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Research Article

Use of molecular scatology to assess the diet of feral cats living in urban colonies

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Abstract

The overpopulation of domestic cats (*Felis catus*) presents a serious concern for wildlife conservationists, animal welfare advocates, public health officials, and community members alike. In cities, free-ranging, unowned cats often form high-density groups (commonly called 'colonies') around human provisioned food sources. While previous diet studies have primarily utilized morphology-based methods, molecular techniques offer a higher resolution alternative. In this study, we used next-generation sequencing techniques to examine the diet composition of feral cats living in five Trap-Neuter-Return colonies located in urban parks on Staten Island, a borough of New York City. We hypothesized that (1) cats living in urban colonies would still consume natural prey despite being regularly fed and (2) that the composition of taxa represented in the diet of each colony would vary, possibly due to differences in prey availability across sites. In total, 16 vertebrate prey taxa were identified in the diet, 13 at the genus level and 3 at the family level. Despite being regularly fed, 58.2% of cat scats contained DNA from natural prey. The diet composition of the cat colonies differed depending on the land cover composition surrounding the colony with the frequency of native prey positively correlated with the proportion of green space and that of non-native prey with developed land cover types. The use of molecular techniques combined with environmental DNA methods offers a promising, non-invasive approach to assessing the diet and consequently, impact of a highly abundant and non-native predator on the persistence of wildlife communities in cities.

Key words: *Felis catus*, next-generation sequencing, molecular scatology, New York City, metabarcoding

Introduction

Domestic cats (*Felis catus*) are one of the world's most widely distributed invasive species, with feral populations established across almost all climate zones from tropical to subarctic (Ogan and Jurek 1997). With an estimated population size of over 100 million across the continental United States, the abundance of free-roaming cats introduces many ecological as well as economic challenges (Winter 2004; Doherty et al. 2017; Lepczyk and Duffy 2018). While any cat, owned or unowned, that is allowed access to the outdoors can and will depredate wildlife, stray and feral cats likely contribute to a substantial proportion of observed wildlife mortalities, introducing a significant challenge to wildlife conservation efforts (Medway 2004; van Heezik et al. 2010; Balogh et al. 2011; Bonnaud et al. 2011; Medina et al. 2011; Loss et al. 2013; Loyd et al. 2013; Maeda et al. 2019).

While many previous feral cat diet studies have concentrated on cats living on islands or in natural environments of high conservation concern (Bonnaud et al. 2011), fewer have focused on feral cats living in highly urban and human

manipulated [i.e. Trap-Neuter-Return (TNR) colonies] settings [but see Lepczyk et al. (2004); Baker et al. (2005, 2008); van Heezik et al. (2010); Thomas et al. (2012); Krauze-Gryz et al. (2017); Piontek et al. (2021) for examples of free-roaming, owned cat diet studies in urban settings]. In cities, widely-used feral cat management practices, such as TNR programs, result in the formation of high-density cat 'colonies' centered around human-provided, communal food sources and shelters (Levy and Crawford 2004). While it is often advertised that feeding reduces/eliminates a cat's reliance on prey (Dietary Biology: What Feral & Stray Cats Really Eat), cats are opportunistic hunters and are known to consume natural prey despite being regularly fed, placing increased stress on the wildlife communities that persist in cities (Woods et al. 2003; Baker et al. 2005; Loyd et al. 2013). In addition, high densities of cats associated with urban colonies may increase the risk of zoonotic transmission of disease—posing a risk to feline, wildlife and human health (Gerhold and Jessup 2013; Lepczyk et al. 2015). These effects may be further exacerbated by alterations to the spatial organization and movement patterns of cats induced by certain aspects of urban living. For example, in urban colonies where resource requirements are eliminated by food subsidies, individual cats may maintain much smaller home ranges and tolerate higher degrees of spatial overlap with other cats in the colony (Haspel and Calhoon 1989; Devillard et al. 2003, 2004; Kaeuffer et al. 2004). Thus, although resource provisioning in association with a TNR program is often presented as a management tool, the presence of colony structures may lead to rapid depletions in local prey species and increased transmission of zoonotic pathogens, resulting in serious consequences for the surrounding environment as well as the well-being of the cats.

Analyses of predator diets from fecal samples have traditionally relied on the morphological identification of undigested remains. However, morphological diet studies are limited by their inability to detect rare prey items, difficulties correctly classifying prey from reference skeletons and the potential to miss soft-bodied organisms, causing predation effort to often be underestimated (Shehzad et al. 2012). For example, previous studies have shown that the use of morphological techniques alone considerably underestimates the rate of consumption for certain taxa including birds as well as overall dietary diversity (Oja et al. 2017; Massey et al. 2021). Although DNA isolated from highly degraded scat may be lower quality, potentially inhibiting the accurate identification of some taxa, a comparison of the two methods revealed that use of molecular techniques results in fewer misassigned species compared to morphological approaches (Massey et al. 2021). In addition, morphological techniques become further limited when undigested remains are rare or entirely absent in the scat, which was common among the scat observed in urban parks (pers. obs L.D.P.). Recent attempts to use isotopic analyses to differentiate between natural prey and anthropogenic food in the diet of free-roaming cats have also had limited success due to high variation in C and N isotope values in cat food (Maeda et al. 2019; McDonald et al. 2020).

Next-generation sequencing techniques offer a higher resolution alternative to both isotopic and morphological approaches (Shehzad et al. 2012; Forin-Wiart et al. 2018). Additionally, the increased detection sensitivities of High-Throughput Sequencing methods require smaller sample sizes compared to traditional morphological approaches (Trites and Joy 2005; Casper et al. 2007). In this study, we aimed to quantify the frequency of occurrence (FOO) of vertebrate prey in the diet of five urban cat colonies on Staten Island, New York City (NYC) using a next-generation sequencing approach. We hypothesized that (1) cats living in urban colonies would still consume natural prey despite being regularly provided with anthropogenic food and (2) while the frequency of natural prey in the diet might be similar across the colonies, the composition of taxa represented in the diet of each colony would vary, possibly reflecting differences in prey availability associated with the dominant land cover type of the surrounding area (Krauze-Gryz et al. 2017).

