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Citation for final published version:

Clare, Elizabeth L., Symondson, William Oliver Christian and Brockett Fenton, M. 2014. An inordinate fondness for beetles? variation in seasonal dietary preferences of night-roosting big brown bats (Eptesicus fuscus). Molecular Ecology 23 (15) , pp. 3633-3647. 10.1111/mec.12519

Publishers page: http://dx.doi.org/10.1111/mec.12519

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An inordinate fondness for beetles? Variation in seasonal dietary preferences of night roosting big brown bats (*Eptesicus fuscus*)

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Key Words: insectivores, species' interactions, molecular diet analysis

Running Title: Molecular analysis of big brown bat diet

Abstract

Generalist species with numerous food web interactions are thought to provide stability to ecosystem dynamics however it is not always clear whether habitat generality translates into dietary diversity. Big brown bats are common across North America and employ a flexible foraging strategy ~~, flying~~ over water, dense forest~~esd areas~~, ~~along~~ forest edges and ~~in~~ rural and urban settings ~~in search of prey~~. Despite this generalist use of habitat, they are paradoxically characterized as beetle specialists. However, hard carapaces may preferentially survive digestion leading to overrepresentation during morphological analysis of ~~faeces~~ diet. ~~and t~~This specialization has not been evaluated independently using molecular analysis and species level identification of prey. We used next generation sequencing to assess the diet of big brown bats ~~from fecal samples collected in 2008 and 2011~~. Beetles were consumed in the highest frequency but Lepidoptera species richness was highest among identified prey. The consumption of species showed strong seasonal and annual variation ~~(2008, χ^2 =20.6, p=0.005, 2011, χ^2 =23.2, p=0.004) but also varied between years ((χ^2 =19.7, p=0.04)~~. While Coleoptera consumption varied ~~seasonally~~, Lepidoptera and Ephemeroptera were relatively constant dietary component ~~in all years and over the entire summer~~. Dietary diversity increased in late summer when insect diversity decreases. Our results indicate that ~~the diet of~~ big brown bats ~~is~~ are dietary ~~omnivorous~~ generalists and, while beetles are an important component of the diet, Lepidoptera are equally

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important, and Lepidoptera and Ephemeroptera are the only stable prey resource exploited. As resources become limited big brown bats may respond by increasing the species richness of prey and thus their connectedness in the ecosystem. This characterization of diet corresponds well with a generalist approach to foraging and this ~~extreme generalist strategy~~ makes them an ~~fundamentally~~ important species in encouraging and maintaining ecosystem stability.

Introduction

Understanding interactions among species is fundamental to assessing the way in which ecosystems function and respond to variation. ~~Predator-prey~~Species' interactions, particularly those involving generalists and omnivores, ~~predators~~ can be ~~particularly~~ important in promoting ecosystem stability. The importance of behavioural flexibility and resource use has been widely modelled in studies of food-web stability. Food webs appear most susceptible to the removal of the most linked (generalist) species (e.g Solé & Montoya 2001). In general, increased behavioural flexibility of species in these networks allows a wider variety of species to interact in response to local resource availability, and this functional redundancy may stabilize ecological networks (Kondoh 2003) e.g. by directly stabilizing both predator and prey population sizes (Singer & Bernays 2003) or via indirect control on lower level food web links (Rosenheim & Corbett 2003). ~~although they are among the most difficult to document.~~

In response to resource limitations, species may compete for resources or alter the prey they choose. Over many generations, resource limitation may drive the evolution of morphological or behavioural specialization and adaptive radiations and sympatric species are thought to evolve and co-exist through partitioning available resources and niche ~~specialization~~ differentiation (Ricklefs 2007). Alternatively, competition for resources may result in increasing

niche flexibility (Grant & Grant 1987; Tebbich *et al.* 2004). ~~Behavioural flexibility is key to ecosystem functioning and is viewed as a stabilizing force in food webs which buffers the impact of species loss (Solé & Montoya 2001; Dunne *et al.* 2004).~~ Spatial-temporal variation in resource use is an important form of behavioural flexibility which is particularly adaptive when resource availability fluctuates.

There are two main ~~hypotheses~~ ways in which ~~attempt to explain how~~ resource distributions ~~are~~ may be related to the stability of food webs. First, increasing complexity within food webs increases their stability and thus highly linked generalists promote ecosystem functioning (Solé & Montoya 2001). Second, generalists that consume resources based on frequency of encounters (Rosenheim & Corbett 2003) (rather than achieving generalism by switching between highly specialized tactics) may respond to resource limitation by increasing the abundance of a particularly resource or increasing their flexibility and consuming a wider variety of resources. In this context~~,~~ behaviourally flexible highly linked omnivores or generalists, that respond to limitations by increasing the variety of prey they consume, may be particularly important components of ecosystem stability and documenting their ecosystem function is vital to understanding ecosystem response to disturbance (Solé & Montoya 2001).

Behavioural flexibility in foraging by insectivorous bats has been well documented and dietary diversity and choices may be influenced by habitat variation (Aldridge & Rautenbach

1987), temporal availability and abundance of prey (Rydell *et al.* 1996), gender (Belwood & Fenton 1976) and or age (Adams 1997). In extreme cases, bats may switch between feeding guilds and trophic levels to supplement diet (e.g. the ~~supposedly~~ nectivorous *Glossophaga soricina* uses unique tactics incorporate insects in its diet (Clare et al. in ~~r~~press~~eview~~)). This degree of flexibility is unusual in a top predator and makes ~~them~~ bats key ecosystem ~~species~~ taxa and excellent models for the study of ecosystem functioning, though their cryptic behaviour makes it extraordinarily difficult to directly document their behaviour in the wild.

A variety of molecular methods have been used to untangle ~~these~~ complex ~~relationships~~ species' interactions (Symondson 2002; King *et al.* 2008), especially next generation sequencing (NGS) methods (Pompanon *et al.* 2012) that can generate millions of ~~prey~~ sequences at relatively low cost. NGS methods can be applied to fragmentary, emulsified or mixed starting materials such as stomach or faecal contents. This approach is based on sequencing in extraordinary volume (so called "sequencing depth") to encompass the complete richness of targets ~~species~~ within a system (Pompanon *et al.* 2012). This contrasts with cloning methods (Zeale *et al.* 2011) that also begin with mixed starting materials but where the discovery of new prey species is based on the inevitably limited number of sequenced clones rather than the NGS mass screening approach. The NGS approach provides a possible solution to the problem of understanding the complexity of interactions between generalist predators and their prey by

analyzing the prey exploited based on large samples. Using this method we can extend our analyses beyond accurately documenting interactions (e.g., Deagle *et al.* 2009, 2010) to testing specific predictions about how species' interactions vary in time and space (e.g., Razgour *et al.* 2011).

Several previous molecular analyses of bat diets have documented temporal variation in resource use. In the first large scale molecular analysis of the diet of an insectivorous bat, Clare et al. (2009) found little evidence that *Lasiurus borealis* (the eastern red bat) in Ontario, Canada exhibited temporal variation in prey consumption. In contrast, molecular analyses of the feces of another species, *Myotis lucifugus* in Ontario (Canada) (Clare *et al.* 2011, Clare et al. ~~in review~~THIS ISSUE) and *Plecotus* in the UK (Razgour *et al.* 2011) showed evidence of temporal variation in diet. In the case of sister species of *Plecotus* this may lead to competition when resources become limited (Razgour *et al.* 2011).

~~The tendency for resident populations of bats to hunt locally and show strong temporal variation in resources use has significant implications for understanding ecosystem dynamics and their response to change.~~ Understanding ecosystem function and the services provided by generalist predators is particularly important when population demography is unstable and this "service" may fluctuate (Blehert *et al.* 2009). *Eptesicus fuscus*, the big brown bat, is common across most of North America and ~~in~~ parts of Central and northern South American and the

Antilles (Simmons 2005). It is one of the bats best known to the ~~general~~ North American public because of its association with rabies (Nadin-Davis *et al.* 2010) and its propensity to roost in buildings (Kurta & Baker 1990). ~~*E. fuscus*~~Individuals ~~may~~ frequently forage within 2 km of their roost (Kurta and Baker 1990), ~~or~~ but may also travel to sites up to 7 km away (Brigham 1991). In some areas, ~~*E. fuscus*~~big brown bats use night roosts as places to temporarily stop and digest prey (Kurta & Baker 1990) so accumulations of droppings at night roosts provide an opportunity to determine which foods are consumed locally.

