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SPECIAL ISSUE: MOLECULAR DETECTION OF TROPHIC INTERACTIONS

The diet of *Myotis lucifugus* across Canada: assessing foraging quality and diet variability

ELIZABETH L. CLARE,* WILLIAM O. C. SYMONDSON,† HUGH BRODERS,‡ FRANÇOIS FABIANEK,§ ERIN E. FRASER,¶ ALISTAIR MACKENZIE,** ANDREW BOUGHEN,†† RACHEL HAMILTON,‡‡ CRAIG K. R. WILLIS,§§ FELIX MARTINEZ-NUÑEZ,§§ ALLYSON K. MENZIES,§§ KALEIGH J. O. NORQUAY,§§ MARK BRIGHAM,¶¶ JOSEPH POISSANT,¶¶ JODY RINTOUL,¶¶ ROBERT M. R. BARCLAY*** and JESIKA P. REIMER***

*School of Biological and Chemical Sciences, Queen Mary University of London, Mile End Road, London E1 4NS, UK, †Cardiff School of Biosciences, Cardiff University, The Sir Martin Evans Building, Museum Avenue, Cardiff CF10 3AX, UK, ‡Department of Biology, Saint Mary's University, Robie Street, Halifax, Nova Scotia, Canada, B3H 3C3, §Faculty of Forestry, Geography and Geomatics, Laval University, 2405 rue de la Terrasse, Quebec, Canada, G1V 0A6, ¶Environmental Science (Biology), Memorial University of Newfoundland Grenfell Campus, 20 University Dr., Corner Brook, Newfoundland and Labrador, Canada, A2H 5G4, **Pinery Provincial Park, Ontario Parks, 9526 Lakeshore Road, R.R. #2, Grand Bend, Ontario, Canada, N0M 1T0, ††Lake St. George Conservation Field Centre, Toronto and Region Conservation, 950 Bethesda Sd. Rd., Richmond Hill, Ontario, Canada, L4E 3G2, ‡‡Department of Biology, Biological and Geological Sciences Building, University of Western Ontario, London, Ontario, Canada, N6A 5B7, §§Department of Biology, University of Winnipeg, 515 Portage Ave., Winnipeg, Manitoba, Canada, R3B 2E9, ¶¶Department of Biology, Laboratory Building, University of Regina, Wascana Parkway, Regina, Saskatchewan, Canada, S4S 0A2, ***Department of Biological Sciences, University of Calgary, 2500 University Drive N.W., Calgary, Alberta, Canada, T2N 1N4*

Abstract

Variation in prey resources influences the diet and behaviour of predators. When prey become limiting, predators may travel farther to find preferred food or adjust to existing local resources. When predators are habitat limited, local resource abundance impacts foraging success. We analysed the diet of *Myotis lucifugus* (little brown bats) from Nova Scotia (eastern Canada) to the Northwest Territories (north-western Canada). This distribution includes extremes of season length and temperature and encompasses colonies on rural monoculture farms, and in urban and unmodified areas. We recognized nearly 600 distinct species of prey, of which ≈30% could be identified using reference sequence libraries. We found a higher than expected use of lepidopterans, which comprised a range of dietary richness from ≈35% early in the summer to ≈55% by late summer. Diptera were the second largest prey group consumed, representing ≈45% of dietary diversity early in the summer. We observed extreme local dietary variability and variation among seasons and years. Based on the species of insects that were consumed, we observed that two locations support prey species with extremely low pollution and acidification tolerances, suggesting that these are areas without environmental contamination. We conclude that there is significant local population variability in little brown bat diet that is likely driven by seasonal and geographical changes in insect diversity, and that this prey may be a good indicator of environment quality.

Keywords: molecular diet analysis, resource use, spatial/temporal variation, species' interactions

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Correspondence: Elizabeth L. Clare, Fax: +44 (0)20 7882 7732; E-mail: e.clare@qmul.ac.uk

Introduction

Molecular techniques are increasingly used to identify species, particularly morphologically cryptic taxa. This

has generated databases of taxonomically validated reference sequences (e.g. BOLD, Ratnasingham & Hebert 2007) to quantify biodiversity (e.g. Hebert *et al.* 2003), detect food market substitutions (e.g. Wong & Hanner 2008; Hanner *et al.* 2011) and improperly labelled food (e.g. Cohen *et al.* 2009). Characterizing ecological connections is more complicated than indexing species' presence (McCann 2007) and the use of reference databases to document interactions (e.g. Smith *et al.* 2006, 2007) has expanded greatly. Molecular techniques provide a powerful means to unravel food webs (Symondson 2002; King *et al.* 2008; Pompanon *et al.* 2012) which cannot be observed. These techniques developed from monoclonal antibody methods (e.g. Symondson & Liddell 1993) to cloning (e.g. Zeale *et al.* 2011; Alberdi *et al.* 2012), and next-generation sequencing (NGS) (Pompanon *et al.* 2012). NGS now dominates these analyses and has been applied to marine systems (Deagle *et al.* 2009, 2010), herbivores (Soininen *et al.* 2009; Valentini *et al.* 2009) and terrestrial insectivores (Bohmann *et al.* 2011; Brown *et al.* 2014). Next-generation sequencing is particularly effective when applied to generalists.

One hypothesis to explain food-web stability is that increased species richness is related to food-web complexity (the number of interactions). When richness is coupled with functional redundancy and behavioural flexibility, food webs become more stable (Solé & Montoya 2001; Kondoh 2003; Dunne *et al.* 2004). Generalism provides the opportunity for flexibility in prey choice and its importance is documented, for example, stabilizing both predator and prey population demography (Singer & Bernays 2003) or indirectly controlling lower food-web links (Rosenheim & Corbett 2003). The main prediction of this hypothesis is that, when resources become limited, flexible consumers become more general in resource use. Dietary flexibility can be driven by limited high quality food, and the necessity to diversify to achieve nutrition, to avoid toxins, to follow resources or minimize foraging risks (Singer & Bernays 2003). Some generalists switch between specialized resources (e.g. omnivory, Clare *et al.* 2013), while others consume food in ratios based on abundance (Rosenheim & Corbett 2003; Bastille-Rousseau *et al.* 2011).

Bats are an ideal group to study dietary flexibility as they occupy multiple trophic levels (carnivores, sanguivores, frugivores, nectarivores, insectivores) and niches (e.g. active hunting, passive listening for prey, fishing, trawling). They are frequently top predators and may consume resources at different trophic levels (e.g. Clare *et al.* 2013). However, they consume resources cryptically (active at night, using high-frequency echolocation) and are thus difficult to observe. Molecular methods provide a solution and are particularly useful in insectivores where thorough mastication of prey limits traditional morphological analyses of faeces (guano) (Kunz & Whitaker 1983) or culled prey remains (e.g. *Nycteris grandis* Fenton *et al.* 1981, 1990). In both cases, identification of prey is limited to order or family and small, soft bodied prey may be overlooked (Clare *et al.* 2009). Molecular analysis permits us to identify prey to species (Clare *et al.* 2009) particularly when coupled with reference libraries (Hebert *et al.* 2003; Ratnasingham & Hebert 2007) that increase precision.