Materials and methods

Site selection

Across NYC's five boroughs, there are an estimated 300 000 to 1 million unowned, free-roaming cats many of which reside in the city's 3100+ TNR colonies (Neighborhood Cats: Colony Database). Urban parks provide a refuge for the city's at least 140 formally designated rare species (McPhearson et al. 2013). With one-third of its acreage dedicated to parks/recreation, Staten Island, a borough of NYC, offered an ideal location to examine the relevance of natural prey to the diet of urban colony cats. Five TNR-managed colonies of feral cats (identified by the presence of multiple cats, evidence of cat feeding and through conversations with local cat colony caretakers) (Lepczyk et al. 2020) located in five separate urban parks within Community District 3 on Staten Island, NYC, were chosen to be part of this study. All protocols involving collection of samples from feral cats were approved by the Columbia University Institutional Animal Care and Use Committee (AC-AABA5461) and all state and local permits to collect samples from parks were obtained prior to sampling.

Sample collection

Fecal samples were collected from both constructed wooden sandboxes designed to mimic litter boxes and from communal latrines (Carrión and Valle 2018) and stored in individual plastic bags. Because the focus of the study was on the diet of free-roaming, unowned cats living in TNR-managed colonies and receiving resource subsidies, a fecal sample was collected if it met the following criteria: (1) resembled cat feces in size and shape, (2) buried under soil/sand/leaves, (3) located in the proximity of other samples meeting the same criteria and (4) discovered in the immediate vicinity of the cat colony. Sites were visited a minimum of three times—at least once between mid-July and mid-August, mid-August and mid-September and mid-September and the end of October 2019. During each collection, the area surrounding the colony was searched extensively to ensure that all scat from the period leading up to collection were removed and to maximize the probability of collecting recently deposited scat upon re-visit. During some collection events, the area surrounding the colony was searched but no scat was found—these sites were re-visited until a total of at least 25 scats distributed approximately evenly across the three collection periods (mid-July to mid-August, mid-August to mid-September and mid-September to end-October) were collected. Samples were stored at −80 or −20°C prior to DNA extraction.

Prey DNA extraction and amplification

DNA extraction was performed using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). Extraction followed the kit's protocol with the following minor adjustments made to ensure an optimal yield of DNA. Following the mixing of 150–

200 mg of each homogenized scat with 1 ml of inhibitEX lysis buffer (Qiagen, Hilden, Germany), all samples were incubated at 56°C for a minimum period of 1 h and up to 12 h for samples collected from sandboxes. Following incubation, samples were centrifuged for 4 min at 13 300rpm to effectively pellet the stool particles. In the final elution step, the samples were eluted in either 100 or 200 µl of elution buffer for a minimum of 15 min and up to 45 min. Concentrations of DNA in each extracted sample were measured using a qubit or spectrometer and samples with concentrations >2 ng/µl were considered for metabarcoding and sequencing. DNA extracted from each scat was stored in individual 1.5 ml tubes at −20°C for short-term storage and −80°C for long-term storage.

To analyze the vertebrate component of the cat diet, the V5 variable region of the mitochondrial 12S gene (73–110 bp) was amplified using a two-step PCR protocol and an approach adapted from De Barba et al. (2014) (Supplementary Table S1). Previous studies have shown that using this primer pair results in both a high barcode coverage index and barcode selectivity index and produces a relatively short fragment, making it a suitable amplification target for degraded DNA (Riaz et al. 2011). The first of the two PCRs amplified an ∼100 bp amplicon of the V5 region of the 12S vertebrate gene through the addition of 12SV5 primers modified in 5′ with the addition of a partial overhang Illumina adapter while the second PCR attached unique barcode sequences to the Illumina adapters attached in the first PCR.

The initial vertebrate amplicon PCR was carried out in a 12.5 µl reaction volume consisting of 6.25 µl KAPA HiFi HotStart ReadyMix, 2 µl DNA, 0.625 µl 10 mM 12SV5 F primer, 0.625 µl 10 mM 12SV5 R primer, 2.5 µl 20 mM blocking oligonucleotides to reduce the prevalence of cat DNA and 0.5 µl PCR grade water. The PCR was run with an initial denaturation step of 3 min at 95°C followed by 40 cycles of 30 s at 98°C, 30 s at 58°C, 10 s at 72°C and a final elongation step for 1 min at 72°C. A PCR negative control (NC) was included in all amplifications to check for potential contaminants. PCR reactions were subjected to gel electrophoresis (2% agarose) at 120 V for 40 min and products of the desired length (∼150–200 bp) were purified using magnetic beads following a previously described protocol (Rohland and Reich 2012) prior to metabarcoding. For two of the vertebrate PCR runs, the NCs produced an amplified product, and as a result, both NCs were included in all following steps and sequenced. For a subset of samples, gel electrophoresis showed non-specific binding of the 12SV5 primers to larger segments of DNA. These samples were re-amplified using the same protocol but with a higher annealing temperature (60°C) to promote more site-specific binding. The NC from this additional PCR run amplified but not to a sufficient concentration for sequencing.

The purified products from the vertebrate amplicon PCR were used as the DNA template for the indexing PCR, which was performed to add unique Illumina XT Nextera indices to Illumina adapter sequences. The index PCR was carried out in a 25 µl reaction volume with 12.5 µl Kapa Hifi Hotstart ReadyMix, 2.5 µl of each Nextera XT index, 2.5 µl DNA template and 5.0 µl PCR grade water. The index PCR was performed with an initial denaturation step of 95°C for 3 min followed by 8 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and a final extension step at 72°C for 5 min. A NC was included in all amplifications to check for potential contaminants. Index PCR products were purified using the aforementioned protocol and attachment of Illumina indices was confirmed by gel electrophoresis on a subset of samples prior to pooling. The concentrations of each sample were measured and recorded using a spectrometer. As spectrometers may overestimate concentrations of DNA, each sample was normalized to a 25 µl volume at 60 nM before 10 µl of each normalized sample was pooled together and mixed thoroughly by pipetting to ensure a high concentration of DNA from each sample in the pool. A 100 µl aliquot was then taken from the pooled sample and sequenced on a MiSeq instrument by paired-end sequencing with a 30% Phi-X spike-in (Genewiz inc., South Plainfield, NJ).

Data processing

Results from sequencing were demultiplexed by Genewiz Inc. (South Plainfield, NJ) and fastq files were processed using the OBITOOLS software, version 1.2.11 (Boyer et al. 2016). Using the 'illuminapairedend' program, the forward and reverse sequences were aligned. Aligned sequences with a quality score <40 were removed using the 'obigrep' program and the 12SV5 primers were trimmed from either end of each read using the 'cutadapt' program (Martin 2011). Unaligned sequences were then filtered out and unique sequences were clustered together using the 'obiuniq' program. Sequences shorter than 73 bp or with a total count of ≤5 occurrences were removed using the 'obigrep' program and PCR and sequencing errors were detected using the 'obiclean' program.

