DIET ANALYSIS OF TEXAS HORNED LIZARDS (*Phyrnosoma cornutum*) IN TWO SMALL TEXAS TOWNS USING MORPHOLOGICAL AND GENETIC METHODS

By

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Abstract

Texas Horned Lizards (*Phrynosoma cornutum*) are considered threatened within Texas, due to their high vulnerability to habitat changes. In particular, horned lizards are believed to be sensitive to the loss of harvester ants (*Pogonomyrmex)*, which make up a large portion of their diet. Thus, dietary studies are important in assessing the reliance of horned lizards on harvester ants . This study focuses on the diets of horned lizards in two populations from Kenedy and Karnes City, TX, to determine if their diets differ from previously studied populations. Additionally, this study explores the effectiveness of genetic analysis in dietary studies of horned lizards. Twenty-three fecal samples from Kenedy and Karnes City, TX were analyzed using a combination of morphological and genetic methods. In morphological analysis, desert termites (*Gnathamitermes tubiformans*) accounted for 50.5% of all prey items observed, while only 4.8% were harvester ants. Furthermore, genetic analysis of 12 samples from Kenedy revealed diets containing 21 different families from 7 orders (Araneae, Coleoptera, Diptera, Hemitptera, Hymenoptera, Isoptera, and Orthoptera). The diversity of prey items and the abundance of termites suggest that the diets of horned lizards from Kenedy and Karnes City differ significantly from previously studied populations. Additionally, genetic sequencing proved to be a useful technique for dietary analysis, due to its ability to accurately distinguish between different taxa.

Introduction

Horned lizards have a variety of behavioral and anatomical adaptations which are essential for ant-based diets (Pianka and Parker 1975). For example, the length of the epipterygoid and mandible are correlated with the percentage of ants normally found in the species' diet (Montanucci 1989). Previous dietary studies suggest that ants may compose between 21 to 99% of the Texas horned lizard diet (Milstead and Tinkle 1969, Eifler et al 2012). In particular, the Texas horned lizard is known to favor harvester ants (*Pogonomyrmex* spp.) (Pianka and Parker 1975). Individual studies have found that harvester ants may contribute as little as 0% to as much as 97% of Texas horned lizard diets (Lemos-Espinal et al 2004, Eifer et al 2012). However, this variation is likely the result of regional variations in prey abundance, as well as small sample sizes. In a study of horned lizard stomach contents of over 300 museum samples, harvester ants accounted for 69% of their overall diet (Pianka and Parker 1975). Additionally, Texas horned lizards have specific adaptations which enable consumption of harvester ants, such as resistance to harvester ant venom and their ability to cover ants in mucous before swallowing to keep from being stung (Schmidt et al 1989, Sherbrooke and Schwenk 2008).

In general, ants have low nutritional value when eaten individually, so species with ant-based diets have to consume large quantities/volumes of ants to have enough nutrition. As a result, individuals would have to spend a large amount of time foraging for ants, which increases predation risk (Eifer et al 2012). This risk could be reduced by consuming larger ant species, since fewer ants would need to be consumed to fulfill

nutritional requirements. Harvester ants are larger than most other ant genera in Texas, nonaggressive, and their mounds are highly conspicuous (Vinson et al 2014). Subsequently, they are easy to find, and a relatively low-threat source of calories, making harvester ants an ideal source of food for horned lizards (Blackshear and Richerson 1999).

Within the state of Texas, the Texas horned lizard (*Phrynosoma cornutum*) is considered threatened, due to their high vulnerability to habitat changes (Hammerson 2007). In particular, they have been particularly susceptible to the loss of harvester ants, development and urbanization of native habitat, invasive fire ants (*Solenopsis invicta*), and the use of pesticides (Hammerson 2007, Henke and Fair 1998, Wolf et al 2013, Donaldson et al 1994). However, it is uncertain which of these factors are the largest contributors to horned lizard declines, making effective management and protection of this species difficult. (Price 1990, Donaldson et al. 1994, Dixon 2000, Henke 2003). Currently, most management strategies focus on protecting horned lizard habitats, but a more informed understanding of ecological threats could allow development of more specific and effective techniques (Henke and Fair 1998).

Previous studies have determined that horned lizard numbers are limited by the density of harvester ants (Whitford and Bryant 1979). This suggests that declining harvester ant populations may be a primary contributor to horned lizard declines (Hammerson 2007). However, few studies have been done to confirm that harvester ants are an essential component of Texas horned lizard diets, especially in areas where they have been declining. Subsequently, dietary studies in these areas could be

invaluable in assessing the importance of harvester ants to horned lizard survival. This study focuses on the diets of horned lizard populations from Kenedy and Karnes City, TX, where there have been several long-term studies exploring the threats which urbanization pose to horned lizards (Wall 2014). In particular, this study aims to determine if the diets of these populations differ significantly from previous dietary studies, and to assess the dietary importance of harvester ants to horned lizard diets in these locations.

