than to direct harm to the animals. Where the species of interest is an invasive pest, such as red imported fire ants (*Solenopsis invicta*) (Lin et al 2011) or brown tree snakes (*Boiga irregularis*) (Vice and Engeman, 2000), concerns for the target species safety is not a consideration. The reverse may be of concern in such instances, where safety to the dog from the targeted species may be of concern. The literature demonstrates that safety to wildlife during surveys has not been a consideration, because risk from a dog interfering with scat, pellets or other sign, results in data loss, not loss of the targeted animal; this consideration was a significant contribution of the DTK9 Program and should be of high importance when using dogs to survey for listed or special status species.

Towards this end, the research presented here lends support to demonstrating the potential for scent discrimination between species by answering important questions about the potential for wildlife species specificity (e.g., only listed bat species) versus a generalized species (e.g., all

bats) detection dog team. Recently published research by Browne et al (2015) lends support towards this end, reporting research that involved a scent discrimination experiment for reptiles. Although this study did not interface canines directly with the reptiles and was conducted in a laboratory setting, success rates at discriminating between different reptile species were high.

The research we report on here was conducted as a means to quickly and efficiently gain knowledge about the potential for a WDD to be trained as a species specialist, or as a species generalist, using analytical chemistry methods. Dogs have the ability to be trained as either; as a species-specialist they would locate and indicate only the specific target species within a broader target class (e.g., only Indiana bat guano among all bats that co-occur) while as a species-generalist they would indicate the presence of any guano from any bat within the survey area. Conducting assessments of dogs' capability requires considerable effort both in time and cost. Most significantly is the fact that multiple canines are needed to deliver sufficient data, and that the dogs require training aids from the target species not only to be trained, but also to be tested. Furthermore, research has shown that detection dog performance varies with numerous factors, including training aspects (Cablk and Sagebiel, 2011), handler bias (Lit et al., 2011), health, among others. All of these factors contribute to considerable expense, time, and effort. Thus an initial investigation using analytical chemistry methods to evaluate volatile organic compounds (VOCs) from bat guano was a logical, less expensive and less invasive first approach.

Evidence from other canine disciplines (human remains detection aka "cadaver dogs") show that a dog trained to find human remains can differentiate and will not indicate on animal remains with proper training, and that they are able to locate any individual within the 'human' target class (e.g., male or female, varying age, varying diet, etc.). This has some scientific support from analytical chemistry, specifically solid phase microextraction (SPME) gas chromatography/mass spectrometry (GC/MS) as well (Cablk et al., 2012). These same laboratory analyses have highlighted overlap in VOC signature categorized as generic 'decomposition' (Rosier et al., 2015). Relevant to guano, we used GC/MS to identify the potential odor molecules present in bat guano sampled from both sexes of multiple species, and in what relative abundances. While this method should not be considered to deliver an absolute formula for what constitutes a signature recognized by a dog, it does provide an assessment of the variability within and among guano from a single species and also across different species. This is an efficient means to gain an objective evaluation of the VOCs of a target species to guide training and testing expectations.

STUDY OBJECTIVES

It was hypothesized that there would be overlap in the VOCs across bat species. For a dog to be trained to locate only the guano from one specific bat species, there must be a unique scent signature that a dog can be trained to such that the dog recognizes all individuals (across sex, age, varying diet, physiological state, etc.) of that species, while differentiating and ignoring guano from other bat species that co-occur (and possibly share some characteristics such as diet or physiological state) within the geographic range of the target species. The results of this type of analysis are important for not only bat species, but for wildlife detection in general, since dogs have been deployed to locate scat of multiple species within a genus (e.g., grizzly and black bear), but have not been trained to exclude one or the other where they co-occur. Whether this is

because it has not been assessed, or has not been proven fruitful and thus not published is unknown.

The objective of the research was to conduct quantitative analyses of VOCs that comprise odor signatures (based on analytical chemistry methods) of guano from different target bat species of interest to inform the potential for training and testing species-specific bat WDDs. To meet this objective the following questions were posed:

1. Is there a difference in guano VOCs between the myotis spp.?
2. To what degree is the guano VOCs from myotis spp. different and to what degree are they similar?
3. In what chemical classes are the species more different and more similar?
4. Is there variability between guano from males and females?
5. To what extent do male and female VOCs vary and in what chemical compounds?
6. How different are non-myotid guano VOCs from myotid samples?
7. Based on the results of these analyses, is it likely that myotis spp. guano is sufficiently different from other bat species guano such that a dog could be trained to be a myotis specialist?
8. What can be inferred from the chemistry results about odor signatures that relate to detectability by dogs?
9. Based on the results of these analyses, is it likely that distinct odor signatures occur such that a canine could be trained as a species-specialist, to detect a single myotis spp.?
10. If the above is true, does it hold for all myotis spp. or just some and which ones?

METHODS

Bat Guano Collection

An initial assessment of 'generic' bat guano was conducted based on a collective sample of guano that represented as many as five bat species. The purpose of this analysis was to inform the analytical chemistry, specifically to identify where in the spectrum of VOCs bat guano was located and to conduct an initial assessment of the range of VOCs within the various classes of compounds. This enabled refinement of the analyses on species specific samples. These generic samples were collected at two different locations known to be currently occupied by numerous bats (Figure 1).

The two locations included (i) a limestone cave located in an open field on private land and (ii) an artificial roost tree located adjacent to a powerline easement on federal land. Permission was obtained prior to entering private land. To collect guano at the cave, large sheets of aluminum foil were placed outside of the entrance of the cave prior to emergence. Biologists returned to the site several hours after peak emergence. Guano pellets were clearly visible on the aluminum foil.

At the artificial roost tree, guano was collected by adhering parchment paper to a previously installed guano-catcher just before sunset. Parchment paper was used instead of aluminum foil under the artificial roost tree due to a concern that sunlight reflecting off the foil may disturb the bats roosting in the structure directly above. Biologists returned several hours after sunset to collect guano, which was clearly visible on the parchment paper.

Guano pellets were collected at each site using a stainless steel tool. The tool was cleaned in distilled water and dried with Chem-wipes in between sites to avoid cross-contamination. Guano pellets from each site were placed in 40 ml amber glass screw top vials with Teflon lined septum caps and overnight mailed to the Organic Analytical Laboratory at DRI for analysis.

Initially, a second limestone cave was also chosen for community sample collection; however, even though bats were observed emerging from the cave entrance, no guano was observed during collection efforts; therefore, it was eliminated from consideration. The lack of guano was likely due to the configuration of the cave entrance and the limited area available for aluminum foil placement.

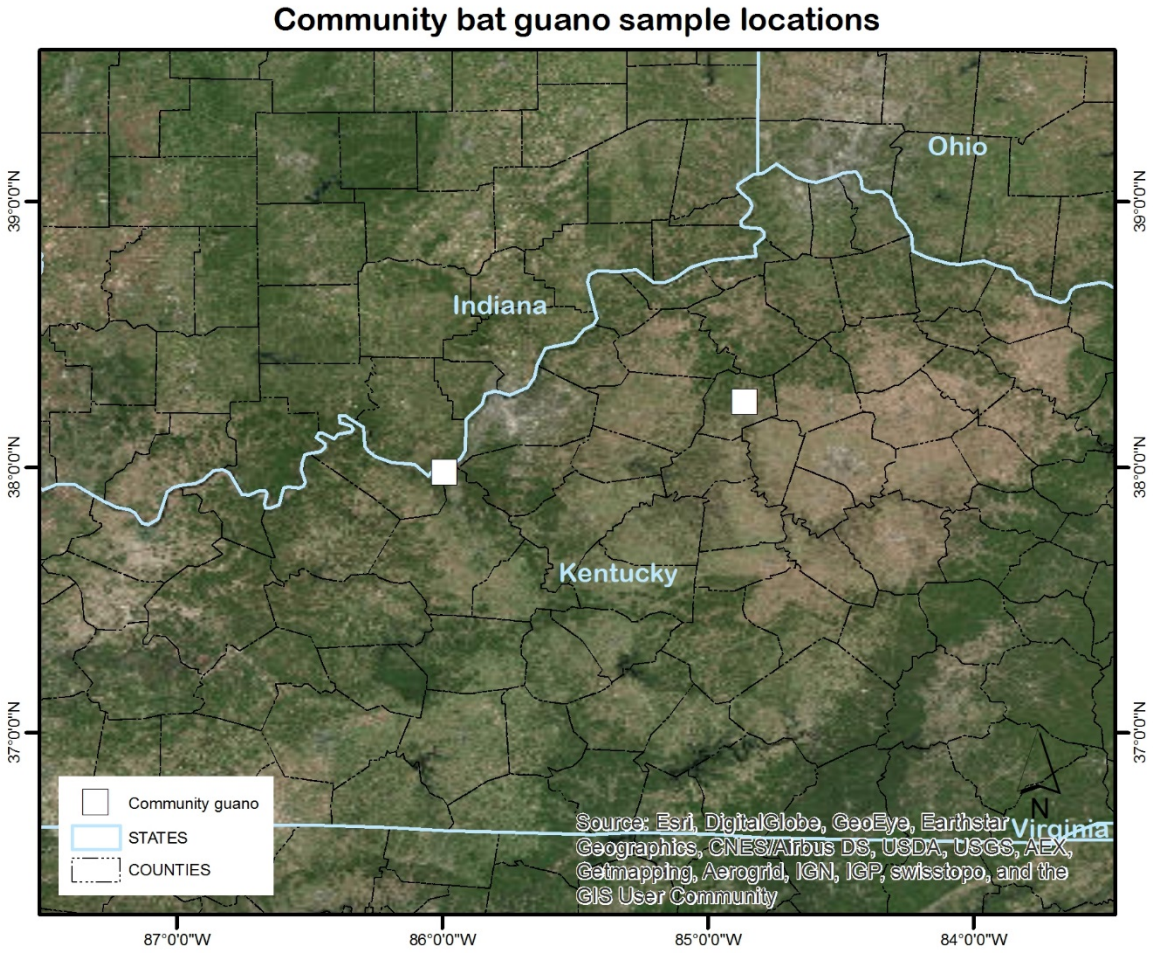


Figure 1. Location of collected bat guano samples. Community Sample is where guano was collected from multiple individuals. All other samples correspond to a particular individual from a certain species.

Two field seasons of bat sampling were conducted during July, August and September of 2014 and again during June, August and September of 2015 using the same method. Individual samples from bats were captured upon exit or entry to their roosts or within travel corridors using mist nets or harp traps. Individuals were sexed, weighed, and placed into a brown paper bag until they deposited at least one guano pellet, upon which time they were released. Individual bats were released no more than 30 minutes after capture regardless of whether they had deposited pellets. Guano pellets were collected from each brown paper bag using a stainless steel tool. The tool was cleaned in distilled water and dried with Chem-wipes in between individual bats to avoid cross-contamination. Guano pellets, typically two to four pellets each, from each individual bat were placed in 40 ml amber glass screw top vials with Teflon lined septum caps and overnight mailed to the Organic Analytical Laboratory at DRI for analysis. Pellets were not handled directly and did not come into contact with anything except the paper bag and the stainless steel tool. Samples were run within 24 hours of receipt as described below. Post-analysis samples were stored in a freezer at DRI.

Guano from individuals from the following 8 species were collected over the two field seasons and analyzed:

1. Indiana bat (*Myotis sodalis*)
2. little brown bat (*Myotis lucifugus*)
3. northern long-eared bat (*Myotis septentrionalis*)
4. gray bat (*Myotis grisescens*)
5. eastern small-footed bat (*Myotis leibii*)
6. Virginia big-eared bat (*Corynorhinus townsendii virginianus)*
7. Tri-colored bat (*Perimyotis subflavus*)
8. Red bat (*Lasiurus borealis*)

It was planned that two samples would be collected from each sex for each species. Actual captures are discussed in the results section.

VOC analysis on guano samples

Upon receipt of bat guano samples, VOC in the head space of each vial were sampled using Supelco (Bellefonte, PA) Carboxen-PDMS (75 µm) solid phase micro-extraction (SPME) fibers VOC were sampled by piercing the Teflon lined silica septa in the vial cap with the SPME needle and exposing the fiber inside the vial for 20 minutes at 20 C. Exposed fibers were analyzed by gas chromatography/mass spectrometry (GC/MS) by desorbing VOC on the SPME fiber in the GC inlet at 300 C for 1 minute. SPME fibers were then reconditioned for 20 minutes at 300 C under a steady stream of ultra-high purity nitrogen gas prior to the next sample collection.

VOC analysis for guano samples collected in 2014 was performed with a Varian 3800 GC with a Saturn 2000 ion trap MS. The GC had a Varian 1177 injector operated in splitless mode at a constant temperature of 300 C. A Varian Factor Four VF-5MS GC column (30 m x 0.25 mm x 0.25 µm) was used. GC oven conditions were as follows: the initial temperature of 35 C was held for one minute, then increased to 80 C at 3 C.min-1, then increased to 120 C at 10 C.min-1, and finally to 260 C at 40 C.min-1 and held for 4 minutes for a total run time of 27.5 minutes.

The MS scanned 40 to 350 a.m.u. from 2 to 26 minutes with an emission current of 10 µamps. Varian Workstation software was used for instrument control and data analysis, along with the NIST mass spectra library for compound identification.

For the 2015 samples, VOC were analyzed with a Bruker 456 GC coupled to a Bruker Scion triple quadrupole MS with an Agilent DB-1 analytical column (60m x 0.32mm x 1um). GC oven conditions were as follows: the initial temperature of 35 C was held for one minute, then increased to 80 C at 3 $C.min^{-1}$, then increased to 120 C at 10 $C.min^{-1}$, and finally to 300 C at 40 $C.min^{-1}$. The MS scanned 40 to 350 a.m.u. from 4 to 43 minutes with an emission current of 80 µamps. Bruker Workstation software was used for instrument control and data analysis, along with the NIST mass spectra library for compound identification.

Data analysis

Principal component analysis (PCA) was used to analyze the VOC data emanating from the guano samples. This method is useful for maximizing capture of variability within a large and complex data set and effectively reducing dimensionality of the data. Visualization of PCA results are both tabular and graphical which is useful to assess separability of the input variable groups (species) based on their VOC constituents.

Standard statistical analyses were run to summarize the VOC components within and among the samples and on bat data. Geographic coordinates of bat survey sites were entered into ArcGIS for display, qualitative and quantitative assessments. Spatial statistics were also run to assess some aspects of the bat data based on sample location within the state.

RESULTS AND DISCUSSION

The community guano was collected on 9 July 2014. One sample was collected in Franklin County and one in Hardin County. The first community sample was collected on sheets of aluminum foil placed at a cave entrance in Franklin County where three different species were suspected: *Myotis grisescens* (Gray bat), *Eptesicus fuscus* (Big brown bat), and *Perimyotis subflavus* (tri-colored bat).

The second generic guano sample was collected on parchment paper placed over a guano catcher immediately below an artificial roost tree in Hardin County within an established, known *Myotis sodalis* (Indiana bat) maternity colony. Bats were audible during collection. Two other species were suspected to be present during collection as well, *Myotis lucifugus* (Little brown bat) and *Myotis septentrionalis* (Northern long-eared bat) although not confirmed as capture was not a component of this collection.

Once the community guano had been assessed, field sampling of individual bats commenced. A total of 22 guano samples from seven different bat species were collected and analyzed between August 4 and September 16, 2014. After conducting the initial analysis on these guano samples it was decided to collect additional samples in the following field season to potentially clarify some possible confusion in the initial results that was thought to have stemmed from the timing of when bats were captured. Thus an additional 15 guano samples were collected and analyzed

from five different bat species between June 17 and September 23, 2015 (Table 1). Table 2 presents the data on each bat captured. Figure 2 shows the geographic locations within the state where bats were captured and sampled.

Table 1. Species from which guano were collected, number of individuals sampled, Status pertains to U.S. Fish and Wildlife Service recognition: E = federally listed 'endangered', T = federally listed 'threatened'; N = not listed.

