Interspecific competition in bats and diet shifts in response to white-nose syndrome

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Abstract. Since the introduction of white-nose syndrome (WNS) in North America, numerous species of bat have dwindled in numbers. These declines observed are often species-specific and thus provides opportunity for a natural experiment to test for shifts in diet through relaxed resource partitioning in bat communities post-introduction of WNS. Acoustic monitoring at locations in Southern Ontario pre- (2009–2011) and post-WNS (2012–2014) introduction showed an increase in activity of big brown bats (Eptesicus fuscus) corresponding to a decline in the activity of little brown bats (Myotis lucifugus). Next-generation sequencing of bat stomachs and guano in Southern Ontario before and after WNS allowed for the characterization of diet changes of these species. Post-WNS, E. fuscus consumed a wider breadth of prey and many of the insect species once consumed by M. lucifugus, including several pest insects. These results suggest that the introduction of WNS has resulted in relaxed interspecific competition within these bat communities in Southern Ontario.

Key words: bats; chiroptera; diet; Eptesicus; exploitative; guano; insectivore; interspecific competition; Myotis; white-nose syndrome.

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INTRODUCTION

Interspecific competition is a driving mechanism in the diversification of species and has remained a focal topic in ecology and evolutionary biology (Meyer and Kassen 2007, Terborgh 2015). For decades, competitive interactions have been investigated in theoretical and empirical studies and are a key consideration in wildlife management strategies (Fryxell et al. 2014). The Lotka-Volterra model of competition predicts that when two or more species coexist within close proximity in nature and share the same basic requirements, they will likely compete for resources, food, territory, or habitat (Zhu and Yin 2009, Schreiber et al. 2011). Interspecific competition has been important for structuring bat communities, particularly in sympatric species which demonstrate niche partitioning (Jachowski et al. 2014). However, competitive coexistence has been demonstrated in bats in Europe (Ashrafi et al. 2011) and in Mexico (Salinas-Ramos et al. 2015). Niche overlap and dietary flexibility have been previously observed in bats of the Pteronotus family (Salinas-Ramos et al. 2015), and there is evidence to suggest that this could also occur for other insectivorous bats. Insectivorous bats have evolved a range of foraging strategies, from generalist to species-specific specialists (Warner 1985, Furlonger et al. 1987). Bat species which share a generalist feeding strategy as thought to be able to coexist through
niche differentiation and the partitioning of resources (Arlettaz et al. 1997, Ashrafi et al. 2011, Clare et al. 2014). This resource partitioning has been observed previously in insectivorous bats, which is may be because of current (or previous) interspecific competition over a limited insect resource (Pianka 1976, Begon et al. 1996). Resource limitation is also likely to be the driving force behind morphological divergence, resulting in the current foraging specialization evident in some bat communities (Freeman 1981, Emrich et al. 2014).

Exploitative competition is defined as an indirect negative effect of individuals on each other that occurs through the use of a shared resource, which depends on resource availability (Tilman 1990, Holdridge et al. 2016). Insectivorous bats Nyctalus noctule (noctule) and Nattereri’s pipistrelle (Pipistrellus nathusii; Roeleke et al. 2018) are among many other species which have been observed to exhibit exploitative competition, including mudsnails (Hydrobiidae; Fenchel and Kofoed 1976); wild dog (Lycaon pictus), spotted hyena (Crocuta crocuta), and lion (Panthera leo); Caro and Stoner 2003). Exploitative competition is difficult to measure without controlled removal experiments, which are exceedingly difficult with bats (Clare et al. 2014). However, natural species-specific removal in response to disease outbreak provides the opportunity to explore this phenomenon.

Since 2006, a devastating disease in eastern North America called white-nose syndrome (WNS) has caused significant species-specific declines in some bat communities (Frick et al. 2015). White-nose syndrome is a fungal infection of the bat skin caused by Pseudogymnoascus destructans and can result in mortality during winter hibernation (Wilcox et al. 2014). In particular, the little brown bat (Myotis lucifugus) was once considered the most abundant and widespread bat in North America (Harvey et al. 2011), but has been dramatically affected by WNS and may face local extirpation in some areas (Russel et al. 2015). Alternatively, the big brown bat (Eptesicus fuscus) is also common in many of the same areas, can contract WNS, but appears to have a higher survival rate and may have some resistance to the disease (Frank et al. 2014). Confirmation of WNS infection is based on the observation of dead or infected bats during their winter hibernation. However, bats can travel great distances from their winter hibernaculum to their summer roosting areas (Norquay et al. 2013), which means that the distribution shown in disease spread maps is likely an underestimate of the actual footprint of the effects of WNS. For this study, all of Southern Ontario is considered to be within the affected range of WNS. Mortality of hibernating bat colonies ranges from 90% to 100% which could lead to local extirpation, and some remnant species may not recover to pre-WNS populations in the short term under any management scenario (Russel et al. 2015).

Myotis lucifugus and E. fuscus are sympatric, with vast similarities in their life history. Both are insectivorous, roost in trees or buildings in the summer, hibernate through the winter primarily in caves or mines (E. fuscus will also hibernate in buildings), and have few predators (Lima and O’Keefe 2013). However, there are physiological and ecological differences between these species which may reflect resource partitioning (Clare et al. 2014). M. lucifugus often prefers to feed over aquatic or wetland areas and sometimes within forest openings, whereas E. fuscus is a flexible hunter and forages in most habitat types including open fields and agricultural areas (Geggie and Fenton 1985, Lacki et al. 2007). For insectivorous bats such as E. fuscus and M. lucifugus, insect availability and thus diet depend heavily upon habitat selection, which could explain evidence of resource partitioning as a function of interspecific competition for this resource (Emrich et al. 2014).

Earlier work on the diet of E. fuscus suggests that this species is more likely to consume hard-bodied Coleoptera than other small insectivorous bat species, evidenced by stronger jaw musculature and bite force (Fraser and Fenton 2007), and by the proportional representation of this order of insects in their diet (i.e., Feldhamer et al. 2009, Thomas et al. 2012 and Clare et al. 2014). Despite having an apparent affinity for Coleoptera, E. fuscus diets consistently also include prey items of Diptera (flies), Lepidoptera (moths), Ephemeroptera (mayflies), Trichoptera (caddisflies), and, to a lesser extent, other insect orders and Arachnida (spiders; Clare et al. 2014, Moosman et al. 2012, Feldhamer et al. 2009).

In contrast to E. fuscus, M. lucifugus often consume a greater proportion of Lepidoptera and
Dipteran (Feldhamer et al. 2009, Thomas et al. 2012, Clare et al. 2013), but also still consume similar species to *E. fuscus* from each of the five main insect orders above (Clare et al. 2011, Mooseman et al. 2012). Both *E. fuscus* and *M. lucifugus* consume a wide variety of species within these orders as generalist feeders, but the direct overlap of diet at the species level is less well understood. Traditional diet studies based on morphological identification of bat gut content or guano (feces) are often incapable of providing genus/species-level taxonomic resolution, and some softer-bodied insects are likely undetectable. Recent advances in DNA sequencing have revolutionized diet analysis by sequencing taxonomic marker genes (e.g., DNA barcodes) directly from DNA extracted from gut contents or feces (Fahn et al. 2016).