The remaining sequences were matched to reference sequences in a database compiled using sequences of target vertebrate taxa from the European Molecular Biology Laboratory and National Center for Biotechnology Information (NCBI) taxonomy databases using the 'ecotag' command in OBITOOLS. Taxonomy for sequences that were not identified to the order and/or family level was resolved via submission to NCBI's megaBLAST tool for taxonomic assignment. The top 10 results per sequence were downloaded and filtered to only include results that had a 95% identity score or higher and query coverage of 89% or greater. Using the R package 'taxonomizr' version 0.5.3 (Sherrill-Mix 2019), accession numbers for each filtered result were converted into taxonomic IDs from which the taxonomy was extracted. Taxonomic assignments for each result were condensed into a single vector with 'Not Applicable' assigned to samples with disagreements between hits. For a subset of sequences that were not identified down to the family level, taxonomy was manually resolved by submitting each sequence individually to NCBI's megaBLAST tool and filtering the results to only include those with a 95% or higher identity score and 89% or higher query cover. If multiple taxa were returned, results were examined for congruency and taxonomy was assigned on the basis of agreement among results, FOO and biological relevance. The cleaned dataset was then filtered to only include reads from vertebrates with a 96% or higher identity match to a reference sequence and a minimum of 10 occurrences across the entire dataset (Coghlan et al. 2012; Sigsgaard et al. 2017).

The number of reads per sample was summed from the raw dataset and merged to the clean dataset. Reads from the same order, family and genus for each sample were then grouped together and summed. If the proportion of summed reads at the order, family and/or genus level represented at least 1% of the total raw reads for the sample, then the corresponding taxon was assumed to be from the diet and not a result of environmental contamination (Elfström et al. 2014). Reads from taxa not meeting the 1% threshold were discarded from subsequent analyses. Diet items that could be attributed to any

anthropogenic source including cat food (i.e. chicken, turkey, beef and fish) or garbage were grouped together and renamed 'catfood'.

Between the two NCs, sequences from three sources met the 1% threshold: cat food, human and cat DNA (Fig. 1 and Supplementary Table S2). In one NC, 95.2% of the reads were from a human source, 1.39% were from a felid and 1.13% were from cat food. Of the 1.13% from cat food, the number of reads from each source (Galliformes and Clupeiformes) did not independently meet the 1% threshold for inclusion. In the other NC, 38.95% of the reads were from a human source and 60% were from cat food, specifically chicken. Because the 12SV5 primers are universal primers and sensitive to all vertebrate DNA, contamination is difficult to control even with good lab practices. While it is possible that the cats may have scavenged on human carcasses, due to its presence in the NC, sequence data matching to human was assumed to have resulted from contamination during collection or environmental contamination and was excluded from the analysis. The presence of sequence data matching to cat food items in the NCs did not hinder future analyses as all cats were fed at least daily and assumed to consume cat food. The presence of cat sequences in the NCs did not affect the diet analyses either as cat DNA was only used to identify the samples as being from cats, in addition to the other aforementioned criteria. As a result, only reads from humans were discarded at this step.

In some samples, DNA from potential non-cat sources (i.e. raccoons, opossums, deer, etc.) met the 1% threshold (Fig. 1 and Supplementary Table S2). While the cats may have fed on the carcasses of some of these species, these taxa are also known to frequent the feeding stations and scavenge in the nearby latrines (pers. obs L.D.P.), making environmental contamination a high probability. However, because the feces were collected only if they met the aforementioned criteria, the presence of sequences from non-target taxa was assumed as environmental contamination and samples were only excluded if they lacked cat DNA entirely.

Diet composition analysis—between colony variations at the family/genus level

The FOO of each diet item was calculated as the number of occurrences of the diet item (maximum of 1 occurrence per sample) divided by the total number of sequenced cat scats. The FOO of each diet item was also calculated at the colony level as the number of occurrences of the diet item divided by the number of cat scats sequenced from the colony.

A Permutational Multivariate Analysis of Variance (PERMANOVA) test was used to determine whether there were significant differences ($P < 0.05$) in diet composition between the five colonies based on the Jaccard dissimilarity of samples containing cat food and/or a prey ($n = 130$) set with 999 random permutations. To confirm that the calculated significance was due to differences between the colonies and not within colony heterogeneity, a dispersion test was performed. The PERMANOVA test and dispersion test were completed using the