Common Name	Species	Code	Status	Males	Females
Gray bat	*Myotis grisescens*	MYGR	E	2	2
Virginia big-eared bat	*Corynorhinus virginianus*	COVI	E	1	1
Northern long-eared bat	*Myotis septentrionalis*	MYSE	T	3	2
Tri-colored bat	*Perimyotis subflavus*	PESU	N	2	2
Little brown bat	*Myotis lucifugus*	MYLU	N	6	3
Indiana bat	*Myotis sodalis*	MYSO	E	3	4
Eastern small-footed bat	*Myotis leibii*	MYLE	N	2	2
Red bat	*Lasiurus borealis*	LABO	N	0	2

Table 2. Data collected on individual bats. Code refers to species as identified in Table 1; sex is F = female, M = male; Age/Sex A = adult, J = juvenile, M = male, F = female; Reproductive condition is PL = post-lactating, NR = non-reproductive, TD = testicles descended, PT = pregnant with twins, P = pregnant, L = lactating.

code	sex	Capture date	Capture time	Age/sex	Weight (g)	Reproductive condition
MYGR	F	4-Aug-14	2240	AF	9.75	PL
MYGR	F	13-Aug-14	2340	AF	9.50	PL
MYGR	M	13-Aug-14	2310	AM	9.25	NR
MYGR	M	14-Aug-14	0030	AM	9.75	NR
COVI	F	15-Aug-14	2123	AF	10.25	PL
COVI	M	16-Aug-14	2345	AM	12.00	NR
MYSE	M	16-Aug-14	2237	AM	5.75	NR
MYSE	M	27-Aug-14	2243	JM	5.50	NR
PESU	M	16-Aug-14	2208	AM	5.25	NR
PESU	M	16-Aug-14	2308	AM	6.00	TD
MYLU	F	15-Aug-14	2055	AF	6.75	PL
MYLU	M	15-Aug-14	2035	AM	6.75	NR
MYLU	M	15-Aug-14	2145	AM	6.25	NR
MYLU	F	15-Aug-14	2025	AF	7.00	PL
MYSO	F	25-Aug-14	2352	JF	7.25	NR
MYSO	M	25-Aug-14	0032	JM	7.00	NR
MYSO	F	26-Aug-14	2137	AF	8.00	NR
MYSO	M	26-Aug-14	2155	AM	7.25	NR
MYLE	M	16-Sep-14	1925	AM	5.00	TD
MYLE	M	16-Sep-14	1940	AM	4.50	-
MYLE	F	16-Sep-14	2117	AF	5.00	NR
MYLE	F	16-Sep-14	2148	AF	4.50	NR
PESU	F	17-Jun-15	2235	AF	7.50	PT
PESU	F	11-Aug-15	2350	AF	7.25	NR
LABO	F	17-Jun-15	2310	AF	15.00	P
LABO	F	17-Jun-15	2221	AF	11.50	L
MYSO	M	10-Aug-15	2028	AM	6.00	NR
MYSO	F	10-Aug-15	2237	AF	8.00	NR
MYSO	F	10-Aug-15	2230	AF	7.25	-
MYSE	M	10-Aug-15	2230	AF	5.90	NR
MYSE	F	11-Aug-15	2225	AF	7.50	NR
MYLU	M	11-Aug-15	2205	AM	8.00	NR
MYLU	F	11-Aug-15	2205	AF	9.50	NR
MYSE	F	13-Aug-15	2155	AF	6.75	PL
MYLU	M	23-Sep-15	2141	AM	9.50	TD
MYLU	M	23-Sep-15	2141	AM	9.50	TD
MYLU	M	23-Sep-15	2141	AM	9.50	TD

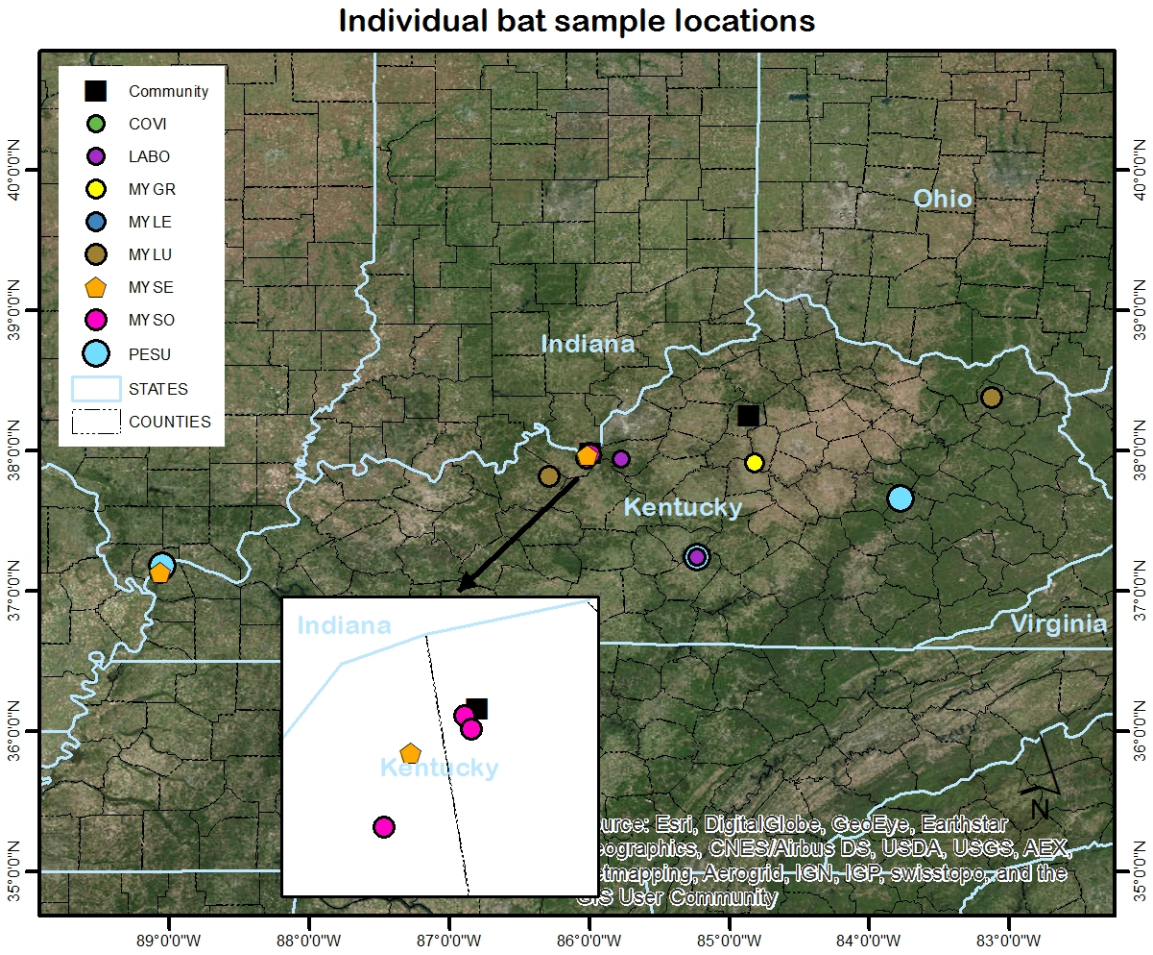


Figure 2. Locations where bat guano samples were collected from individual bats.

The guano was collected from sample locations that spanned the entirety of the state. In several instances guano was collected from individual bats at locations that were much farther than an individual would have traveled to forage. For example, MYSE guano was collected from bats captured at locations 200 km (~124 miles) between the two field seasons. LABO guano samples in 2015 were collected 91.4 km (~59 miles) apart. MYLU guano was collected from across the state in three different locations, as far as 539 km (~224.8 miles) apart between the two field seasons. In 2015 MYSO samples were collected at locations 285 km (~177 miles) from where they were collected in 2014 while in 2015 PESU guano samples were collected at two locations 339 km (~210.6 miles) apart. Other species such as COVI, MYGR, and MYLE were only captured at single locations.

The land use setting surround bat capture locations varied widely. MYGR were sampled in forests that were interspersed with agricultural land use (Figure 3) while COVI were captured in a contiguous forested landscape (Figure 4). LABO were captured on the edge of contiguous forest with agricultural and rural developed matrices as shown in Figure 5. MYLE were captured in a forest agricultural matrix (Figure 6), along with several other species including MYLU. The other two capture sites for MYLU are shown in Figure 7. Similarly, Figure 8 shows two sites in

addition to those shown in Figure 6 (MYLE capture locations) where MYSE were captured. PESU were captured in three locations, one that was the same site shown in Figure 4, one of which coincides with the LABO site shown in Figure 5, the other one shown in Figure 9. The landscape is an agricultural forested matrix near some industrial complex associated with a waterway. MYSO survey locations (Figure 10) were both contiguous forests and a matrix of forest and agriculture.

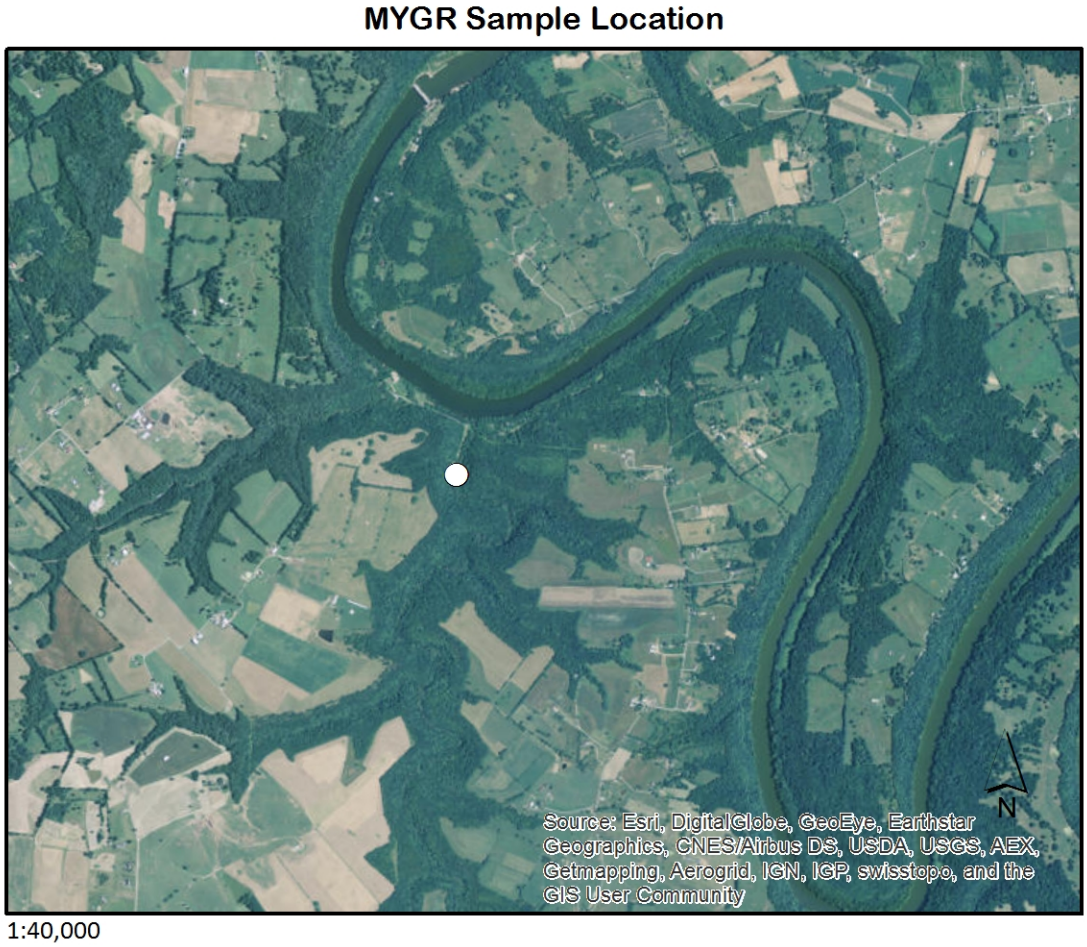


Figure 3. MYGR were captured in a forest-agricultural matrix landscape.

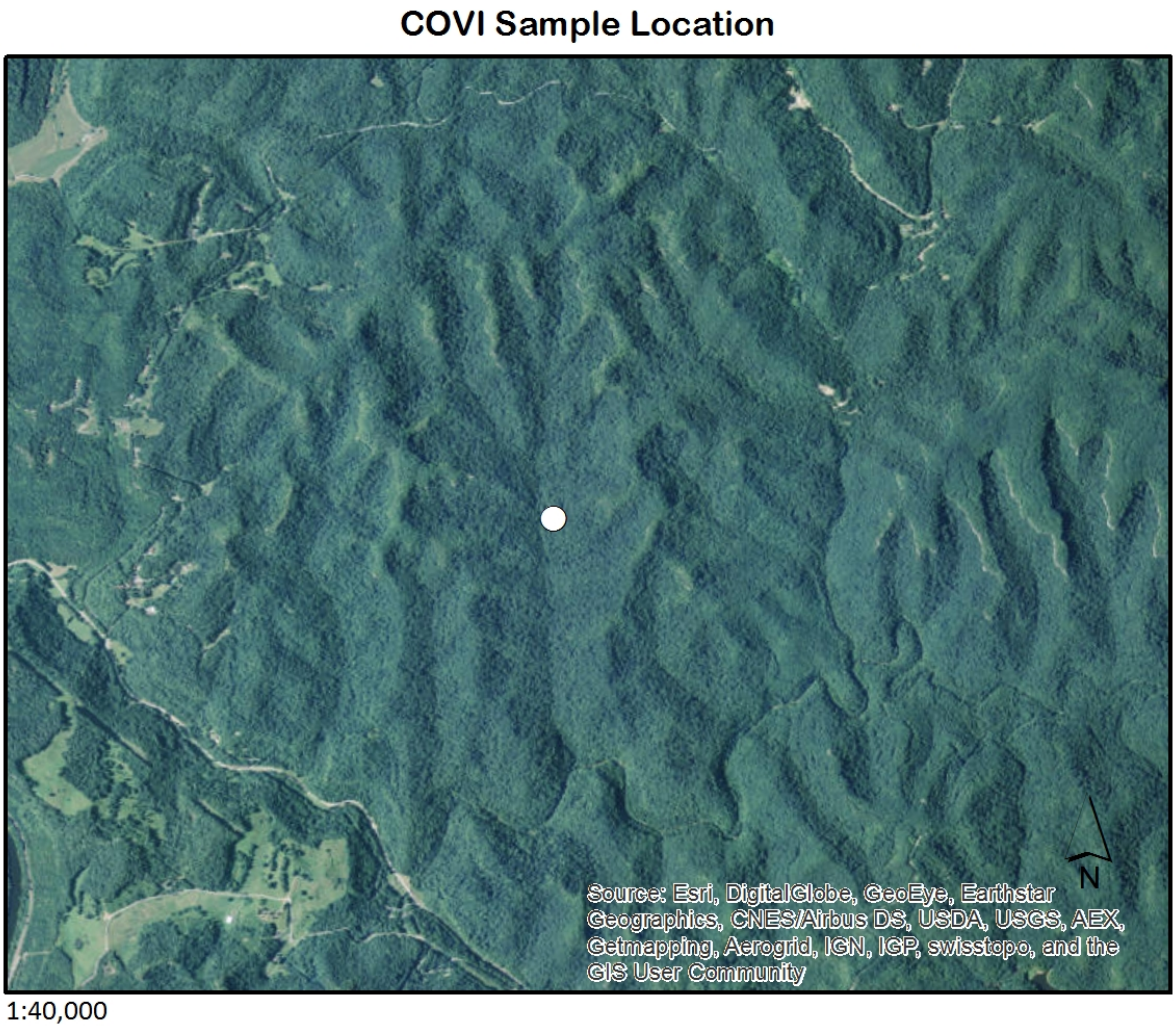


Figure 4. COVI were captured in a contiguous forest landscape

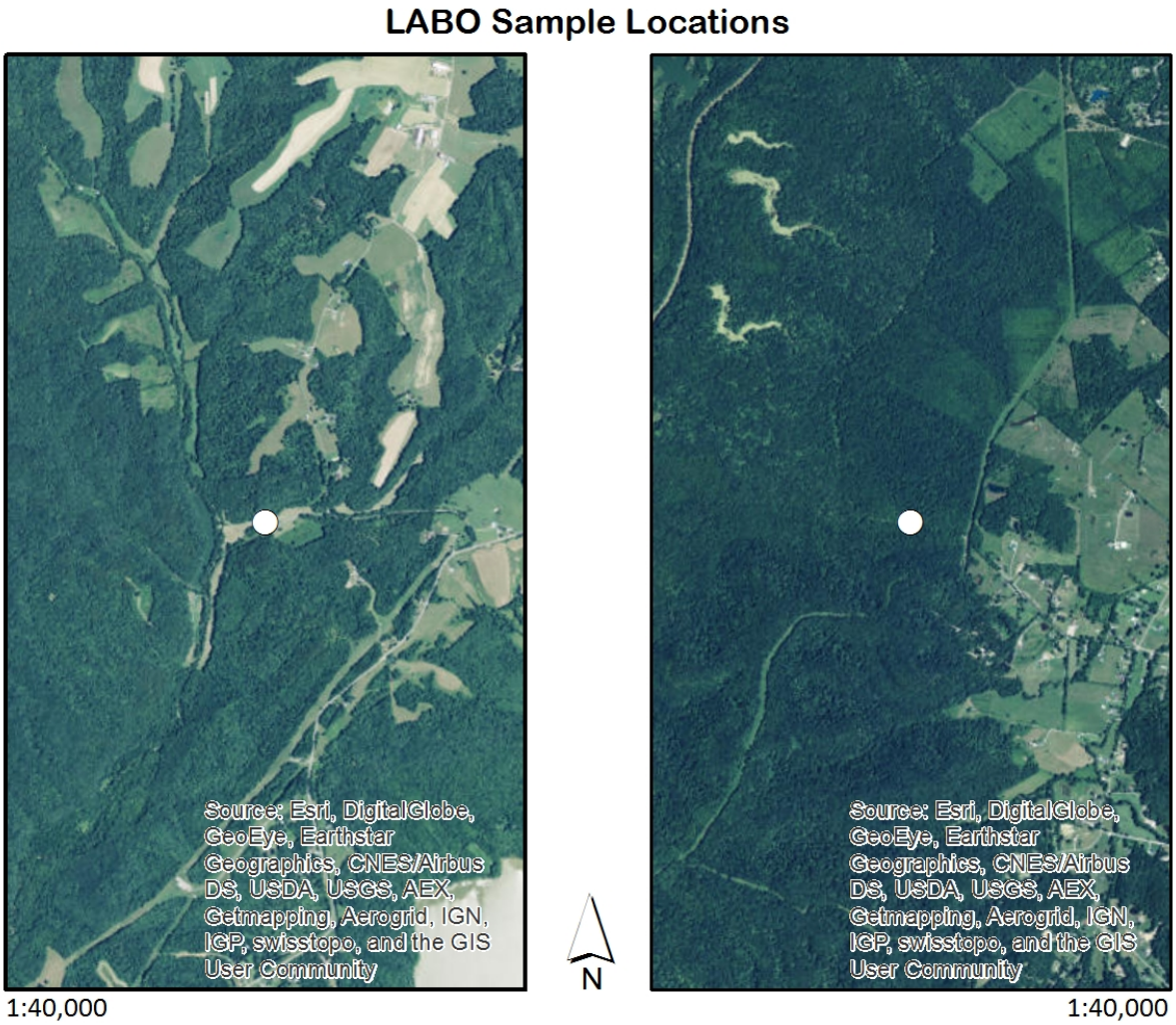


Figure 5. LABO were captured on edges between contiguous forests and patchwork matrix landscapes.