Establishing a dietary baseline for both *E. fuscus* and *M. lucifugus* enables changes in diet to be detected in response to the effects (such as competitor removal) of WNS (Clare et al. 2014). Hence, the loss of one competitor (e.g. *M. lucifugus*), measured using acoustic recording devices (bat detectors) as a proxy for relative abundance, should increase the availability of preferred resources for other competitors (e.g. *E. fuscus*) and accordingly result in increased population numbers. Additionally, the loss of a competitor should lead to expansion in the diet breadth of the unaffected species. A previous study by Jachowski et al. (2014), regarding pre- and post-WNS bat activity for *M. lucifugus* and *E. fuscus* (among other bat species), indicated increased activity in *E. fuscus* with declines of *M. lucifugus* and suggested that an overall reduction in interspecific competition and relaxation of niche partitioning occurred post-WNS.

Insectivorous bats are known to aid insect control (Maine and Boyles 2015, Tuttle 2015). For example, a recent study has successfully excluded bats from crop fields and demonstrated that the insect control from bats does, in fact, improve crop growth and quality (Maine and Boyles 2015), while another study has shown that the apple orchard pests, including the codling moth (*Cydia pomonella*), oriental fruit moth (*Grapholita molesta*), and plum curculio (*Conotrachelus nenuphar*), which were expected to be consumed by *E. fuscus* were not identified as part of their diet (Long et al. 2013). A previous study reviewed the economic importance of bats in agriculture and highlighted the economic cost of bat loss through WNS (Boyles et al. 2011). A single colony of *E. fuscus* (150 individuals) can consume nearly 1.3 million pest species per night (Whitaker 1995) and a single *M. lucifugus* individual is estimated to consume 4–8 g of insects per night (Anthony and Kunz 1977). The importance of insect control by bats has dramatically heightened due to the emergence of WNS in North America and the observed and predicted loss of so many bats in the landscape (Frick et al. 2010, Boyles et al. 2011, Alves et al. 2014). The predicted value of bats in the cotton-dominated agroecosystem is estimated at $22.9 billion/year and if current declines of key bat species including *M. lucifugus* continue, economic losses are likely to be substantial and ongoing (Boyles et al. 2011). The loss or significant decline in one or more species of bats could impact prevalence of both pest (i.e. agricultural) and beneficial insect populations.

Exploitative competition theory leads to the prediction that competitive release due to the loss of *M. lucifugus* will lead to an increase in *E. fuscus* and expansion in their dietary breadth. Hence, *M. lucifugus* should be recorded less frequently and *E. fuscus* should be recorded more frequently following the introduction of WNS, as a result of competitive release. The competition hypothesis also leads to the prediction that the diet of *E. fuscus* should expand to include broader species richness, more overlap with prey items that were also consumed by *M. lucifugus*, and an increase in the frequency of pest insects in the diets of *E. fuscus* individuals.

**METHODS**

Acoustic survey of bat activity

Monitoring stations (total = 13) were established in Southern Ontario, based on proximity to suspected roosting sites, feeding areas, or commuting corridors for bats in a variety of habitat types (woodland, open grassland, waterbodies) across the landscape (Fig. 1A; Table 1). Monitoring station Hald was situated outside of a building that was known as a nursery roost for *E. fuscus*, but as a large building, it could also have provided a roost for *M. lucifugus*. All survey stations were located within areas where WNS was prevalent.
Each station consisted of an AR125 microphone (Binary Acoustic Technology (BAT)) paired with an FR125 recorder (Binary Acoustic Technology), collectively called the BAT detector to enable detection of bat species at these locations. The BAT detectors were set to record nightly from at least 30 min before sunset to at least 30 min after sunrise. They were set to trigger a recording when a sound within the range of 15–90 kHz with an intensity of at least 18 dB was detected. When triggered, the BAT detector would continue recording for at least 5 s and then stop recording if there were at least 5 s of no sounds in this range or a maximum recording length of 15 s. Sound files were recorded in wavpack (.WV) format, later converted to wave (.WAV) using a decompression package, and were sorted into a folder for each night of recording (a night includes the following morning). Nights on which all or some of the night was not recorded because of power failure or other circumstances were excluded from further analysis.

White-nose syndrome was first confirmed in North America (four caves in New York State) in February 2006 (U.S. Fish and Wildlife Service 2017) and spread at a rate of approximately 200–400 km per year, reaching Ontario and Quebec in 2010, and New Brunswick and Nova Scotia in 2011 (Government of Canada 2012). The decline of bat communities in Southern Ontario was not observed until the end of 2012. Therefore, in this study, pre-WNS refers to data collected prior to the WNS decline and post-WNS is data collected after declines were first observed in Southern Ontario. Pre-WNS data were collected between 2009 and 2011, during time periods when bats would be active on the landscape. The study period for each station was replicated with data collected at the same station, at post-WNS between 2012 and 2014 (to 2015 for Dun).

Acoustic data were filtered using the program SCAN’R (version 1.7.3; Binary Acoustics Technology, Tucson, Arizona, USA) by tracking the noise floor of the recording and searching for burst sound energy that is greater than the noise by at least 6 dB. The data were further processed by removing low intensity sounds from 15 kHz to 25 kHz and between 70 kHz and 90 kHz, as well as removing calls from Myotis sodalis, a species not present in the surveyed locations.

### Table 1. Information on the different acoustic and guano monitoring stations sampled in this study for both *Eptesicus fuscus* and *Myotis lucifugus*.

| Station code | Acoustic/guano | Corresponding bat species         |
|--------------|----------------|-----------------------------------|
| Bru1         | Acoustic       | *E. fuscus* and *M. lucifugus*    |
| Bru2         | Acoustic       | *E. fuscus* and *M. lucifugus*    |
| Bru3         | Acoustic       | *E. fuscus* and *M. lucifugus*    |
| Bru4         | Acoustic       | *E. fuscus* and *M. lucifugus*    |
| Cam          | Acoustic       | *E. fuscus* and *M. lucifugus*    |
| Dun          | Acoustic       | *E. fuscus* and *M. lucifugus*    |
| Grey         | Acoustic       | *E. fuscus* and *M. lucifugus*    |
| Hald         | Acoustic       | *E. fuscus* and *M. lucifugus*    |
| Hur1         | Acoustic       | *E. fuscus* and *M. lucifugus*    |
| Hur2         | Acoustic       | *E. fuscus* and *M. lucifugus*    |
| Lam1         | Acoustic       | *E. fuscus* and *M. lucifugus*    |
| Lam2         | Acoustic       | *E. fuscus* and *M. lucifugus*    |
| Mid          | Acoustic       | *E. fuscus* and *M. lucifugus*    |
| BRU          | Guano          | *E. fuscus*                       |
| CAMB         | Guano          | *E. fuscus*                       |
| DUNN         | Guano          | *E. fuscus*                       |
| GREY         | Guano          | *E. fuscus*                       |
| HALD         | Guano          | *E. fuscus*                       |
| NORF         | Guano          | *E. fuscus*                       |
| SAND         | Guano          | *M. lucifugus*                    |