In general, dietary studies can be informative about a variety of behavioral and ecological factors impacting a species (Blackshear and Richerson 1999, Whitford and Bryant 1979). Many previous dietary studies of Texas horned lizards have relied on the contents of stomachs and intestines, which requires removing individuals from wild populations (Blackshear and Richerson 1999, Lemos-Espinal 2004, Pianka and Parker 1975). However, analysis of scat is non invasive, and can reveal virtually the same information, making it a practical alternative for threatened species. Additionally, horned lizard scat is highly conspicuous, making it easy to find and sample. Because chitin is not easily digested, exoskeletons of many prey items are still relatively intact in horned lizard scat, and can potentially be identified using morphology. Many dietary studies utilize this method for the estimation of prey frequency and volume (Zeale et al 2011). However, this requires extensive knowledge of insect morphology, and can be very time consuming. Additionally, although the individual pieces of the exoskeleton are still intact, they are usually broken apart such that you cannot identify which pieces belong together. Furthermore, morphological methods are limited to species with hard

exoskeletons, due to high decomposition of soft-bodied prey (Zeale et al 2011). Subsequently, genetic sequencing is more reliable for species identification than morphological dietary studies, since it does not rely on specific anatomical features (Zeale et al 2011). Due to these limitations of morphological studies, this study also explores the usefulness of genetic sequencing for diet analysis of Texas horned lizards.

Methods

We had two survey sites in Kenedy (28.8191° N, 97.8486° W) and Karnes City (28.8850° N, 97.9008° W), Karnes County, Texas (Figure 1). These two small towns, roughly six miles apart, have been the site of ongoing horned lizard research by TCU faculty and students since 2013. In this study, samples were taken from 6 survey sites, 4 in Karnes City, and 2 in Kenedy. Sites were chosen based on recorded lizard presence from previous field seasons (Wall 2014). Four of the samples utilized in morphological analysis were collected in urban alleyways between residential properties in Karnes City (Figure 2a). The remaining 4 samples from Karnes City and 15 from Kenedy were collected in open fields or municipal parks (Figure 2b,c). Survey sites were irregular in shape and size with area ranging from 0.07-1.11 hectares. Samples were collected between May 28th and June 2nd, 2015. This study analyzed a total of 23 samples: 11 using morphological analysis, 5 using genetic analysis, and 7 using both methods.

Survey sites were censused by 2-5 people slowly walking 2 meter wide line transects until the entire area of the site had been searched. These surveys lasted from 30 minutes to 2 hours depending on the size of the survey site, number of searchers, and number of horned lizards and scat encountered. Searches were generally conducted between 8:00-11:00 am and 5:00-8:00 pm, when horned lizards are typically most active (Moeller et al. 2005). We collected the following data for each survey: time of day, number of searchers, site type (field, alley, or mixed), survey site, study town (Kenedy or Karnes City), and total search time. All intact scat (Figure 3) were collected and placed in 1.0 or 3.0 mL of 8 M Urea buffer solution (depending on size of scat) and their location marked via GPS (Garmin GPS72).

Figure 1. Map of Texas with Karnes County in red.

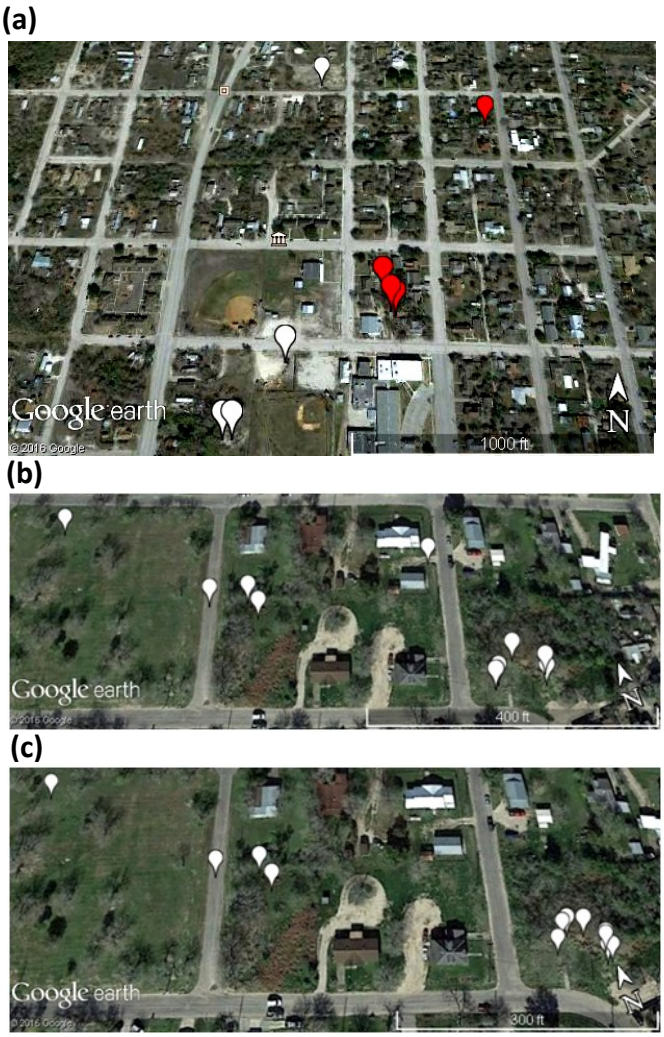

Figure 2. Maps of collection sites of samples used in this study. Red markers indicate collection in an alleyway. White markers indicate collection in a field or park. (a) Samples used for morphological analysis from Karnes City. (b) Samples used for morphological analysis from Kenedy. (c) Samples used for genetic analysis.