Carter *et al.* (2006) showed a proof of the concept by amplifying chicken DNA from the faeces of white-winged vampire bats (*Diaemus youngi*). The first full molecular analysis of bat diet assessed predator/prey relationships between *Lasiurus borealis* and Lepidoptera (Clare *et al.* 2009) by sequencing DNA directly from residual prey fragments. Cloning and prey-specific primers were developed (Zeale *et al.* 2011) and used to uncover a novel hunting strategy of *Barbastella barbastellus* (Goerlitz *et al.* 2010) and the diet of *Plecotus macrobullaris* (Alberdi *et al.* 2012). These methods have rapidly been replaced by NGS (Bohmann *et al.* 2011; Razgour *et al.* 2011; Clare *et al.* 2014; Emrich *et al.* 2014) which is faster and more cost effective.

Myotis lucifugus, the little brown bat, was one of the most common and widespread bats in North America, though populations are in decline due to white nose syndrome (Frick *et al.* 2010). They have a distribution from Alaska, through southern Northwest Territories, the prairies, Ontario, Quebec and the Maritime provinces in Canada, and south through the continental United States and northern Mexico (Fenton & Barclay 1980). Arthropod consumption by bats (including *Myotis lucifugus*) varies by species and season (tied to temporal changes in prey abundance and reproductive cycle) (Kunz *et al.* 2011), and by age (Fraser & Fenton 2007). At peak metabolic demand during lactation, little brown bats may consume more than their body mass in prey each night (Kurta *et al.* 1989) and thus potentially provide a significant ecosystem service through insect consumption (Boyles *et al.* 2011). They are generalists consuming insects of low prey hardness (Freeman 1981) mostly emerging from aquatic systems, for example, Diptera and Trichoptera (Belwood & Fenton 1976; Freeman 1981; Ober & Hayes 2008), although adult females consume more Lepidoptera and Trichoptera (Belwood & Fenton 1976).

Myotis lucifugus' tendency to forage over water provides a means to assess foraging-location quality. In this context, our reference to foraging habitat/location quality refers to both type of habitat (such as moving or still water) and also to the potential acid and pollution content of the aquatic system. Benthic macro-invertebrates are frequently used as environmental indicators. Their pollution tolerance (e.g. organic pollutants, acidification)

and habitat requirements have been documented (Hilsenhoff 1988). If we consider bats as a sampling mechanism, species-level diet analysis provides data for assessing the quality of foraging areas without complicated, potentially invasive methods such as radio tracking bats to locate foraging followed by mass insect sampling. Thus, while bats may not be used as a method of general habitat assessment (their sampling is biased by perceptual characters and preferences etc.), their diet can provide us with information on specific areas they have visited.

Clare *et al.* (2011) performed the first molecular analysis of little brown bat diet in three locations in Southern Ontario. They identified 66 prey species and noted a shift from consumption of Diptera early in the summer to Ephemeroptera in mid and late summer. There was evidence of local diet variation that allowed inferences about foraging-location quality. There is evidence that diet diversity is a function of location; populations in northern Ontario have greater dietary variability than those in southern areas (Belwood & Fenton 1976). The range of little brown bats in Canada includes areas of high and low insect species richness. If prey themselves are a limited (and limiting) resource, as prey richness decreases, the null hypothesis is that predators should similarly consume a lower species richness; however, if abundance is high, diet may change little or predators may adopt a more general strategy and consume a wider variety of prey (higher values of Simpson's diversity index, Simpson 1949).

Our study had two objectives. First, we assessed variability of little brown bat diets across Canada, over the summer and between years, and tested the hypothesis that they have a high degree of dietary variability across location and time. Second, we used the identity of prey to make inferences about habitat, based on known habitat requirements and pollution tolerances of the prey. We tested four predictions about diet: (i) latitude has an effect on diet; (ii) temporal patterns of prey exploitation across the summer are stable from year to year; (iii) there is a significant shift from the consumption of species of Diptera to Ephemeroptera associated with phases of the reproductive cycle; and (iv) species-level analysis of prey provides criteria for assessing foraging-location quality and yields quantitatively different insights than ordinal-level analysis.

Methods

Sample collection

We collected guano under maternity roosts of *Myotis lucifugus* across Canada (Fig. 1) during three periods, including pregnancy (early summer = May to mid-June), lactation (middle summer = mid-June to mid-July) and postlactation (late summer = mid-July to September). Collections in Ontario were performed in 2009 (at Clinton, the Pinery), 2009 and 2011 (Lake St. George) and in 2011 for all other locations. Sampling was performed weekly in Ontario throughout the summer (fine-grained analysis) and during the three established periods in other locations (see Fig. 1 for details). Additional material was collected at two locations in Quebec but due to sampling differences and difficulties with molecular analysis we include this only as a supplement (see details in Data S1 and S2, Supporting information) for comparison. We adopted the definitions of seasons from Clare *et al.* (2011) (see Data S3, Supporting information for collection dates and locations). We froze samples or preserved them in high-percentage ethanol (70–100%). Because we collected samples from colonies rather than individuals, the volume of material was substantial (exceeding half a litre per week by volume in some cases) and reflected deposition by many individuals (potentially exceeding a thousand in some locations), we analysed a random subset of the pellets from each collection (volume c.1.5 mL of guano or c.50 pellets, hereafter a 'sample').

DNA extraction, amplification and sequencing

We extracted DNA from homogenized samples using the QIAmp DNA Stool Mini Kit (Qiagen, UK) following manufacturer's instructions with modifications from Zeale *et al.* (2011), further modified as follows: (i) to encompass more individuals and thus greater prey diversity we used approximately 50× more starting material and (ii) we extended the first centrifuge step (Zeal step 4) to 3 min to aid in pelleting the particulate material. Extracted DNA was stored at −20 °C prior to amplifications.

We tested DNA extractions success using the primers ZBJ-ArtF1c and ZBJ-ArtR2c (Zeale *et al.* 2011). We then amplified each sample using a modified fusion-primer version for the Roche FLX sequencer (Bohmann *et al.* 2011) consisting of a Lib-L adaptor, the key sequence, a unique 10 bp DNA sequence (MID) and the original primer sequence (ZBJ-ArtF1c or ZBJ-ArtR2c). In our design (following Brown *et al.* 2014; Clare *et al.* 2014), MID sequences were used on both forward and reverse primers allowing fewer primers to be used to resolve the same number of samples (i.e. rather than 100 unique forward MID tagged primers for 100 samples, 10 unique forward and 10 unique reverse MIDs can yield the same resolution power) while reducing primer costs. We assigned each sample a unique primer combination so all sequences could be identified to original samples.