Fig. 1. Map of (A) acoustic monitoring locations for all 13 stations (Bru1, Bru2, Bru3, Bru4, Cam, Dun, Grey, Hald, Hur1, Hur2, Lam1, Lam2, and Mid) and (B) guano collection roost locations for all seven locations (BRU, CAMB, DUNN, GREY, HALD, NORF, and SAND).
least the value set by the Trigger Level. At the same time, the scan will reject all sound energy at frequencies lower than the minimum frequency cutoff setting or higher than the maximum setting, thus removing non-bat files. Acoustic files are then converted to WAV format and passed through a second filter program called Scrubber (version 5.2; Sonobat, Arcata, California, USA), to further remove noise files using frequency (Hz) filters. A subset of the files removed by both filters were reviewed manually, and it was estimated that less than 1% of removed files contained recognizable bat calls, but even these files were very poor quality and likely could not be identified to the species level. Species classification of the files was conducted using the Sonobat NNE package (version 3.2.1; Sonobat, Arcata, California, USA). The resulting species classifications were totaled for each night then analyzed for average number of passes pre- and post-WNS using Microsoft Excel and Program R (R Core Team 2015).

Bat capture and tracking

Potential bat roosts were located through contact with bat specialists, bat eviction companies, church managers, and through a capture and tracking program. In the Dunnville area (DUNN) where we have the most consistent, long-term acoustic data, no local roosts were known. Therefore, a capture and tracking program was undertaken to find roosts where bat guano from our two target species could be collected in this area. The capture and handling of bats were conducted under a license (WSCP number 1076739), issued by the Guelph District Ministry of Natural Resources and Forestry. A scientific license to conduct this research on Grand River Conservation Authority was also granted. All animals were handled carefully and ethically in accordance with these permits and licenses. Capture and handling of bats followed accepted WNS decontamination protocols. There was no mortality or injury of the bats handled.

The capture program was conducted from 5 July to 12 July 2014. Capture events were carried out at Byng Conservation Area (5 July 2014), the Morningstar Cottage (6–9 July 2014), Dunnville Marsh Area 1 (10 July 2014), Dunnville Marsh Area 2 (11 July 2014), and Rock Point Provincial Park (12 July 2014). At each site, we installed three to five mist net sets. These included one, 12 m wide × 8 m high (three nets stacked), one 9 m wide × 8 m high, at least one 6 m wide × 5 m high (two nets stacked) and some single nets (3 m high) of various widths. Netting effort was calculated based on one 6 m wide single net for one hour = 1 net-hour. The nets were opened at 30 min past sunset to avoid incidental capture of birds and were checked at a 10-min frequency. Once a bat was found within the net, it was carefully extracted and placed into a paper bag for processing. During processing, we identified the species, sex, age (adult or juvenile), physical size measurements, and indicators of health, and a bat band (Porzana) of the appropriate size was placed on the forearm of the bat with a unique identification number. Upon capture of an E. fuscus individual, a very high frequency (VHF) radio transmitter was glued to the bat using Skin Bond surgical cement. For up to 3 d after affixing the transmitter, the bat was located at its roost using the truck-mounted and handheld receivers (Advanced Telemetry Systems). Once this E. fuscus roost was identified and permission was granted by the landowner, it was added to the list of roosts at which guano samples could be collected.

Collection of guano

Guano was collected from locations known to have roosting bats or sites where bats had been found roosting through capture and tracking, but generally occur over a similar geographic region as the acoustic monitoring stations (Fig. 1B; Table 1). At each roost, paper was placed under the bats at the start of the sampling period (typically the start of each month). In each sampling period, 4–20 subsamples were collected at each roost. Subsamples were stored in 95% ethanol at −20°C prior to processing for molecular analysis. Samples from the E. fuscus roosts in the Cambridge area were pooled (CAMB), and samples from the E. fuscus roosts in Dunnville were also pooled (DUNN). The Sandilands roost (SAND), south of Cambridge, was not grouped with other Cambridge roosts because it was a M. lucifugus roost.

Collection of stomachs

Stomachs from E. fuscus individuals were extracted from bat carcasses submitted to the Canadian Wildlife Health Cooperative at the University of Guelph, which had been collected across Southern Ontario. In total, 44 E. fuscus
stomachs were available from 2011 or earlier (pre-WNS), and 46 were available from 2012 to 2014 (post-WNS). Each of these bats was dissected to extract the stomach and intestine (collectively referred to as stomach contents). The equipment was sterilized between dissections of each bat. The stomach was placed in 95% ethanol and stored at −20°C until molecular processing. No stomach samples from M. lucifugus were possible to obtain.

**Next-generation sequencing**

The guano was dried in an air-circulating incubator set at 56°C. Stomachs were pulverized using a 5.8” drill bit spun at 400 revolutions per minute for a period of 30 s in forward and 30 s in reverse. A 1.2-mL Lysing Matrix tube was filled with guano (approximately 8–10 pellets depending on pellet size) or stomach tissue from one individual and 760 μL of buffer T1 and 75 μL of Proteinase K (from the NucleoSpin Tissue Kit). Thereafter, all guano and stomach samples were treated the same. The Lysing Matrix tube was placed in a shaker set to shake at 6.0 m/s for a period of 40 s to homogenize and liquefy the sample.

The DNA extraction was conducted using a NucleoSpin Tissue kit (Macherey-Nagel, Bethlehem, Pennsylvania, USA), following instructions for the standard protocol for human or animal tissue and cultured cells. The final step where pure water was eluted from the filter was conducted using 30 μL of molecular biology grade water heated at 70°C to elute the DNA, rather than 100 μL of buffer BE (as described in the instruction manual) to concentrate the eluted DNA. The extracted DNA was then stored at −20°C. Two non-overlapping mini-barcode fragments within the standard COI DNA barcode region were amplified with two primer sets in a two-step PCR amplification regime (Hajibabaei et al. 2011, Gibson et al. 2015). The BR5 fragment (~330 bp) was amplified using the following primers, previously optimized for use with a broad range of arthropod orders: B CCIGAYTRGCIT-TYCCIGG (Hajibabaei et al. 2011) and R5 GTRATIGCCICGCTTARIAC (Gibson et al. 2014). The second fragment F230 (~230 bp) was amplified using F GGTCAACAATCATAAAGATAITGG (Folmer et al. 1994) and F230_R CTTATRTRTTTTATIGGIRAIGC (Gibson et al. 2015). The first PCR used COI-specific primers and the second PCR involved Illumina-tailed primers. The PCRs were assembled in 25 μL volumes. Each reaction contained 2 μL DNA template, 17.5 μL molecular biology grade water, 2.5 μL 10 × reaction buffer (200 mmol/L Tris–HCl, 500 mmol/L KCl, pH 8.4), 1 μL MgCl2 (50 mmol/L), 0.5 μL dNTPs mix (10 mmol/L), 0.5 μL forward primer (10 mmol/L), 0.5 μL reverse primer (10 mmol/L), and 0.5 μL Invitrogen’s Platinum Taq polymerase (5 U/μL). The PCRs were initiated at 95°C for 5 min, followed by a total of 30 cycles of 94°C for 40 s, 46°C (for both primer sets) for 1 min, and 72°C for 30 s, and a final extension at 72°C for 5 min and held at 4°C. Amplicons were purified using Qiagen’s MiniElute kit and eluted in 30 μL water. The purified amplicons from the first PCR were used as templates in the second PCR with the same amplification condition used in the first PCR with the exception of using Illumina-tailed primers in a 30-cycle amplification regime. All PCRs were undertaken using Eppendorf Mastercycler, and negative control reactions (no DNA template) were included in all experiments. PCR products were visualized on a 1.5% agarose gel to check the amplification success. All generated amplicons were dual indexed, pooled, and sequenced on multiple Illumina MiSeq flowcells using V2 MiSeq sequencing kits (250 × 2; FC-131-1002 and MS-102-2003). All sequences are available in Dryad (https://doi.org/10.5061/dryad.dnf2z34wb).