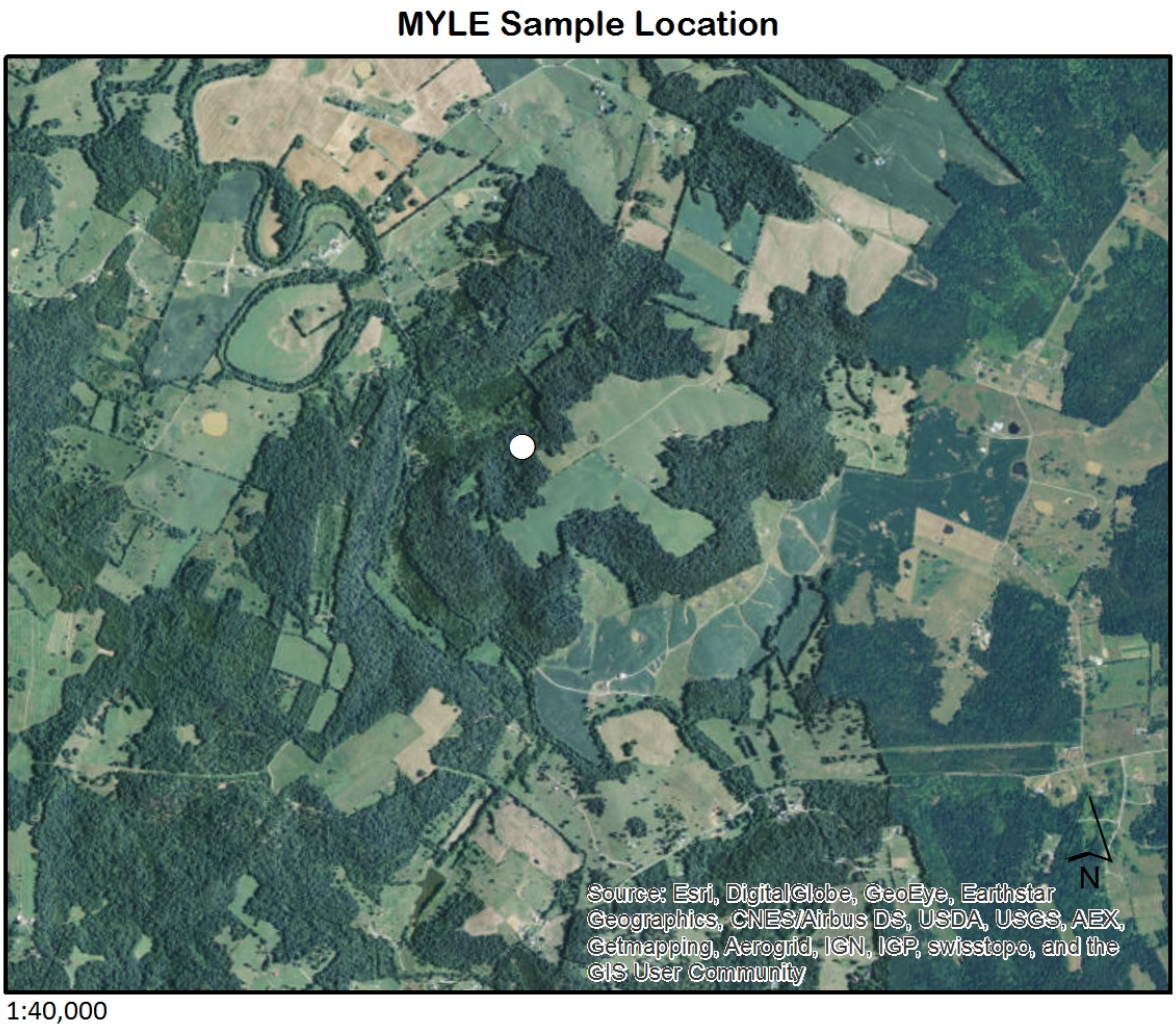
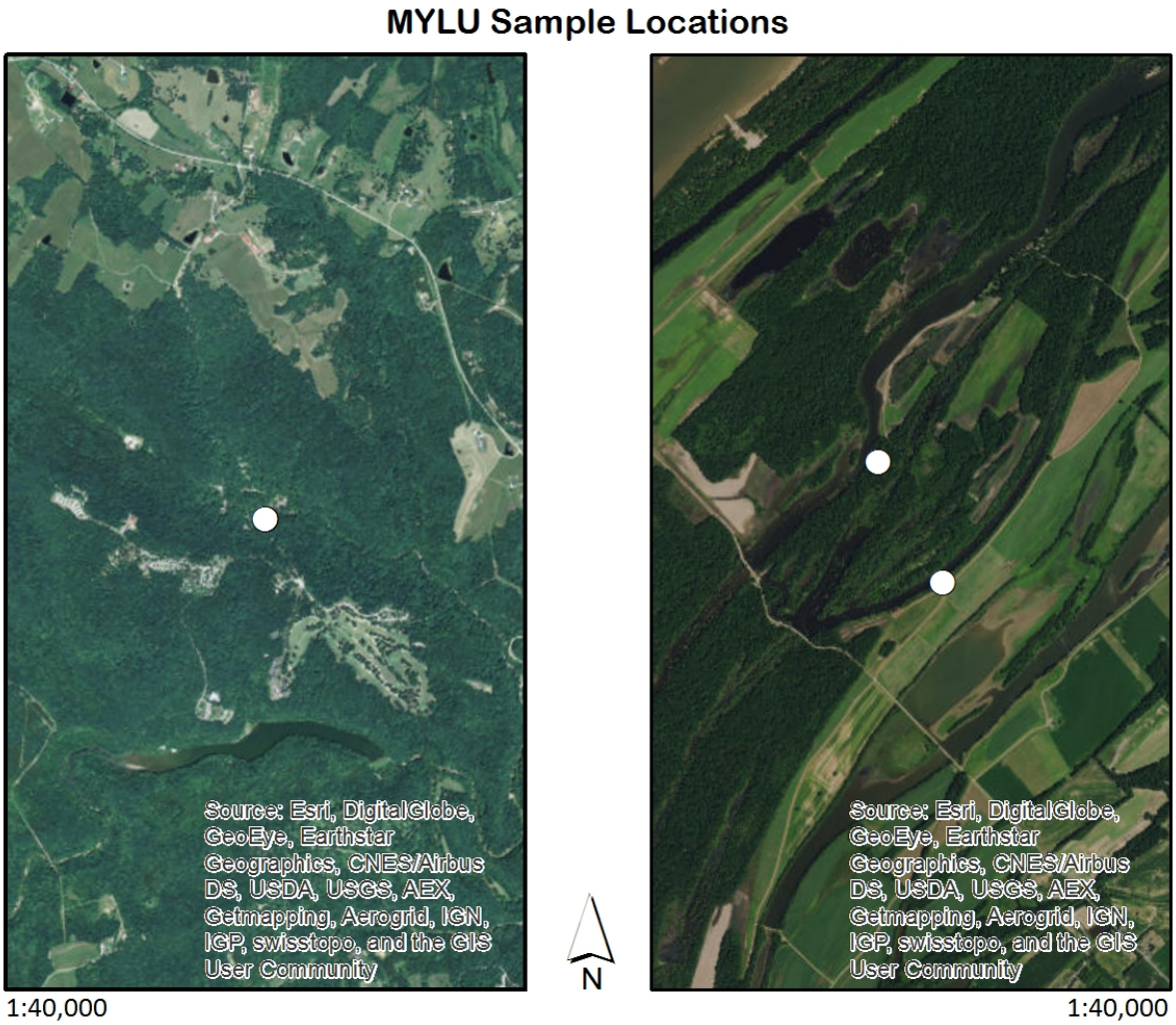


Figure 6. MYLE were captured in a forested agriculture matrix landscape.



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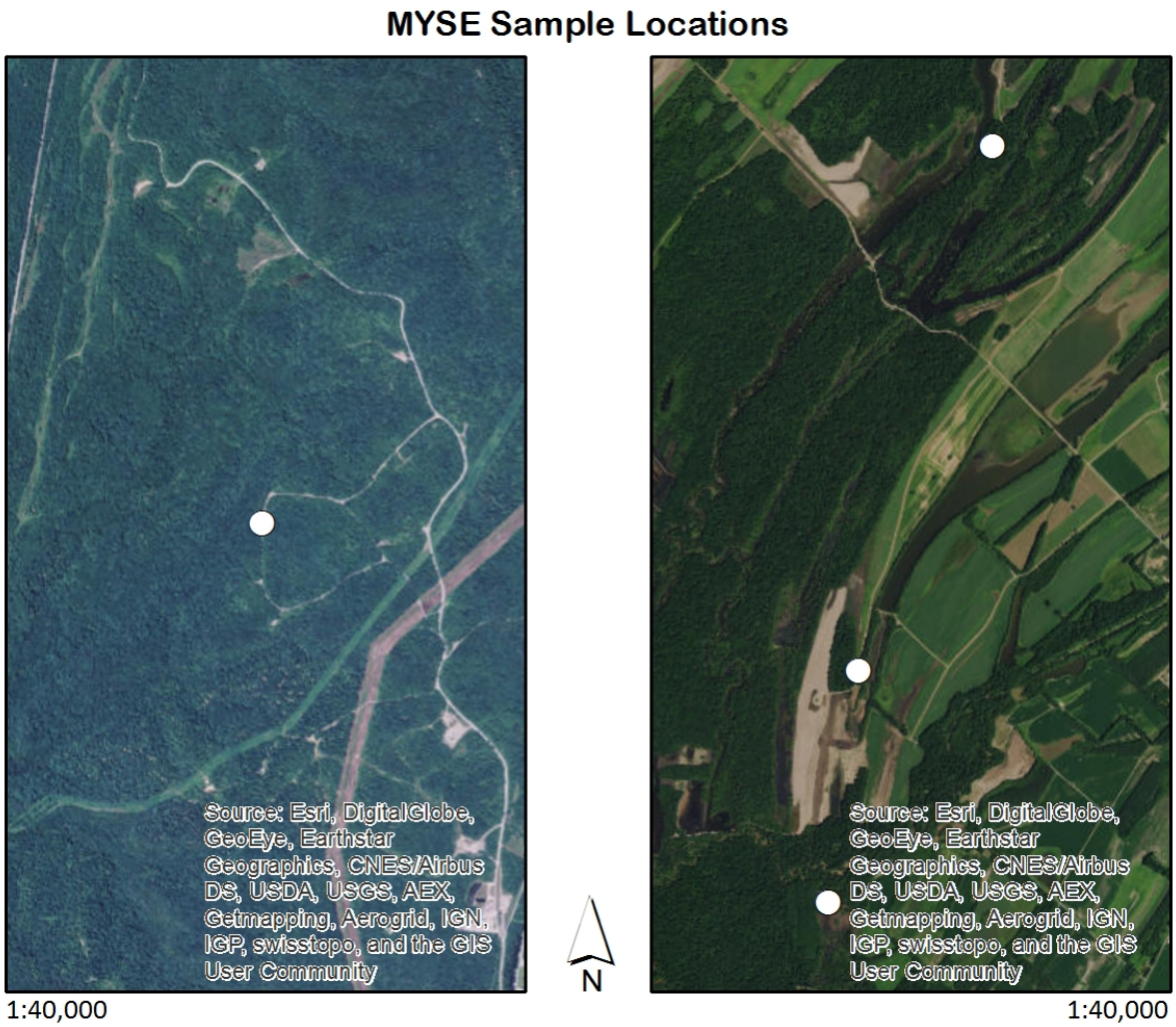

Figure 8. MYSE capture locations, in 2015 (right) and 2014 (left). These are in addition to the same sight shown in figure 6, where these were among multiple species captured.

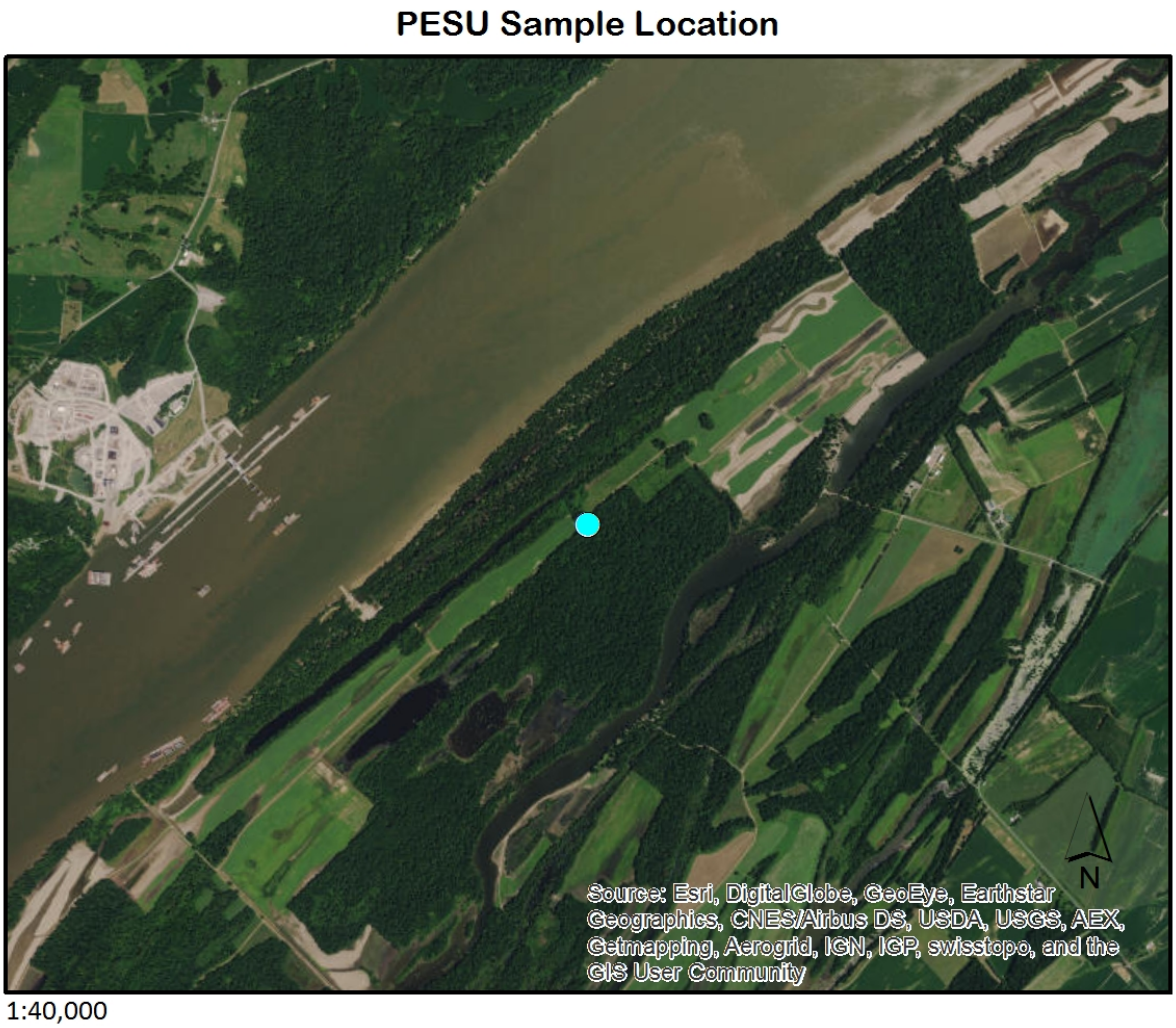

Figure 9. One of the PESU capture sights. The other sites are shown in figures 4 and 5, respectively.