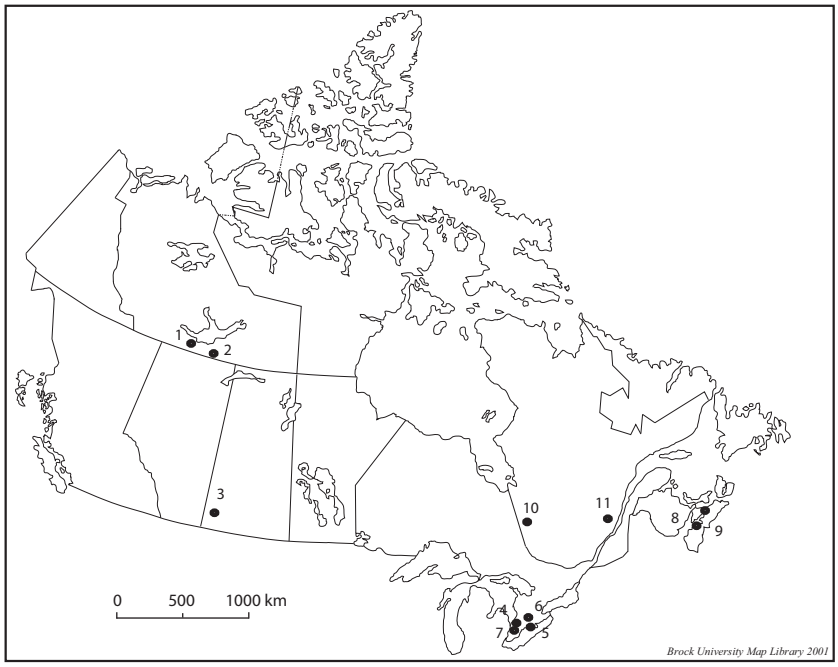

Fig. 1 Distribution of sampling sites across Canada. Samples in Northwest Territories (n = 5) were collected at sites in Kakisa (1) and Salt River (2) (considered as one unit in statistical analysis). Samples in the prairies (n = 3) were collected between Medicine Hat (Alberta) and Swift Current (Saskatchewan) (3). Samples in Ontario were collected in Clinton (4) (n = 14), Long Point (5) (n = 7), Lake St. George (6) (2009 n = 18, 2011 n = 7) and Pinery Provincial Park (7) (n = 4). Samples in Nova Scotia (n = 8) were collected at sites in Martock (8) and Tatamagouche (9) (considered as one unit in statistical analysis). Samples in Quebec were collected at Aiguebelle National Parks (10), Jacques-Cartier National Park and Montmorency Research Forest (11). (Map Modified from: Canada Outline Map. St. Catharines, Ontario: Brock University Map Library. Available: Brock University Map Library Controlled Access http://www.brocku.ca/maplibrary/maps/outline/North_America/canadaNONAMES.pdf (Accessed April 2, 2013).)

We performed PCRs as described by Bohmann *et al.* (2011) in a 20 μL reaction containing 1 μL of template DNA using Qiagen multiplex PCR kits (Qiagen, UK) with the following modifications. We did not use Q solution (from the kit) or BSA (as suggested by Bohmann *et al.* 2011). We visualized PCR products on a 1.5% agarose gel and quantified them following Brown *et al.* (2014) and mixed approximately equal molar quantities of each sample. We size-selected products using a QIAquick Gel Extraction kit (Qiagen, UK) and quantified the final PCR mix using a Qubit dsDNA BR Assay Kit (low sensitivity with a Qubit Fluorometer, Invitrogen life technologies).

We concentrated the final product to 10 μg/μL in molecular grade water. Sequencing was conducted at the Liverpool Center for Genomic Research (University of Liverpool) using a ¼ plate, Lib-L chemistry on a Roche 454 GS FLX+ sequencing system (Roche Applied Sciences).

Sequence analysis

We analysed sequences using Galaxy (https://main.g2.bx.psu.edu/root, Giardine *et al.* 2005; Blankenberg *et al.* 2010; Goecks *et al.* 2010). We screened all recovered sequences for those longer (>180 bp) or shorter (<100 bp) than expected, collapsed all sequences to unique haplotypes, split the file by forward and reverse MIDs, removed primers, MIDs and adaptors and excluded rare haplotypes (<2 copies).

We clustered the sequences into molecular operational taxonomic units (MOTU) in jMOTU (Jones *et al.* 2011) and tested thresholds from 1 to 10 bp. A graph of recovered MOTU vs. threshold (not shown) suggests a 4 bp cut-off was most appropriate (Razgour *et al.* 2011).

We compared representative sequences for each MOTU to the BOLD database (www.barcodinglife.org) following criteria modified from Razgour *et al.* (2011): 1a = match to one species or several species in a genus (100% similarity), most conservative taxonomy kept; 1b=good match (>98% similarity), but could belong to a congener showing a higher sequence match; 2 = match to more than one species (>98%), only one of which is present in the sampling range (that taxonomy kept); and 3 = close match (as above) to several species from different genera, or to a reference sequence which lacks a full taxonomic record. In these cases, the most conservative taxonomy (normally family) was kept (note this

is not an identification to higher level taxonomy, but a match meeting criteria 1b but retaining ambiguity in the assignment due to multiple similar matches or incomplete data in the reference collection).

We also estimated the identity of all MOTU (including unidentified MOTU) using the methods of Emrich *et al.* (2014) and the programme MEGAN (Huson *et al.* 2011). See Emrich *et al.* (2014) for details of that procedure and a brief discussion.

Ecological analysis

We divided our collections into the three time periods. We conducted ecological analyses in PAST (Hammer *et al.* 2001) on species and order-level data with *P*-values estimated by permutation. We compared the Simpson's diversity indices for identified prey among locations (sequential Bonferroni correction) and among summer sampling periods, and estimated the magnitude of the effect (effective number of species), where differences were statistically significant, following Jost (2006). We compared species richness from paired weekly samples from the high-density sampling sites at Clinton (rural monoculture farming area) and Lake St. George in 2009 (environmentally variable conservation area). We computed rarefaction curves for all data.

We compared the proportion of each order in the diet (proportion = frequency of occurrence of that order/total occurrences, where an occurrence is an identified MOTU in a sample) among locations and among sampling periods using a χ^2 frequency test with *P*-values computed using a Monte Carlo simulation with 2000 replicates in R 2.15.1 (R Development Core Team: R: A language and environment for statistical computing 2008).

We use the recovered species to evaluate the foraging location of the populations using the Hilsenhoff Biotic Index for organic pollutants developed for the western Great Lakes (Hilsenhoff 1988) and the Fjellheim & Raddum (1990) index for acid tolerance.

Results

Sequence processing

We recovered 167 562 sequences. After filtering, these were resolved into 10 792 unique haplotypes that could be assigned to an original sample. We clustered these into molecular operational taxonomic units (MOTU) and examined a representative sequence from each cluster. We removed six MOTU as contaminants (nearest similarity was identified as a nonprey item e.g. bacteria). The remaining 566 MOTU were used in further analysis and represent a mean of ≈9 species per sample.