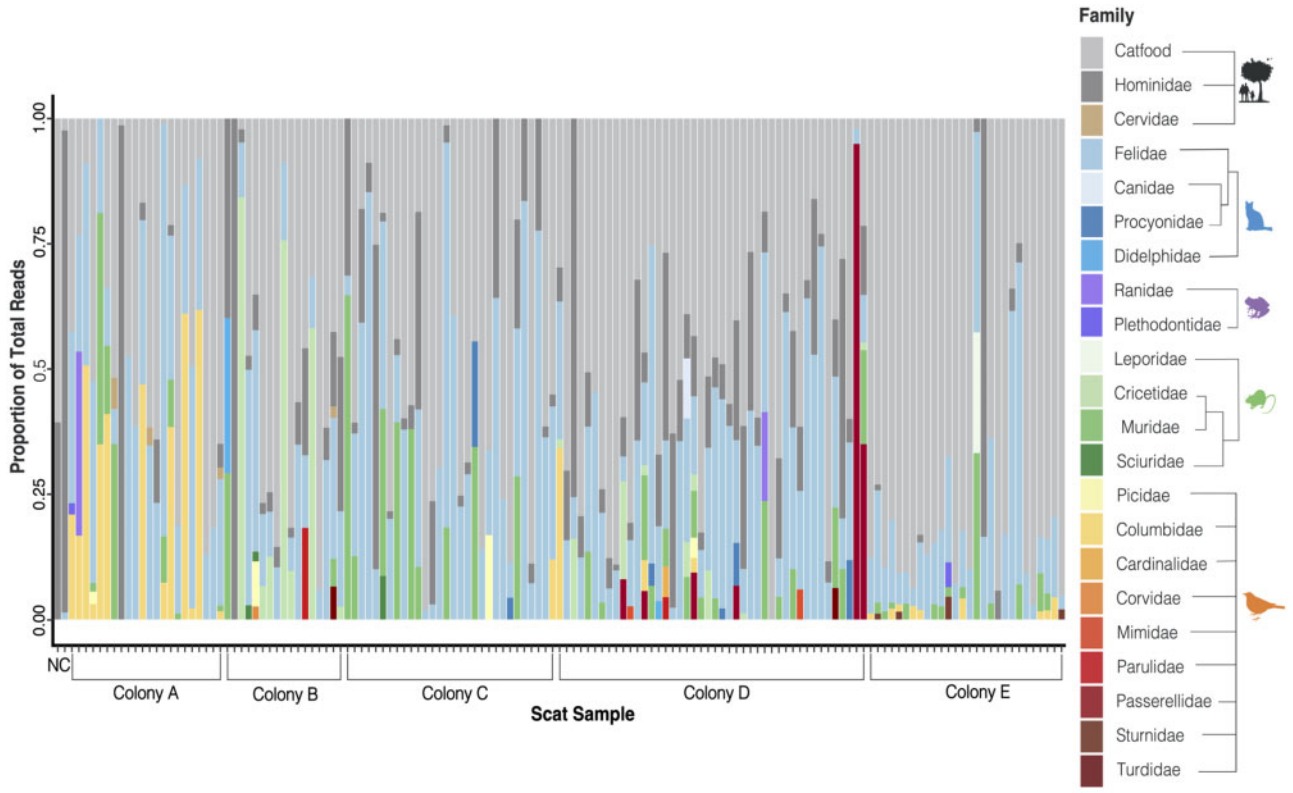

Figure 1: Proportion of sequence reads (>1%) per scat and NCs belonging to the lowest shared taxonomic unit among all reads (family)
Not including taxa related to cat food, 21 families representing at least 22 genera were detected in the scats. Each scat is indicated by a tick mark along the X-axis and scats collected from the same colony (A–E) are grouped together by brackets. In subsequent analyses, reads belonging to cat (Felidae), human (Hominidae), deer (Cervidae), opossum (Didelphidae), raccoon (Procyonidae) or dog (Canidae) were removed resulting in a total of 16 prey taxa remaining, 13 of which were resolvable to the genus level and 3 of which were identifiable only to the family level. A breakdown of the proportion of reads per scat belonging to the lowest resolvable taxonomic unit and separated by taxonomic grouping (amphibians, birds, medium-sized mammals and small mammals) is provided in the supplementary Fig. S1.

Table 1 Number of scat samples collected from each cat colony during each collection period (mid-July to mid-August, mid-August to mid-September, mid-September to end-October) and number of scats that were successfully sequenced from each collection

	Colony A			Colony B			Colony C			Colony D			Colony E		
	Date	# Collected	# Sequenced	Date	# Collected	# Sequenced	Date	# Collected	# Sequenced	Date	# Collected	# Sequenced	Date	# Collected	# Sequenced
Mid-July to mid-August	7 August 2019	11	8	22 July 2019	1	1	13 August 2019	13	10	8 July 2019	5	5	8 August 2018	13	9
										11 July 2019	2	2	14 August 2019	5	4
				14 August 2019	6	4				29 July 2019	4	3			
Mid-August to mid-September	21 August 2019	10	8	22 August 2019	3	1	23 September 2019	9	9	5 September 2019	18	18	19 August 2019	9	9
Mid-September to end-October	11 October 2019	14	6	11 October 2019	9	5	11 October 2019	6	6	11 October 2019	11	10	23 September 2019	7	6
				5 November2019	7	6	5 November 2019	5	5	5 November 2019	7	6			
Total (collected/sequenced)		35/22			26/17			33/30			47/44			34/28	

R package 'vegan' version 2.5-6 (Oksanen 2019). To identify prey taxa significantly ($P < 0.05$) affiliated with the diet of each colony, a species indicator analysis was run with 9999 random permutations using the R package 'Indicspecies' version 1.7.8 (Caceres 2020). This test identified significant correlations between certain taxa and colony groupings by determining the point biserial correlation coefficient, which is mathematically equivalent to the Pearson correlation coefficient.

Diet composition analysis—representation of non-native vs. native prey

The FOO of non-native prey in the diet of each colony was calculated as the number of occurrences of non-native prey, which included the family *Columbidae* and the genera *Mus* and *Sturnus*, divided by the total number of prey occurrences in the cat scat sequenced from each colony. The FOO of native prey in the diet of each colony was calculated as the number of occurrences of all remaining native prey taxa divided by the total number of prey occurrences in the samples sequenced from the colony. PERMANOVA, dispersion and species indicator tests were performed following the steps outlined above to determine whether there were significant ($P < 0.05$) differences in the representation of non-native and native prey types among the diet of the colonies.

To assess possible relationships between the land cover of the surrounding area and the representation of non-native vs. native prey in the diet (hypothesis 2), the representation of different land cover types within a 400 m radius of the colony feeding station was extracted from the 2016 National Land Cover Database (NLCD), a 30-m resolution raster dataset that classifies land cover into 16 classes based on a modified Anderson Level II Classification System (Homer et al. 2020) . Analyses were conducted in a projected geographic coordinate system, EPSG 2263 North American Datum of 1983/Universal Transverse Mercator zone 18 N (NAD83/UTM zone 18 N) using Google Earth Engine (Gorelick et al. 2017). The 400 m buffer was chosen based on the previously reported home range size and maximum distance observed from the colony feeding site for cats living in similar urban colony structures (Haspel and Calhoon 1989; Nutter 2005; Pillay et al. 2018). Two derived land cover classes were obtained by merging the extracted 2016 NLCD land cover types—the proportion developed land, calculated as the sum of the proportion of developed (low intensity), developed (medium intensity) and developed (high intensity) NLCD land cover classes and the proportion green space, calculated as the sum of the proportion developed (open space), deciduous forest, woody wetlands, emergent herbaceous forest, barren land (rock/sand/clay) and shrub/scrub NLCD land cover classes.