While many bats used mixed foraging habitats (and sympatric species may overlap in their foraging (Furlonger *et al.* 1987)) some general trends in habitat use are apparent for the common species in the study site (Ontario Canada). For example, horay bats and ~~Eastern~~ eastern red bat~~s~~s often forage in cover or along edges (Furlonger *et al.* 1987) and frequently in concentrations of insects at street lights (Hickey & Fenton 1996; Acharya & Fenton 1999), while the three small *Myotis* bats ~~little brown bats mainly forage~~are much more habitat restricted and forage over riparian systems~~water~~ (Furlonger *et al.* 1987~~,~~) very close to their roosts (Clare *et al.* 2011) more commonly than the othe ~~Clare *et al.* 2011)~~species.~~.~~ In contrast, ~~Big~~ big brown bats appear to employ ~~one of the most~~a very ~~general~~ flexible ~~forage strategies~~strategy with no significant habitat associations (Furlonger *et al.* 1987)~~s~~. Acoustic monitoring has measured big brown bat activity in all rural settings ~~They have been reported to fly over~~ including over water,

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forested areas, along edges and at street lights ~~and in both rural and urban settings~~ (Geggie & Fenton 1985; Furlonger *et al.* 1987) and they are the only species to make significant use of urban areas (Furlonger *et al.* 1987). Although they employ a generalist strategy across these landscapes, big brown bats have been paradoxically called beetle specialists (Coleoptera; e.g., Freeman 1981; Ober and Hayes 2008; Agosta et al. 2003). However, the ~~alleged~~ observed preference for beetles (as high as 96% of diet by mass, Agosta *et al.* 2003) has not been challenged by accurate species-level identification of their prey. The hard carapace of beetles may preferentially survive digestion leading to an overrepresentation of these taxa during morphological analysis of prey remains in faeces. Molecular analysis has been demonstrated to accurately identify small, soft bodied prey and rare taxa (Clare *et al.* 2009) and it presents an excellent diagnostic tool ~~to assess the complete~~providing species-level analysis of the diet of big brown bats. These data will ~~and~~ answer the question of whether their generalist foraging strategy translates into a far more general diet than previously documented.~~?~~

We assessed the diet of a group of night-roosting big brown bats during ~~their colony establishment periods in~~the summer of both 2008 and ~~in~~ 2011 (total of 25 weeks). The roost was located in a forested patch along the Grand River in Cambridge Ontario. The bats roosted in an overhanging section of soffit and fascia. Continuous observation and inspection of the home showed no evidence that the bats roosted in the home during the day however they ~~. *E fuscus*~~

foraged in the area in the apparent absence of other bat species and fresh guano (faeces) accumulated each night. Given this, ~~We~~ we concluded that we were sampling faeces left by night-roosting bats rather than a permanent colony. If the bats whose diets we documented fed locally, we predicted that the diet would vary reflecting local availability of prey.

We tested three hypotheses about the diet of this species. First, we tested the hypothesis that resident big brown bats exhibit seasonal variation in their diet reflecting a preference for local feeding, tracking generally established fluctuations in prey abundance. Second, we tested the hypothesis that variation in diet between years is minimal so that overall dietary diversity and seasonal variation in diet are stable across years. Finally, we tested the prediction that ~~E fuscus is~~big brow bats are beetle specialists by estimating the relative importance of prey groups in the diet of this species over two years of monitoring. We also compared ordinal level analysis of prey (as traditionally conducted during morphological dietary analysis) with analysis at the species level made possible using molecular methods.

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Materials and Methods

Sample collection, DNA Extraction, Amplification and Sequencing

We placed collection sheets under the colony for weeklong periods between May and September in 2008 and again in 2011. We collected the accumulated faeces weekly and stored it

at -20°C prior to analysis for a total of 25 weeks of monitoring (15 weeks of continuous monitoring in 2008 and 10 weeks in 2011 reflecting differential colony establishment times in the two years, we observed no variation in sequencing success due to length of storage).

For each weekly sample we selected a minimum of 30 faecal pellets and homogenized them as a single unit to ensure they were well mixed – hereafter we refer to these homogenized pellets as a "sample". From each sample we extracted DNA using the QIAmp DNA Stool Mini Kit (Qiagen, ~~Valencia, CA~~UK) according to manufacturers instructions with the modifications suggested by Zeale *et al.* (2011). In addition we made the following protocol additions; 1) to encompass community diet in each sample, rather than the diet of a single individual bat, we used many (at least 30) faecal pellets rather than just one giving a volume of 1-1.5ml of starting material and 2) we extended the first centrifuge step (Zeale step 4) to 3 minutes to aid in pelleting the particulate material produced by this large volume. We stored the extracted DNA at -20°C prior to PCR amplification.

We tested all DNA extractions using unmodified primers ZBJ-ARTF1c and ZBJ-ArtR2c from Zeale *et al.* (2011) to confirm extraction success. We then amplified each sample using fusion primers (Figure 1) adapted for the Ion Torrent platform (Ion Torrent, Life Technologies). These primers consisted of adaptor sequences (forward adaptor and trP regions) a unique DNA sequence (MID) used to bioinformatically separate sequences for analysis and the original primer

sequence ZBJ-ARTF1c and ZBJ-ArtR2c from Zeale *et al.* (2011) as required for the sequencing platform. In our design we follow Brown et al. (THIS ISSUE) and used unique combinations of 10 bp MID sequences on both the forward and reverse primer for each pooled sample. This design allows fewer primers to be used to resolve the same number of samples (called sequence libraries) (e.g., rather than 100 unique forward MID tagged primers for 100 samples, 10 unique forward and 10 unique reverses~~d~~ can yield the same resolution power). This reduced the cost of primers. We sequenced only in the forward direction ~~did not use bi-directional sequencing but sequenced only in the forward direction~~ further reducing the number of required primers.

We carried out PCRs ~~reactions~~ following the amplification reaction described by ~~(~~Bohmann *et al.* (2011) in a 20 ul reaction containing 1ul of template DNA using Qiagen multiplex PCR kits (Qiagen, UK) as described with the following modifications: we did not use either Q solution (from Qiagen) or BSA (as suggested by Bohmann *et al.* 2011). We visualized all PCR products on a 1.5% agarose gel. We quantified the PCR products by measuring the relative luminescence of 1ul of PCR product on a 1.5% agarose gel stained with ethidium bromide (validated using Qubit low sensitivity dsDNA BR Assay Kit on a Qubit Flurometer, Invitrogen Life Technologies~~)~~). We pooled equal molar quantities of each PCR product and then size selected and purified these using a QIAquick Gel Extraction kit (Qiagen, UK). We quantified the final mixed PCR product using a Qubit dsDNA BR Assay Kit (low sensitivity)

with a Qubit Flurometer (Invitrogen Life Technologies). Sequencing of the product was conducted at the University of Bristol Genomics facility (School of Biological Sciences, Bristol UK) on an Ion Torrent personal gene machine (PGM) (Ion Torrent, Life Technologies) using a 316 chip and 300 bp chemistry. PCR products were quantified for sequencing using a Bioanalyzer (high sensitivity kit, Agilent Technologies).

Sequence Analysis

We separated forward and reverse MIDs, clipped primer and adapter sequence and filtered sequences using the Galaxy platform (https://main.g2.bx.psu.edu/root, (Giardine *et al.* 2005; Blankenberg *et al.* 2010; Goecks *et al.* 2010) and Bioedit (T. Hall, http://www.Mbio.ncsu.edu/bioedit/bioedit.html). We filtered all recovered sequences for rare haplotypes (represented by <2 copies) and sequences much longer (>175 bp) or shorter (<100 bp) than the expected 157 bp amplicon.

We clustered the sequences into molecular operational taxonomic units in the program jMOTU (Jones *et al.* 2011) and tested thresholds from 1-10 bp. A graph (not shown) of recovered MOTU vs. threshold suggests that a 4 bp cut-off was most appropriate for this data set (see ~~(~~Razgour *et al.* 2011). We extracted representative sequences for each MOTU ~~and edited~~

~~and aligned them manually to remove indels and to match~~to compare with reference sequences from known insect ~~sequences~~taxa.