Figure 3. Image of horned lizard scat

Morphological Analysis

To conduct morphological analysis, samples were filtered using vacuum filtration and dried, before being sorted. Using a dissecting scope, remains were sorted, counted, and grouped based on taxonomic order and physical appearance. Hymenopterans were sorted into categories based on morphological characteristics of the head (henceforth referred to as 'morphs'). Objects which were not informative to identification were discarded. If possible, morphs were identified by comparison with field samples collected during the summer, as well as using AntWeb (https://www.antweb.org/) and the Texas A&M 'Common Ant Genera of Texas' ID guide (Vinson et al 2014). During identification, we focused on heads which had mandibles or other attached body parts (Figure 4). We then determined the composition of each sample, and estimated the relative contribution of each morph to the total insect count.

To estimate volume, we measured dimensions (to 0.01 mm) of up to 10 heads for each morph. Length and width were measured at the largest length and width of the

head, not including mandibles (Figure 5). Previous studies of *Solenopsis* have found that head size is correlated with overall body size (Tschinkel 2013). Subsequently, we used the average product of the length and width of each head as a rough estimate of volume for hymenopteran and isopteran morphs. Proportions were estimated by dividing the contribution of each morph by the total sum of all morphs. Volume was only estimated for ants and termites. Unfortunately, we were unable to calculate the volumetric contribution of coleopterans or hemipterans because we have not found a reliable way to predict the body volume of ants in relation to beetles. We analyzed 18 samples using this method, 10 from Kenedy, and 8 from Karnes City. Both locations were included, so that diets of the two regions could be compared to one another, as well as populations from other studies.

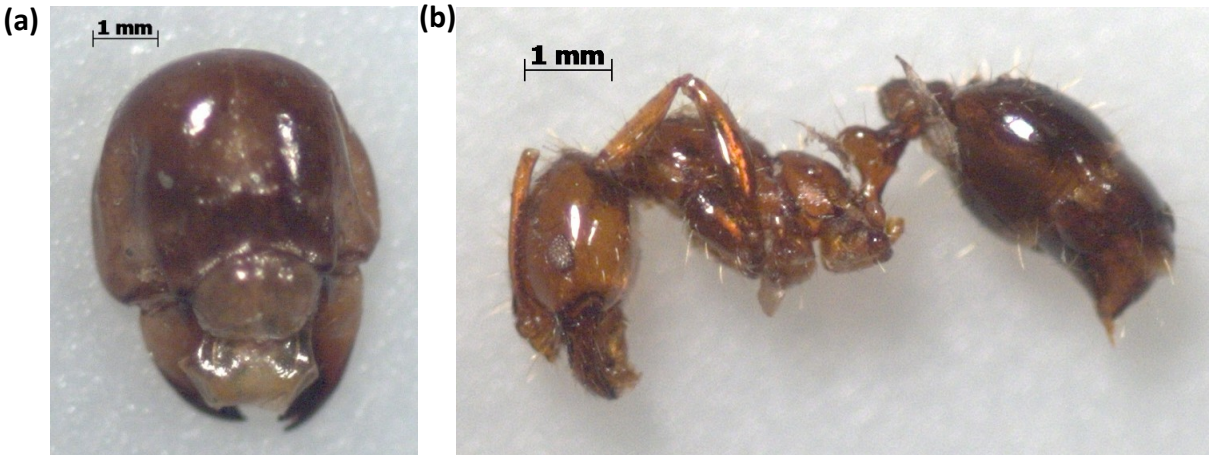

Figure 4. Examples of remains that were used for morph identification. (a) *Gnathamitermes tubiformans* head (b) *Solenopsis* body

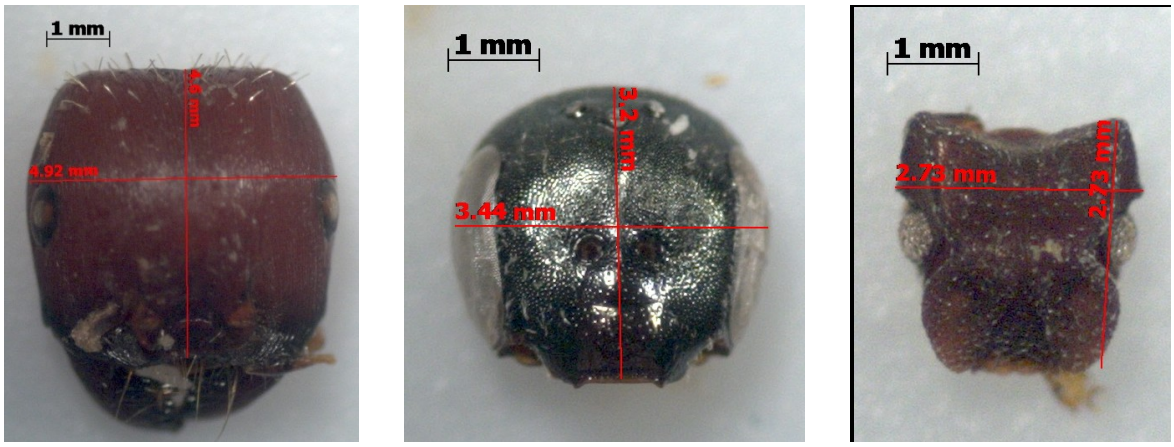

Figure 5. Images taken for morphological analysis, with dimensions used for volumetric calculations of (a) harvester ants (b) wasps, and (c) *Cyphomyrmex.*