Diet composition analysis—rarefaction analysis and assessment of dietary diversity

To evaluate the validity of the sampling effort represented by the composition of each colony's diet, incidence-based rarefaction and extrapolation curves were constructed using sample size-based methodologies. Using a taxa-by-sampling-unit matrix with the sampling unit defined as a single scat, rarefaction curves were constructed using the iNext software in R (Chao et al. 2016) and parameterized to estimate taxon richness per site with respect to sample size. The extrapolation was extended to 45 samples to account for both the smallest and largest sample sizes and set with 100 replicate bootstrapping runs to estimate 95% confidence intervals. Estimates of taxon

richness were calculated based on the methods proposed in Chao (1984, 1987) using the ' ChaoSpecies' function in the 'iNext' package in R.

Results

Sample collection and DNA extraction/amplification

Scat was collected from each colony on a minimum of 3 and maximum of 6 occasions between July and November 2019 with a minimum of 1 and maximum of 18 scats collected per date (Table 1). The total number of scats collected from each colony ranged between 26 and 47 (Table 1). DNA was extracted from 175 scats and 141 scats (80.6%) and 2 NCs amplified to sufficient concentrations and were successfully sequenced (Fig. 1). The proportion of samples collected that were successfully sequenced from each colony ranged between 62.9% and 93.6% (Table 1). Following read filtering/cleaning and taxonomic assignment, seven samples were identified as non-cat and removed resulting in 134 total scats included in subsequent diet analyses (Supplementary Table S2).

Overall diet composition

Not including the taxa that could be contributed to cat food (i.e. cow, chicken and fish), the cat scat contained DNA from 22 different vertebrate taxa, 16 of which were determined as probable prey items (i.e. not due to environmental/human contamination or possible scavenging on carcasses) (Fig. 1). Of the 16, 13 were identifiable to the genus level and 3 were only resolvable to the family level. In total, 58.2% of scat samples contained DNA from at least one of the 16 prey taxa. Cat food was present in 94.3% of the scat samples and the number of prey items per scat ranged from zero to five. Colony D (10 taxa) and colony B (7 taxa) had the most taxon rich diets while colonies A (5 taxa), C (4 taxa) and E (5 taxa) had the least taxon rich diets. The incidence-based rarefaction curves reached an asymptote for colonies A, C and E between 20 and 30 samples indicating sufficient sampling effort for these sites (Supplementary Fig. S2a). However, the curves for colonies B and D did not reach an asymptote with an estimated 12 taxa missing (Supplementary Fig. S2a). The extrapolated curves predicted that colony B's diet was the most taxon rich followed by colony D. The diets of colonies A, C and E were predicted to be both similarly taxon rich as well as less taxon rich overall. However, overlap between the confidence intervals of the extrapolated curves suggest that further analysis is needed to confidently state differences. The estimated sample coverage for colonies A, C, D and E were in excess of 90% indicating that the most abundant taxa were likely present in the samples analyzed (Supplementary Fig. S2b). Sampling effort for colony B was not as thorough with a sample coverage just below 85% (Supplementary Fig. S2b).

Diet composition by colony at family/genus level

Across all sites, natural prey occurred at similar frequencies in the diet (Table 2) (colony A = 54.5%, colony B = 48.3%, colony C = 29.4%, colony D = 48.4% and colony E = 52.8%). The PERMANOVA test confirmed statistically significant dissimilarity between the compositions of prey taxa in the diet of each colony (PERMANOVA $F_{4,125} = 5.1417$, $P < 0.001$). The significant variation was because of between colony variation and not within colony dispersion ($F_{4,125} = 1.391$, $P = 0.249$). The presence of *Peromyscus*, *Sciurus*, *Sturnus*, *Melospiza*, Columbidae or *Mus* contributed significantly to the dissimilarity between colonies with the presence of *Peromyscus* (%FOO, B = 46.7%; Species Indicator Test, rphi = 0.485, $P < 0.05$) (Table 3) and *Sciurus* (%FOO, B = 13.3%; Species Indicator Test, rphi = 0.27, $P < 0.05$) (Table 3) associated with colony B, presence of *Sturnus* (%FOO, E = 14.8%; Species Indicator Test, rphi = 0.36, $P < 0.05$) (Table 3) correlated with colony E, presence of *Melospiza* correlated with colony D (%FOO, D = 13.9%; Species Indicator Test, rphi = 0.34, $P < 0.005$) (Table 3), presence of Columbidae associated with colonies A and E (%FOO, A = 61.9%; %FOO, E = 33.3%; Species Indicator Test, rphi = 0.53, $P < 0.005$) (Table 3) and presence of *Mus* associated with all colonies except colony B (%FOO, A = 38.1%; %FOO, C = 25%; %FOO, D = 34.9%; %FOO, E = 48.2%; Species Indicator Test, rphi = 0.27, $P < 0.05$) (Table 3). With the exception of colony D, colonies with a high FOO of the genus *Mus* had no occurrences of the other mouse genus, *Peromyscus* (Fig. 2b and Table 3). Similarly, a high frequency of the family Columbidae was also paired with a low or no frequency of song birds (Fig. 2b and Table 3).

Diet composition by prey type (native vs. non-native)

Overall, the scat samples contained DNA from 3 non-native prey taxa, including the genera *Mus* and *Sturnus* and the family Columbidae, and 13 native prey taxa. While some of the Columbidae sequence reads may have been from native mourning doves (*Zenaida macroura*), of the eight scats containing reads from the family Columbidae, seven had a sub-threshold number of reads identified to the genus *Columba* and none had reads matching to the genus *Zenaida*. Thus, sequences belonging to this family were assumed to be from the genus *Columba* of which only non-native *Columba livia* (feral pigeon) are found in New York (Supplementary Table S3). In total, non-native prey was present in 44.03% of the fecal samples and native prey occurred in 25.4% of the scat samples. Of the samples containing native prey taxa, 11.8% contained DNA from two different native prey taxa and 5.9% had occurrences of three different native prey taxa totaling 42 occurrences of native prey.