We compared these representatives to the reference database in BOLD (www.barcodinglife.org) and extracted identifications based on four criteria modified from (Razgour *et al.* 2011). Confidence 1a = match to one species or several species in a genus (100% sequence similarity) most conservative taxonomy kept; confidence 1b = good match (>98% sequence similarity) but could belong to a congener if the database is updated with something with a higher sequence match; confidence 2 = match to more than one species (>98.5%) only one of which is known to be present in sampling range (that taxonomy kept) and confidence 3 = close match (as above) to several species from different genera, or to reference sequence which itself lacks a full taxonomic record. In these cases, the most conservative taxonomy (normally family) was kept (Note: this is not the same as an identification to higher level taxonomy, but an acknowledgement of a match meeting the criteria of 1b but retaining an ambiguity in the proper assignment due to either multiple similar matches or incomplete data in the reference collection). In addition, we estimated the identity of all prey (including unidentified MOTU) using the methods of Emrich et al. (THIS ISSUE) and the programme MOTU. See Emrich et al. (THIS ISSUE) for details of that procedure and a brief discussion.

Ecological Analysis

We divided our collections into three temporal periods: early (May13th - June 20th) middle (June 20th –Aug 6th) and late (Aug 6th - September 9th) (periods in 2011 varied by one or two days to equalize time periods and based on colony establishment) following (Clare *et al.* 2011) to coincide with the observed periods of pregnancy, lactation and post lactation for this species in Ontario. Ecological analyses were conducted using the program PAST (Hammer *et al.* 2001) on species and order level data with p-values estimated by permutation. We compared the Shannon and Simpson diversity indices for identified prey between years and between early~~m~~ middle and late summer within years and estimated the magnitude of the effect where differences were statistically significant following Jost (2006). We computed rarefaction curves for these data with 95% confidence intervals.

We compared the frequency of consumption for each order (number of species consumed and the frequency of each species consumption) and each species (with its frequency) between years and between early, middle and late summer within years using a χ^2 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).

Results

Prey Identification and Dietary Composition

We recovered ~3.5 million DNA sequences from the Ion Torrent 316 chip of which ~50% were produced for this study (the chip was shared). Of these, processing for quality (sequence length, recoverable MIDs and primers), collapsing to unique haplotypes and splitting files by MID left a total of 32,212 unique haplotypes from all 25 weeks of collection (Data archived in Dryad ~~Dryad:…~~). Processing through jMOTU reduced these to 221 molecular operational taxonomic units (MOTU) at the 4 bp threshold.

Using representative sequences of the 221 MOTU (species of prey) we identified 158 (71%), with most identifications at the species level, through comparison to existing reference databases of known insect DNA barcodes for the area. Of these, species of Lepidoptera, Diptera and Coleoptera dominated the diet, with species of Coleoptera representing the highest frequency of consumption while Lepidoptera were present in the highest taxonomic richness (Table 1).

Seasonal and Annual Variation in Prey Consumption by order

Using order level taxonomy ~~(ignoring genus and species)~~only, the consumption of species showed strong seasonal variation in 2008 (χ^2 =20.6, p=0.005) and in 2011 (χ^2 =23.2, p=0.004) (Figure 2) although the pattern of variation was inconstant between years and the overall consumption of prey differed significantly between the two years (χ^2 =19.7, p=0.04;

Figure 3). In 2008 the early summer diet was dominated by species of Diptera (43% of prey consumed) while the importance of Coleoptera increased throughout the year peaking at 32% in the late summer. In 2011, Coleoptera were dominant in both early (39%) and mid (38%) summer diet while Diptera increased from 20-26% over the year. Consumption of Lepidoptera was relatively constant within and between years varying between ~18-29% of the diet at all times (Figure 2, Figure 3). Similarly the consumption of Ephemeroptera~~ns~~ was low but consistent across the summer and years representing 8-13% of the identified prey diet at all times (Figure 2, Figure 3).

Despite variation ~~in how prey resources are used~~in the consumption of prey orders, we found no change in the diversity of diet between years (Shannon index 2008=1.57, 2011= 1.60, p=0.55, Simpson index 2008=0.78, 2011= 0.77 p=0.41; Figure 4 and 5). Within years, we detected a significant increase in the diversity of prey consumed between early and late summer in 2008 (Shannon index early=1.4, late= 1.5, p=0.036, Simpson index early=0.71, late= 0.77 p=0.007) though significance was lost in the Shannon index comparison after a sequential Bonferroni correction (Figure 4, Figure 5). The magnitude of the difference in the effective number of species consumed was close to 10% between the early summer and either middle or late summer. In 2011 the diversity of prey consumed was significantly higher in late summer than in either early (Shannon index early=1.5, late= 1.7, p=0.009, Simpson index early=0.75,

late= 0.81 p=0.014) or mid (Shannon index early=1.4, late= 1.7, p=0.001, Simpson index early=0.73, late= 0.81 p=0.003) summer (Figure 4, Figure 5) with the magnitude of the difference estimated at 9% and 7.5% respectively. In both cases, the effective number of species in the diet appears to increase by as much as 10% from early in the summer (when bats are pregnant) to late summer (when bats are preparing to hibernate).

Seasonal and Annual Variation in Prey Consumption by MOTU

Using MOTU as a proxy for species level taxonomy, dietary diversity also was constant between years (Shannon index 2008=4.6, 2011= 4.4, p=0.54, Simpson index 2008=0.99, 2011= 0.95 p=0.41) (Figure 4, Figure 5). In 2008 we detected a significant increase in the diversity of prey consumed between early and mid summer (Shannon index early=3.8, mid= 4.1, p=0.032, Simpson index early=0.97, mid= 0.98 p=0.021) though significance is lost in the Shannon index after a sequential Bonferroni correction (Figure 4, Figure 5). We also detected an increase in diversity between early and late summer (Shannon index early=3.8, late= 4.1, p=0.013, Simpson index early=0.97, mid= 0.98 p=0.012) (Figure 4). We detected no significant trend in diversity changes in 2011 after sequential Bonferroni correction (Figure 4, Figure 5).

We recovered a similar analysis of prey diversity from MEGAN (Figure 6) which suggest that unidentified prey are relatively dispersed among the consumed insect groups.

Dietary Species of Interest

A number of prey species consumed by big brown bats are interesting in the context of "ecosystem services" provided by bats. Big brown bats ate human pests such as *Culex pipens* (the common house mosquito), *Simulum* spp. (the black flies) and *Polistes* spp. (the paper wasps). Paper wasp nests were observed under the same overhang as the bat roost. It is possible this record represents a secondary contamination event, however the nest was not located over the collection surface. Bats also ate some species such as Trichoptera that emerge from water. Other aquatic insects *Caenis spp.* and *Maccaffertium mediopunctatum* are generally found over moving water habitats in this area of their distribution (Clare *et al.* 2011).

Discussion

Dietary Richness and Diversity

The most striking conclusion from our analysis is the confirmation that ~~Eptesicus fuscus~~big brown bats rely~~ies~~ heavily on Coleoptera although, at most, beetles constituted about 40% of dietary richness. ~~This contrasts strongly with the~~Previous analyses have reported 42-96% consumption by volume in some studies (e.g. Agosta *et al.* 2003). While abundance is not ~~necessarily~~ proportional to species richness, they are often related, particularly when a predator