Diet of little brown bats

Through comparison to the reference library, we identified 211 MOTU to species using criteria 1a, 1b and 2 (Data S1, Supporting information), hereafter referred to as species. We also identified an additional group of MOTU using criteria 3 but consider them as provisional identifications. Of the identified occurrences (defined above), ≈45% were Lepidoptera, ≈34% Diptera, ≈11% Ephemeroptera, ≈6% Trichoptera and ≈4% Coleoptera (Fig. 2). An additional nine species were found to be Araneae (four species), Hemiptera (one species), Hymenoptera (one species), Megaloptera (two species) and Neuroptera (one species). The most common prey were two species of Chironomids (Diptera): *Dicrotendipes tritomus* and *Paracladopelma winnelli* found in 29% and 22% of samples, respectively, and two species of Ephemeroptera: *Caenis youngi* and *Caenis amica* found in 28% and 22% of samples, respectively (note that *Caenis* are difficult to separate morphologically or genetically and multiple cryptic species are suspected, and thus, the actual identity of species within this genus should be considered an estimate due to taxonomic limitations). A single species was identified as prey in all sampled locations, a moth, *Hydriomena* (Lepidoptera, Geometridae). However, *Hydriomena* contains species with overlapping DNA barcodes (shared haplotypes at COI), and thus, this identification may correspond to more than one species. We recovered a similar analysis of prey diversity from MEGAN which suggests that

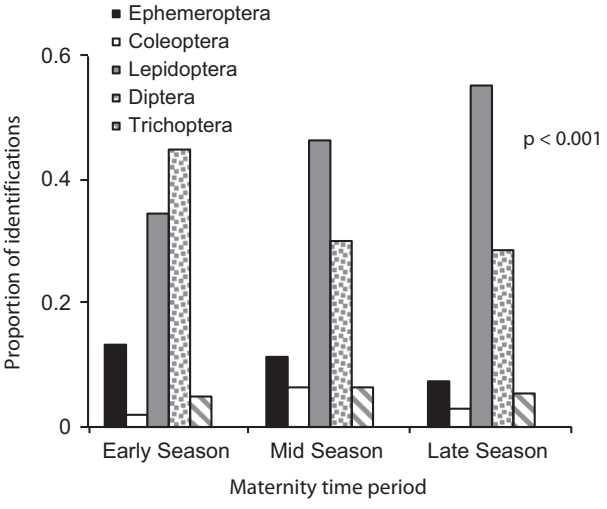

Fig. 2 Seasonal diversity in prey consumed by *Myotis lucifugus*. The proportion of each prey group in the diet varied significantly across seasons. Diptera dominated the early season diet, while Lepidoptera become more important in the middle and late seasons. Proportion = frequency of occurrence of that order/total occurrences, where an occurrence is an identified molecular operational taxonomic unit in a sample.

unidentified prey are relatively dispersed among the consumed insect groups.

Many of the prey consumed provide specific information on the type and quality of the aquatic system; the most sensitive taxa, including families Glossosomatidae, Ephemerellidae and Corydalidae and genera *Lemnephilus, Agrypnia* and *Phryganea*, were consumed in both the Northwest Territories and Lake St. George (for a site-by-site analysis see Table 1).

Spatial-temporal variation in resource use

Considering species from the five main prey groups (Ephemeroptera, Coleoptera, Lepidoptera, Diptera and Trichoptera) with all data pooled, the proportion of consumption varied significantly among periods ($\chi^2 = 26.89$, $P = 0.0005$, Fig. 2). In early summer, the diet was dominated by Diptera (45% of occurrences) though their presence decreased throughout the summer (30% in mid-summer, 29% in late summer). In contrast, Lepidoptera increased from 35% of occurrences in early summer, to 46% in mid-summer and 55% in late summer. The frequency of occurrence of Ephemeroptera, Coleoptera and Trichoptera remained stable. We did not observe a switch from consumption of Diptera to Ephemeroptera as previously reported (Clare *et al*. 2011).

Prey use varied significantly among locations ($\chi^2 = 119.69$, $P = 0.0005$, Fig. 3). In some locations (Northwest Territories, Lake St. George 2009), the main prey were Lepidoptera and Diptera, while in other locations (e.g. Lake St. George 2011) prey consumption was dominated by Lepidoptera. These differences do not appear to reflect sampling intensity; the three most heavily sampled locations (Clinton, Lake St. George 2009 and 2011) showed different patterns of prey use.

Despite difference in prey consumption, Simpson Index measures did not indicate a significant difference in dietary diversity among locations (Fig. 4) except at Pinery Provincial Park in Ontario. When considered at the ordinal level, diversity of prey at Pinery was particularly low. This pattern was different when considering species (MOTU) level resolution; diversity estimates were more even, and bats at Pinery had high diversity. Saturation of rarefaction curves (Fig. 5) indicates sampling reached a plateau in ordinal-level identifications, while species-level identifications were still increasing almost linearly (Fig. 5C,D). Diversity estimates at ordinal and species level were not correlated ($r = 0.27$, $P = 0.18$). Latitude did not correlate with diversity at the ordinal ($r = 0.43$, $P = 0.15$) or species ($r = -0.11$, $P = 0.4$) levels.

Diversity estimates varied significantly among seasons (early = 0.66, mid = 0.67, late = 0.60) with a nearly significant reduction in dietary diversity observed between early and late season ($P = 0.05$) and a significant reduction between mid and late season ($P = 0.031$) (Fig. 6), reflecting reductions in the effective numbers of species of 14% and 20%, respectively.

Table 1 Approximate habitat assessments based on the lowest scoring (least tolerant to pollution or acidification) taxa identified in the diet of bats at each location

Location	Example taxa	Pollution tolerance[†]	Acid tolerance	Maximum quality
NWT	Glossosomatidae	0	Low	
	Ephemerellidae	1		Low organic pollution
	Heptagenia sp		Low-med	No acidification
Lake St. George	Glossosomatidae	0	Low	
(Ontario)	Ephemerellidae	1	High	Low organic pollution
	Corydalidae	0		No acidification
Clinton	Helicopsychidae	3		
(Ontario)	Tipulidae	3		Trace organic pollution
	Isonychia	3		
Long Point	Leptoceridae	4		Some organic pollution
(Ontario)	Phryganeidae	4		
Nova Scotia	Leptoceridae	4		
	Phryganeidae	4	High	Some organic pollution
	Stenacron	4		
	Molanna sp.		Low	No Acidification
Pinery	Chironomidae	6		Some organic pollution
(Ontario)	Psychodidae	10		
	Phryganeidae	4	High	Possibly acidified
Saskatchewan	Chironomidae	6		Likely organic pollution*

*Little data available.

[†]Hilsenhoff index goes from 0 (low) to 10 (high) tolerance.