Genetic Sequencing

We collected fresh fecal pellets of Texas horned lizards (easily recognized by the cylindrical shape of the feces) and preserved the samples in 1.0 mL 8M Urea buffer (10 mM Tris pH 7.5, 125 mM NaCl, 10 mM EDTA, 1% SDS, 8 M urea) until extraction (Asahida et al. 1996). We selected 12 samples from Kenedy for genetic analysis. For this methodology, we did not include samples from Karnes City, since this part of the study was focused on testing methodology, rather than making direct comparisons between populations. DNA extraction followed the protocol outlined in the QIAamp DNA Stool Mini-kit (Qiagen Genomics, Valencia, CA). A negative control was made with each round of extraction to ensure non-contamination of reagents. Extractions were conducted in an extraction dedicated AirClean® 600 PCR workstation. For fecal DNA samples, PCR reactions were conducted in a separate room from DNA extractions in a PCR dedicated AirClean® 600 PCR workstation. Negative controls were used in all PCR reaction batches.

A part of the mitochondrial cytochrome oxidase I gene was amplified using arthropod specific primers ZBJ-ArtF1c and ZBJ-ArtR2c developed by Zeale et al. (2011).

Because DNA becomes highly fragmented during digestion, this primer pair was designed to target a small 157 bp region, which had enough taxon-specific qualities to provide species resolution of prey items (Zeale et al 2011). During the course of this study we found these primers had low affinity for ants and termites, due to a base pair mutation in the last position of the forward primer which is essential to ensure amplification. Subsequently, we designed two additional sets of primers to amplify ant and termite DNA (AntF: 5'AGA TAT TGG AAT TYT ATA TTT YAT TYT WGC WHY YTG, AntR: 5'ACW AGR AAR TTT GCA AAK CCT CC; TerF: 5' AGA TAT TGG AAC ATT ATA TTT YGT ATT YGG AGC, TerR: 5' ACN ART CAR TTT CCR AAH CCW CC). The primers were designed to target the same region as Zeale et al. (2011), based on ant and termite sequences obtained from Genbank. Sequences were selected from genera which we had identified from morphological analysis, or are known to exist in south Texas. The downloaded sequences were aligned to sequences obtained from the ZBJ primer pair in MEGA 6.0 (Tamura et al. 2013) using Muscle (Edgar 2004). We then identified the target binding sites for the ZBJ primers, and designed sequences based on shared base pairs between the ant and termites sequences for these binding sites. We then entered the forward and reverse sequences into Primer3 (Untergasser et al. 2012), to set annealing temperatures and ensure the primers would not form hairpins or dimers.

Polymerase chain reactions (PCR) (10µL) contained 2 µl DNA, 0.5 µM of each primer, 2X BSA, 1X Qiagen Multiplex PCR Master Mix with HotStarTaq, Multiplex PCR buffer with 3mM $MgCl_2$ pH8.7, and dNTPs. Reactions were cycled in an ABI 2720 thermal cycler. The cycling parameters were one cycle at 95°C for 15 min, followed by

40 cycles of 30s at 94°C, 90s at 55°C (ZBJ and termite primers) or 50°C (ant primer), 90s at 72°C, and then a final extension at 72°C for 5 minutes. Products were gel purified and ligated into pGEM-T vectors (Promega) then transformed into JM109 competent cells. Bacterial colonies which contained the inserts were white, while those that did not were colored blue. At least 12 colonies for each scat sample were picked and amplified directly using vector-specific primers (F: CGACTCACTATAGGGCGAATTG, R: CTCAAGCTATGCATCCAAGG). Unincorporated nucleotides and excess primers were removed from PCR products using *rSAP* and *ExoI* (New England Biolabs) according to manufacturer protocols.

PCR products were then bi-directionally sequenced using the vector primers and ABI Big Dye Terminator Cycle Sequencing v3.1 Chemistry (Life Technologies). Sequences were electrophoresed on an ABI 3130XL Genetic Analyzer (Life Technologies); edited, put into contigs, and trimmed using Sequencher v5.0 (Gene Codes USA). Sequences were then aligned in MEGA 6.0 (Tamura et al. 2013) using Muscle (Edgar 2004) and the primer sequences were removed resulting in a 157 bp fragment. All sequences were translated and checked for stop codons which would indicate the presence of nuclear mitochondrial pseudogenes (*numts*). Sequences were then annotated using the Barcode of Life Data System and NCBI Basic Local Alignment Search Tool research databases. Results from the BOLD Systems database were given priority as they were obtained from vouchered DNA reference sequences. Identifications to order, family, genus or species were made when sequence matches were greater than 85.9%, 91.0%, 94.9% and 99.3% respectively (Zeale et al. 2011). In several cases, we then searched BugGuide

(http://bugguide.net/) for images of the taxa identified via sequencing, to help with morphological analysis. Additionally, we compared the diversity of taxa identified using genetic sequencing to those from morphological analysis, to determine the usefulness of this methodology to assessing biodiversity.