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cannot effectively make prey choices at the species level. It is very likely that consumption varies significantly between areas and years, at least in part due to local conditions. ~~In this case,~~ ~~w~~We furthermore strongly suspect ~~that~~ big brown bats ~~are unlikely to~~cannot obtain enough information to discriminate subtle species level characteristics, but ~~likely~~ make choices about prey ~~perhaps~~ based on acoustic detection of size, flight speed, flight path, and acoustic properties generated by hard carapaces revealed through echolocation (see also Brigham & Saunders ~~(~~1990~~,)~~ ~~and~~ Barclay & Brigham ~~(~~1994~~)~~). It is also very probable~~y~~ that the flexibility exhibited by big brown bats leads to significant regional differences in diet thus the relative importance of beetles may vary greatly by location (e.g. ~~(~~Brigham 1990). Agosta *et al.* (2003) argued that richness of diet increases when prey themselves become more limited, presumably because resource limitation provides incentive for a more generalist strategy. Our data also suggest~~ed~~ that dietary richness increased in the late summer. In this case, increases in dietary richness measured at the species level here, and estimates made for richness measured by abundance (Agosta *et al.* 2003) may be in agreement that diet is responding~~-~~ to local insect population fluctuations. This presumes that insects themselves are a limited resource and there may be some evidence for this in late summer of temperate bats' active periods. Razgour *et al.* (2011) observed increased dietary partitioning between cryptic bat species late in the year and speculated that a drop in insect abundance led to increased resource competition driving a temporally constrained niche

specialization. ~~Records~~ Trends of flight periods of adult beetles in Ontario obtained from the Canadian National Collection (CNC, Ottawa Ontario, Canada) for many species in this dataset suggest a local flight peak in mid summer corresponding to the lactation period of bats. For example, *Ecyrus dasycerus*, and *Cymatoder bicolor* peak in June and July, *Ampedus semicinctus* peaks in May-July, *Graphisurus fasciatus* and *Monochamus notatus* peak in June – August, and *Melanotus similis* peaks in April-August (Bruce Gill, CNC Ottawa, personal communication). We hypothesize that if beetle [insect] species richness and diversity undergoes a significant local decrease in late summer coinciding with increased dietary needs of bats as they approach hibernation, this could drive increases in dietary richness without the bats actively choosing new prey at the species level. As such, it is an effect of insect phenology, rather than predator choice. Interestingly, records for a wider geographic area show peaks for these same insect species often extend into late fall (Yanega 1996) in other areas thus the affect may be local to this part of the bats' and beetles' range. An additional contributing factor to this pattern is the emergence of juvenile bats that may be less discriminatory in their prey choice. Their appearance co-insides with this drop in insect richness and abundance and both factors may cause an increase in dietary richness.

In addition to the importance of Coleoptera in the diet, Diptera, Lepidoptera, and Ephemeroptera were often eaten. While Diptera (primarily chironomids) and Coleoptera varied

364 in importance over time, Lepidoptera and Ephemeroptera were consistent components of the
365 bats' diet across years. As such, they may be an underappreciated stable resource supporting the
366 population while other insect groups fluctuate in importance.

367 Several ~~One~~ important data considerations need to be taken into account. First, ~~is that~~
368 within a sample, prey ~~are~~ were measured simply by their presence (quantification is not
369 possible). We used larger pools of guano to maximize potential biodiversity by increasing the
370 number of contributing predators. However, a rare item and a common item would both be
371 recorded as "present" in that sample. A large sample size may control for the potential for
372 overrepresentation of rare prey (or underrepresentation of common prey) in any one sample,
373 though it is not a correction that can be empirically assessed. Another consideration is that
374 Lepidoptera dominates the reference collection, as this has been a major campaign of the various
375 DNA barcode consortia. As such, there is likely an identification bias towards Lepidoptera which
376 must be considered, though our estimates based on MEGAN (Figure 6) did not vary greatly
377 suggesting this is not a significant factor. Finally, we have no basis to conclude whether the bats
378 have consumed prey groups (e.g. moths) in accordance with abundance, however the authors
379 have evaluated the diet of 5 sympatric species of bat (manuscript published and in preparation)
380 and none has revealed beetles as such a strong dietary component, thus we suggest this is a true

preference for this predator on this prey rather than a case of encountering more in the environment.

Temporal variation in diet

Increases in dietary diversity as the summer progressed, suggests a relationship between diet and known changes in species richness in prey availability. Seasonal limitations in prey cause shifts to more generalist behaviour in several groups. For example, spiders are normally unselective in their choice of prey because they are in a state of suboptimal nutrition and cannot afford to be selective, however, when prey are in excess, they become more selective (Riechert & Harp 1987). We may have recorded a similar effect raising implications for the relationship between diet and hibernation. Hibernation and torpor success is directly influenced by dietary components. For example, in marmots, diets deficient in essential fatty acids led to decreased length of torpor (Florant *et al.* 1993) and a diet high in n-6 polyunsaturated fatty acids (PUFA) increases the frequency and duration of torpor, decreases body temperature and decreases mass loss during inactivity, and the ratio of n-6 to n-3 PUFAs may be key (Ruf & Arnold 2008). However, insectivorous bat diet is naturally very low in PUFA and, though they do apparently preferentially select insects which maximize PUFA intake, the goal may be to optimize rather than maximize PUFA (Munro & Thomas 2004). Coleoptera and Trichoptera are very high in the

399 essential fatty acid linoleic acid (Schalk & Brigham 1995) and the richness of these in the diet of
400 big brown bats increased in the fall in 2008 and 2011 ~~respectively~~. This may reflect an increase
401 reliance on prey which can supply high quality diet in preparation for hibernation when body
402 mass may increases by as much as 50% (Beer & Richards 1956), though the relationship
403 between dietary fats and hibernation is complex.

404 Our observations of an increase in dietary diversity contradict the pattern that dominates
405 in little brown bats across Canada (Clare *et al.* ~~in review~~THIS ISSUE). In ~~this~~ that species,
406 diversity peaked in mid summer and was significantly reduced in late summer. This difference
407 likely reflects the degree of habitat flexibility between these two predators. Little brown bats
408 hunt ~~mainly~~ more frequently over water. In ~~this~~ that case, reduced flexibility in habitat choice
409 may reduce the diversity of their diet. Little brown bats may rely on greater volume of more
410 limited prey richness, while big brown bats exploit a wider variety of prey.

411 We documented dietary variation across the summer but also between years which
412 strongly supports of the importance of long term monitoring. Had our analysis been limited to a
413 single year we would not have uncovered that the seasonal patterns of prey use change between
414 years. For example, Diptera accounted for half of the dietary prey in early 2008 and decreased in
415 importance throughout the year but the opposite trend occurred in 2011 where they increased in

416 importance. Similarly, considering only inter-annual variation in diet would not have uncovered
417 the seasonal variation in prey use.
418
419 *Ordinal vs. species level analysis of diet.*
420 We compared dietary trends using data restricted to ordinal level identifications (as
421 would have been obtained using traditional fecal analysis by morphology) to species (MOTU)
422 level taxonomy available using molecular methods. There are two main advantages of molecular
423 dietary analysis. First, it automates the identification process reducing the need for specialized
424 training. While some mammalogist have specific entomological qualifications, many~~Most~~
425 traditional analyses of diet are conducted by ~~mammalogists~~ individuals without this
426 specialization ~~(not entomologists)~~ and thus the skills required for proper identification of ~~these~~
427 insect fragments under a microscope must be obtained on an *ad hoc* basis for analysis. While the
428 bioinformatics of NGS analysis similarly require training, molecular analysis in general is less
429 specialized and most molecular labs can perform these steps without additional training. Second,
430 the resolution is much higher using molecular analyses (Clare *et al.* 2009) providing much more
431 information from the same samples with less effort and we ~~which~~ are ~~is~~ more likely to document
432 the presence of rare prey items (Clare *et al.* 2009). While these differences may be important in
433 terms of demonstrating behavioural flexibility which stabilizes ecosystem functioning, it may not

be important in terms of energetics when these are consumed in low frequency (Table 1). It is particularly interesting that, while the trends we found are similar between the different levels of analysis (Figure 4, Figure 5), our ability to detect statistical significance differed. This is largely due to the variability of the data. For example, while diversity increased between early and mid summer in 2008 (Figure 4) we only detected significant differences in the species-level analysis (Figure 4d). Similarly, diversity increased in late 2011 but this was only significant in ordinal level analysis. Rarefaction curves (Figure 5) are more revealing in species level analysis as the data quickly plateaus in ordinal level analysis. The key then is to recognize the advantage of species-level resolution, while keeping in mind that the bats themselves may not be acquiring the same information acoustically. Concurrent analyses at both levels may be the most revealing. We used both the Shannon and Simpson diversity indices though we only report data for the Simpson indices in figures. The Simpson index is less sensitive to the inclusion of rare records. Species-level analysis of diet almost always leads to the detection of many rare taxa (Bohmann *et al.* 2011), indeed that is one of its advantages (Clare *et al.* 2009), thus the Simpson index is likely more appropriate for these data, while both the Shannon and Simpson index could be used at the ordinal level where the detection of new taxa has reached a plateau (Figure 5).