The PERMANOVA test revealed statistically significant dissimilarity between the frequency of anthropogenic food, non-native prey and native prey in the diet of each colony

Table 2: %FOO of natural prey (not including cat food or taxa that may have been present due to environmental contamination or scavenging), avian prey, small mammal prey, non-native prey and native prey in the diet of each colony (A–E)

Colony	% frequency of occurrence				
	Natural prey	Avian prey	Small mammal prey	Non-native prey	Native prey
A	54.5	58.3	33.3	87.5	12.5
B	48.3	28.6	71.4	7.1	92.9
C	29.4	20	80	80	20
D	48.4	37.5	60	45	55
E	52.8	46.4	50	92.9	7.1

Table 3: %FOO of each prey taxon in the diet of each colony (A–E)

Diet item				Frequency of occurrence (%)				
Group	Family/genus	Most likely species	Prey type	A $n=21$	B $n=15$	C $n=28$	D $n=43$	E $n=27$
Other	Cat food	NA	Anthropogenic	95.2	100	85.7	97.7	92.6
Birds	*Columbidae*	*Columba livia* (feral pigeon)	Non-native	61.9	0	3.57	6.97	33.3
	Sphyrapicus	*Sphyrapicus varius* (yellow-bellied sapsucker)	Native	4.8	6.7	0	2.33	0
	Colaptes	*Colaptes auratus* (Northern flicker)	Native	0	0	3.6	0	0
	Turdus	*Turdus migratorius* (American robin)	Native	0	6.7	0	2.3	0
	Parulidae	Unknown warbler	Native	0	6.7	0	2.3	0
	Melospiza	*Melospiza melodia* (song sparrow)	Native	0	0	0	13.9	0
	Cardinalis	*Cardinalis cardinalis* (Northern cardinal)	Native	0	0	0	2.3	0
	Corvidae	*Cyanocitta cristata* (blue jay)	Native	0	6.7	0	0	0
	Dumetella	*Dumetella carolinensis* (gray catbird)	Native	0	0	0	4.7	0
	Sturnus	*Sturnus vulgaris* (European starling)	Non-native	0	0	0	0	14.8
Small mammals	*Mus*	*Mus musculus* (house mouse)	Non-native	38.1	6.7	25	34.9	48.2
	Peromyscus	*Peromyscus leucopus* (white-footed mouse)	Native	0	46.7	0	20.9	0
	Sciurus	*Sciurus carolinensis* (eastern gray squirrel)	Native	0	13.3	3.6	0	0
	Sylvilagus	*Sylvilagus floridanus* (Eastern cottontail)	Native	0	0	0	0	3.7
Amphibians	*Desmognathus*	*Desmognathus fuscus* (Northern dusky salamander)	Native	4.8	0	0	0	3.7
	Lithobates	*Lithobates catebeianus* (American bullfrog)	Native	4.8	0	0	2.3	0

Prey taxa (not including cat food or taxa that may have been scavenged or present due to environmental contamination) were classified as either non-native prey or native prey. The most likely species was determined by examining the number of species in each genus present in New York (Supplementary Table S3). The frequency of occurrence of each prey taxon was calculated as the number of sequenced cat scats from a colony containing that taxon divided by the total number of sequenced cat scats from the colony (n).

(PERMANOVA $F4{,}125 = 7.85$, $P < 0.001$). The dispersion test confirmed that the significance detected by the PERMANOVA test was due to between colony variation and not within colony dispersion ($F4{,}125 = 2.44$, $P = 0.2$). The presence of non-native prey or native prey contributed significantly to the dissimilarity between colonies with the presence of non-native prey associated with the diet of cats residing in colonies A and E (%FOO, A = 87.5%; %FOO, E = 92.9%; Species Indicator Test, rphi = 0.515, $P < 0.0001$) (Fig. 3a and Table 2) and the presence of native prey correlated with the diet of cats living in colonies B and D (%FOO, B = 92.9%; %FOO, D = 55%; Species Indicator Test, rphi = 0.48, $P < 0.0001$) (Fig. 3a and Table 2). No significant associations were found between cat food and any given colony, which was expected as every colony consumed cat food at a high frequency.

Our assessment of the land cover composition in the 400 m area surrounding each colony showed that the dominant (>50%) land cover type differed between colonies. Developed land was the dominant land cover type surrounding colonies A, C and E (A = 72.47%, C = 77.74%, E = 70%) (Fig. 3b and Supplementary Table S4) while green space dominated the area surrounding colonies B and D (B = 56.53%, D = 70.1%) (Fig. 3b and Supplementary Table S4). Colonies A, C and E also consumed non-native prey more frequently than native prey whereas the opposite was true for colonies B and D (Fig. 3a and Table 2). However, due to the limited number of colonies included in this study ($n = 5$ colonies), we were unable to identify statistically significant correlations between %FOO native vs. non-native prey and the dominant land cover type surrounding each colony.

Discussion

In support of our first hypothesis, we found that despite being provisioned with food on a regular basis, urban colony cats will still supplement their diet with natural prey. In support of our second hypothesis, the FOO of certain taxa varied between scat collected from different colonies, with non-native taxa more abundant in scat collected from colonies A, C and E and native prey more common in scat collected from colonies B and D. We also noted that green space was the dominant land cover type in the area surrounding colonies that more frequently consumed native prey (B and D) whereas non-native prey was more common in the diet of colonies located in more heavily