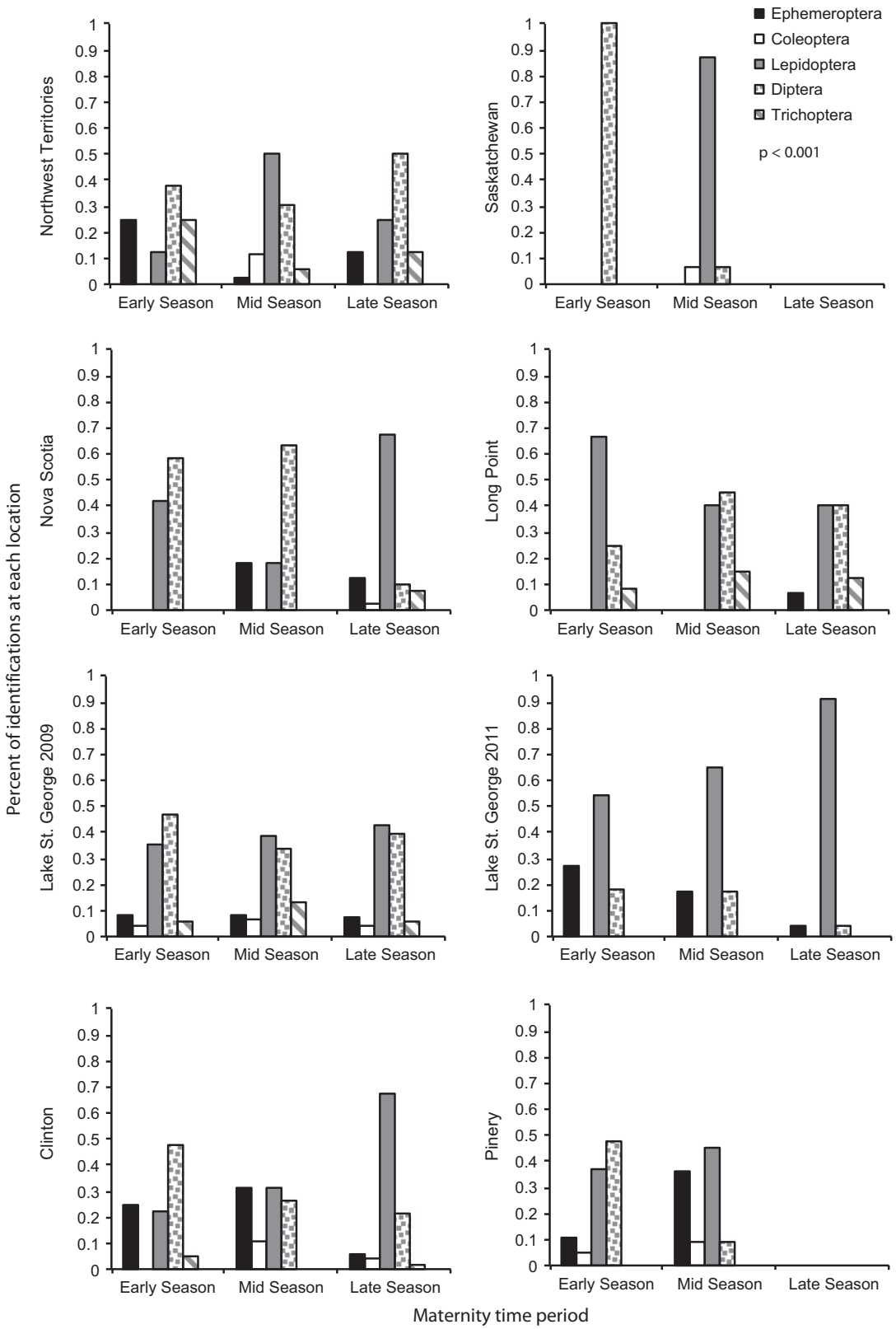

Fig. 3 Seasonal diversity in prey consumed by *Myotis lucifugus* at 8 locations across Canada. The proportion of each prey group composing the diet varied significantly across seasons and with location. Proportion = frequency of occurrence of that order/total occurrences, where an occurrence is an identified molecular operational taxonomic unit in a sample.

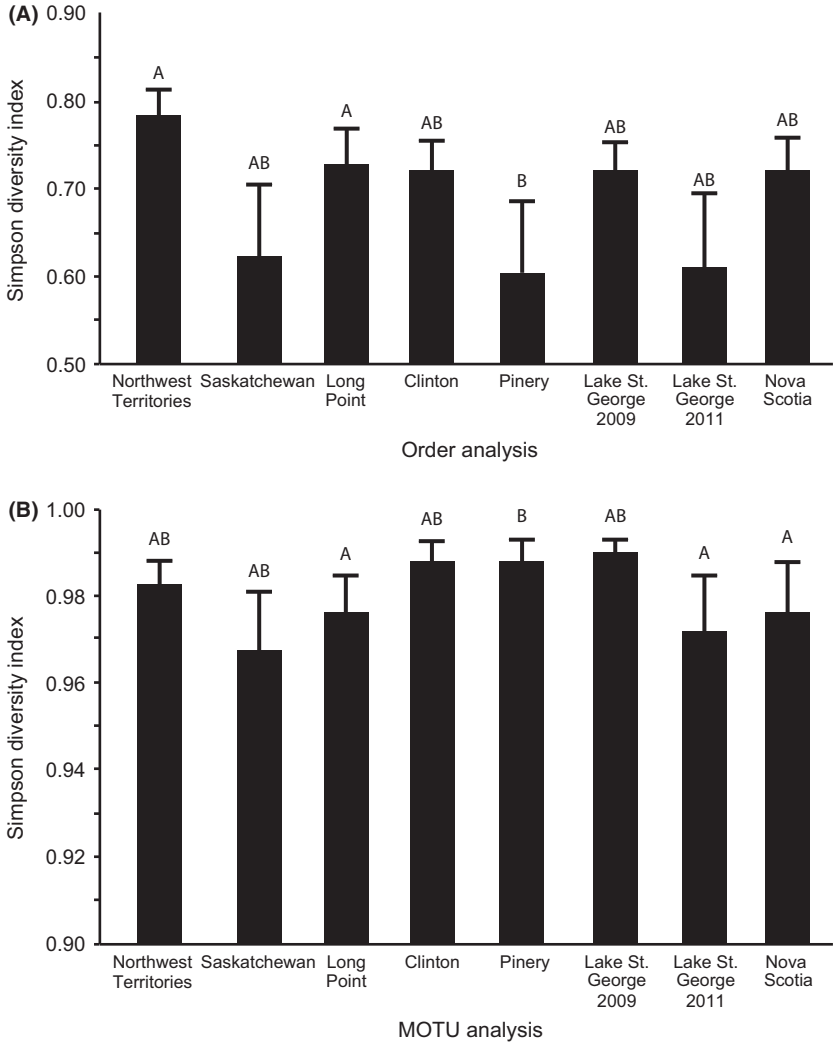

Fig. 4 Estimates of *Myotis lucifugus'* dietary diversity with 95% confidence intervals, based on the Simpson diversity index on data restricted to ordinal-level taxonomy (A) and using molecular operational taxonomic units as a proxy for species (B).

We sampled the same colony at Lake St. George in 2009 and 2011. In 2009, we estimated that this colony consisted of several thousand individuals, although this number declined slightly in 2011 likely due to white nose syndrome (Frick *et al.* 2010). Sampling at this location was performed during matched weeks between the 2 years, but we observed remarkable difference in the spatial/temporal pattern of prey use. In 2009, prey use mirrored that observed across all locations (Fig. 2), while in 2011, Diptera represented a minority of prey, Lepidoptera dominated all seasons (91% in late season), and no Coleoptera or Trichoptera were consumed.