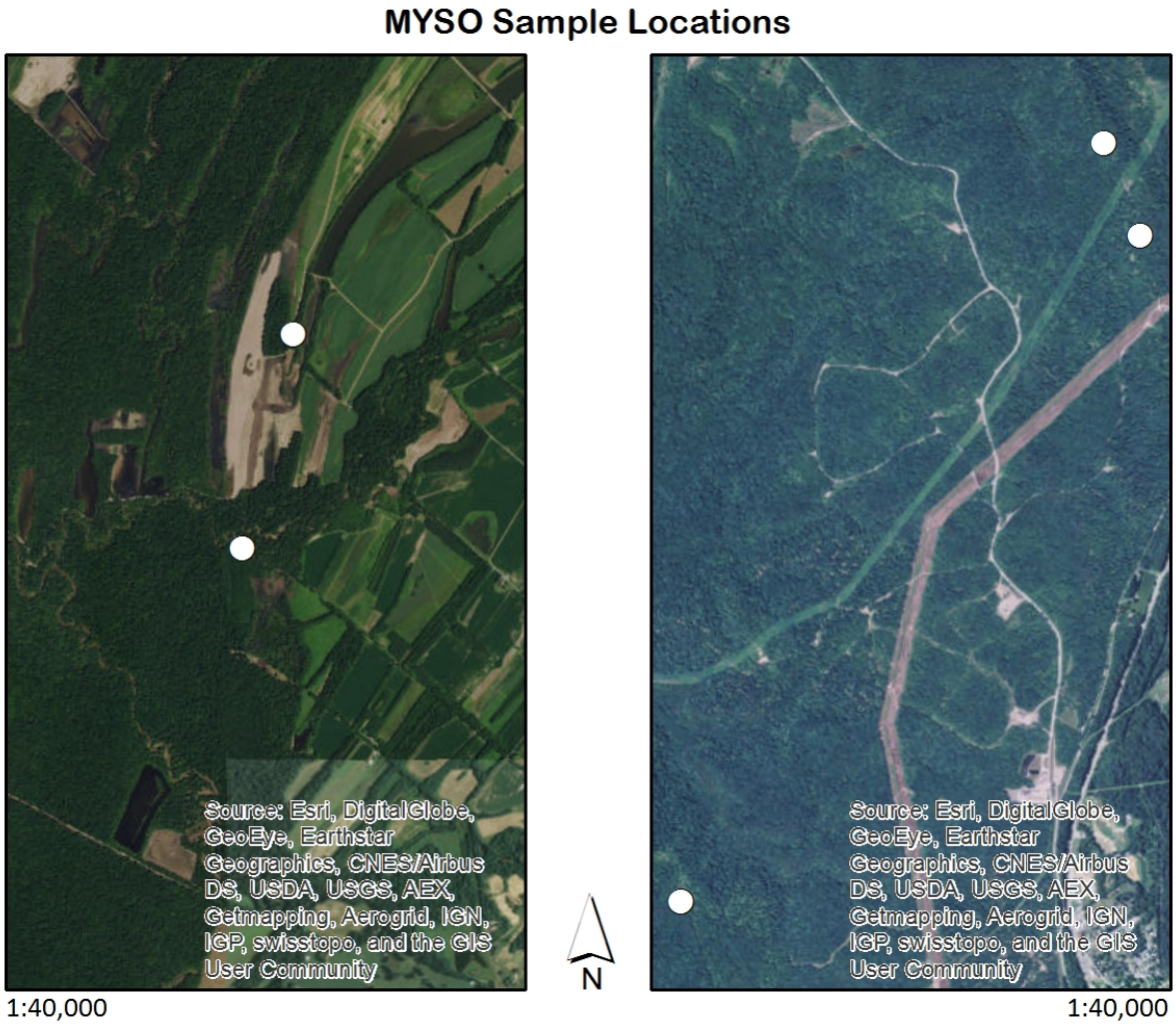

Figure 10. MYSO were captured at two general locations. In 2014 (right) the location was in a fairly contiguous forested landscape, while in 2015 (left) it was a forest agricultural matrix.

Global Moran's I calculated on individual bat weights returned a random distribution over the survey locations ($I = 0.029$, $z = 0.44$, $p = 0.66$). As shown in Figure 11 there was not a significant difference in physical weight of the bats across those captured and sampled (ANOVA, $DF = 3$, $F = 0.74$, $p = 0.54$), however bat weight varied significantly by species (ANOVA, $DF = 7$, $F = 19.07$, $P < 0.0001$) (Figure 12).

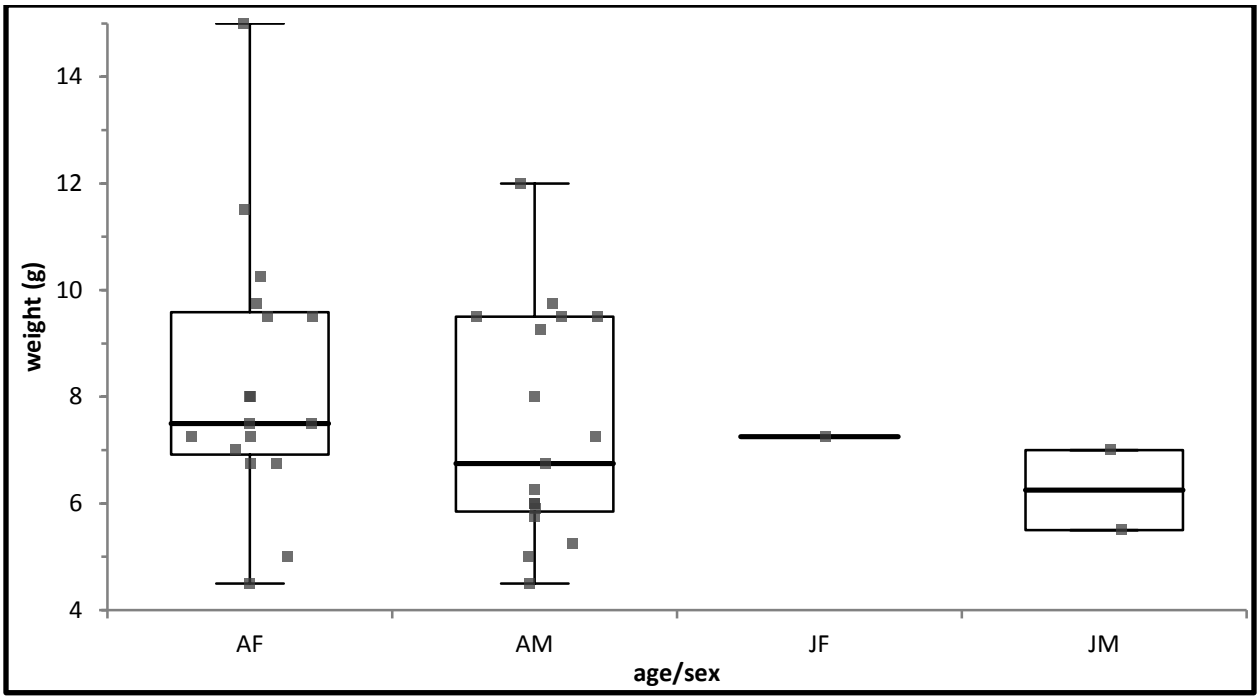

Figure 11. Box plots showing distribution of bat weight in grams by age and sex. AF = adult female, AM = adult male, JF = juvenile female, JM = juvenile male.

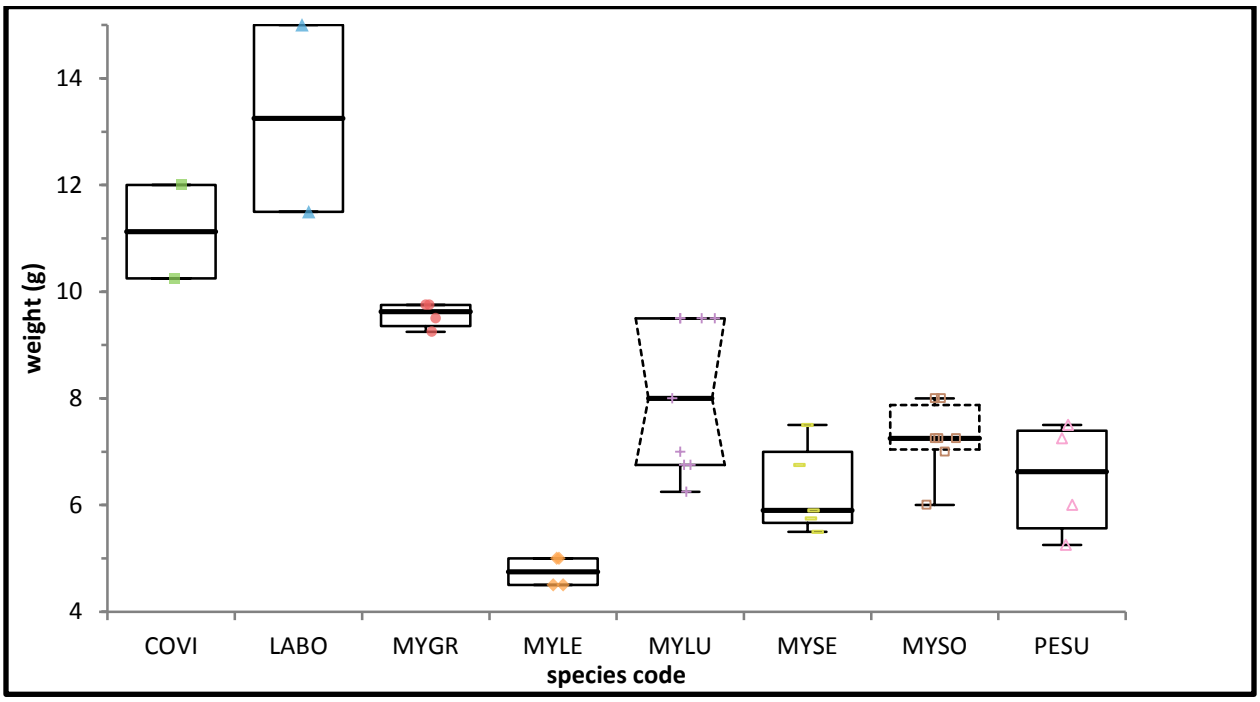

Figure 12. Bat weights (g) at time of capture varied by species.

2014 Guano VOC analysis results

Community samples excluded, a total of 32 compounds were identified from the individual bat guano samples. These can be lumped into general categories as: nitrogen-containing, sulphide, ether, hydrocarbon, aldehyde, alcohol, ketone, aromatic or phenolic. Phenolics were present in every guano sample (n=22). Hydrocarbons comprised the second most abundant (n=17) volatile followed by nitrogen-containing compounds (n = 15) and sulphides (n = 13). Aldehydes were found in 9 samples, ethers in only 3 samples and the remaining categories (alcohol, ketones and aromatics) were present in 2 samples. The VOCs originated from urine, fats, protein and vegetable decomposition. Vegetable decomposition is presumed to be insect digesta, from prey. Guanidine and methyl-guanidine were reported (a primary amine) which would be expected in guano. These results are consistent with diet and metabolism, although could also be contributed to shedding of cells from the bat intestinal system. Nonetheless, there was a VOC signature that was readily captured using the analytical chemistry instrumentation, thus indicating odor would be present and would differ between samples. The absolute amounts and relative proportional composition for each sample are shown in Figure 13.

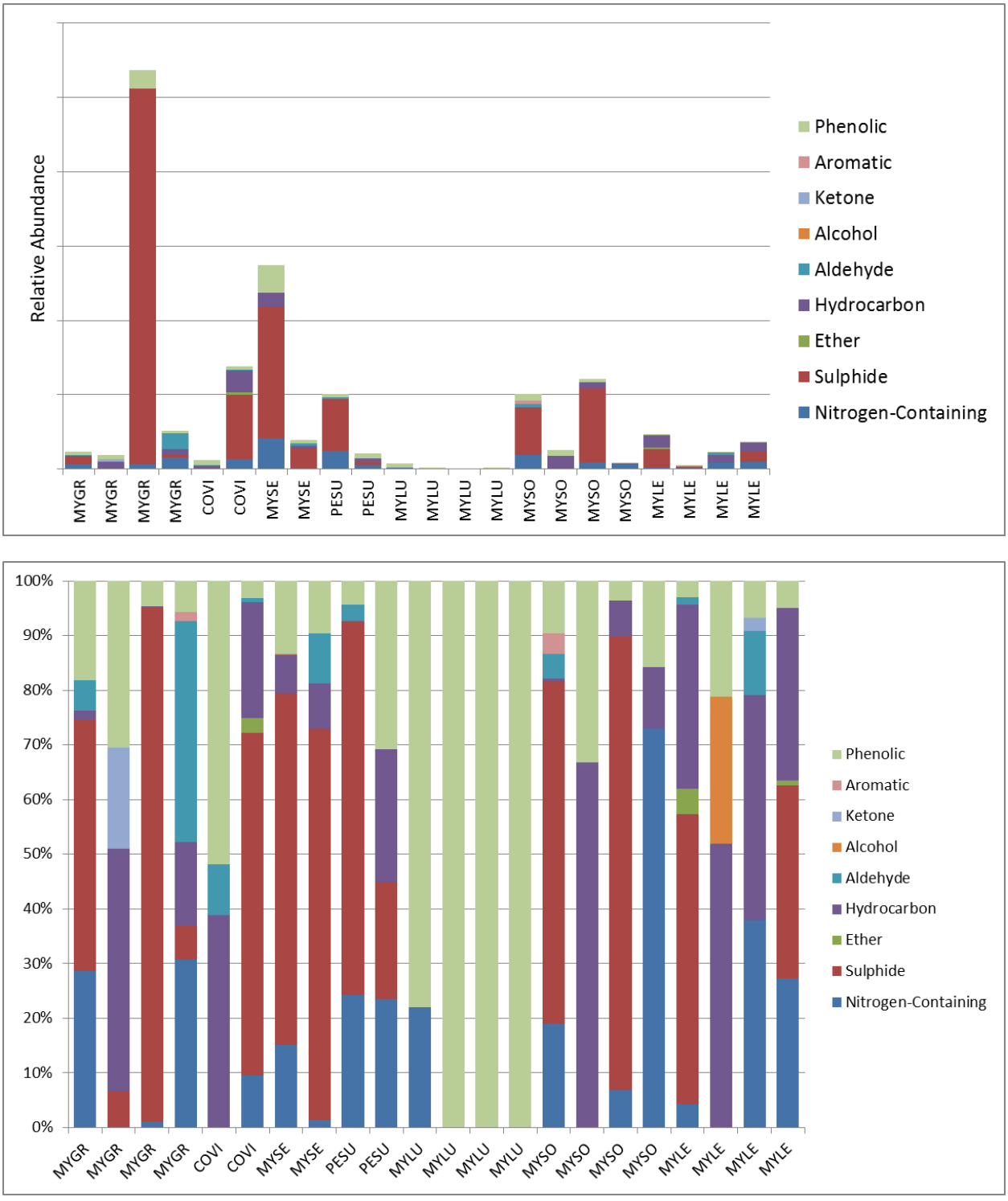

Figure 13. Composition of VOCs from guano collected in 2014 by general chemical category for each individual bat guano sample analyzed.

Principal component analysis results from 2014 samples on the categorized data are shown in Figure 14, which plots the mean score for each bat species. Means are calculated over 9-dimensional space but can only be presented here in 2D. Typically the first two or three components from PCA account for 85% or more of the variability of the input data set. In the 2014 data set, however, the first three components explained only 61% of the variability, with the first two components explaining ~45% of the input data variance.

The first component was heavily influenced by nitrogen-containing compounds, sulphides, and phenolics. The second component was heavily weighted by ethers, hydrocarbons and aromatics. The PCA biplot of the first two components show that in general, the guano presented sufficient variability between species to be differentiated. Admittedly, the 2014 data set is unbalanced having only sampled two non-myotids and one species within each genus of those two. The *Myotis* guano VOCs were sufficiently different enough to distinguish among the different *Myotis* species. PESU and MYGR were more similar to each other than any other of the species analyzed. COVI were distinguishable from the myotids, although less different from MYSO than the others. These results would indicate that in fact it would be expected that a canine could be trained as a species-specific guano detection dog, although PESU and MYGR differentiation might be questionable.