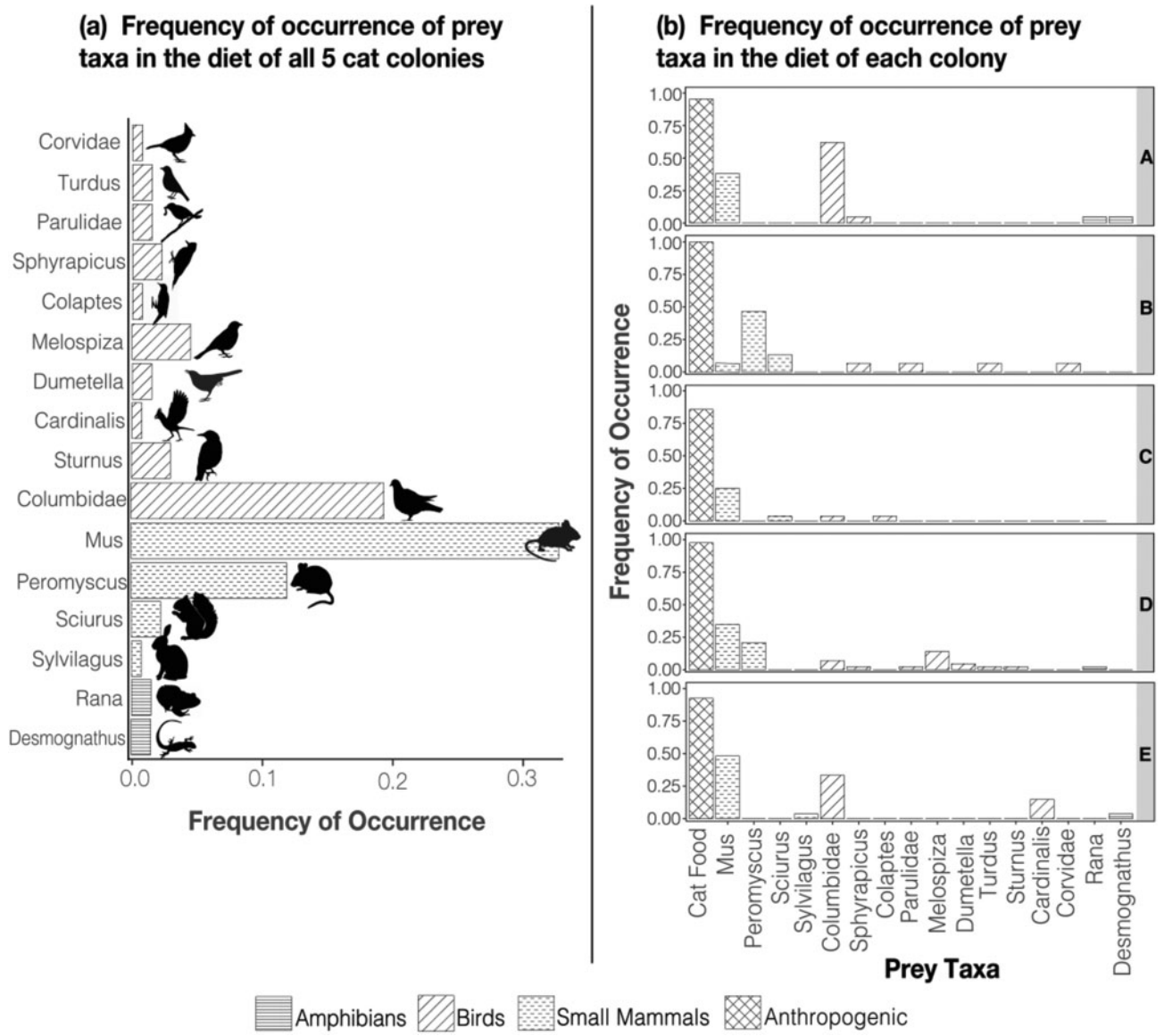

Figure 2: FOO of prey taxa in the diet of all colonies combined (**a**) and separated by colony affiliation (**b**, A–E)

Prey taxa were identified to the family/genus level and patterned by taxonomic group. While a cat could consume multiple individuals belonging to the same prey taxon, the number of occurrences/prey taxon/scat was limited to a maximum of one occurrence to avoid potential biases in sequence amplification.

developed areas (A, C and E). We suggest that these differences may in part be explained by variations in the prey community composition of each park related to differences in the land cover surrounding each colony. However, because of the limited number of colonies included in our study, we were not able to test these trends statistically. Future studies should incorporate the abundance of native vs. non-native prey present in different land cover types across the urban landscape to assess how a colony's location may predict its risk to local biodiversity.

While the process of urbanization is often assumed to result in biotic homogenization, research on rodent (Cavia et al. 2009) and avian (Aronson et al. 2014) communities indicate that urban areas harbor diverse species assemblages, with vegetation structure serving as a strong determinant of a city's ability to support a multiplicity of fauna (Sims et al. 2007; McKinney 2008; Faeth et al. 2011; La Sorte et al. 2020). Thus, the presence of high-density cat colonies near/in urban green spaces is of particular concern when seeking to maintain and promote a city's biodiversity (Lepczyk et al. 2020). As opportunistic predators, the diet of cats generally reflects the composition and availability of the different prey species in the area (Duffy and Capece 2012; Loyd et al. 2013; Széles et al. 2018). We found that variations in the diet composition of cats are present even among colonies located in the same urban neighborhood and predictably reflect our understanding of urban rodent and avian community dynamics, which are broadly influenced by each prey taxon's response to varying degrees of urbanization (Nupp and Swihart 1996; Anderson 2003; Crooks et al. 2004; Wilder and Meikle 2005; McKinney 2006; Gomez et al. 2008; Palacio et al. 2018). Thus, the identification of high-risk zones to inform initial targets for removal and/or increased surveillance to prevent colony establishment may be possible using predictors, such as