For all 257 samples, 37,331,865 Illumina reads were generated from both COI amplicons. For each sample, the forward and reverse raw reads for the BE fragment and the F230 fragment were merged with SEQPREP software (https://github.com/jstjohn/SeqPrep) requiring a minimum overlap of 25 bp and no mismatches, resulting in 17,392,577 total paired-end reads (mean—77,301 reads/sample). All Illumina paired-end reads were filtered for quality using PRINSEQ software with a minimum Phred score of 20, window of 10, step of 5, and a minimum length of 150 bp. A total of 16,800,094 of both paired BE reads and paired F230 reads (mean—74,667 reads/sample) were retained for further processing. USEARCH v6.0.307 (Edgar 2010) with the UCLUST algorithm was used to dereplicate and cluster the remaining sequences using a 98% sequence similarity cutoff. Chimera filtering was performed using USEARCH with the de novo UCHIME algorithm. At each step, cluster sizes were
retained, singletons were retained, and only putatively non-chimeric reads were retained for further processing. Membership in each cluster for each sample was recorded as an operational taxonomic unit (OTU) sequence abundance matrix.

An OTU was defined as a cluster of at least 10 sequences in each sample with a minimum of 98% similarity to any COI reference sequence in the GenBank and BOLD databases. Assigned operational taxonomic units (AOTUs) were defined as clusters of sequences which all assigned to the same taxonomic classification, at the genus, family, or order level. If there were more than one cluster of sequences assigned to the same taxa, these were pooled together. Only those AOTUs within the class Insecta were carried forward for further analysis.

Despite the recent improvements in molecular sampling techniques and the ability for highly accurate species-level classification, there is still debate whether the number of sequences of a particular AOTU obtained within each sample is representative of abundance (Deagle et al. 2013, Pompanon et al. 2012). Therefore, we did not use the number of sequences as an indicator of abundance, but rather quantified abundance based on the frequency of occurrence of that AOTU across multiple samples.

Since guano pre-WNS was not available, we used raw sequence data from Clare et al. (2011, 2013) for *M. lucifugus* and Clare et al. (2014) for *E. fuscus*. The sequences underwent a BLAST (Basic Local Alignment Search Tool) against GenBank and BOLD and followed the same criteria for AOTU presence as the guano or stomach samples. The data from each of these were pooled so as to determine whether each prey AOTU was present or absent in the diet of bats from each study, rather than frequency of occurrence in samples.

A list was developed of the frequency of occurrence of each AOTU within the samples of each of the following groups: pre-WNS stomach (*n* = 44, *E. fuscus*), post-WNS stomach (*n* = 46, *E. fuscus*; Clare et al. 2011, *n* = 1, due to pooled dataset of *M. lucifugus*; Clare et al. 2013, *n* = 1, pooled dataset of *M. lucifugus*; Clare et al. 2014, *n* = 1, pooled dataset of *E. fuscus*), NORF (*n* = 20, *E. fuscus* roost subsamples), HALD (*n* = 23, *E. fuscus* roost subsamples), DUNN (*n* = 37, *E. fuscus* roost subsamples combined from various roosts), CAMB (*n* = 22, *E. fuscus* roost subsamples combined from various roosts), GREY (*n* = 18, *E. fuscus* roost subsamples), BRU (*n* = 23, *E. fuscus* roost subsamples), and SAND (*n* = 24, *M. lucifugus* roost subsamples).

**Statistical analysis**

Acoustic data classified as *E. fuscus* and *M. lucifugus* were pooled for pre-WNS and post-WNS for each of these two species to quantify overall changes in bat activity. Permutational multivariate analysis of variance (PERMANOVA) was conducted in PRIMER (v. 6; Clarke and Gorley 2006) to compare activity of both *E. fuscus* and *M. lucifugus*, considering two factors as main sources of variance: station (fixed, 13 levels: Bru1, Bru2, Bru3, Bru4, Cam, Dun, Grey, Hald, Hur1, Hur2, Lam1, Lam2, and Mid) and WNS status (fixed, two levels, pre and post). Significant differences were investigated using a posteriori pair-wise test. *P* values in the PERMANOVA and pair-wise tests were obtained from Monte Carlo asymptotic distributions, because of the restricted number of unique permutations (Anderson and Robinson 2003). For the Dun acoustic station with data across three years of pre-WNS and four years of post-WNS, the data from those years were pooled for comparison. The mean bat passes per year was also plotted for each of the seven years at Dun for each species to look at annual changes.

For the diet data, the mean number of AOTUs within each of the major insect orders was calculated to compare differences between *E. fuscus* stomachs pre-WNS and post-WNS. A rarefaction curve was created from AOTUs using the iNext package (Hsieh et al. 2015) and the Chao function (Chao et al. 2014, Cayuela et al. 2015) in Program R (R Core Team 2015). Through this analysis, the overall AOTU richness was calculated where the rarefaction curve reaches an asymptote (estimated total diet AOTU richness) and has a 95% confidence interval. The difference between the estimated richness of stomachs pre-WNS compared with post-WNS. The same analysis was conducted to describe differences in estimated richness from the guano of *E. fuscus* at the different roosts.

Lists of insect taxa identified to the species level were created for all guano and stomach...
samples for both species of bat and the lists created from cross-referencing the sequences from Clare et al. (2011, 2013, 2014). These lists were checked against lists of known pest insects from OMAFRA (2015), and mosquito species from Giordano et al. (2015) and Cywinska et al. (2006). The list of species identified in the diet of bats was also compared to the beneficial insect species listed in OMAFRA (2009). Changes in the frequency of pest and beneficial insects were then compared from pre-WNS to post-WNS.