Results

Morphological Analysis

Results of morphological analysis are displayed in Table 1. We observed 4,593 prey items from Coleoptera (2.5%), Hemiptera (0.4%), Hymenoptera (46.3%), and Isoptera (50.5%). Desert termites (*Gnathamitermes tubiformans*) were the most abundant prey item. Termites occurred in 15 of the 18 samples, and accounted for 50.5% of all prey items consumed. When compared to Hymenoptera, termites composed 71.0% of the volume of prey. Conversely, ants accounted for only 46.0% of the prey items found. We classified ants into 26 different morphs, which we equated to different genera. Of these, 19 had 10 or fewer individuals, and were classified as 'other large ants' or 'other small ants' (Table 1). Only 5 of these morphs were identified, including carpenter ants (*Camponotus,* N=5), pharaoh ants (*Monomorium pharaonis*, N= 4), *Cyphomyrmex* (N=1), fungus ants (*Trachymyrmex*, N=4), and *Pachycondyla* (N=1). The remaining 14 groups were unidentified. Of these 19 morphs, 12 morphs were only found in a single sample.

Dorymyrmex (8.6% by number, 7.0% by volume) and *Aphaenogaster* (26.9% by number, 10.6% by volume) were the most abundant ant taxa. However, these numbers

may be artificially high, due to the presence of multiple species in these groups. Harvester ants (*Pogonomyrmex*) were only found in 12 of the 18 samples, composing 4.8% of the diet by number and 7.6% by volume. Other fairly common ants included *Phediole* (1.0%, 0.6% vol), little black ants (*Monomorium minimum*; 0.8%, 0.2% vol), and *Tapinoma* (1.7%, 0.8% vol). Several samples contained fire ants, which accounted for 1.3% of the total diet by number and 0.8% by volume. A number of samples contained what we concluded to be wasps, which composed 0.3% of prey items and 0.2% of volume. Similarly, beetles (*Coleoptera*) and true bugs (*Hemiptera*) made up 2.5% and 0.4% of the prey items, respectively.

There was a high degree of variability in the number of termites and ants consumed in Kenedy and Karnes City (Table 2). However, there was not a significant difference in the medians of harvester ants (Mann-Whitney U Test, $W_{0.05(2),\ 10,8}$=105.0, P=0.40) and termites (Mann Whitney U Test, $W_{0.05(2),10,8}$=77.5, P=0.13) between the two towns (Figure 6). Conversely, when comparing all 18 samples, the median number of termites, harvester ants, and other ants differed significantly (Kruskal-Wallis test, H_2=21.70, P<0.001, N=54). This suggests that the abundance of these prey are not equal within the diets of horned lizards in these populations (Figure 7), and that harvester ants made up a reduced proportion of their diet.

Table 1. Cumulative number and proportion (P) of arthropods (N = 18 scat) using morphological analysis. Any species ID with N<10 was placed into either 'Other large ants' or 'other small ants'. Size categorization was based on size relative to harvester ants. Note that volume was not calculated for beetles or hemipterans.

Prey Item	Number of samples	Number	P[Number] (number/total)	Volume(mm^2)	P[Volume] (volume/total)
Desert Termites	15	2,320	0.505	50,770.28	0.710
Harvester Ants	12	221	0.048	5,441.92	0.076
Phediole	6	44	0.010	412.48	0.006
Wasps	7	13	0.003	170.26	0.002
Dorymyrmex	17	395	0.086	4,978.64	0.070
Aphaenogaster	16	1,237	0.269	7,595.70	0.106
Fire Ant	6	59	0.013	592.46	0.008
Little Black Ants	7	38	0.008	109.12	0.002
Tapinoma	2	80	0.017	538.14	0.008
Other Large Ants	7	22	0.005	603.37	0.008
Other Small Ants	8	30	0.007	327.40	0.005
Coleoptera	13	117	0.025	--	--
Hemiptera	8	17	0.004	--	--
Total	18	4,593	1.000	71,539.77	1.000

Table 2. Number and proportion (P) of harvester ants and termites in each sample.

Sample	Location	Number of Harvester Ants	P[Harvester Ant]	Number of Termites	P[termite]
F1	Kenedy	0	0.000	109	0.901
F2	Kenedy	29	0.644	5	0.111
F3	Kenedy	1	0.018	1	0.018
F4	Kenedy	5	0.060	0	0.000
F5	Kenedy	22	0.349	2	0.032
F7	Kenedy	23	0.307	0	0.000
F9	Kenedy	15	0.326	0	0.000
F15	Kenedy	3	0.010	223	0.748
F17	Kenedy	0	0.000	201	0.866
F20	Kenedy	5	0.025	169	0.862
F21	Karnes City	52	0.272	88	0.460
F22	Karnes City	0	0.000	76	0.567
F23	Karnes City	0	0.000	675	0.859
F30	Karnes City	3	0.013	58	0.246
F32	Karnes City	62	0.224	201	0.728
F34	Karnes City	1	0.001	4	0.006
F40	Karnes City	0	0.000	81	0.386
F62	Karnes City	0	0.000	427	0.526