The most heavily sampled locations were Clinton (n = 14 weeks) and Lake St. George in 2009 (n = 18 weeks). Of these, 13 sampling weeks were common and could be directly compared (difference reflects differential colony establishment). Although not significant, there is a trend towards higher species richness at Lake St. George in 2009; mean prey species richness was 20 species/sample compared with a mean of 17 in Clinton (Fig. 7), although the number of species was higher in only 8 of 13 weeks.

Discussion

Our goal was to examine variation in resource use by bats across Canada and to use these data to infer foraging-location quality. While at the most northern sampling location (NWT) bats consumed prey evenly between orders, there was no consistent pattern of consumption among locations. Intensive sampling of populations in different locations in Ontario across 2 years indicated significant spatial/temporal variation in prey use even in small geographic scales. We did not observe a seasonal shift between the consumption of Diptera and Ephemeroptera. Analyses at the species level showed different patterns than at the ordinal level, indicating that species-level resolution provides novel insights into dietary analysis.

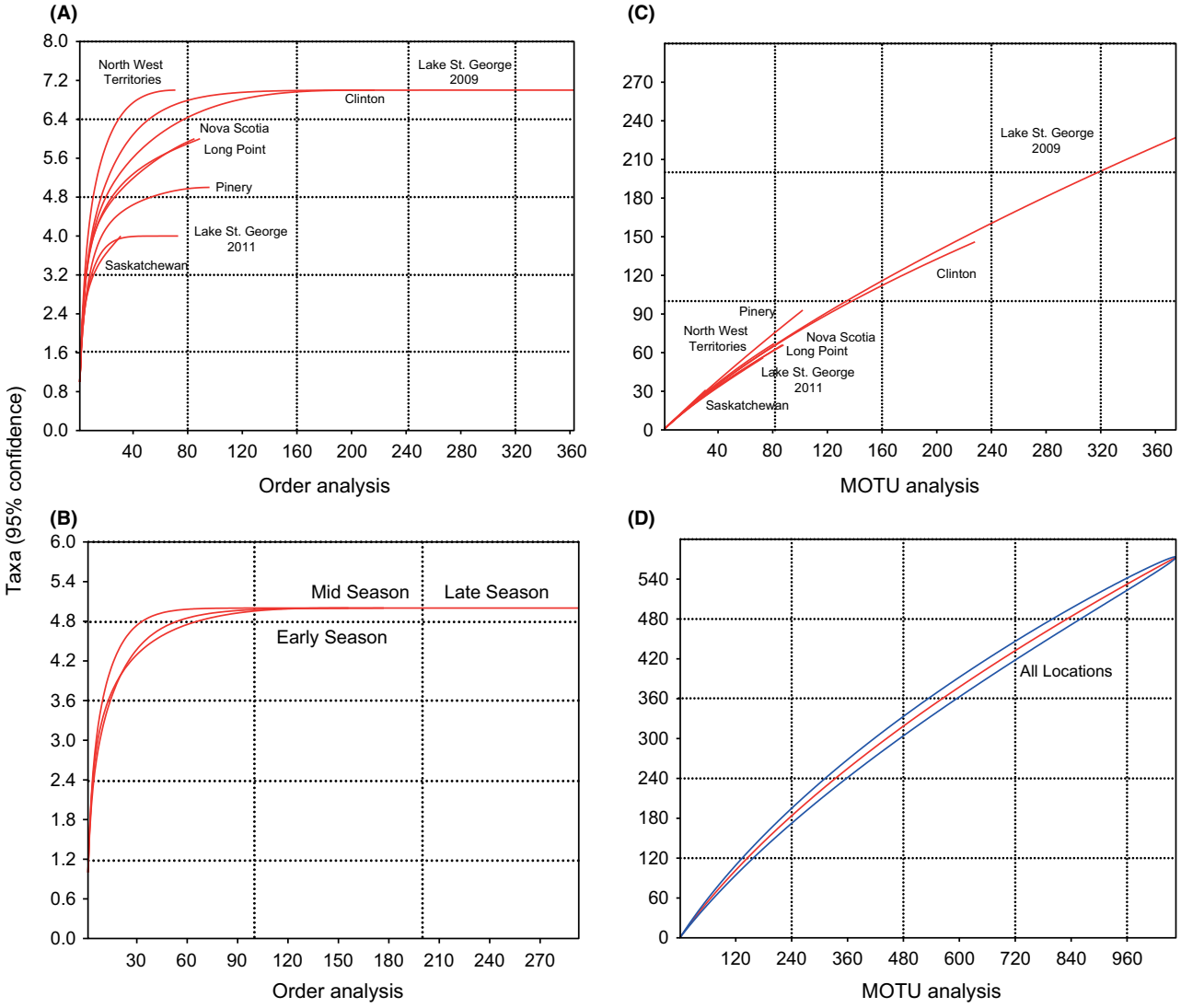

Fig. 5 A comparison of rarefaction curves for operational taxonomic units at the order (A, B) and species (C, D) level. Lines are mean estimates (A, B, C) or mean with 95% confidence levels (D) based on permutations.

Spatial variation in diet across Canada

When we combined data from all locations, Diptera dominated the diet in the early season but were replaced by Lepidoptera in the mid- and late seasons. This pattern was prominent at Lake St. George (2009) and the NWT, but variable at other locations. The reliance on Diptera in the early season agrees with previous morphological (Belwood & Fenton 1976; Freeman 1981; Ober & Hayes 2008) and molecular (Clare *et al.* 2011) analyses. Diptera are an important prey group in both species richness and dietary abundance. We found no evidence to support the reported heavy reliance on Trichoptera, but found more species of Lepidoptera than expected. This may reflect the overabundance of Lepidoptera within the reference collection, biasing the number of taxonomic identities reported. It is possible that Trichoptera represent a large number of the 'unknowns' within our sample although our estimations using MEGAN indicate that unknowns are relatively dispersed among taxonomic groups (Fig. 8).

Traditional morphological analyses are based on estimating abundance of prey groups in any given sample. Lepidoptera are frequently identified from scales and small morphologically cryptic species may be lumped into a single unit or overlooked. One advantage of molecular analysis is the routine detection of rare prey (Clare *et al.* 2009). However, as molecular analyses cannot estimate abundance, biomass or volume within a sample, rare and common items are both 'present'. For example, haplotype number does not reflect abundance,

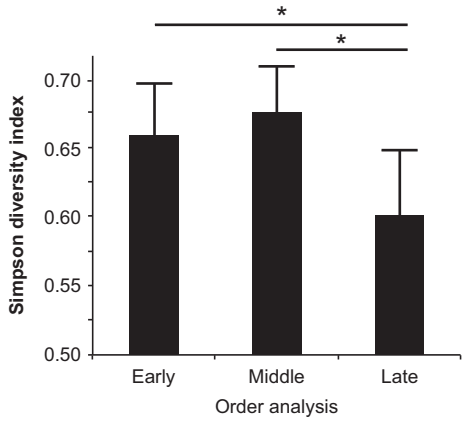

Fig. 6 Estimates of *Myotis lucifugus'* dietary diversity with 95% confidence intervals based on the Simpson diversity index from three seasons. Early season = females are pregnant, middle season = females are lactating, late season = young are independent.