**RESULTS**

**Capture and tracking**

Bat capture and tracking in 2014 was carried out in Dunnville, one of which capture locations corresponding to the Dun acoustic station. In total, thirteen bats were captured, all of which were *E. fuscus*. Of the thirteen bats, three were adult male, six were adult female, four were juvenile male, and none were juvenile female. All bats were photographed and released at the site of capture with no evidence of harm. None of the bats displayed evidence of wing membrane damage that could have resulted from WNS infection (all were Reichard’s Wing Score of 0; Reichard 2009). Four of these bats, captured at four different locations, were tracked to their roost. The distance between the capture site and the roost for these bats was approximately 360, 830, 1080, and 2700 m. Two of the roosts were accessible, in which there was a large accumulation of guano and several bats observed, indicating that the colony was well established at these locations. At the other two roosts, the *E. fuscus* individual was identified at the building due to the VHF tag response, but the population and guano samples were not collected because the roost could not be accessed.

**Acoustic analysis**

Across thirteen stations, a total of 960 and 1187 nights of data were collected pre-WNS and post-WNS, respectively. The Dun station provided most acoustic data consisting of 395 nights (over three years) for pre-WNS and 601 nights (across four years) for post-WNS measurements.

Overall, the decline of *M. lucifugus* activity pre-WNS to post-WNS across all stations was 81.72% (Fig. 2A). At Dun, there was 99.9% decline of *M. lucifugus* between 2010 and 2014 (Fig. 3A). Significant decline in *M. lucifugus* activity was observed across ten of the thirteen stations, with decline ranging from 14.5% to 98.7% (Figs. 4A, 5). At some stations, there was an observed decline in activity of both *E. fuscus* and *M. lucifugus* (Bru1, Grey, Hald, Hur2, and Mid; Fig. 5). The mean activity level of *E. fuscus* (number of bat passes per night across all monitoring stations) from pre- to post-WNS increased by 27.5% overall (Fig. 2B). When investigating the differences in mean nightly bat passes at each station for *E. fuscus*, the trend was inconsistent (Fig. 4B).

The results of the PERMANOVA carried out between pre- and post-WNS revealed the presence of significant difference in activity for *M. lucifugus* (*P(MC) < 0.001*) but not *E. fuscus* (*P(MC) = 0.057*; Tables 2, 3). Regarding activity at different stations, there was significant difference in *M. lucifugus* (*P(MC) < 0.001*) and *E. fuscus* (*P(MC) < 0.05*) activity across stations and similarly the interaction between station and WNS status was also significant for both species (*P(MC) < 0.05*). Pair-wise PERMANOVA results for station comparisons individually and nested with pre- or post-WNS status for both species can be found in supplementary material (Appendix S1: Tables S3–S14). Results of pair-wise PERMANOVA for pre- vs. post-WNS was not significant for *E. fuscus* (*P(MC) = 0.057*; dissimilarity = 38.5%; Table 4 but was for *M. lucifugus* (*P(MC) < 0.001*; dissimilarity = 42.7%; Table 4). Within each station, there were some significant differences between bat activity pre- and post-WNS (Table 5). For *E. fuscus*, stations Cam (*P(MC) = 0.0135*) and Hur1 (*P(MC) = 0.0435*) were significant, whereas for *M. lucifugus*, activity pre- and post-WNS was significantly different at sites Bru1 (*P(MC) = 0.0062*), Dun (*P(MC) = 0.0001*), Hald (*P(MC) = 0.002*), and Mid (*P(MC) = 0.0263*; Table 5).

**DNA-based diet analysis**

Monthly collection of guano was conducted at all seven roosts or roost areas, including SAND. There was less guano available at the individual roosts in DUNN in the early part of the season (prior to capture and tracking), so these roosts were pooled for the DUNN samples. Similarly, guano accumulation at the individual CAMB roosts was lower at the end of the season;
therefore, CAMB area roosts were also pooled. Of 345 guano samples collected across all roosts, 167 were processed through next-generation sequencing, which included a subset of samples from each month at each roost area. Next generation resulted in 1058 AOTUs from *E. fuscus* roosts, with each site ranging from 99 (GREY) to 214 (DUNN) AOTUs (Table 2). The mean percent of the number of AOTUs within each insect order at each of the roosts is depicted in Fig. 6A, including SAND (*M. lucifugus*-specific site). Coleoptera was in very low proportion for *M. lucifugus* (SAND) as expected, but the proportion of Coleoptera varied across *E. fuscus* roosts with the lowest at Demarce and Burke. The lower proportion of Coleoptera is apparent at Demarce and Burke, but the difference is not statistically significant. Diptera were also in lower proportion at Demarce, Burke, and Cambridge (the most northern roosts), and these three roosts had the highest proportion of Ephemeroptera and Trichoptera, most similar to SAND.

The number of AOTUs identified at a particular roost is not an exhaustive list of all insects consumed by the bats at that roost. Therefore, rarefaction curves were created to determine the total richness of insects consumed (Fig. 6B). Associated with the rarefaction curves is a calculation of the total estimated richness of prey items, provided in Table 6. HALD (365 AOTUs; 95% CI [300, 473]) and DUNN (482 AOTUs; 95% CI [373, 666]) showed high estimated richness relative to BRU (254 AOTUs; 95% CI [195, 360]), GREY (209 AOTUs; 95% CI [152, 323]), CAMB (258 AOTUs; 95% CI [210, 346]), and NORF (242 AOTUs; 95% CI [176, 379]). Of all AOTUs classified to the species level, observed in *M. lucifugus* (total 253 species), seven were recorded pre-WNS in *E. fuscus* in Clare et al. (2014) and 37 were observed in post-WNS stomachs. However, 58 of these species from *M. lucifugus* diets were observed in post-WNS stomachs and 97 were observed in at least one of the post-WNS roost diets.

Of the stomach samples extracted from *E. fuscus*, 44 were from pre-WNS and 46 were from post-WNS. The mean proportion of insects eaten by order was not significantly different between pre-WNS and post-WNS samples (Fig. 6B).

Using rarefaction curves (Fig. 7), there is a significant difference in the overall richness within the samples, with the estimated richness greater for post-WNS samples (627 AOTUs; 95% CI [532, 769]), than for pre-WNS samples (560 AOTUs; 95% CI [458, 718]), Table 2). All taxa identified to the species level are listed in Appendix S1: Table S2. The AOTU richness of stomach samples was higher than that of guano samples, suggesting that sampling from stomachs is more sensitive in detecting dietary breadth.
Fig. 3. Changes in mean bat passes per night at the Dun acoustic station across years for (A) *Myotis lucifugus* and (B) *Eptesicus fuscus*.