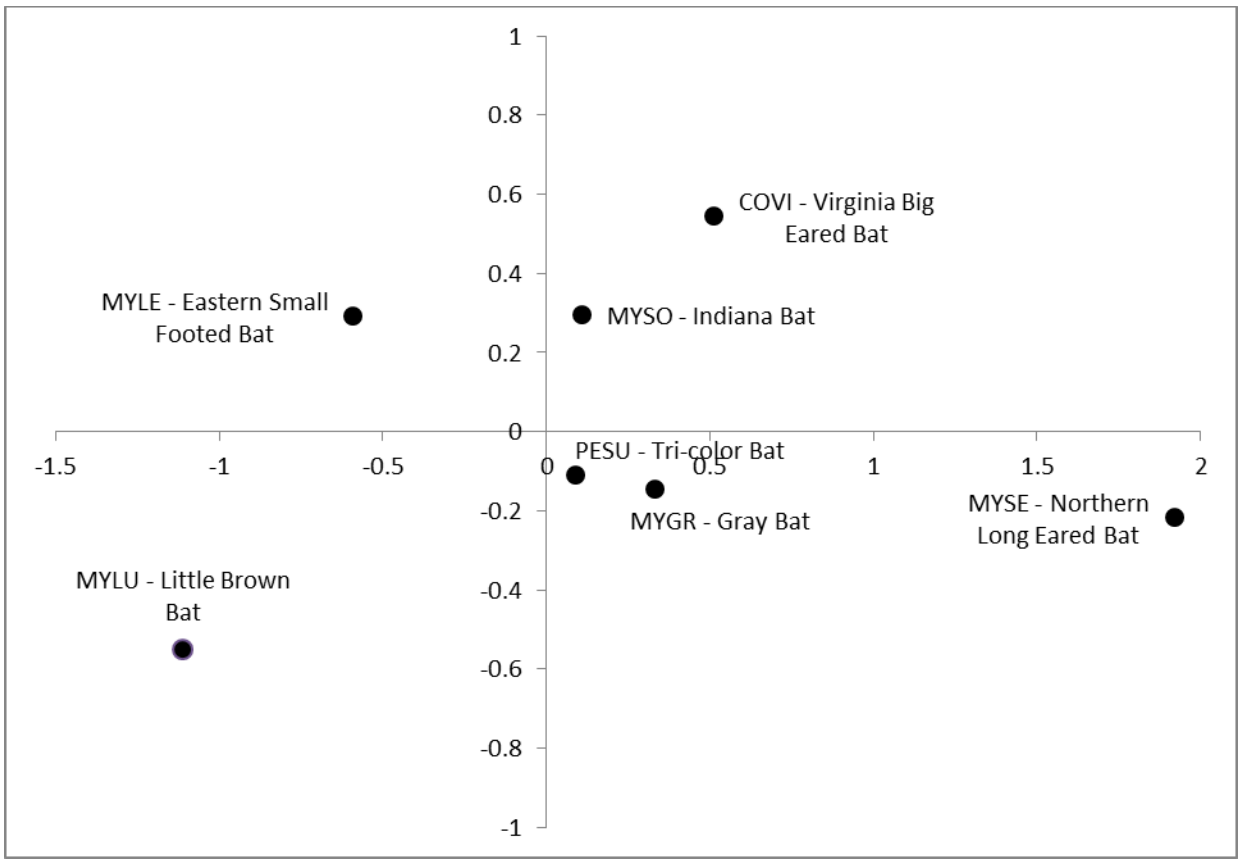

Figure 14. PCA plot of the mean scores for the bat guano sampled in 2014 based on general VOC compound categories.

For comparison, PCA results from the un-categorized data set, including all 32 compounds, explained 63% of the variability in the original data in the first three components. The first two components explained ~51% of the variance.

2015 Guano VOC analysis results

A total of 23 compounds were identified from the individual bat guano samples. For comparison with the 2014 data, these were also lumped into general categories as: nitrogen-containing, sulphide, ether, hydrocarbon, aldehyde, alcohol, ketone, or aromatic. No aldehydes or phenolics were identified in the 2015 samples, although they were present in samples from the prior year. Sulphides (n=13) and alcohols (n = 12) were the most abundant volatiles followed by nitrogen-containing compounds (n = 10) and ketones (n = 9). The absolute amounts and relative proportional composition for each sample are shown in Figure 15.

The first two components explained over 90% of the variability of this dataset, with the first component being heavily weighted by aromatics, ketones and nitrogen-containing compounds. The second component was heavily weighted towards sulphides. Because the primary interest was in the pooled data set, limited analyses are presented on the 2015 guano data as a standalone data set.

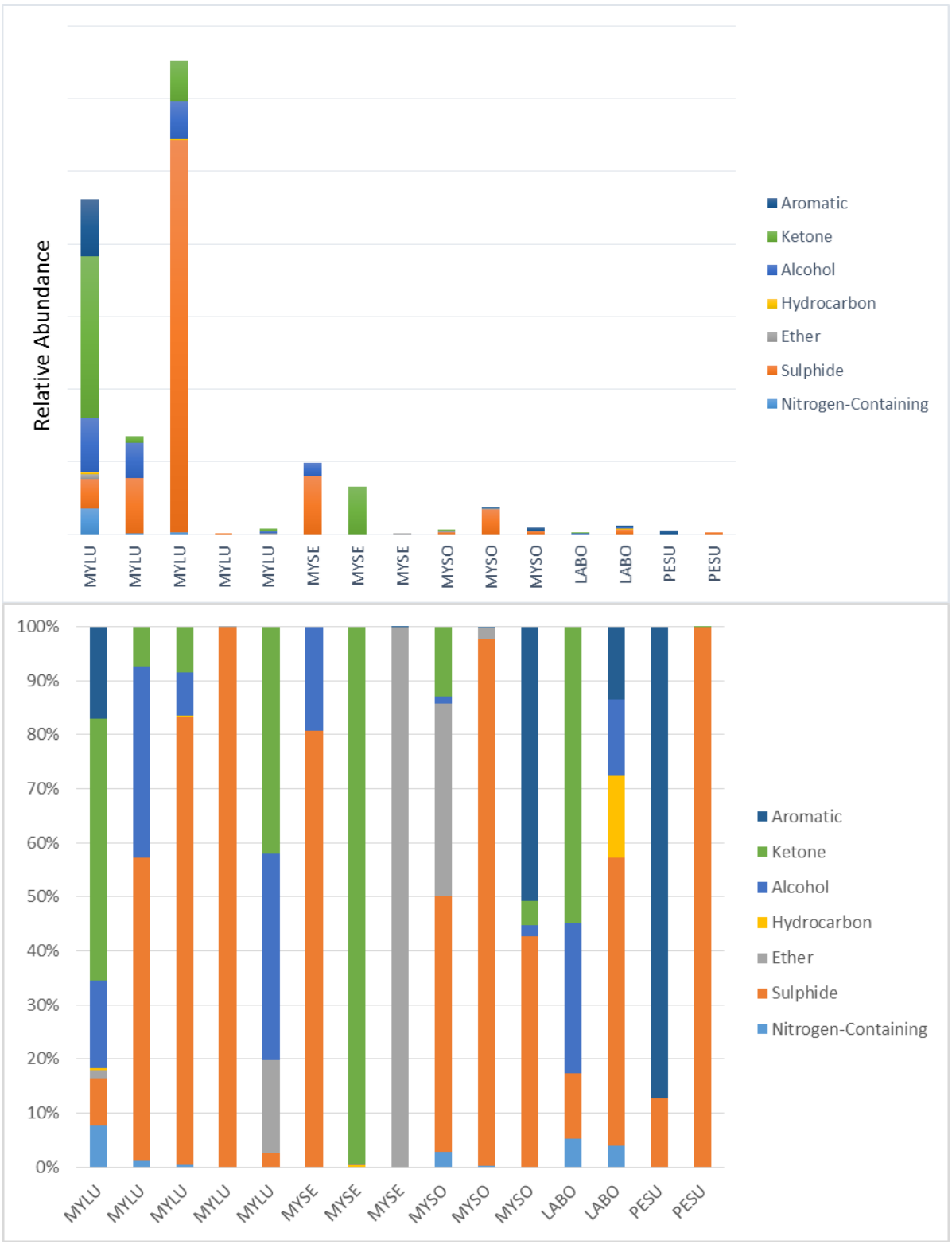

Figure 15. Composition of VOCs from guano collected in 2015 by general chemical category for each individual bat guano sample analyzed.

Combined results over both field seasons

The VOCs reported in the guano samples were contributed by the expected components, namely fats, proteins, urine, and some vegetation decomposition. Sulphides (n = 25) and nitrogen-containing compounds (n = 25) were dominant in the guano samples across all species and from both years of sampling. These compounds are primarily from the digestive breakdown of proteins, although they have other sources in the diet. For example, chitin is a polymer that makes up much of the exoskeletons of insects and contains nitrogen, carbonyl, and alcohol functionality in its structure. Hydrocarbons (n =21) were also commonly found in the guano over both years. These primarily come from fats in the diet, although some could come from plant leaf waxes in the diet of insects that the bats consumed. It is unclear why no phenolics or aldehydes were present in the 2015 samples, particularly given that phenolics were reported in every sample in 2014. Although different instruments were used for 2014 versus 2015 samples, the mass spectrometer detectors themselves would not likely have been a source of variability in the results. The instrument used in 2015 was more sensitive than the one use in 2014, which should have resulted in lower detection limits for all compound classes. However, the analytical columns used by each instrument were different and could have had some effect on what compounds were ultimately detected. In 2014, the column installed in the gas chromatograph was a Varian VF-5MS, which is slightly more polar than the Agilent DB-1 column installed in the instrument used for the 2015 samples. The DB-1 column provides better resolution for nonpolar VOCs than a VF-5MS. Being more polar, the VF-5MS may have better retained and resolved phenolics and aldehydes due to their more polar properties, thereby improving their odds of being detected. That said, the overall effect should not have resulted in an entire class of compounds being eliminated in the analysis.

The phenolics could come from protein digestion, specifically from the amino acid tyrosine, and from the breakdown of the plant polymer lignin in the insects' diet. This raises the question of whether or not the diet of the insects subsequently ingested by the bats were different between sample years. Possible sources of variability could come from a number of factors including phenology of the vegetation the insects ate, time of day they were eaten, soil nutrient conditions and water content of the plant matter eaten. In addition the timing of the insect meals may have had an impact. If the insect had just eaten when it itself became prey, that might lead to more or less of these compounds being present with the assumption that longer time between the meal of the insect and the time that insect was eaten by a bat would correlate with lower levels of the phenolic compounds. Ketones and alcohols, on the other hand, appeared in only one or two samples in 2014 but were quite abundant in 2015. The sources of ketones and alcohols are varied from proteins to chitin to some types of fat digestion. Since there was an effort in 2015 to capture more animals coming back to the roost, as opposed to those leaving the roost, the state of digestion for the animals may have influenced the VOC in the excreta. In humans, for example, it is well known that under low energy states, ketone and aldehyde compounds are produced as a normal part of metabolism. Something similar may be occurring in the bats. Table 3 presents the categorical results for both years presented in tabular form.

ANOVA calculated on these data returned no significant difference in the number of VOC categorized compounds ($DF = 7$, $F = 0.78$, $p = 0.61$) across years by species. Levene test for equal variances returned a significant difference in the variability of the number of categorized VOC compounds by species ($F = 2.46$, $DF = 7/29$, $p = 0.041$). The proportion of VOCs by

category are shown in Figure 16. PCA results for sex differences in compounds are shown in Figure 17. There is a difference between the sexes, and it appeared that there was an outlier in the male data set that might have skewed the results. This bat was a male MYLU captured in Breckinridge County on 23 September 2015 at the same location as sampled the previous year in 2014 where four MYLE were captured. Two other male MYLU were captured during the same effort in a harp trap with Bird-X, captured re-entering B & O Cave. The weights on all three bats are 9.5 g. There were no notes indicating anything remarkable about this individual. The VOC profile from this individual shows volatiles in all categories except for aldehydes and phenolics, which none of the samples from 2015 had. The logical explanation is a dietary difference, whether this bat ate different insects or more insects, although we have no data to conduct an assessment. Excluding this individual from the analysis returned a similar result (Figure 17). Female guano returned fewer nitrogen-containing compounds, ethers, hydrocarbons, aldehydes and phenolics than males. There was no difference in sulphides or aromatics between males and females.

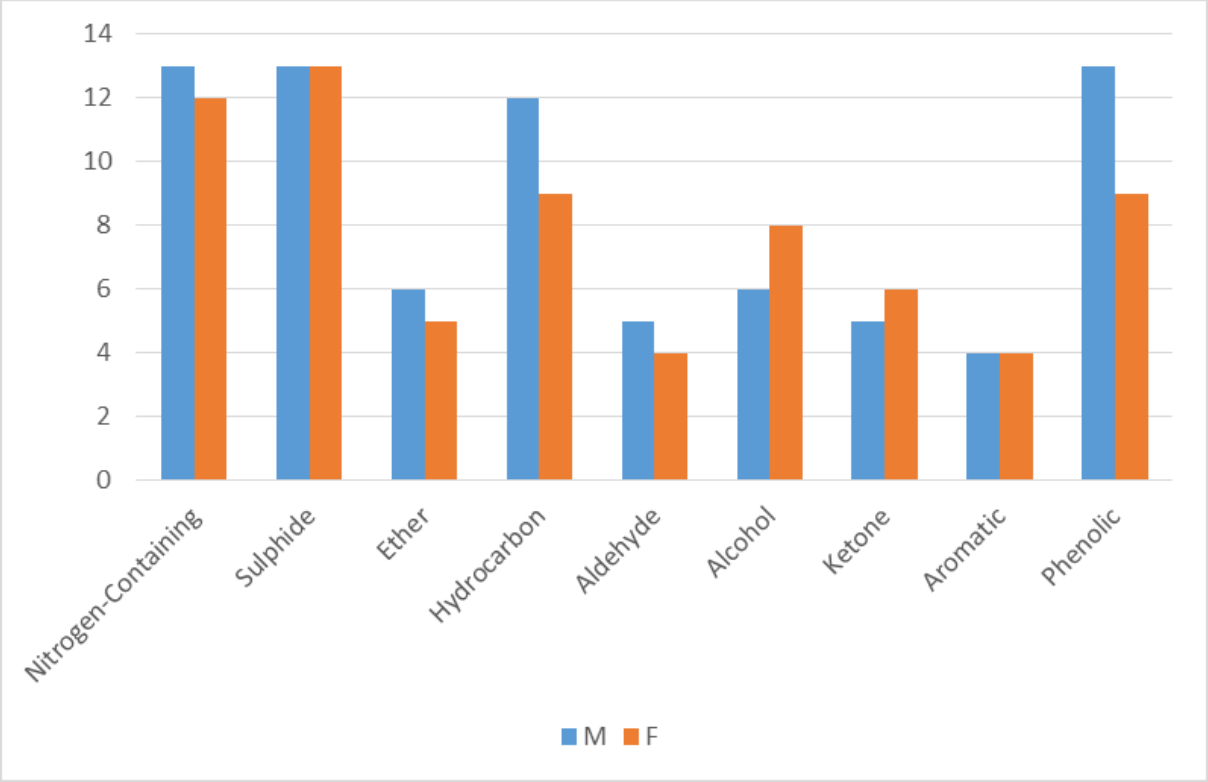

Figure 16. Proportion of compounds per VOC category by sex.

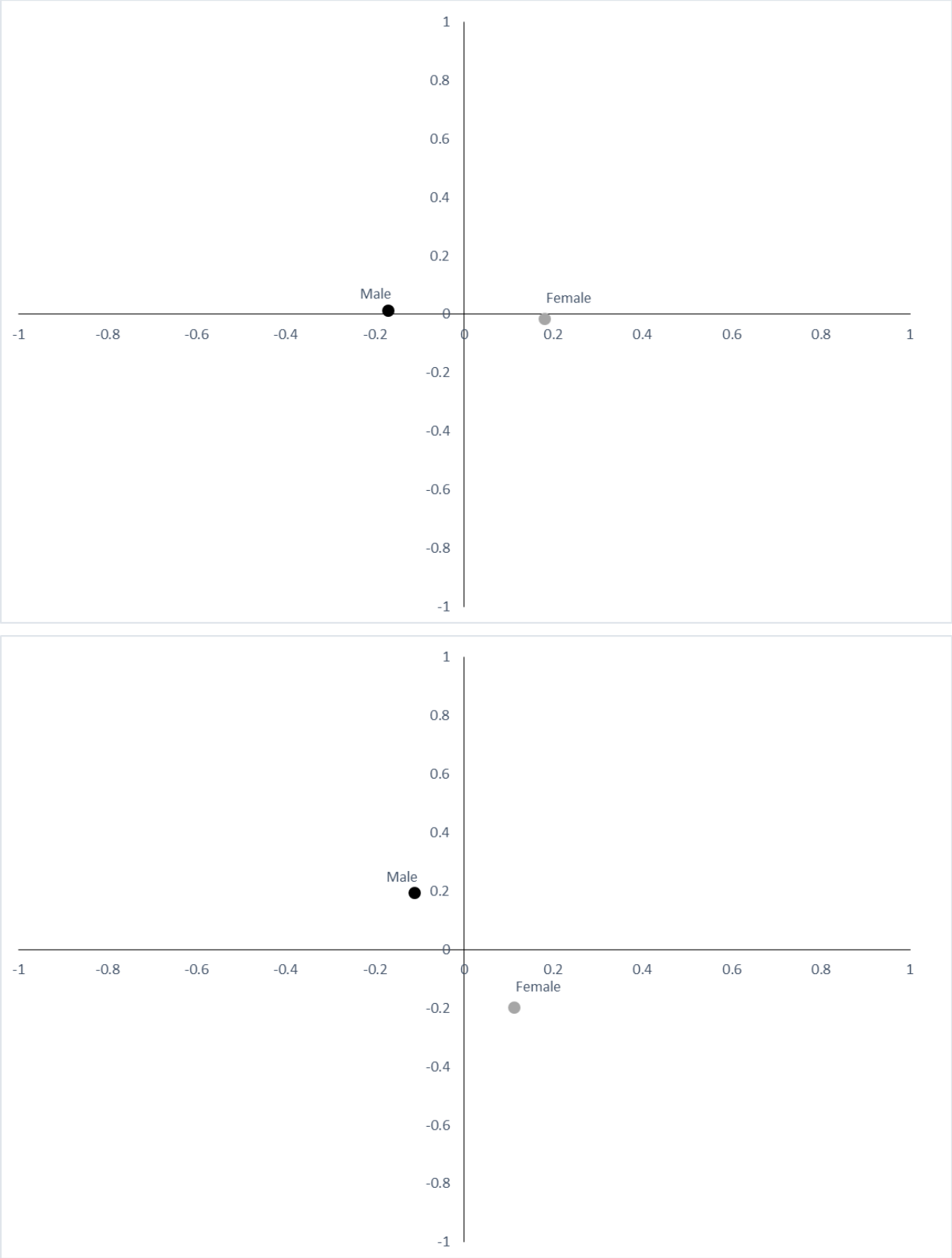

Figure 17. PCA results for data coded to male versus female data. Top graph is all data while the bottom graph excludes a sample that was thought to be an outlier.