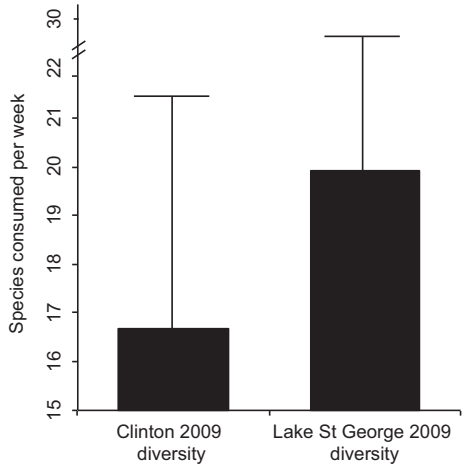

Fig. 7 Weekly species richness in the diet of *Myotis lucifugus* for the two most heavily sampled sites, at Clinton and Lake St. George in 2009, showing a trend of higher mean species richness with 95% confidence intervals in bats at Lake St. George, which is also an area where prey has a lower pollution tolerance suggesting higher quality habitat.

MID tags, primers and adaptors influence sequencing, sequencing direction can produce different results and biases in sequencing are not consistent between runs even using the same PCR products (Pompanon *et al.* 2012; Deagle *et al.* 2013; Piñol *et al.* 2013). A large sample size may control for overrepresentation of rare prey (or underrepresentation of common prey); however, there is a trade-off between increasing the volume of material analysed (the pooling method here) to increase our assessment of biodiversity and the potential for skew with presence and absence records, though it is not a correction that can be empirically assessed.

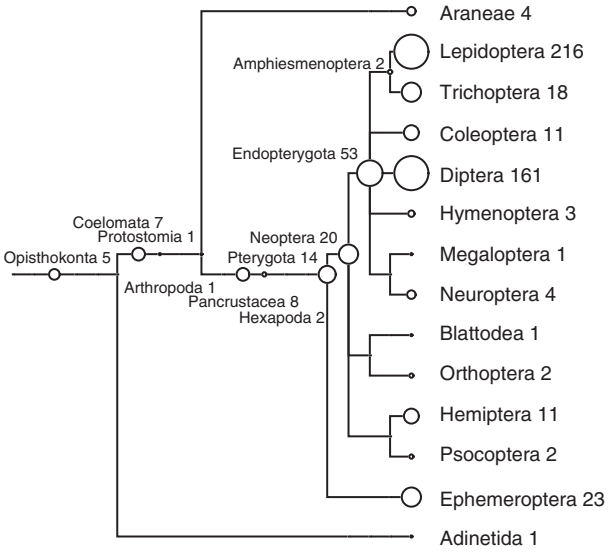

Fig. 8 A schematic of prey species consumed including all molecular operational taxonomic units(MOTU) (including those that could not be identified using a reference database). Identifications have been made by BLAST score and are limited to hypothesis at the order level. Values at nodes or tips represent the number of MOTU assigned. Node size is scaled to the number of assignments. See Emrich *et al.* (2014) for additional details.

While we cannot estimate sample-based abundance, molecular analysis allows us to measure species richness and frequency across samples. While richness within an order can be related to abundance, there are important exceptions. Mass-emerging prey such as mayflies (Ephemeroptera) may be extraordinarily abundant but low in species richness. In our analysis, Lepidoptera may appear as the most important food source because they are more speciose, while mayflies may be underrepresented. The abundance of Lepidoptera may also reflect previous observations that females consume more Lepidoptera than males (Belwood & Fenton 1976); all of the colonies we sampled were maternity groups dominated by females and their offspring. The results from Quebec, based on males (Data S2, Supporting information), indicated more Diptera which may support this conclusion.

We observed significant spatial variation in diet. We use Simpson's Index which is less sensitive to rare events that frequently occur in species-level analysis (Bohmann *et al.* 2011; Razgour *et al.* 2011). Our estimates of diversity were not correlated with latitude and not related to sample size. The Saskatchewan and Pinery colonies had the lowest sample sizes (and could not be sampled in late season at all) but differ in patterns of prey use. Both were low in diversity at the ordinal level but so was Lake St. George (2011) which had one

of the largest sample sizes. Significant spatial variation in resource use is unsurprising across such a wide geographical area; however, it was also similarly variable within southern Ontario and between years. This matches previous observations (Clare *et al.* 2011) supporting the view that these bats responded to local variation in environment and prey. As such, predicted declines in the populations of little brown bats (Frick *et al.* 2010) may have locally-specific effects on insect populations.

The main assumption of the correspondence between insect diversity and diet is that resources themselves are limiting. Although little brown bat colonies may consume thousands of insects in a night, it is not clear whether their populations are large enough to significantly reduce local populations of insects.

Temporal variation in diet

We observed a significant decrease in dietary diversity in late season when the effective reduction in species richness was 20%. This contrasts with a matching analysis of big brown bats (*Eptesicus fuscus*) (Clare *et al.* 2014) for which dietary diversity rose sharply in late season. These inverse patterns may reflect nonoverlapping resource use by these predators. Big brown bats are a flexible hunter that appears to forage in most habitat types (Geggie & Fenton 1985; Furlonger *et al.* 1987) and consumes large numbers of beetles, moths and flies (Clare *et al.* 2014). Insect diversity falls in late season just as both species must store fat for hibernation. While big brown bats may compensate by exploiting a wider variety of habitats (and thus prey), increasing their dietary diversity, little brown bats may simply consume a greater volume of more limited prey. Habitat selection by bats strongly influences insect availability, and thus diet, and may explain apparent resource partitioning among many species (Emrich *et al.* 2014). Current or historical competition for resources is also possible, but makes the assumption that resources are limiting. There is little direct evidence that competition drives patterns of resource use because this cannot be assessed without controlled removal experiments, which are exceedingly difficult with bats.

Clare *et al.* (2011) observed a significant shift from consumption of Diptera in early season to Ephemeroptera in middle and late season. The same pattern was not observed here in any location, including in the same samples originally analysed by Clare *et al.* (2011). This likely reflects a difference in methodology. Clare *et al.* (2011) sequenced DNA directly from fragments of prey removed from guano under microscopic dissection. The advantage of this technique is that the user can preferentially attempt to maximize the taxonomic richness of the sample, but it is likely biased towards the detection of less-digestible prey (Razgour *et al.* 2011). Because Clare *et al.* (2011) took efforts to sample a large number of guano pellets, they also assumed that each fragment represented a different capture, and thus, frequency was calculated directly from the recovered sequences. NGS provides an automated method to maximize the diversity of prey recovered, but does not allow for the same assumption of independence of each haplotype. The fragment and sampling method employed by Clare *et al.* (2011) is a hybrid between traditional morphological analysis and NGS and may be more similar to abundance-based methods. This is only likely to cause significant difference when the taxa are mass-emerging species found in high abundance but low species richness, such as Ephemeroptera. NGS may underestimate the importance of this prey group, while the fragment method may overestimate them if the assumption of independence between fragments is not met. In addition, our methods used short amplified regions (157 bp) compared with Clare *et al.* (2011) who used full DNA barcodes of ≈657 bp. Short primers may limit taxonomic resolution in some cases but increase the likelihood that degraded DNA will be amplified. Different primers will always have different binding affinities and this may partially explain specific prey differences between these two analyses.