Fig. 4. Changes in mean bat passes per night at each station (Bru1, Bru2, Bru3, Bru4, Cam, Dun, Grey, Hald, Hur1, Hur2, Lam1, Lam2, and Mid) for (A) *Myotis lucifugus* and (B) *Eptesicus fuscus*.
A list of 242 insects, including 67 mosquito species, was cross-checked against those species identified in the guano and stomachs of E. fuscus and M. lucifugus, including those reported in Clare et al. (2011, 2013, 2014), each study was pooled for presence or absence of species). A total of 37 insect pest species were identified in guano and stomachs across all samples, with 14 of these being consumed by M. lucifugus and 35 being consumed by E. fuscus overall. Of these, only two leaf-miner species, *Argyresthia canaden시스* and *Argyresthia thuiella*, were consumed by *M. lucifugus*, but not found in *E. fuscus*. Of the 35 species identified in the diet of *E. fuscus*, 20 were identified from pre-WNS (Clare et al. 2013, or stomachs), and 33 were identified from post-WNS (stomachs or roosts). Only two of the 35 pest species observed in *E. fuscus* were not observed in post-WNS diets: the Western Bean Cutworm (*Striacosta albicosta*) and a Mosquito (*Culiseta inornata*), both of which were found in only one sample of *E. fuscus* pre-WNS. Of the 14 species identified in the diet of *M. lucifugus*, eight were consumed by *E. fuscus* pre-WNS and nine were consumed by *E. fuscus* post-WNS. A

![Graph showing percentage change in mean number of bat passes per night for both *Myotis lucifugus* (black) and *Eptesicus fuscus* (white) pre- and post-white-nose syndrome (WNS) for each acoustic monitoring station.](image)

**Fig. 5.** Percentage change in mean number of bat passes per night for both *Myotis lucifugus* (black) and *Eptesicus fuscus* (white) pre- and post-white-nose syndrome (WNS) for each acoustic monitoring station.

### Table 2. Permutational multivariate analysis of variance (PERMANOVA) results for comparisons of *Eptesicus fuscus* activity by station, white-nose syndrome (WNS) status, and their interactions, computed with Monte Carlo (MC) tests.

| Source          | df | SS   | MS    | Pseudo-F | P(perm) | P(MC) |
|-----------------|----|------|-------|----------|---------|-------|
| Station         | 12 | 22794| 1899.5| 2.1286   | 0.0103* | 0.0043*|
| WNS status      | 1  | 2639.7| 2639.7| 2.9582   | 0.0584  | 0.0569 |
| Station × WNS status | 11 | 16331| 1484.6| 1.6637   | 0.0537  | 0.0475*|
| Res             | 67 | 59788| 892.7 |          |         |       |
| Total           | 91 | 1.0092E5 |     |          |         |       |

**Notes:** Fixed factors: station (13 levels); WNS status (pre or post; two levels). Number of permutations: 9999, df, degrees of freedom; SS, sum of squares; MS, mean squares; P(perm), significance; P(MC), significance after Monte Carlo correction.

* *P* < 0.05.
mosquito species (*Aedes vexans*) was observed in *M. lucifugus* diets and all *E. fuscus* diets, suggesting that it is frequently consumed by both bat species. Pest insects consumed by *M. lucifugus* and found only in post-WNS samples of *E. fuscus* include the following: (1) A fruit tree leaf roller (*Archips argyrospila*) was found in one guano sample at HALD; (2) red-banded leaf roller (*Argrotaenia velutinana*) was found in guano samples at HALD and BRU; (3) onion maggot (*Delia antiqua*) was found in post-WNS stomachs, NORF, DUNN, and CAMB; (4) June Beetle (*Phyllophaga futilis*) was found at all *E. fuscus* guano roosts, but not in post-WNS stomachs; and (5) Tarnished plant bug (*Lygus lineolaris*), which was found in all *E. fuscus* guano sampling sites except GREY and in both pre-WNS and post-WNS stomachs but was not found in any sample from our study of *M. lucifugus*. This species is considered to be an important native agricultural pest in Ontario (Broadbent et al. 2006).

**Beneficial insects**

None of the 22 beneficial insect species listed in OMAFRA (2009) were identified in the list of insects consumed by bats in this study, and no bees (known pollinators) or dragonflies or damselflies (known predatory insects) were identified. Although the species listed in OMAFRA (2009) were not found in any bat diet, other species within this family were identified. In particular, two lacewing species (Neuroptera family) were identified in the bat diets, *Hemerobius atrifrons* and *H. stigma*. Both of these insects are considered to be predatory on other insects; their ecology is not well known. Ground beetles in the family Carabidae are considered to be caterpillar hunters. Although the Carabidae species listed in OMAFRA (2009) were not found in any bat diet, other species within this family were identified (Appendix S1: Table S2). The species most frequently identified are *Stenolophus comma*, a predator on codling moth larvae and apple maggot pupae which was not identified in *M. lucifugus* diet, and *Stenolophus ochropezus*, found in all sampling groups for *M. lucifugus* and *E. fuscus* except SAND and BRU.

**Discussion**

The overall trend of acoustic activity of *E. fuscus* in response to the decline of *M. lucifugus* was significantly positive with six of the 13 stations show statistically or biologically significant

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### Table 3. Permutational multivariate analysis of variance (PERMANOVA) results for comparisons of *Myotis lucifugus* activity by station, white-nose syndrome (WNS) status and their interactions, computed with Monte Carlo (MC) tests.

| Source           | df  | SS    | MS    | Pseudo-F | P(perm) | P(MC) |
|------------------|-----|-------|-------|----------|---------|-------|
| Station          | 12  | 33834 | 2819.5| 4.8368   | 0.0001**| 0.0001**|
| WNS status       | 1   | 5755.2| 5755.2| 9.8728   | 0.0005**| 0.0005**|
| Station × WNS status | 11  | 11633 | 1057.5| 1.8141   | 0.0281* | 0.0285*|
| Res              | 67  | 39057 | 582.94|          |         |       |
| Total            | 91  | 1.0091E5 |       |          |         |       |

**Notes:** Fixed factors: station (13 levels); WNS status (pre or post; two levels). Number of permutations: 9999. df, degrees of freedom; SS, sum of squares; MS, mean squares; P(perm), significance; P(MC), significance after Monte Carlo correction.

* P < 0.05.

** P < 0.001.

### Table 4. Pair-wise permutational multivariate analysis of variance (PERMANOVA) results for *Eptesicus fuscus* and *Myotis lucifugus* activity pre- vs. post-white-nose syndrome (WNS), computed with Monte Carlo (MC) tests.

| Species   | Groups       | Pseudo-F | P(perm) | Unique perms | P(MC) | % Dissimilarity |
|-----------|--------------|----------|---------|--------------|-------|----------------|
| *E. fuscus* | Pre-, Post-WNS | 2.95805  | 0.0537  | 9954         | 0.0567| 38.464         |
| *M. lucifugus* | Pre-, Post-WNS | 9.8728   | 0.0004  | 9945         | 0.0004*| 42.726         |

* P < 0.05.
increase, individually, which supports the prediction of a competitive interaction through niche overlap as was previously suggested by Jachowski et al. (2014). The stomach samples collected throughout southern Ontario showed an increase in diet richness following the introduction of WNS, supporting the predictions arising from the competition hypothesis of an increase in richness of *E. fuscus* prey items following competitive release. This conclusion is further supported by an increase in the presence of insect pests in *E. fuscus* diet following the introduction of WNS.