Habitat and foraging behaviour inferred from dietary analysis

The species richness of prey consumed by big brown bats included both terrestrial insects and those that emerge from water (e.g., mayflies and caddisflies) supporting the view that *E. fuscus* is a generalist. This reflects the fact that there is little evidence of preference for habitat(s) (Geggie & Fenton 1985; Furlonger *et al.* 1987). Species level dietary analysis provides a unique opportunity to infer habitat parameters non-invasively without radio tracking (Clare *et al.* 2011, Clare et al. THIS ISSUE). Consumption of *Caenis* and *Maccaffertium mediopunctatum* suggest that these bats hunt over a small, fast-moving stream located 100 m of the night roost. Similarly the bats appear to have eaten paper wasps whose nests were adjacent to the night roost providing a direct benefit to the homeowners who actively tried to discourage the wasps. The strong reliance on beetles and moths indicates that most hunting is not over water (unlike little brown bats) although some insects (e.g., Coleoptera genus *Agonum*) prefer damp habitats.

Implications of resource competition

Resource partitioning is thought to be common and may result from previous (or ongoing) competition between consumers. This rests on the assumption that resources such as food are limited and therefore limiting. While it is obvious that many bats eat different insects (e.g., eastern red bats and Lepidoptera (Clare *et al.* 2009), little brown bats and emerging aquatic insects (Clare *et al.* 2011), big brown bats beetles + a variety of other prey) it is not clear why

these divisions occur. The alternative hypothesis is that habitat preferences based on morphology (e.g. (Freeman 1981) lead to partitioning of insect resources in the absence of any food resource limitation or current competition (Emrich et al. THIS ISSUE). For a true test of these hypotheses, at least one potential competitor must be excluded to determine whether the behaviour of the others changes. The continuing spread of white nosed syndrome (WNS) (Cryan *et al.* 2010) and its lethal impact on bats that hibernate underground may provide a natural test of this hypothesis. Although ~~*Eptesicus fuscus*~~big brown bats appear to be somewhat resilient to infection and mortality, several sympatric species of *Myotis* species are not and are predicted to be locally extirpated by 2020 (Frick *et al.* 2010). Our study represents an important baseline of information about diet based on a pre-WNS population (2008) and ~~effected~~ affected (but not post-WNS) population (201~~0~~1). If resource competition exists between bat species in this community, these measures may be used to assess both competition between predators and population responses of prey as predators are lost from the ecosystem, particularly if measured across a broad geographic region.

Conclusions

Our observations confirm that the flexible foraging strategy of big brown bats corresponds to a generalist diet. Extreme seasonal and inter-annual fluctuations in diet highlight

the importance of continguous monitoring for accurate dietary characterization. We confirm the importance of beetles in the diet, but also highlight that, while this resources appears to fluctuate, Lepidoptera and Ephemeroptera are stable dietary components and may be an important buffer in times of resource limitation. Our observation that, in response to resource fluctuations, these bats become even more flexible and increase taxonomic diversity of prey, highlights their importance as a highly connected ecosystem components promoting stability in response to disturbance.

Acknowledgements:

We wish to thank landowners Allison Lupton and Geoff Somers for their co-operation with sample collection. Professors Gareth Jones and Colin Lazarus provided laboratory support. This study was made possible by funding from K.M. Molson Foundation and the Natural Sciences and Engineering Research Council of Canada through grants to MBF and ELC. We thank many colleagues for advice and suggestions on analysis and manuscript content.

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625

626 **Data Accessibility:** Sequence files are a read me file are available in Dryad~~…. (accession/doi~~

627 ~~available on publication)~~: doi:10.5061/dryad.t30bh

628

629 **Author Contributions:** ELC and MBF conceived of the project and provided funding. ELC

630 conducted all field, molecular and bioinformatics analysis. ELC and WOCS contributed to

631 molecular protocol designs. ELC, WOCS and MBF wrote the manuscript.

632

633 **Figure Legends**

634 Figure 1: Primer design used in this study for uni-directional sequencing on the Ion Torrent
635 sequencing platform. Our forward primer included a "key" region. The key is the only difference
636 between the design of the forward primer in Roche 454 Lib-L chemistry (Roche, Basel,
637 Switzerland) and the Ion Torrent platform primer A (Ion Torrent, Life Technologies). Including
638 this "key" permits us to use the same primers on both platforms and does not interfere with
639 amplification or sequencing thus it was included here but is not required.

640

641 Figure 2: Seasonal variation in dietary prey consumption. The proportion of each prey group
642 composing the diet varied significantly across the summer and between years. A) In 2008
643 Diptera dominated early while Coleoptera become more important in the late summer. B) In
644 2011 Coleoptera were more important earlier in the year but decreased towards late summer.

645

646 Figure 3: Overall consumption of prey groups was similar in both 2008 and 2011. Coleoptera,
647 Diptera and Lepidoptera dominated the diet of *Eptesicus fuscus*. Consumption is calculated as
648 presence or absence within a sample x frequency among samples.

649

650 Figure 4: Estimates of dietary diversity based on the Simpson diversity index. Data restricted to
651 ordinal level taxonomy shows variation between early, middle and late summer in A) 2008 and
652 B) 2011 though overall diversity C) was the same between years. Similarly trends are observed
653 in species-level data D), E) and F).
654
655 Figure 5: A comparison of rarefaction curves for operational taxonomic units at the order (A, B,
656 C) and species (D, E, F) level. Red lines indicate mean estimates while blue lines indicate the
657 95% confidence level from permutations.
658
659 Figure 6: A schematic of prey species consumed including all MOTU (including those that could
660 not be identified using a reference database). Identifications have been made by BLAST score
661 and are limited to hypothesis at the order level. Values at nodes or tips represent the number of
662 MOTU assigned. Node size is scaled to the number of assignments. See Emrich et al. (in press)
663 for additional details.
664

665 Table 1: Prey species identified in the diet of night roosting *Eptesicus fuscus.* Frequency refers to
666 presence or absence in each weekly sample.

Class	Order	Family	Genus	Species	Similarity	Frequency
Arachnida	Aranea	Theridiidae	*Parasteatoda*	*sp.*	100	1
Insecta	Coleoptera	Carabidae	*Agonum*	*placidum*	100	1
			Amara	*apricaria*	100	1
			Amphasia	*sericea*	99.33	2
			Calosoma	*frigidum*	99.36	1
			Harpalus	*pennsylvanicus*	99.31	3
			Notiobia	*terminata*	100	16
			Ophonus	*puncticeps*	100	8
			Platynus	*cincticollis*	100	1
			Platynus	*sp.*	98.72	3
			Poecilus	*lucublandus*	100	8
			Selenophorus	*opalinus*	100	1
			Stenolophus	*comma*	99.36	4
			Stenolophus	*ochropezus*	98.08	1
			Trichotichnus	*vulpeculus*	100	1
			Trichotichnus	*sp.*	100	6
		Cerambycidae	*Ecyrus*	*dasycerus*	100	1
			Graphisurus	*fasciatus*	100	1
			Monochamus	*notatus*	100	1
			Monochamus	*sp.*	100	2
		Cleridae	*Cymatodera*	*bicolor*	98.08	2
		Elateridae	*Ampedus*	*semicintus*	100	2
			Melanotus	*similis*	100	2
		Silphidae	*Nicrophorus*	*pustulatus*	100	1
		Tenebrionidae	*Tenebrio*	*sp.*	99.21	4
	Diptera	Chaboridae	*Unknown*	*sp.*	99.36	1
			Unknown	*sp.*	99.36	4
		Chironomidae	*Ablabesmyia*	*americana*	99.36	1
			Dicrotendipes	*tritomus*	100	7
			Paracladopelma	*winnelli*	99.33	3
			Tanytarsus	*mendax*	100	1
			Tanytarsus	*sp.*	98	1
			Xenochironomus	*zenolabis*	99.35	1
			Unknown	*sp.*	100	1
			Unknown	*sp.*	100	1
			Unknown	*sp.*	99.31	2
		Culicidae	*Culex*	*pipiens*	99.07	1
			Unknown	*sp.*	100	1
		Peiciidae	*Unknown*	*sp.*	100	6
		Psychodidae	*Unknown*	*sp.*	100	1
			Unknown	*sp.*	100	1
			Unknown	*sp.*	100	6