Figure 18 presents the results of the PCA analysis run on the categorized VOCs for both survey years combined. This is a plot of the mean scores for each species. PC1 explained 0.565 of the variability in the data, PC2 added 0.153 variance for a cumulative proportion of 0.718 (Table 4). Figure 19 shows the corresponding correlation monoplot. Figure 20 shows the scores for the individual bat samples, the means of which are shown in Figure 18.

The first component was heavily influenced by nitrogen-containing compounds, ketones and aromatics. The second component was heavily influenced by sulphides, followed by alcohols and aromatics. Coefficients are presented in Table 5. These results show differences between species, particularly between MYLU and LABO from the other species. These two species were heavily weighted with sulphides, alcohols, and hydrocarbons. MYSE, MYSO, COVI, PESU, and MYLE all fall within a relatively tight grouping in the transformed space, indicating aldehydes and phenolics having a significant role in the VOCs. MYGR, while influenced by similar VOCs, is still different enough from the other species sampled to be considered 'different'. With the inclusion of the second field season the data response indicates that some species are highly likely to be differentiable by odor signature, while others could present some challenges. Ultimately we cannot make predictions about whether a canine could differentiate between some of the species that appear to be similar at least from an analytical chemistry perspective, such as MYSE versus MYSO, however the data do suggest that there are enough differences in the VOCs that it could be possible. It should be noted that some MYSO and MYSE samples were collected in geographically nearby locations.

From the correlation monoplot (Figure 19) aldehydes and phenolics are correlated, which might suggest they originate from the same source. In that case, it is likely they are dietary in origin and since they are not correlated to the nitrogen compounds, and might not be protein, then we suggest that these compounds come from plant material in the insects the bats ate, which would transform phenolics from lignin and aldehydes from fats/waxes. The relatively short vector lengths indicate that their variability is not well captured in the first two components, or that they simply lacked much variability between samples. The compounds in the lower left quadrant are fairly well correlated with variability captured in the first two components. From a chemistry perspective these would all be protein based since that is a logical origin. The upper left indicates origin from chitin or a similar structure that is high in sulfides and would also have alcohols. The hydrocarbons may be from associated waxes. Thus it appears that the compounds captured come from not only the primary food source of the bats, but the primary food source's digesta as well.

As with any study, these results are framed by the context of the analytical approach. The analytical technique chosen for this study, SPME collection with GC/MS analysis, necessarily eliminates some compounds that either cannot be collected on the SPME fiber or cannot survive the GS/MS analysis. For example ammonia or some very light primary amines, could not be detected via this method. However, those compounds, and others, may be present in the headspace over bat guano and thus would be available to a detector dog. This is why we can say that even if there is only a slight difference in the VOC profile of two species' guano, it still might be possible that a properly trained and handled detector dog might be able to differentiate them.

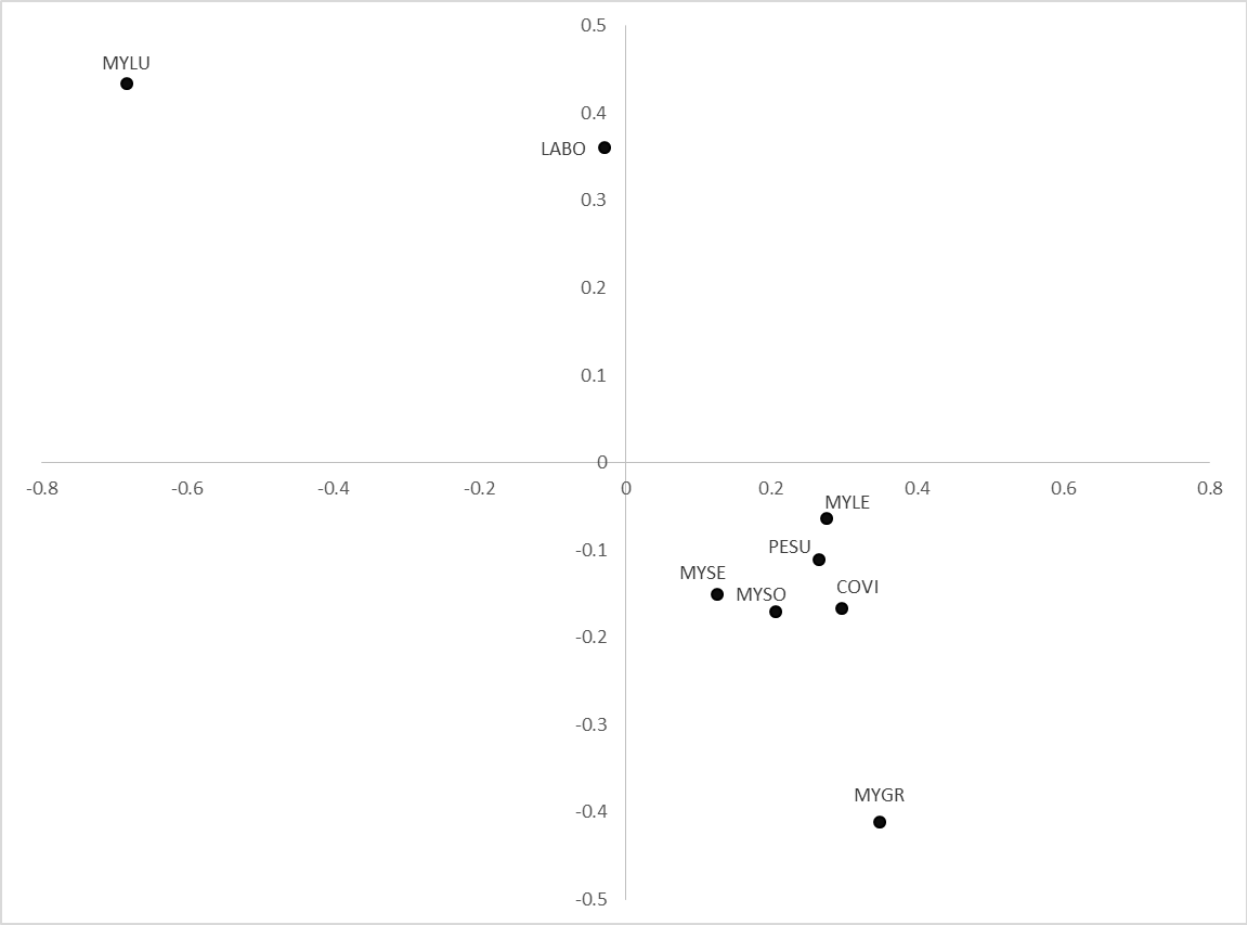

Figure 18. PCA correlation biplot of mean scores of bat guano collected over both field seasons. The first two components explain almost 72% of the variability of the data set.

Table 3. Summary of VOCs present (“X”) in guano samples for the survey years, by species and sex (M = male; F = female).

year	spp	sex	N-Containing	Sulphide	Ether	Hydrocarbon	Aldehyde	Alcohol	Ketone	Aromatic	Phenolic
2015	MYLU	M	X	X	X	X	0	X	X	X	0
2015	MYLU	M	X	X	X	0	0	X	X	0	0
2015	MYLU	M	X	X	0	X	0	X	X	0	0
2015	MYLU	F	0	X	X	0	0	0	0	0	0
2015	MYLU	M	0	X	X	0	0	X	X	0	0
2015	MYSE	F	X	X	X	0	0	X	0	0	0
2015	MYSE	F	X	0	0	X	0	X	X	0	0
2015	MYSE	M	X	0	X	0	0	0	0	X	0
2015	MYSO	F	X	X	X	0	0	X	X	0	0
2015	MYSO	F	X	X	X	0	0	0	0	X	0
2015	MYSO	M	0	X	0	0	0	X	X	X	0
2015	LABO	F	X	X	0	0	0	X	X	0	0
2015	LABO	F	X	X	0	X	0	X	0	X	0
2015	PESU	F	0	X	0	0	0	X	0	X	0
2015	PESU	F	0	X	0	0	0	X	X	0	0
2014	MYGR	F	X	X	0	X	X	0	0	0	X
2014	MYGR	F	0	X	0	X	0	0	X	0	X
2014	MYGR	M	X	X	0	X	0	0	0	0	X
2014	MYGR	M	X	X	0	X	X	0	0	X	X
2014	COVI	F	0	0	0	X	X	0	0	0	X
2014	COVI	M	X	X	X	X	X	0	0	0	X
2014	MYSE	M	X	X	0	X	0	0	0	0	X
2014	MYSE	M	X	X	0	X	X	X	0	0	X
2014	PESU	M	X	X	0	0	X	0	0	0	X
2014	PESU	M	X	X	0	X	0	0	0	0	X
2014	MYLU	F	X	0	0	0	0	0	0	0	X

year	spp	sex	N-Containing	Sulphide	Ether	Hydrocarbon	Aldehyde	Alcohol	Ketone	Aromatic	Phenolic
2014	MYLU	M	0	0	0	0	0	0	0	0	X
2014	MYLU	M	0	0	0	0	0	0	0	0	X
2014	MYLU	F	0	0	0	0	0	0	0	0	X
2014	MYSO	F	X	X	0	X	X	0	0	X	X
2014	MYSO	M	0	0	0	X	0	0	0	0	X
2014	MYSO	F	X	X	0	X	0	0	0	0	X
2014	MYSO	M	X	0	0	X	0	0	0	0	X
2014	MYLE	M	X	X	X	X	X	0	0	0	X
2014	MYLE	M	0	0	0	X	0	0	0	0	X
2014	MYLE	F	X	0	0	X	X	X	X	0	X
2014	MYLE	F	X	X	X	X	0	0	0	0	X
		count	25	26	11	21	9	14	11	8	22

Table 4. Eigenvalues and variance for the PC components for the 2014-2015 combined data.

Component	Eigenvalue	Proportion	Cumulative proportion
1	5.085	0.565	0.565
2	1.381	0.153	0.718
3	0.993	0.110	0.829
4	0.946	0.105	0.934
5	0.277	0.031	0.965
6	0.172	0.019	0.984
7	0.099	0.011	0.995
8	0.043	0.005	1.000
9	0.003	0.000	1.000

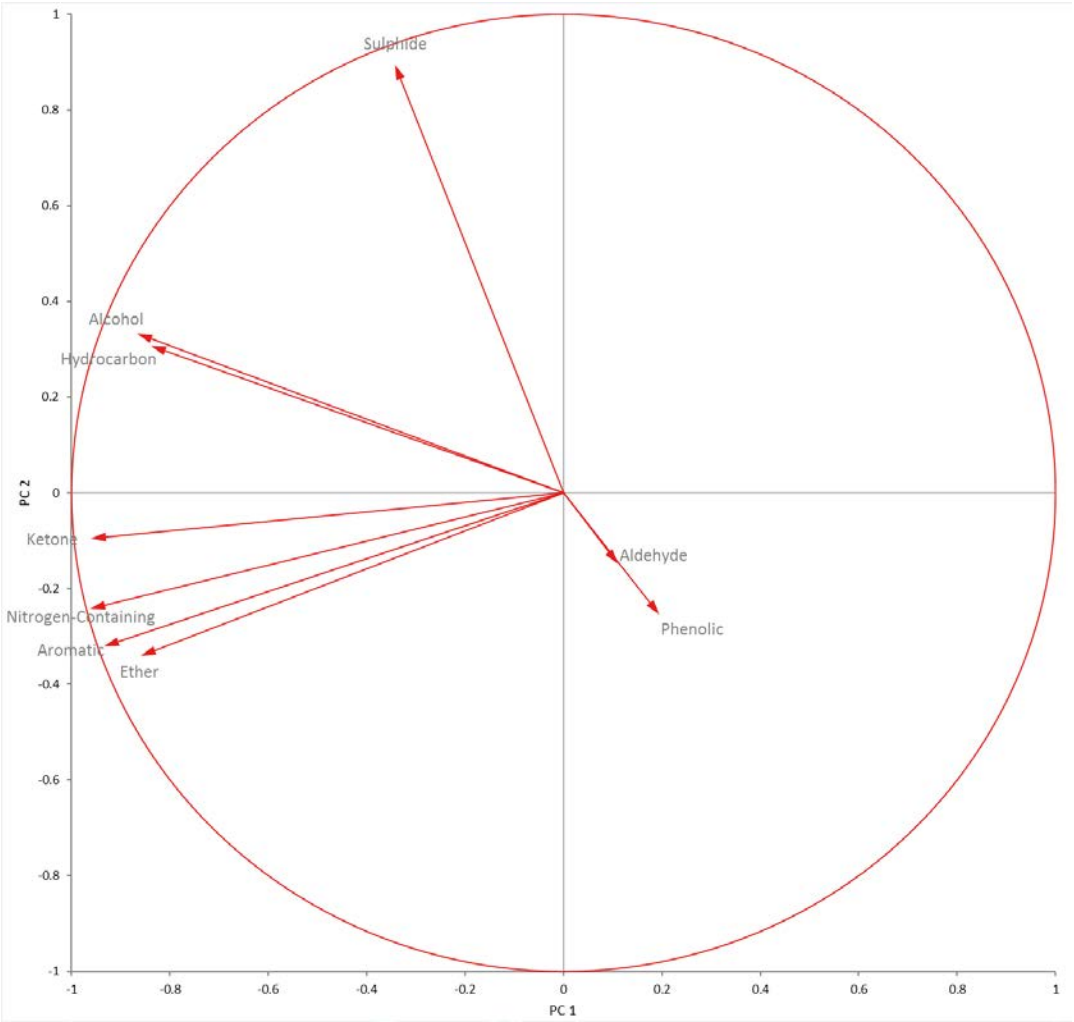

Figure 19. Correlation monoplot for guano VOC categories from the combined data set.

Figure 20. Individual scores for the bat guano samples collected over both field seasons.

Mean location in PC space indicates significant differences between the VOCs reported off of the different bat species, however mean values projected into 2-D feature space merit assessment of the individual spread of guano samples. Because the first two components capture almost 72% of the variability of the guano VOC data, plotting these two components makes sense.

The geographic distribution of sampling might have had an effect on the VOCs, or alternatively it might not. In the case of PESU there were four samples collected, two in each of the survey years, at three different locations as far as ~340 km apart. The VOCs reflect a distribution that is relatively similar compared with the other species. As seen in Figure 21, the spread of the VOCs in PC space are within the range of the other species. In the 2014 samples, this species reported nitrogen-containing compounds, sulphides and phenolics, but in 2015 there were no nitrogen-containing compounds, but alcohols, ketones and aromatics appeared.

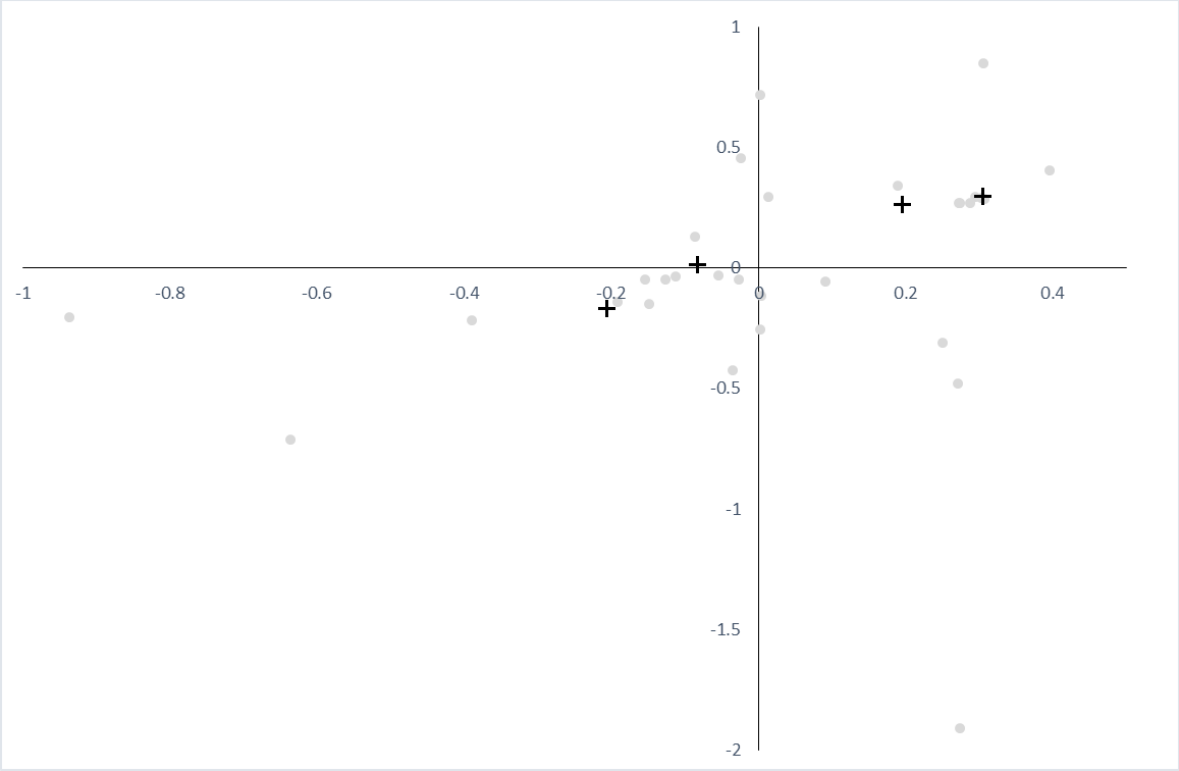

Figure 21. PCA score plot of PESU (+) compared with all other species (gray circles).