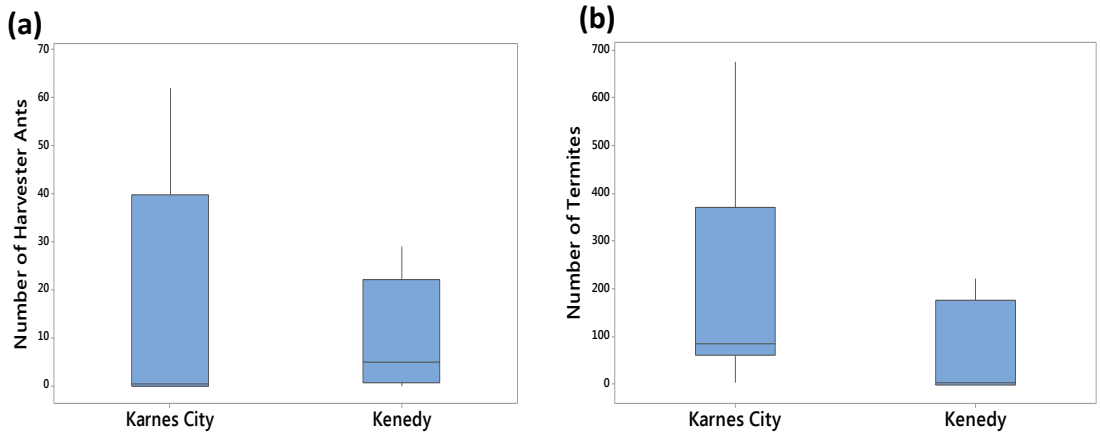

Figure 6. Box and whisker plots of frequency of prey items from Karnes City and Kenedy. (a) Number of harvester ants heads in each sample. (b) Number of termite heads in each sample. Whiskers correspond to upper and lower quantiles. Boxes represent the middle quantiles and the median.

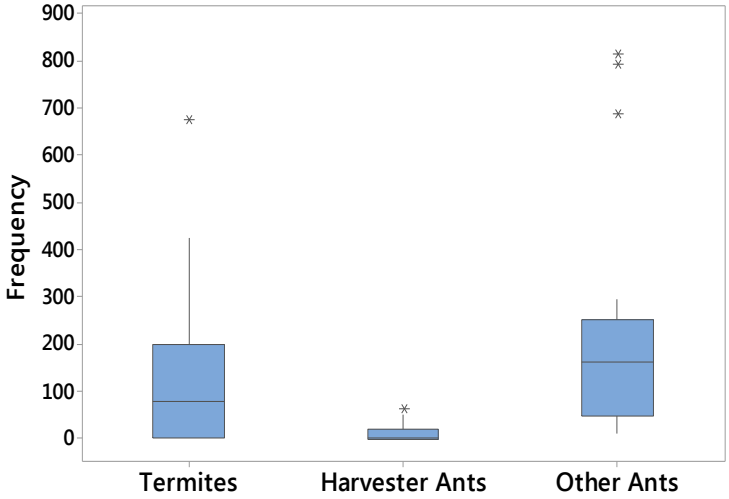

Figure 7. Box and whisker plot of number of termites, harvester ants, and other ants in all samples (N=18). Whiskers correspond to upper and lower quantiles. Boxes represent the middle quantiles and the median.

Genetic Analysis

Using genetic methods, we analyzed 12 samples using three primers pairs with a total of 137 clones (91 ZBJ, 30 Ant, 16 Ter). We were able to identify 21 families from the orders Aranae, Diptera, Lepidoptera, and Orthoptera, in addition to those identified from morphological analysis (Coleoptera, Hymenoptera, and Hemiptera) (Table 3). Of the 33 unique sequences, 11 were matched to the species level (cutoff 99%), 21 were matched to genus level (cutoff 94.9%), and only one (*Latridiidae,* 93.59% matching) was matched to family level (cutoff 91%) (Table 3). The ZBJ primer described in Zeale et al (2011) yielded the highest diversity of sequences- with 7 species, 17 genera, 19 families, and 6 orders. Conversely, the Ant primer had 2 species, 6 genera, 3 families, and 3 orders. Four of these genera (*Calligrapha, Phediole, Solenopsis,* and *Zygogramm*) were only found using the Ant primer pair. Using the termite primer pair, we were able to identify 4 species, 5 genera, 4 families, and 5 orders. Three of these genera (*Conozoa,*

Eurosta, and *Gnathamitermes)* were only identified using the termite primer. The high number of families and genera identified with the ZBJ primer pair may simply be a result of higher numbers of clones. Based on the sampling curve of ZBJ, the number of new families discovered tapers off around 8 clones (Figure 8a). However, this could also be because few samples had more than 8 clones successfully sequenced. Conversely, all sequences using the ant primer were discovered using only 4 clones (Figure 8b). The sampling curve of the termite primer suggests that more taxa may have been discovered if more than 8 clones were sequenced (Figure 8c).

Table 3. Results of genetic sequencing methods.