	Sarcophagidae	*Unknown*	*sp.*	100	1
	Simuliidae	*Simulium*	*gouldingi*	100	1
	Tachinidae	*Zaira*	*sp.*	98.72	1
	Tipulidae	*Tipula*	*furca*	100	5
		Tipula	*sp.*	100	1
		Unknown	*sp.*	100	1
		Unknown	*sp.*	99.36	1
		Unknown	*sp.*	100	1
		Unknown	*sp.*	99.36	1
		Unknown	*sp.*	98.64	2
Ephemeroptera	Caenidae	*Caenis*	*youngi*	100	1
		Caenis	*sp.*	99.35	11
	Ephemeridae	*Hexagenia*	*atrocaudata*	100	1
		Hexagenia	*limbata*	100	1
	Heptageniidae	*Leucrocuta*	*maculipennis*	100	2
		Maccaffertium	*mediopunctatum*	98.72	9
	Isonychiidae	*Isonychia*	*bicolor*	100	1
		Isonychia	*sp.*	100	15
		Isonychia	*rufa*	99.36	6
Hemiptera	Miridae	*Lygus*	*lineolaris*	100	1
Hymenoptera	Vespidae	*Polistes*	*sp.*	100	2
Lepidoptera	Blastobasidae	*Holcocera*	*immaculella*	100	2
	Coleophoridae	*Coleophora*	*versurella*	98.72	1
	Crambidae	*Herpetogramma*	*phaeopteralis*	100	2
		Petrophila	*bifascialis*	100	1
	Elachistidae	*Agonopterix*	*robinella*	100	1
	Erebidae	*Idia*	*sp.*	100	2
	Gelechiidae	*Coleotechnites*	*sp.*	98.72	3
		Ezoteleia	*dodecella*	99.36	1
		Filatima	*pseudacaciella*	100	1
	Geometridae	*Hydriomena*	*impluviata*	100	6
	Lasiocampidae	*Malacosoma*	*disstria*	99.36	15
	Limacodidae	*Lithacodes*	*fasciola*	100	1
	Lyonetiidae	*Corythophora*	*sp.*	100	1
	Noctuidae	*Elphria*	*sp.*	100	1
		Spodoptera	*sp.*	100	3
		Zale	*galbanata*	100	1
	Pyralidae	*Sciota*	*virgatella*	100	2
	Tineidae	*Amydria*	*sp.*	99.36	1
	Tortricidae	*Acleris*	*chalybeana*	100	1
		Clepsis	*clemensiana*	99.26	1
		Cnephasia	*geitalana*	98.08	1
		Eucosma	*derelecta*	98.72	1
		Eucosma	*sp.*	99.31	1
		Phtheochroa	*birdana*	100	3

		Pseudoexentera	*cressoniana*	99.36	3
		Pseudoexentera	*maracana*	99.36	1
		Sparganothis	*pettitana*	100	1
Mantodea	Mantidae	*Mantis*	*religiosa*	100	3
Megaloptera	Corydalidae	*Chauliodes*	*sp.*	98.67	20
Neuroptera	Hemerobiidae	*Hemerobius*	*stigma*	100	4
		Unknown	*sp.*	100	1
Orthoptera	Gryllidae	*Gryllus*	*pennsylvanicus*	100	1
Trichoptera	Helicopsychidae	*Helicopsyche*	*borealis*	100	1
		Cheumatophsyche	*campyla*	100	1
		Cheumatophsyche	*sp.*	100	1
		Cheumatopsyche	*campyla*	99.36	5
		Macrostemum	*zebratum*	98.69	1
	Leptoceridae	*Ceraclea*	*transversa*	100	1
	Limnephilidae	*Limnephilus*	*sp.*	99.36	1

667

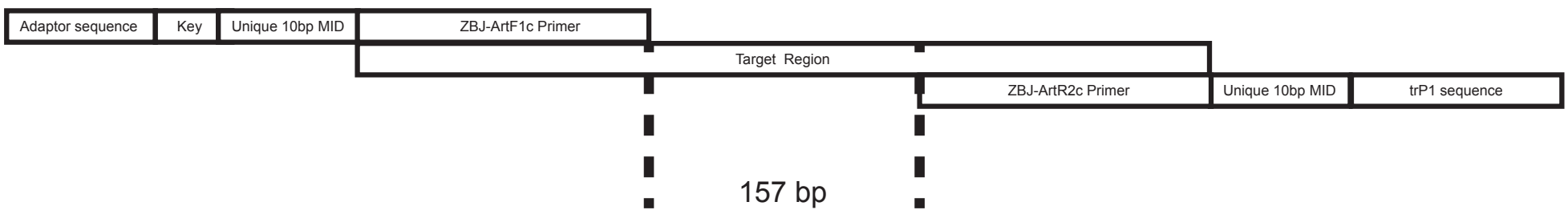
Forward Fusion Primer

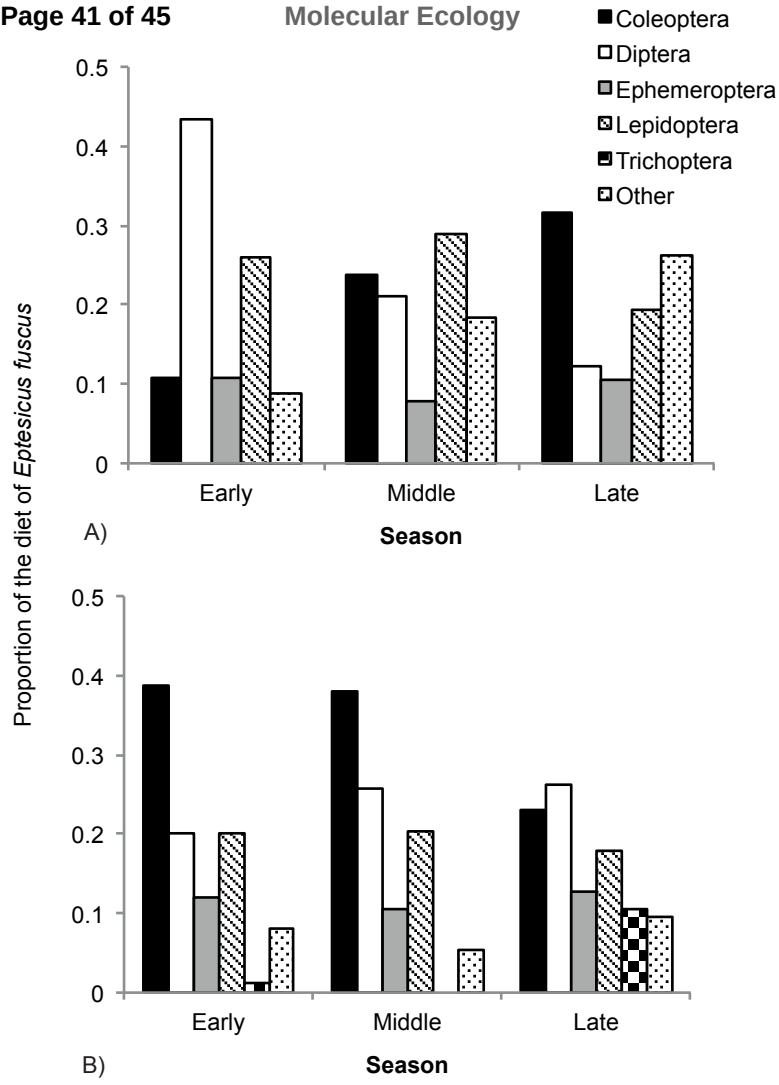
Adaptor sequence	Key	Unique 10bp MID	ZBJ-ArtF1c Primer