MYLU, on the other hand, exhibited a large variability in VOCs, with two outlier guanos as shown in Figure 22. Samples were taken in both 2014 (4) and 2015 (5) in two different locations. The difference likely stems from the paucity of VOCs that were reported in 2014. It was this species that brought the team to consider the timing of capture of bats, when it was observed that the guano analyzed resulted in only a few compounds, primarily phenolics. We hypothesized that there may have been a difference in what was excreted by a bat that was physically hungry, versus one that was returning to roost with a ‘full belly’. In 2015 the samples returned numerous compounds in all but aldehydes and phenolics. Subsequent sampling in 2015 was conducted in

the central and western part of the state, leaving open the question of how geography affects diet, which affects VOCs. From a canine scent perspective, some MYLU guano would be easily generalized, while other guanos may not be recognized as belonging to the species. In this instance a generalist bat guano canine would likely present a better option for detection to reduce misses.

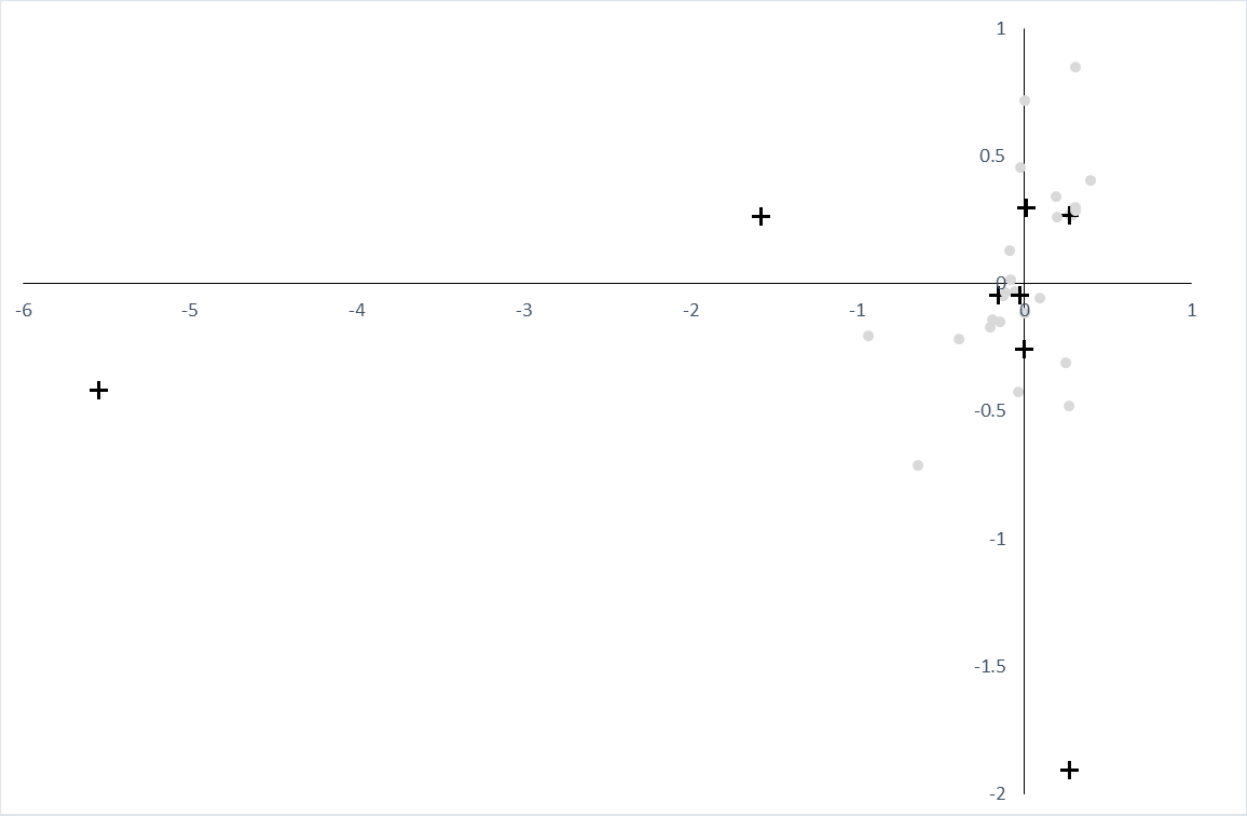

Figure 22. Score plot for MYLU (+) compared with all other species (gray circle).

MYSO and also the primary focal species of this analysis, showed similar results to the other myotis species and with PESU (Figure 23). Generally the VOCs clustered in all four quadrants indicating that the odor would require some generalization with overlap of other species. There was some variability in the guano VOCs within and among years. No ethers were reported in 2014, but were in 2015. As with all 2015 samples, there were phenolics in 2014 but not 2015, and only one sample from a female had aldehydes in 2014 otherwise aldehydes were absent in this species. Alcohols and ketones were present in 2015 but not 2014.

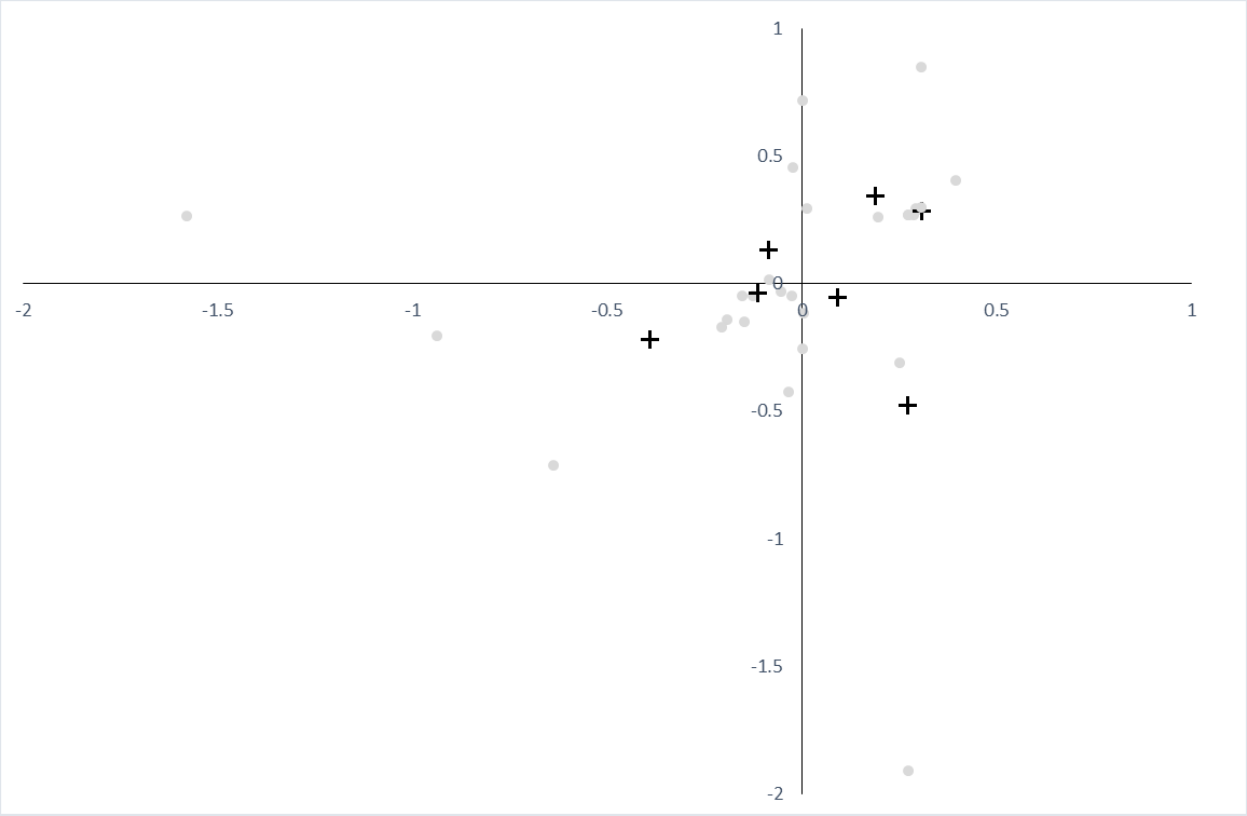

Figure 23. Score plot for MYSO (+) compared with all other species (gray circle).

MYSE showed more variability in VOC responses in both component axes, although there is some clustering in PC1. This species was sampled in two distinct areas, in central and western Kentucky, over both field seasons. A distinct geographic difference is not apparent in the data (Figure 24), although there is one outlier. No sulphides, alcohols or phenolics were reported in the 2015 data.

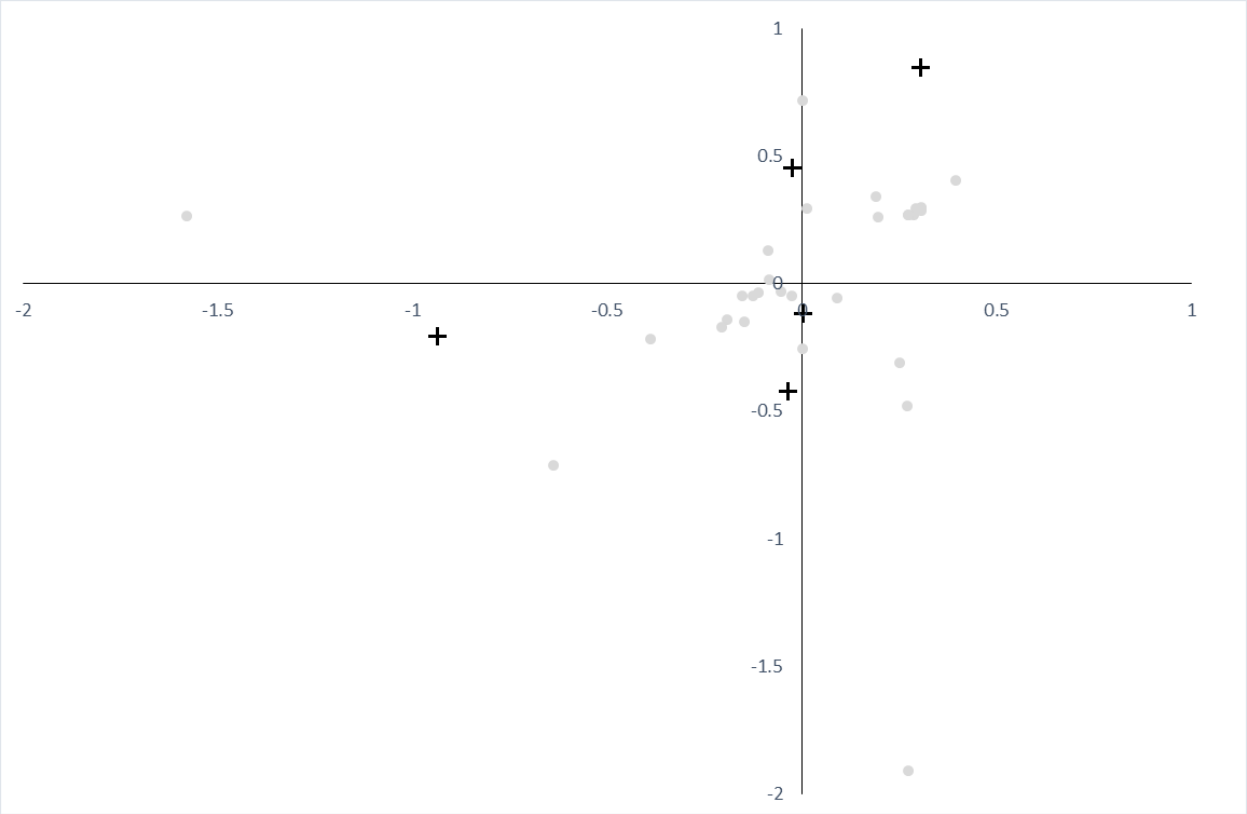

Figure 24. Score plot for MYSE (+) compared with all other species (gray circle).

LABO samples were collected at two different locations only in the 2015 field season. Both samples were from female bats. Numerous VOCs were reported from these samples, although the individual samples fall in different quadrants of the first two PC components (Figure 25). Both samples had nitrogen-containing compounds, sulphides and alcohols, and as with the other 2015 samples there were no aldehydes or phenolics. Neither guano reported ether.

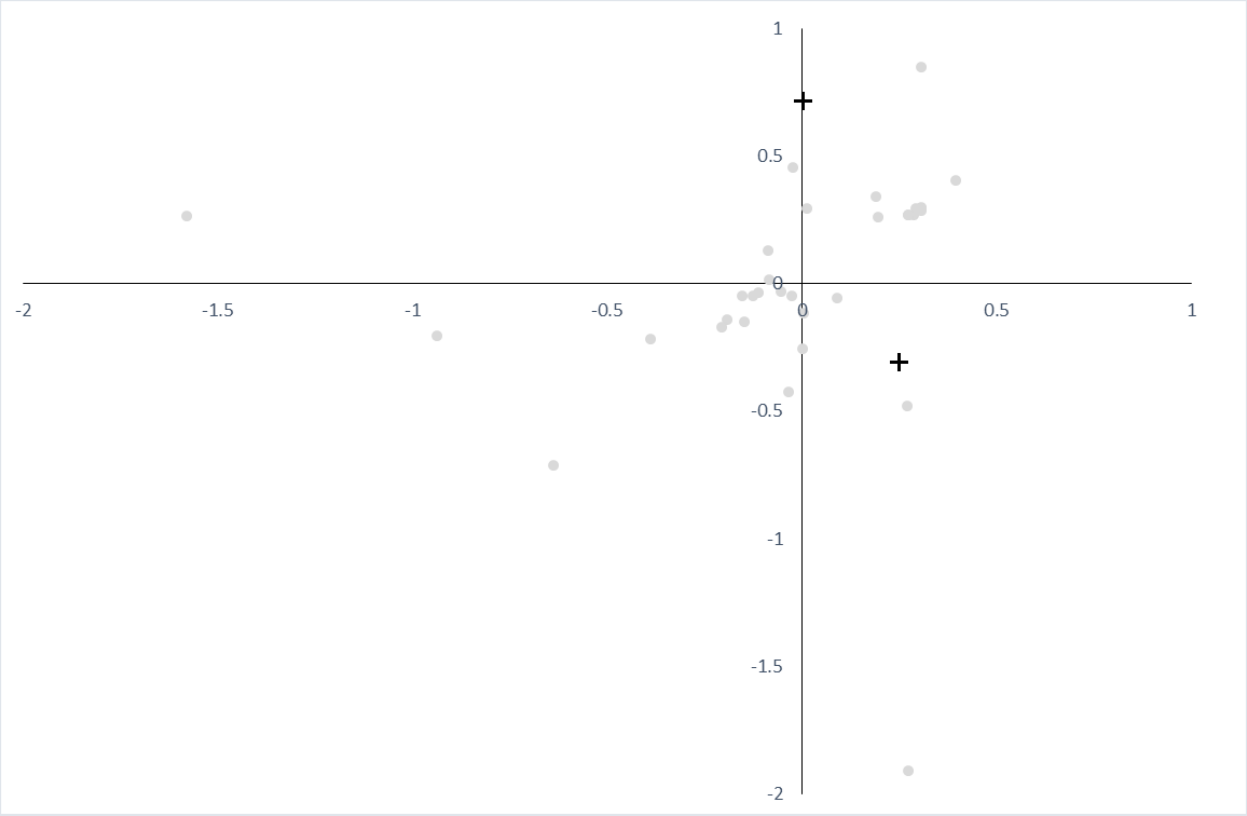

Figure 25. Score plot for LABO (+) compared with all other species (gray circle).

COVI were captured only in 2014. The score plot in Figure 26 shows the individual samples in feature space, one of which is male and the other female. Neither sample reported alcohols, ketones or aromatics. The female guano lacked nitrogen-containing compounds, sulphides and ethers. The female was captured earlier in the evening at 2123, while the male was captured later at 2345 which also suggests a ‘full belly’ phenomenon.