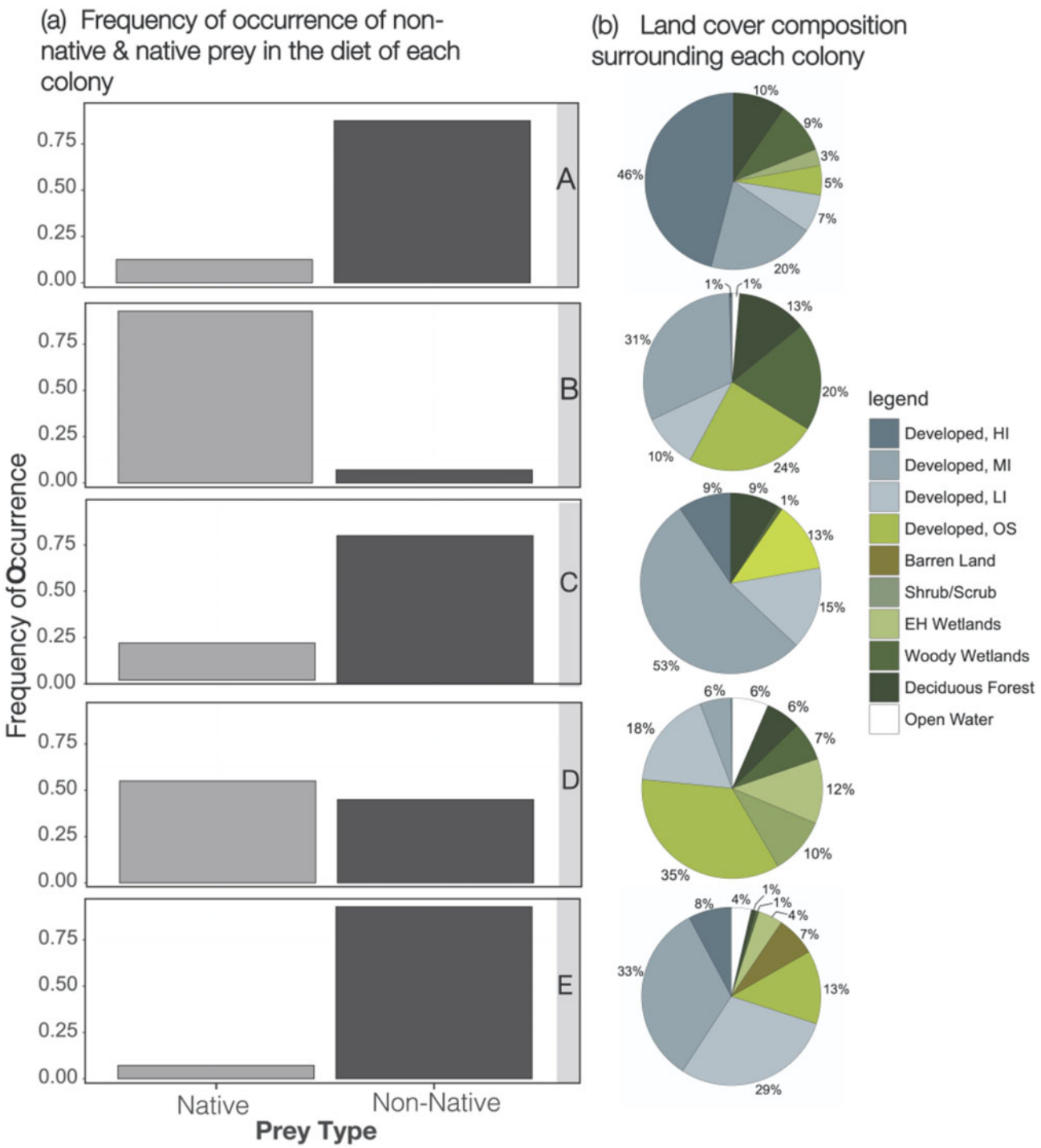

Figure 3: FOO of non-native vs. native prey in the diet of each colony (**a**) alongside the land cover composition of the area surrounding each colony (**b**)

The FOO of each prey type was calculated as the number of occurrences of native or non-native prey divided by the total number of prey occurrences from each colony (A–E). Pie charts represent the proportion of each land cover class from MLRC's 2016 NLCD Land Cover database within a 400 m radius of the colony's feeding station. Developed land cover types are colored in shades of blue–gray and abbreviated as follows: HI—high intensity, MI—medium intensity, LI—low intensity. Land cover types associated with green space/natural areas are colored in shades of green/brown and abbreviated as follow: OS—open space, EH—emergent herbaceous. Proportions are rounded to the nearest whole percent.

colony diet composition, surrounding habitat type and the predicted abundance of different prey species associated with certain habitat types.

While our study design was effective at characterizing the diet of a small sample of urban cat colonies, additional data on the colony age structure and size, local prey community composition and abundances, and the diet of additional colonies would allow for a more robust determination of the factors influencing diet composition. In addition, patterns of seasonal prey abundance (i.e. avian seasonal migratory patterns) are also

likely to impact cat diet composition, and our sampling effort was limited to late-summer/fall in the northern hemisphere. Thus, additional sampling over multiple seasons should be incorporated into future studies (Krauze-Gryz et al. 2017). Furthermore, our study was limited to investigate the vertebrate component of cat diet and it is important to note that cats may also hunt and consume invertebrates (Dickman and Newsome 2015).

While molecular methods offer a promising advancement to diet analyses, prey quantification via molecular technology remains difficult and the number of sequences cannot be used as a reliable metric for quantity due to biases in sequence amplification (Shehzad et al. 2012; Zarzoso-Lacoste et al. 2016; Forin-Wiart et al. 2018). As a result, molecular methods are limited to estimate the FOO of certain prey species and cannot provide reliable estimates of prey biomass or volume, which are needed to approximate the relative importance of a prey item to the diet (Klare et al. 2011; Hervías et al. 2014). Therefore, our approach was restricted to estimating the presence or absence of vertebrate prey in each scat. Presence/absence data may either result in an underestimation of the relevance of a prey species to the diet as each occurrence is assumed to represent a single predation event or may overestimate the relevance to the diet if DNA from the same predation event is reflected in multiple scats. To minimize the risk of potentially overestimating the relevance of certain prey genera to the diet, samples were collected at multiple intervals and multiple locations over a 5-month period. However, a more thorough, although costly, approach would be to sequence unique molecular markers and determine the number of samples deposited by the same individual allowing for confirmation that the time between collections was sufficient to allow for complete digestion as well as to verify the number of unique cats represented by the sampling effort (Menotti-Raymond et al. 1999). In addition, while molecular methods, morphological techniques and isotopic analyses all provide an estimate of the consumption frequency of different prey items, an understanding of the abundance of the prey (availability) is also needed to assess the importance of different prey to the predator's diet as well as the overall effect of predation on the prey population.

In conclusion, the use of molecular scatology to determine the diet of feral cats offers a promising approach to addressing the impact that these highly abundant and invasive predators have on urban ecosystems. While previous studies have quantified the diet of feral cats by classifying undigested prey remains based on morphology, diet items are often sorted into broad categories, such as rodents, birds and amphibians (Yip et al. 2014). Although there are benefits to this method (i.e. estimating prey biomass), the technique is also prone to missing rare prey items, underestimating prey diversity and misclassifying prey taxa (Zarzoso-Lacoste et al. 2016; Oja et al. 2017; Massey et al. 2021). While feral cats are a well-established threat to biodiversity, reliance on these traditional methods may severely underestimate the risk they pose to wildlife. This limitation is of particular concern in urban settings where the identification of undigested remains in scat may be obscured by an overabundance of anthropogenic food in the diet. Molecular approaches not only improve the resolution of diet analyses by allowing for genus and even species level classifications but, with the addition of universal primers and metabarcoding techniques, are also capable of simultaneously detecting multiple taxonomic groups of organisms from many scats at a relatively low cost (Shehzad et al. 2012; Forin-Wiart et al. 2018; Massey et al. 2021).

Our findings show that urban colony cats consume native wildlife in city parks despite being regularly fed. Thus, it is vital that policies regarding the management of NYC's feral cat population and/or the conservation of the city's wildlife communities consider the impact that these highly abundant predators likely have on the surrounding environment despite the efforts of current management programs (i.e. TNR, resource provisioning).

Supplementary data

Supplementary data are available at *JUECOL* online.

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Conflict of interest statement. None declared.

Data availability

The datasets generated and analyzed as part of this study are available from the corresponding author upon request.

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