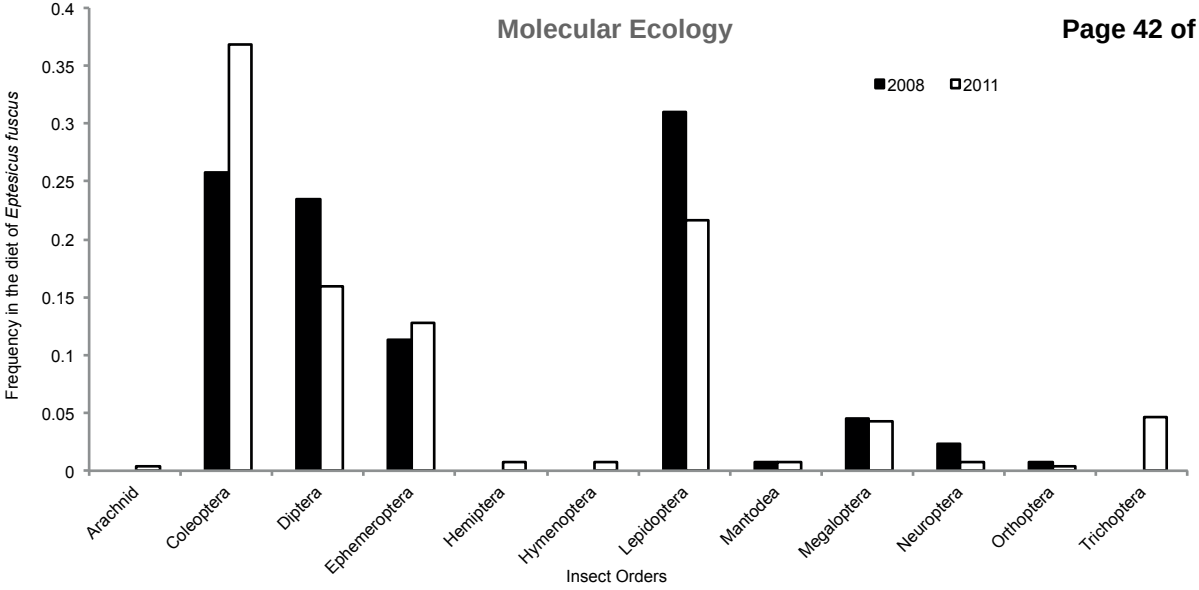
Reverse Fusion Primer

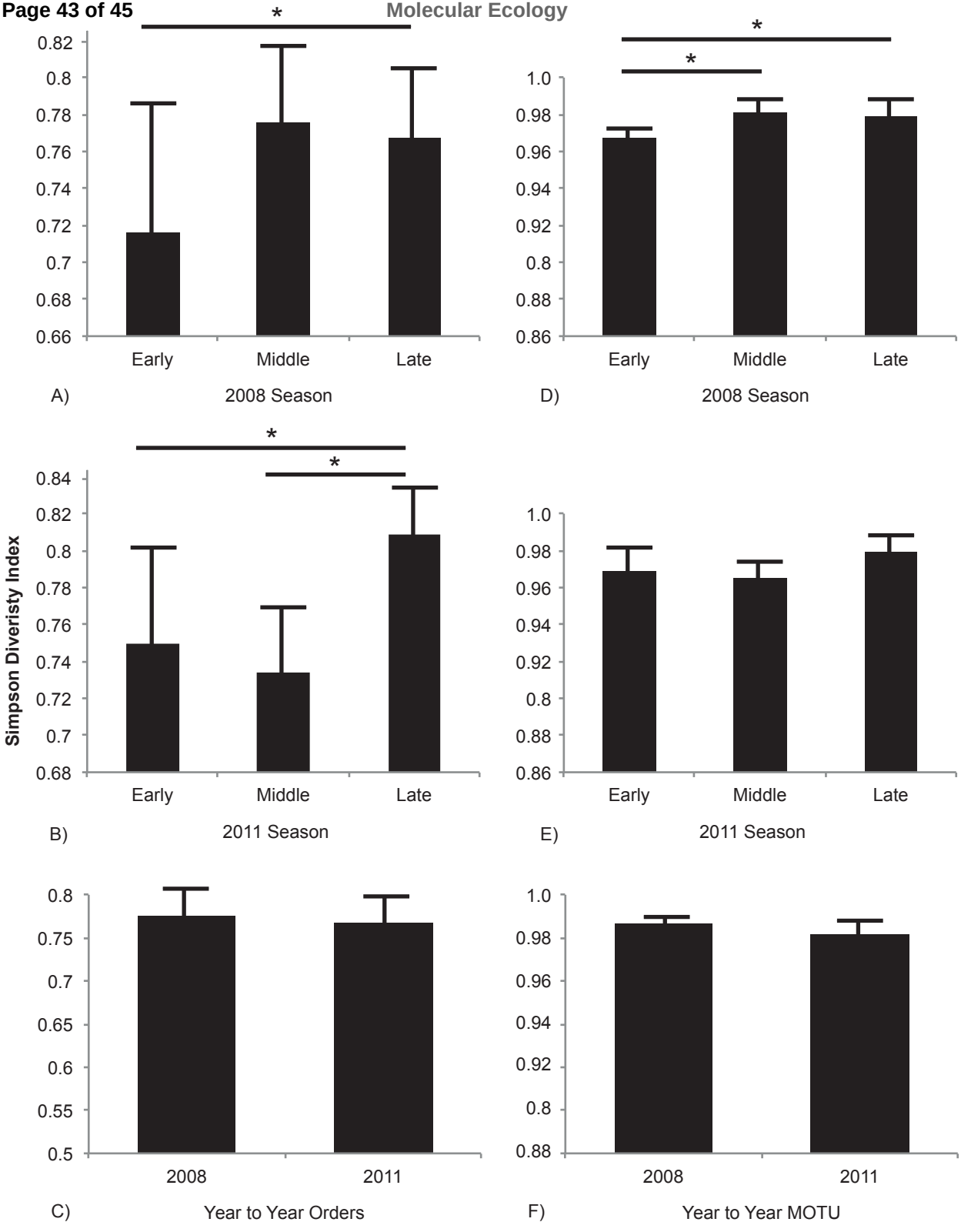
trP1 sequence	Unique 10bp MID	ZBJ-ArtR2c Primer

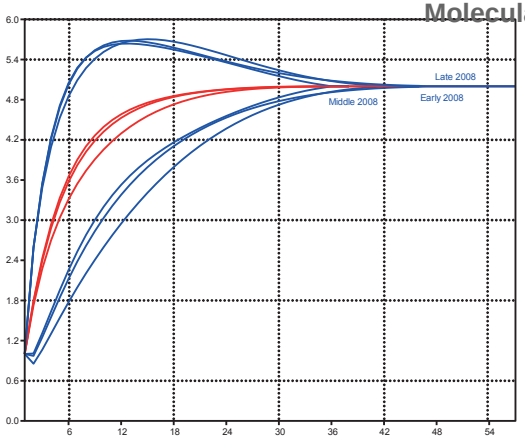
Resulting Amplicon



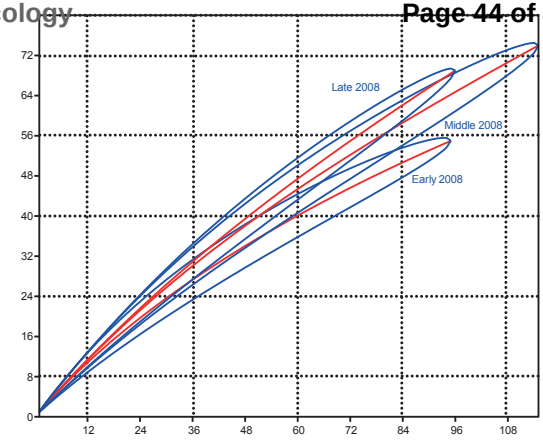

Coleoptera
Diptera
Ephemeroptera
Lepidoptera
Trichoptera
Other
Proportion of the diet of *Eptesicus fuscus*
0.5
0.4
0.3
0.2
0.1
0
Early
Middle
Late
A)
Season
0.5
0.4
0.3
0.2
0.1
0
Early
Middle
Late
B)
Season


0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Frequency in the diet of *Eptesicus fuscus*
2008
2011
Arachnid
Coleoptera
Diptera
Ephemeroptera
Hemiptera
Hymenoptera
Lepidoptera
Mantodea
Megaloptera
Neuroptera
Orthoptera
Trichoptera
Insect Orders