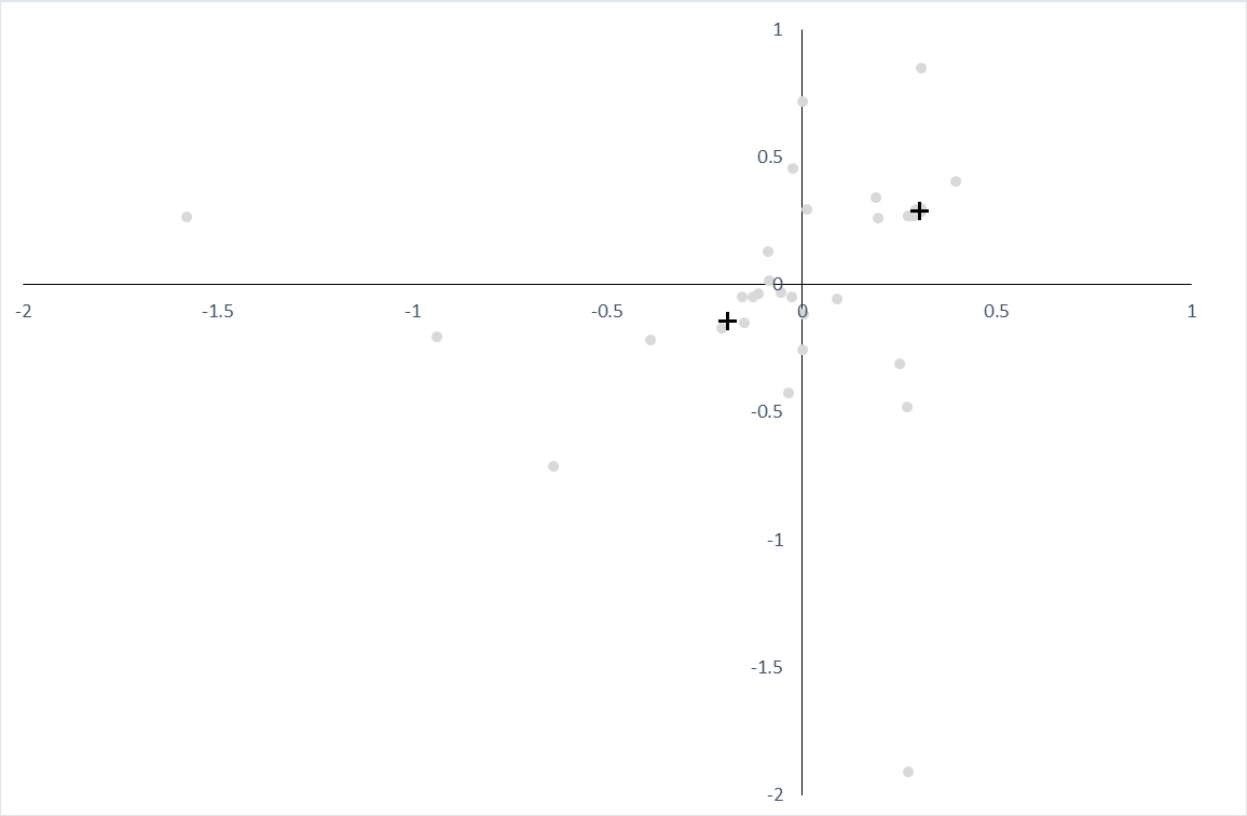

Figure 26. Score plot for COVI (+) compared with all other species (gray circle).

MYGR were captured in the 2014 field season only. The samples reported nitrogen-containing compounds, sulphide, hydrocarbons and phenolics but no ethers or alcohols. The score plot in Figure 27 shows significant variability between individual bats. This was initially unexpected given that all samples were collected at the same location. One possible explanation for the observed variability is that MYGR nightly summer foraging range can be quite large compared to other myotids, and the variability directly relates to variability in diet.

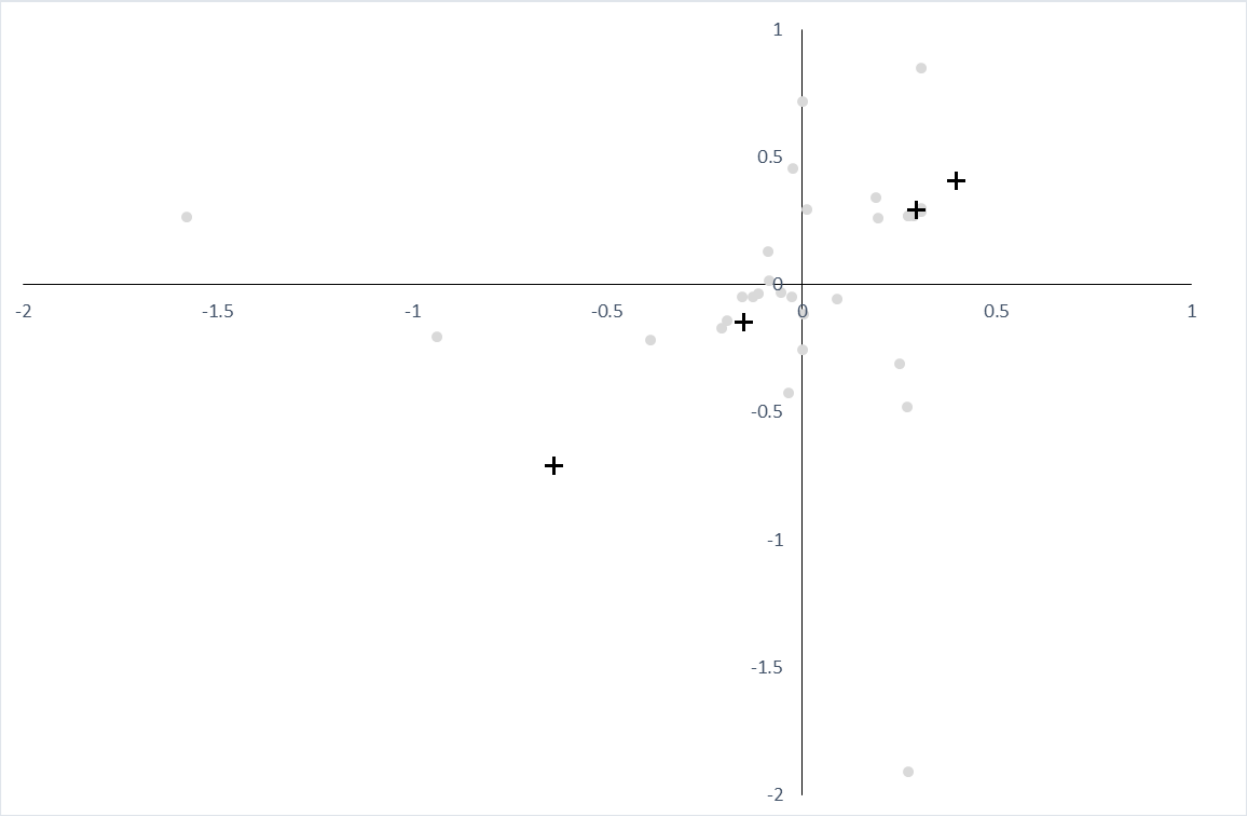

Figure 27. Score plot for MYGR (+) compared with all other species (gray circle).

MYLE were captured and samples were collected only in 2014. As shown in Figure 28 the data cluster, however these are not male – female differences. These samples were taken at the same location. No aromatics were reported in any of this species' guano.

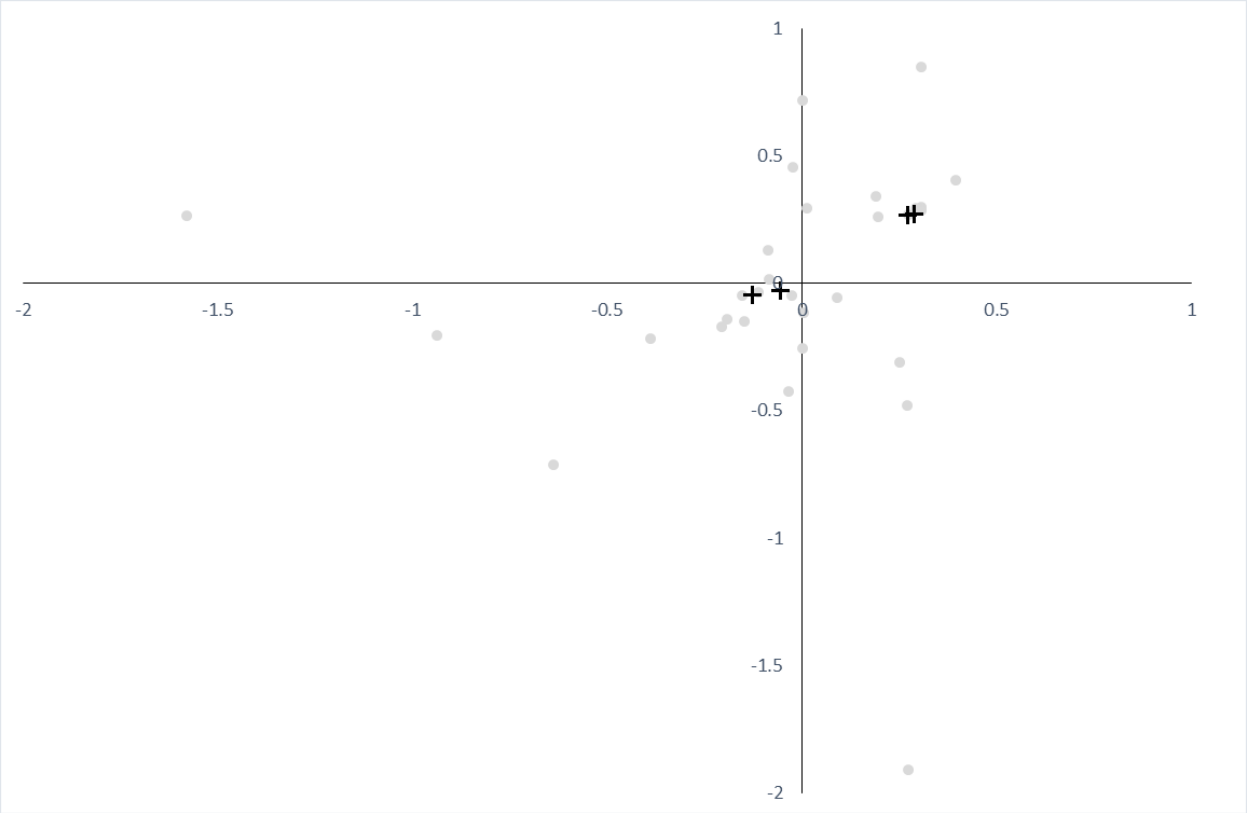

Figure 28. Score plot for MYLE (+) compared with all other species (gray circle).

Shapiro-Wilk test results for normality indicated the data were normally distributed between male and female counts of number of compounds per guano sample (female $W = 0.94$, $p = 0.32$; male $W = 0.94$, $p = 0.27$). ANOVA was run on the combined data set to determine if there were significant differences in the numbers of compounds produced by males versus females. Results showed no difference ($F = 0.10$, $p = 0.7537$) in means and Brown-Forsythe test for dispersion showed no significant difference in variance ($F = 0.89$, $DF = 1/35$, $p = 0.35$).

Taken in combination, there exists overlap between the samples and there is some relationship between geography, which likely relates to diet, and the VOCs from guano. This is not a predictable relationship, however, as not all bats captured at the same location produced similar VOCs and there was variability within species between survey years.

CONCLUSIONS

There were several positive outcomes from this research. First, the method was demonstrated to be effective, and this included learning the best approach to safely capture and handle the bats as they emerged or returned to their roosts. Careful consideration must be taken with samples to be analyzed in a GC-MS to reduce the likelihood of contamination. We were also able to demonstrate that the guano VOCs of interest were stable enough to persist through collection, shipping, receiving, and analysis, a process that generally took 24-48 hours to complete.

The significance of these method developments relate directly to training of bat guano detection dogs. Detection dogs of any discipline require pure source training aids to learn and maintain specificity to their target odor. Direct collection of guano using the methods described here showed machine detectable VOCs produced by guano stored in the glass vials, which provides support for producing viable training aids by bat species. If a machine can detect the VOCs from the samples, a dog can. What we cannot comment on with respect to training aids, however, is the length of time that the guano would be viable as a training aid. Our samples were frozen once they were analyzed so future analysis is an option to assess the temporal component to training aids.

Regarding the possibility for differentiating bat species based on guano VOCs, and possibly odor, we had posed the following questions. Based on the results of our analyses the summary conclusions are as follows:

1. Is there a difference in guano VOCs between the *Myotis* spp.?

There are differences in guano VOCs such that it may be expected that a canine could differentiate between species. However, there exists variability within species to suggest that training aids (guano) would need to be considered carefully. The primary consideration would be to provide the canine with guano collected from the geographic location where surveys would occur. For canines deployed to cover a wide range of geography, a similarly diverse constituent guanos would be advised.

2. To what degree is the guano VOCs from *Myotis* spp. different and to what degree are they similar?

The bat species sampled all eat insects, and while diets may vary somewhat between species and across ranges, ultimately the excreta are comprised of similar digesta. Thus it was not surprising to see similarities in classes of compounds. The exception was the difference in phenolics and aldehydes not identified in any of the 2015 samples. Because we were able to detect insect digesta, having been digested by bats, it is possible that the differences reported are both primary diet differences, and dietary differences of prey. Timing of the digestion of insect prey and forage along with timing of sampling of bat guano post-digestion are likely candidates to explain differences and variability.

3. In what chemical classes are the species more different and more similar?

There was a lot of overlap among the chemical classes which is expected, given the habitat

similarities of the survey locations in and the relatively similar diets as well. Notable classes and chemical species included MYSE that had nitrogen containing VOCs in all samples for both years. PESU showed sulphide compounds in all samples for both years as well. These were the only two such occurrences. MYSO had nitrogen containing and sulphide compounds across both years, although not in all samples. There are notable absences as well, such as the ether compounds that were present in MYLU but which were absent among other species for both years of data. These class differences suggest that the odor profiles too will be different.

4. Is there variability between guano from males and females?

There was variability in the components from guano of males and females, and our analysis suggests differentiation based on VOCs may be possible, if species specificity is ignored. We did not have sufficient sample size to assess both species specificity and sex in terms of VOC based separability.

5. To what extent do male and female VOCs vary and in what chemical compounds?

There were differences in the compound categories that were reported from samples by sex, although the source of those differences are difficult to identify with certainty from our analysis. The obvious possibility, diet, is worth further investigation because presumably males and females are eating similar diets, although perhaps there are some differences that have yet to be explored. If there is no dietary difference, then it should be a biological difference tied to sex. Therefore while we might hypothesize that there may be an olfactory difference between the sexes, there would likely be other factors related to sex (e.g., hormones) that could affect odor too. These compounds are not measured in the GC-MS instrumentation so we cannot contribute much to a discussion surrounding them.

6. How different are non-myotid guano VOCs from myotid samples?

This varied by species and also varied with the addition of a second year of data collection. LABO returned VOCs that were quite different from any other species, as did two of the myotids, MYGR and MYLUs. None of these species were similar in their VOCs to any other. On the contrary, the rest of the species sampled returned VOCs that clustered similarly in PCA analysis, indicating more similarity between them.

7. Based on the results of these analyses, is it likely that *Myotis* spp. guano is sufficiently different from other bats species guano such that a dog could be trained to be a *Myotis* specialist?

Based on the 2014 data alone, MYSO, MYLE, MYLU, and MYSE would be differentiable, with some potential for confusion between MYGR with MYSE. Results were less clear with the combined data set over two years of sampling, which showed that MYLU and MYGR are clearly different from other species. It is possible that there is sufficient variability in the other three *Myotis* species to differentiate them from each other (MYSE, MYSO, MYLE) via olfaction.

8. What can be inferred from the chemistry results about odor signatures that relate to detectability by dogs?

Clearly there exist VOCs that would comprise an odor signature that a canine could detect, since our instruments were able to detect them. With that said, it is important to remember that the results of analytical chemistry cannot be assumed to be the equivalent mixture of what a dog uses to recognize 'target odor'. For complex targets such as guano, it remains unknown what a dog's recognized odor signature would be. Because we were able to capture and report VOCs that made logical sense from a biological and chemical perspective, it seems quite reasonable to expect a dog could not only be trained to recognize bat guano as a target odor, but possibly differentiate species.

9. Based on the results of these analyses, is it likely that distinct odor signatures occur such that a canine could be trained as a species-specialist, to detect a single *Myotis* spp.?

Qualified yes, for certain species as discussed above more so than others, but results of this analysis suggest species specific bat guano detection dogs are possible. The results we present here represent the analytical chemistry component only and are no guarantee that *any* dog with *any* handler could be proficient at the task. It would require proper training and a correct plan of reinforcement on searches with dogs in the field to establish and maintain species-specificity.

10. If the above is true, does it hold for all *Myotis* spp. or just some and which ones?

MYLU and MYGR, as well as LABO, would be likely candidates for species-specific guano detection dogs based on the combined field data, while the 2014 data suggest that any of the species sampled might produce sufficiently different odor signatures. The 2014 data suggest somewhat contradictory results for MYGR, which returned a similar odor composite to PESU. The 2014 data also shows that MYSO would be distinct from the other *Myotis* species. Based on the combined data sets, MYSO and MYSE would have the potential to be similar from an odor perspective, with less likely overlap with other *Myotis* species.

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