Methodological advances and species versus ordinal-level data

We used two specific methodological advances in our analysis. To separate samples after sequencing, NGS uses incorporated tags in primers. These tags are often called MIDs or 'barcodes' (although we do not use this term to avoid confusion with DNA barcodes as per Hebert *et al.* (2003)). Using MIDs on forward primers, each sample can be amplified with a unique forward primer and subsequently separated. However, for very large sample sizes, this becomes costly. As introduced (Brown *et al.* 2014), we incorporated MIDs in both forward and reverse primers so that each sample can be assigned a unique combination of MIDs (e.g. 10 forwards and 10 reverses = 100 unique combinations). This technique significantly reduces primer costs without impacting sequencing performance. Second, rather than extracting DNA from a single guano pellet (or even half a pellet as in some publications) we extracted DNA from a pool of pellets totalling 1–1.5 mL by volume. This roughly translated into 20–50 pellets per sample (depending on size). Previous analyses have estimated a mean of 5 taxa per pellet (Bohmann *et al.* 2011), while we recovered a mean of nine per sample. In this study, each 'sample' is, in effect, an assay of diet in what is

likely dozens of individuals. The disadvantage of this method is that larger volume extractions lead to more PCR inhibitors that may complicate reactions. However, this also provides two specific advantages. In general, it leads to greater taxonomic richness in the resulting sequencing run. More specifically, insectivorous bats have a very fast gut transit time with prey passing as fast as 35 min after ingestion (Buchler 1975). As such, any single pellet may be low in prey richness. Morphological analyses normally examine many dozens of pellets to estimate diet and we have incorporated this method. As discussed earlier, large sample sizes may control for the potential for overrepresentation of rare prey though this may explain our lower than expected measures of Ephemeroptera.

Molecular methods allow us to go beyond traditional ordinal-level assessments, available from morphological analysis, to establish species-level taxonomic assignments of prey. It is particularly interesting that when we remove these data, some dramatic changes (e.g. estimates of diversity in Pinery) can be observed. This is largely due to saturation of ordinal-level collections, while species-level data have not reached a plateau.

Environmental indicators and foraging assessment

Benthic macro-invertebrates are frequently used as environmental indicators of the quality of a water system (Hilsenhoff 1988; Fjellheim & Raddum 1990; Lenat 1993). The analysis of diet from bats foraging over these locations provides a direct (non-invasive) method to infer the quality of their foraging location. This method is more specific than a general insect survey as it assesses where the bat has been rather than where it may have been. Insect tolerance estimates vary by season and area (see a comparison of Wisconsin and North Carolina, Lenat (1993)), but we can make a number of observations from our data using the Hilsenhoff Biotic Index for organic pollutants developed for the western Great Lakes (Hilsenhoff 1988) and the Fjellheim & Raddum (1990) index for acid tolerance (extrapolating from related species) and inferences about other Canadian regions (Table 1).

Among the Trichoptera, Hydropsychidae, Leptoceridae and Phryganeidae have moderate pollution tolerances of 4, while Helicopsychidae have a tolerance of 3 and Glossosomatidae a tolerance of 0. Glossosomatidae also have a low tolerance for acidification. Leptoceridae and Phryganeidae were eaten by bats in the Northwest Territories, Nova Scotia, Long Point and Lake St. George (2009), while Helicopsychidae occurred in the diet at Clinton. The pollution intolerant glossosomatides were eaten in the Northwest Territories and Lake St. George (2009). Diptera in the family Tipulidae have a tolerance of 3 and were also found at Clinton. The Ephemeroptera family Ephemerellidae has a pollution tolerance of 1. These were detected in the Northwest Territories and Lake St. George (2011); the Megaloptera family Corydalidae has a pollution tolerance of 0 and was detected in Lake St. George (2009). Species of *Molanna* may be acid intolerant and were detected in Nova Scotia.

While habitat specificity of many macro-invertebrate species declines (or becomes more variable) at higher latitudes (Lenat 1993), our observations suggest that bats at Clinton forage in good quality habitat (Helicopsychidae and Tipulidae both have tolerance = 3). However, there is convincing evidence that the sites in the Northwest Territories and Lake St. George have an excellent quality habitat with little apparent organic pollution (species with tolerance of 0 and 1 detected frequently) or acidification. This might be expected for the remote Northwest Territories locations (which are far from major human modification), but is less expected for Lake St. George, which lies on the edge of the greater Toronto area. The presence of prey with low pollution tolerances at Lake St. George in 2009 and 2011 demonstrates the stability of this site and may be an indication of the effectiveness of small-scale conservation efforts even in areas near intensive urban modification.

Some macro-invertebrates are relatively good indicators of habitat type. Species in the Trichoptera genera *Agrypnia* and *Traenoides* were identified in Northwest Territories, Long Point and Lake St. George. They are associated with pond or lake-like habitats in northern parts of their range. We have previously confirmed that the Lake St. George bats hunt in the vicinity of Lake St. George (a very small water body) <300 m from the roost site. It is likely that the Long Point bats are hunting along the shores of Lake Erie, and the Northwest Territories population may be using any of hundreds of variously sized water bodies.

Summary

In response to resource fluctuations, species may move to track prey or adapt to match local variability. The little brown bat, *M. lucifugus*, occupies a broad niche, foraging over aquatic systems. Species-level identifications of benthic macro-invertebrate prey serve as environmental indicators and allow us to use information about diet to directly measure the quality of the foraging habitat. In total, we recorded nearly 600 species of prey consumed by this predator and present one of the largest and most geographically diverse molecular dietary analyses to date. With these data, we demonstrate seasonal, regional and interannual variation in

little brown bat diets across Canada which is independent of latitude. We identify two locations where the prey consumed are particularly intolerant to organic pollution or acidification and thus locations where foraging habitat is of high quality, even when in the vicinity of high-density urban development.

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E.L.C., H.B., F.F., E.F., A.M., A.B., R.H., C.W., F.M., A.M., K.N., M.B., J.P., J.R., R.M.R.B., J.P.R. designed and conducted field research. E.L.C. conducted the molecular analysis. W.O.C.S. contributed to molecular protocols. All authors contributed to manuscript production.

Data accessibility

All DNA sequencing reads and an explanatory 'read me' file along with BLAST scores for figure 8 have been placed in Dryad: doi:10.5061/dryad.67g2q.

Supporting information

Additional supporting information may be found in the online version of this article.

Data S1 All taxonomic matches are 1 or 1a unless indicated with a* = level 2.

Data S2 Procedures for Quebec samples.

Data S3 Sampling dates.