*
0.82
0.8
0.78
0.76
0.74
0.72
0.7
0.68
0.66
Early
Middle
Late
A)
2008 Season
*
*
1.0
0.98
0.96
0.94
0.92
0.8
0.88
0.86
Early
Middle
Late
D)
2008 Season
*
*
Simpson Diveristy Index
0.84
0.82
0.8
0.78
0.76
0.74
0.72
0.7
0.68
Early
Middle
Late
B)
2011 Season
1.0
0.98
0.96
0.94
0.92
0.8
0.88
0.86
Early
Middle
Late
E)
2011 Season
0.8
0.75
0.7
0.65
0.6
0.55
0.5
2008
2011
C)
Year to Year Orders
1.0
0.98
0.96
0.94
0.92
0.8
0.88
2008
2011
F)
Year to Year MOTU

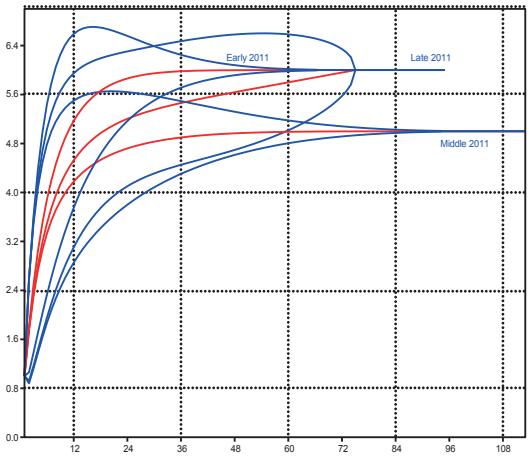


A)

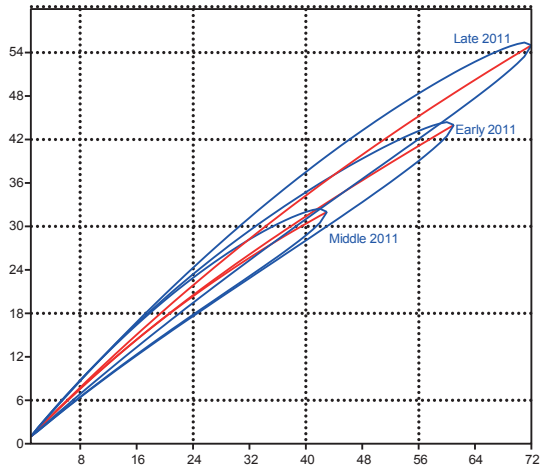


D)

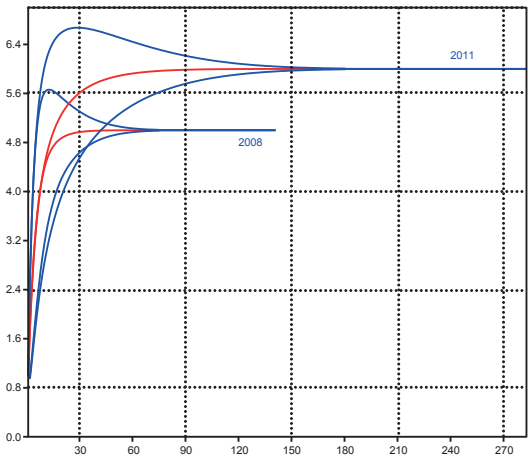
Taxa (95% confidence)



B)

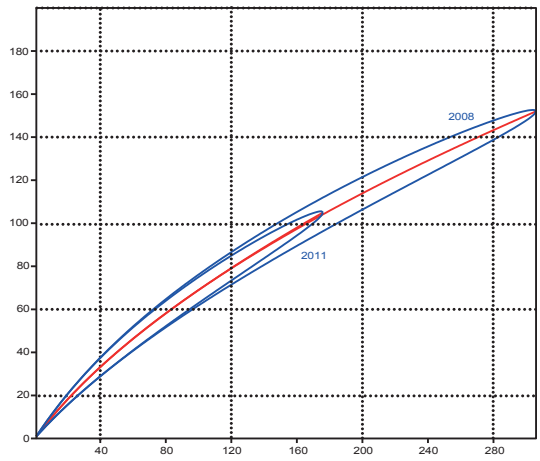


E)



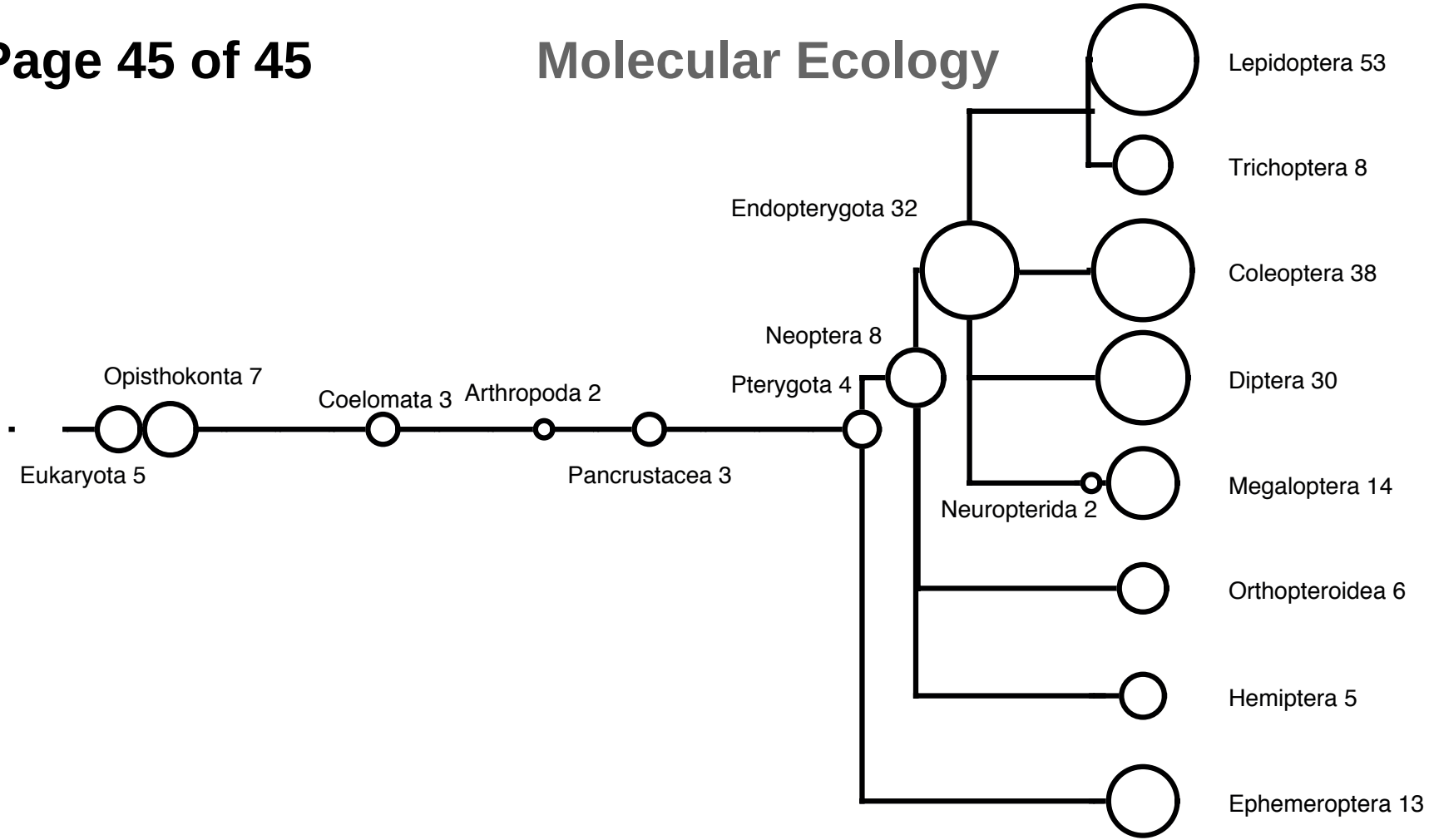
C)

Order Analysis



F)

MOTU Analysis


Lepidoptera 53
Trichoptera 8
Endopterygota 32
Coleoptera 38
Neoptera 8
Diptera 30
Opisthokonta 7
Pterygota 4
Coelomata 3
Arthropoda 2
Megaloptera 14
Neuropterida 2
Orthopteroidea 6
Hemiptera 5
Pancrustacea 3
Eukaryota 5
Ephemeroptera 13