Order	Family	Genus	Species	Number of Clones	Number of samples	Percent matched	Primer
Arachnida							
Araneae	Linyphiidae	*Tennesseellum*	*formica*	1	1	100%	ZBJ
Insecta							
Coleoptera	Anthribidae	*Trigonorhinus*	*alternatus*	3	1	100%	ZBJ
	Brentidae	*Kissingeria*	*nearamaurum*	4	1	96.15%	ZBJ
	Carabidae	*Colliuris*	*pensylvanica*	1	1	99.3%	ZBJ
	Carabidae	*Harpalus*	*reversus*	1	1	98.92%	Ter
	Carabidae	*Harpalus*	*affinis*	1	1	98.77%	ZBJ
	Carabidae	*Selenophorus*	*ellipticus*	1	1	98.72%	ZBJ
	Carabidae	*Selenophorus*	*pedicularius or planipennis*	47	8	97.87-100%	ZBJ, Ter
	Chrysomelidae	Unknown	unknown	1	1	96.79%	
	Chrysomelidae	*Stator*	*vittatithorax*	8	2	96.55%	ZBJ
	Chrysomelidae	*Zygogramma*	*conjuncta pallida*	6	2	96.79%	Ant
	Chrysomelidae	*Calligrapha*	*californica coreopsivora*	4	2	96.79%	Ant
	Curculionidae	*Hypera*	*postica*	8	2	99.32-100%	ZBJ
	Latridiidae	*Corticaria*	*rubripes*	1	1	93.59%	ZBJ
Diptera	Ceratopogonidae	*Culicoides*	*lahontan*	1	1	95.24%	ZBJ
	Culicidae	*Anopheles*	*atroparvus*	1	1	98.77%	ZBJ
	Lauxaniidae	*Homoneura*	Unknown	3	1	95.18%	ZBJ
	Psychodidae	Unknown	Unknown	1	1	97.59%	ZBJ
	Sciaridae	*Pseudolycoriella*	*bruckii*	1	1	96%	ZBJ
	Tachinidae	*Neomintho*	Unknown	1	1	96%	ZBJ
	Tephritidae	*Eurosta*	*solidaginis*	1	1	99.32%	Ter
Hemiptera	Unknown	Unknown	Unknown	10	2	93.59-100%	All
	Lygaeidae	*Neortholomus*	*scolopax*	1	1	99.36%	Ant
Hymenoptera	Crabronidae	*Cerceris*	Unknown	3	1	96.03%	ZBJ
	Formicidae	*Pheidole*	*BEB03*	2	2	95.24%	Ant
	Formicidae	*Pheidole*	*bicarinata*	1	1	95.33%	Ant
	Formicidae	*Phediole*	*diversipilosa*	3	1	100%	Ant
	Formicidae	*Solenopsis*	unknown	16	4	99.36-100%	Ant
Isoptera	Termitidae	*Gnathamitermes*	*tubiformans*	1	1	100%	Ter
Lepidoptera	Apatelodidae	*Apatelodes*	*torrefacta*	1	1	96%	ZBJ
	Noctuidae	*Feltia*	*subterranea*	1	1	100%	ZBJ
	Tortricidae	*Endothenia*	*gentianaeana*	9	1	96.15%	ZBJ
Orthoptera	Acrididae	*Conozoa*	*carinata*	1	1	95.06%	Ter

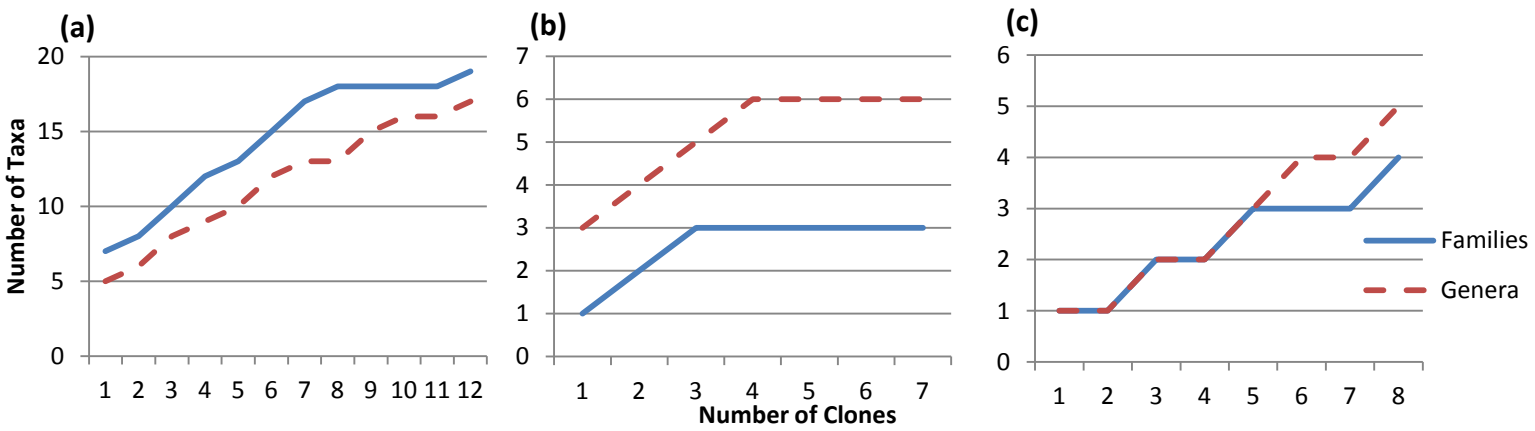

Figure 8. Sampling curves. Number of taxa identified per clone using (a) ZBJ, (b) Ant, and (c) termite primers.

Discussion

Using both morphology and DNA sequence data, we were able to detect a wide diversity of prey items, including ants, wasps, true bugs, beetles, weevils, moths, flies, grasshoppers/crickets, spiders, and termites. Of these, only ants, wasps, beetles, true bugs, and termites were identified using morphology, whilst all were detected with genetic methods. This was consistent with the findings of previous dietary studies, which found a wide diversity of prey, including coleopterans, orthopterans, hemipterans, and a variety of hymenopterans. (Blackshear and Richardson 1999, Lemos-Espinal et al 2004, Milstead and Tinkle 1969).