Replicated acoustic monitoring and especially long-term stationary acoustic monitoring provide an effective means to track long-term changes in bat activity as a surrogate for bat abundance. While these data could have some degree of autocorrelation because stations were re-sampled in different years, a repeated-measures calculation was not conducted. The detectors and automated species classification programs have limitations in their ability to discriminate bats in flocks with multiple individuals, because of poor sound quality or atypical search-phase calls (Adams et al. 2012), but this bias was consistent across the entire study. There is potential that additional passes of these species were recorded and classified to a higher level (i.e. *Myotis* spp.), or misclassified as another species (i.e. *E. fuscus* being misclassified as *Lasionycteris noctivagans*), but those recordings were consistently excluded from the dataset for this analysis and therefore the effect of this exclusion should be consistent. There is also the potential that

### Table 5. Mean bat activity (pre- and post-WNS) with percentage change, *t*-value, P(perm), number of unique permutations, and P(MC) results for nested pair-wise permutational multivariate analysis of variance (PERMANOVA) for both *Eptesicus fuscus* and *Myotis lucifugus* at each acoustic monitoring station.

| Station | Pre-WNS mean | Post-WNS mean | % Change | *t* | P(perm) | Unique perms | P(MC) |
|---------|--------------|---------------|----------|----|---------|--------------|-------|
| **E. fuscus** | | | | | | | |
| Bru1    | 3.43         | 0.53          | -88.4    | 2.153 | 0.0205  | 10           | 0.2169 |
| Bru2    | 30.37        | 37.57         | +23.70   | 0.1099 | 0.7995  | 10           | 0.8516 |
| Bru3    | 4.13         | 19.81         | +379.50  | 2.1325 | 0.3288  | 3            | 0.2762 |
| Bru4    | 29.78        | 79.22         | +166.00  | 7.9772 | 0.3364  | 3            | 0.0680 |
| Cam     | 1.40         | 13.82         | +886.30  | 8.5990 | 0.0226  | 91           | 0.0135* |
| Dun     | 11.66        | 20.79         | +78.20   | 2.4367 | 0.0972  | 9950         | 0.0963 |
| Grey    | 14.08        | 0.86          | -93.90   | 4.5544 | 0.1959  | 10           | 0.1233 |
| Hald    | 40.87        | 24.17         | -40.90   | 0.6874 | 0.5963  | 10           | 0.4845 |
| Hur1    | 9.30         | 40.03         | -330.50  | 14.1812 | 0.3299  | 3            | 0.0435* |
| Hur2    | 30.88        | 24.06         | -22.10   | 0.51093 | 0.6642   | 3            | 0.5901 |
| Lam1    | 9.92         | 57.80         | +482.50  | N/A   | N/A     | N/A          | N/A   |
| Lam2    | 19.54        | 15.00         | -23.20   | N/A   | N/A     | N/A          | N/A   |
| Mid     | 67.83        | 28.93         | -57.30   | 1.1033 | 0.2979  | 10           | 0.3839 |
| **M. lucifugus** | | | | | | | |
| Bru1    | 5.67         | 0.05          | -99.20   | 22.6509 | 0.1048  | 10           | 0.0062* |
| Bru2    | 190.46       | 76.40         | -59.90   | 0.2565 | 0.6011  | 10           | 0.7649 |
| Bru3    | 1.00         | 0.14          | -86.10   | 4.4403 | 0.3342  | 3            | 0.163   |
| Bru4    | 6.87         | 5.28          | -88.50   | 11.4515 | 0.3376  | 3            | 0.0771 |
| Cam     | 0.21         | 0.09          | -56.00   | 1.2166 | 0.3585  | 17           | 0.3035 |
| Dun     | 45.99        | 5.28          | -88.30   | 26.9050 | 0.0001** | 9938         | 0.0001** |
| Grey    | 48.02        | 0.70          | -98.50   | 6.4653 | 0.1959  | 10           | 0.0673 |
| Hald    | 7.53         | 0.10          | -98.70   | 45.0523 | 0.0996  | 10           | 0.002*  |
| Hur1    | 3.85         | 3.29          | -14.30   | 1.8986 | 0.6633  | 2            | 0.2998 |
| Hur2    | 60.77        | 6.11          | -89.90   | 1.3188 | 0.3388  | 3            | 0.3681 |
| Lam1    | 0.08         | 0.00          | -100.00  | N/A   | N/A     | N/A          | N/A   |
| Lam2    | 0.00         | 0.00          | N/A      | N/A   | N/A     | N/A          | N/A   |
| Mid     | 3.88         | 0.04          | -99.10   | 14.2558 | 0.0992  | 7            | 0.0263* |

*Note:* WNS, white-nose syndrome; P(perm), significance; P(MC), significance after Monte Carlo correction.

* P < 0.05.

** P < 0.001.
other species were misclassified as *E. fuscus* or *M. lucifugus*, but this should also be uncommon and consistent across the analysis. There could be other environmental factors such as climate change, roost availability, or anthropogenic disturbance which can change the activity measures, and overall population of bats over time or at specific locations. Since the effects of WNS on *M. lucifugus* have happened very rapidly, the change in bat activity is considered to be reflective mostly of those effects from WNS, rather than other environmental conditions.

From our data, we can deduce that it is likely *M. lucifugus* in Southern Ontario declined following the introduction of WNS, based on summer activity patterns, as predicted by the
competition hypothesis. *E. fuscus* activity levels increased overall, but not consistently across all locations. The significant decline of *E. fuscus* at Mid, Grey, and Bru1 was opposite to the general trend. These locations may have suffered local disturbances (i.e., roost eviction), resulting in localized population declines and thus reduced activity, or there could have been less foraging overlap and therefore relaxed interspecific competition between *E. fuscus* and *M. lucifugus* at these locations (Kunz 1974, Jachowski et al. 2014). DUNN showed a lower level of activity in 2012 and 2014, but significantly higher activity in 2013 and 2015, suggesting that there could be significant inter-annual variation in local bat activity, mediated through temperature or resource availability, which could explain some inconsistency in the results across stations (Syme et al. 2001, Krüger et al. 2014).