Although morphological analysis is useful for quantifying the abundance of prey items, it has limitations for species identification. Although exoskeletons are not completely broken down during digestion, our study confirmed that they are frequently broken apart. When identifying ant genera in particular, not having intact exoskeletons makes identifying genera particularly difficult, as they are commonly distinguished by very specific anatomical characteristics. In several cases, we were forced to try and

identify ants using only their heads. As a result, certain groups (particularly *Dorymyrmex* and *Aphaenogaster*) almost certainly contain multiple genera, since heads from a number of different genera are similar size and have similar morphological features. In some cases, uncertainty in morphology was resolved by DNA sequences. For samples that were both sequenced and sorted, we were able to successfully identify items observed in morphological analysis using our results from sequencing. This was especially useful for beetles, which had quantitatively few individuals, but produced a high number of sequences.

The diets of these populations appear to differ substantially from other populations, primarily because of the abundance of desert termites, which were not observed in previous studies (Blackshear and Richardson 1999, Lemos-Espinal et al 2004, Milstead and Tinkle 1969, Pianka and Parker 1975). There are a variety of reasons for why termites could be an attractive food source. Like harvester ants, termites are highly abundant in our study area. When foraging, they build long tubes of mud on the surface of the soil and grasses leading to sources of food (Nash et al 1999). When these tunnels break, it exposes them to predation, which could make them an easily accessible food source for horned lizards. Additionally, the exoskeletons of termites are less rigid than ants, and may be easier for horned lizards to digest. Furthermore, studies of desert termites show that there is a positive correlation between rainfall and desert termite activity (Nash et al 1999). According to the National Weather Service, Karnes County received substantially more rainfall in 2015 than normal. Thus, it is possible that

the high abundance of termites in the samples could simply be a result of high seasonal abundance, rather than any dietary or behavioral adaptation.

Conversely, harvester ants do not appear to be an essential part of the overall diet of the populations analyzed in this study, although they may still be essential to the diets of other populations. Previous surveys of the study sites have found that harvester ant density for Kenedy and Karnes City is similar to harvester ant density in rural areas (unpublished data, Crist and Wiens 1996). This suggests that the low number of harvester ants observed in this study is not due to low harvester ant abundance in the area. Additionally, it is possible that populations in Kenedy and Karnes City may act more opportunistically than other populations, depending on the availability of other prey species.

In general, horned lizard species are not known to prey upon introduced ant species, and other species of horned lizard can experience declines in body condition when they do (Suarez and Case 2002). Although imported fire ants (*Solenopsis invicta)* are not a traditional prey item of Texas horned lizards, they were observed in several samples, but only accounted for 1.3% of the prey items observed. Since they are small, the low abundance of fire ants suggests they were not eaten for dietary reasons. In all but one sample, there were less than 12 ants. This was consistent with the observations of Webb and Henke (2003), where horned lizards were observed eating fire ants in self defense when there were just a few ants. However, for larger numbers of ants, horned lizards would use methods such as flee-and bury, rather than consumption.

In this study, genetic sequencing was more effective than morphological analysis at identifying the diversity of prey items. Using three different primer pairs, we were able to detect significantly more taxa than morphological methods. However, the designed ant and termite primers were only marginally successful for their intended purpose. Although we designed the primer pairs to match known sequences, the primers may still have had a low affinity for ant and termite DNA, respectively. The ant primer only successfully amplified two genera of ant, *Solenopsis* and *Phediole*. Similarly, we were able to detect desert termites using DNA, but only from a blue colony, which would normally indicate the DNA insert was absent. However, both primers yielded several new taxa of prey than had not been observed with the original ZBJ primer. Additionally, these results are consistent with the fact that sequencing cannot be used as an indicator for relative abundance of prey items. As such, although genetic analysis is informative for taxa identification, it cannot be used to estimate relative importance of prey species, and should be used in conjunction with morphological analysis.

Conclusions

The results of this study suggest that genetic dietary analysis is more specific for identification of species than morphological analysis. However, morphological analysis is still necessary to accurately estimate the frequency and proportion of each species. The combination of both methods seems to give the most accurate depiction of the overall diet, since both methods discovered taxonomic groups which were not detected by the other.

One of the objectives of this study was to assess the diet of horned lizards in Kenedy and Karnes City. For these samples, desert termites were the most abundant prey item. It is uncertain if this diet is normal, or the result of seasonal overabundance of termites. In any case, the abundance of termites suggests that the diets of horned lizard populations in Kenedy and Karnes City are substantially different than those analyzed in previous studies. We were not able to detect a difference in the frequency of harvester ant or termite consumption between Kenedy and Karnes City. Our results also suggest that harvester ants are relatively insignificant in the diets of these populations, which could be the result of prey availability or unique dietary preferences of these populations.

A variety of further studies are needed to determine the significance of this study. The methods used in this study should be applied to samples from previous, drier years, to determine if there is a correlation between termite consumption and rainfall. Additionally, a variety of insect surveys should be conducted in Kenedy and Karnes City, especially with respect to termites, to determine if the abundance of prey items is indicative of their abundance in the study area. Furthermore, collecting field samples would allow construction of a morphological and genetic database, which would help substantially with identification. The designed ant and termite primers should be modified until they can consistently be used to identify ants and termites. This may require changing sequences of the primer pairs, or potentially targeting a different region of the cytochrome oxidase I gene. Lastly, a methodology needs to be developed to accurately estimate body volume of hymenopterans and isopterans based on head

size, so that volume estimations can be expanded to include other taxa, such as coleopterans and hemipterans.

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