Three stations (Cam, Lam2, and Bru4) all had low levels of activity of *M. lucifugus* pre-WNS (Appendix S1: Table S1). Despite the low activity level of *M. lucifugus*, there was still a significant decline at Bru1, Dun, Hald, and Mid and Bru3 and a corresponding significant increase of *E. fuscus* at Cam. Where no *M. lucifugus* were observed either pre-WNS or post-WNS at Lam2, the change in *E. fuscus* was not significant. In contrast, the

| Station | Total subsamples | Observed AOTUs | AOTU richness estimator | Est S.E. | 95% lower | 95% upper |
|---------|-----------------|--------------|------------------------|---------|----------|----------|
| NORF    | 20              | 112          | 242.421                | 49.425  | 175.611  | 379.401  |
| HALD    | 23              | 200          | 365.052                | 43.193  | 299.665  | 473.336  |
| DUNN    | 37              | 214          | 482.18                 | 72.732  | 373.096  | 666.058  |
| CAMB    | 22              | 152          | 258.028                | 33.617  | 209.807  | 346.475  |
| GREY    | 18              | 99           | 208.587                | 41.526  | 152.453  | 323.672  |
| BRU     | 23              | 127          | 253.51                 | 40.536  | 195.553  | 360.468  |
| SAND    | 24              | 154          | 334.188                | 54.665  | 254.728  | 476.328  |
| Stomachs Pre-WNS | 44 | 277 | 560.13 | 65.171 | 458.376 | 718.969 |
| Stomachs Post-WNS | 46 | 339 | 627.629 | 59.382 | 532.655 | 769.182 |

Fig. 7. Rarefaction curves for (A) all *Eptesicus fuscus* roosts (filled circle: BRU; filled square: DUNN; filled triangle: CAMB; cross: GREY; open square: HALD; star: NORF) and the *Myotis lucifugus* roost (line: SAND), and (B) pre-white-nose syndrome (WNS) (filled triangle) and post-WNS (filled circle) stomachs of *Eptesicus fuscus.*
M. lucifugus activity level at Hur1 declined by only 14.5 percent, which was not considered a significant decline, but the activity level of E. fuscus still increased significantly, by 330%.

At the known E. fuscus roost (Hald), there was a significant decline of M. lucifugus, but also a small decline of E. fuscus (though not statistically significant). It is possible that E. fuscus may have chosen to use other roosts for this year, as this species is documented for being flexible with roosting habitats (Kunz 1982, Brigham 1991, Agosta 2002), or this species could have spent less time near the roost, resulting in reduction of acoustic activity detected. There could also have been attempts to exterminate bats at this roost (i.e. a pail of water was found within the roost that had two dead bats) or local landscape disturbance, which would have resulted in the reduced activity at this site (Agosta 2002, Gehrt and Chelsvig 2003).

Collection of bat guano and DNA metabarcoding was an effective method to develop a comprehensive list of taxa consumed by these bats, but despite a significant number of samples collected and processed, the total AOTUs did not approach the estimated total richness calculated through rarefaction curves. Next-generation sequencing of DNA has proven superior to traditional methods of morphological prey identification for the determination of prey richness (Whitaker et al. 2009), but DNA processing methods are still not suitable for directly quantifying the abundance of prey items in the bat diet (Swift et al. 2018). Therefore, future studies should focus on ways to calculate the volume of each prey item in a sample, which can then be extrapolated to determine the impact of bats feeding on insects. Greater insect prey richness was found in bat stomachs, likely because the contents of the stomachs have been less digested and degraded than in guano. This indicates that these two species of bat have a considerably broad insect diet, although many taxa could be consumed rarely, indicating a combination of specialized and opportunistic foraging (Clare et al. 2011, 2014). Proportional representation of insect orders varied considerably across roosts, indicating that the breadth of the diet of bats is locally driven, as suggested in Clare et al. (2011).

The diet of bats at all E. fuscus roosts continued to have a high proportion of Coleoptera (supporting the conclusions of Clare et al. 2014) relative to the M. lucifugus roost pre- and post-WNS, although this was lower at GREY and BRU (the two most northerly roosts). In contrast, GREY and BRU had the highest relative proportion of Ephemeroptera of all roosts, also similar to M. lucifugus at SAND. These two roosts have two of the lowest richness of AOTUs of all E. fuscus roosts, but most closely resemble the diet assemblage of M. lucifugus at the insect order level. These data suggest that E. fuscus showed an increased richness of diet after the introduction of WNS, potentially due to the increased abundance of food items previously consumed by other bats. Also, the AOTU richness of stomach samples was higher than that of guano samples, suggesting that sampling from stomachs could be more sensitive in detecting dietary breadth, which has been observed in other studies (e.g. Clare et al. 2009, 2014). These data do not account for the availability of insect prey on the landscape. The assumption is that prey was equally available to these bats over time pre-WNS to post-WNS. The same is considered across roosts for data collected in 2014 where it is assumed that the richness of insect prey available is spatially the same. Future studies could be conducted to test the abundance of insects on the landscape and how they have changed over time. A change in insect abundance of those species which are prey of bats could indicate the strength of population pressure of bats on insects and how that may have changed as a result of the loss of bats from WNS.

To compare species consumption overlap between M. lucifugus and E. fuscus, the most comparable samples are pre-WNS and post-WNS stomachs of E. fuscus, in which there was an increase from 37 to 58 species in common with M. lucifugus. Clare et al. (2014) found only seven species from the list generated for M. lucifugus, while 97 insect species were identified in the diet of E. fuscus across all post-WNS roosts. These data support the prediction that there is an increase in the overlap of prey items between E. fuscus and M. lucifugus in response to the decline of M. lucifugus (Clare et al. 2014). Further analysis could use radio isotopes of the guano samples (Salvarina et al. 2013, Royer et al. 2015), to characterize differences in the places where each species of bat is foraging based on differences in isotope ratios for insects which
feed over terrestrial landscapes vs. aquatic landscapes (Lam et al. 2013).

Our study showed that both *E. fuscus* and *M. lucifugus* consumed insect pest species, although these data cannot be used to quantify the abundance of these species in the diet or the overall effect on the population of these insect pests (Swift et al. 2018). Also, of the insect pests consumed by *M. lucifugus*, there was an increase in the number of these consumed by *E. fuscus* in response to the decline of *M. lucifugus*. The observed increase in the number of pest insect species consumed by *E. fuscus*, suggests that diet expansion by *E. fuscus* (due to competitive release), could help to fill the niche of insect pest control that was previously afforded by *M. lucifugus*. This apparent niche expansion by *E. fuscus* is important regarding the rescue effect (release), could help to relevant to pest control efforts. Very few benefits of the insects consumed by bats is not well known.

Further research is needed to quantify the overall ecological effect of WNS on the bat community assemblage, considering the effects of exploitative interspecific competition. This will be critical in modeling the potential for population recovery of *M. lucifugus* and other species, if they are able to overcome the direct effects of WNS. This study provides an extensive list of insect species which could be affected by changes in predation, but further research is needed to understand the overall impact on those insect populations, especially with respect to insect pests. Although DNA metabarcoding cannot currently quantify the magnitude of insect consumption, it nonetheless demonstrates that bats play a potential role in the control of insect pests and possible effects on beneficial insects. As described above, further research is needed on the biomass of each of these insects in the environment and how much of that biomass is actually consumed by bats to determine whether there is an actual population control service provided by bats and how that has changed with differences in the proportion of different bat species on the landscape.

Understanding competitive interactions in nature requires extensive studies of different types of systems. Those studies are often completed in controlled lab settings. The study here took advantage of a unique event in the environment, had no controls on the system, but was still able to detect significant changes in two bat species as a function of competitive release. Future research should build in these findings and better quantify the strength of the competitive interaction and what that means for bat population recovery and insect control.

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