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Title: Who's for dinner? High-throughput sequencing reveals bat dietary differentiation in a biodiversity hotspot where prey taxonomy is largely undescribed.

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Running Title: Bat dietary diversity in a biodiversity hotspot.
Abstract:

Effective management and conservation of biodiversity requires understanding of predator-prey relationships to ensure the continued existence of both predator and prey populations. Gathering dietary data from predatory species, such as insectivorous bats, often presents logistical challenges, further exacerbated in biodiversity hotspots because prey items are highly speciose yet their taxonomy is largely undescribed. We used high-throughput sequencing (HTS) and bioinformatic analyses to phylogenetically group DNA sequences into molecular operational taxonomic units (MOTUs) to examine predator-prey dynamics of three sympatric insectivorous bat species in the biodiversity hotspot of south-western Australia. We could only assign between 4-20% of MOTUs to known genera or species, depending on the method used, underscoring the importance of examining dietary diversity irrespective of taxonomic knowledge in areas lacking a comprehensive genetic reference database. MOTU analysis confirmed that resource partitioning occurred, with dietary divergence positively related to the ecomorphological divergence of the three bat species. We predicted bat species’ diets would converge during times of high energetic requirements, i.e., the maternity season for females and the mating season for males. There was an interactive effect of season on female, but not male, bat species’ diets, although small sample sizes may have limited our findings. Contrary to our predictions, females of two ecomorphologically similar species showed dietary convergence during the mating season rather than the maternity season. HTS-based approaches can help elucidate complex predator-prey relationships in highly speciose regions, which should facilitate the conservation of biodiversity in genetically uncharacterised areas, such as biodiversity hotspots.
Introduction

To effectively manage and conserve biodiversity, it is critical to understand predator-prey relationships so that both predator and prey populations can be conserved. This is becoming increasingly important as continuing habitat loss and degradation may lead to trophic collapse (Dobson et al. 2006). Accurate dietary studies can contribute greatly to understanding predator-prey relationships and can also provide integral knowledge concerning foodwebs and trophic interactions, which in turn influence ecological processes such as niche partitioning and inter-specific competition (Amarasekare 2008; Pompanon et al. 2012; Roughgarden 1983). Determining the dietary requirements of species through direct field observations is often difficult and time-consuming (Williams et al. 2012), particularly in regions where prey are highly speciose and undersampled. Tropical and southern temperate biodiversity hotspots, which support the highest number of globally threatened species (Bohm et al. 2013; Myers et al. 2000), typify regions where most prey species are taxonomically undescribed and their DNA sequences unknown (Bohmann et al. 2011; Fonseca 2009). Biodiversity hotspots are estimated to harbour over 40% of the world’s insects, most of which are undescribed, and conservative estimates suggest at least 22% are threatened (Fonseca 2009). The poor taxonomic knowledge of prey items, such as invertebrates, may hamper conservation efforts by limiting identification of important prey items for many species.

Recent advances in molecular technologies have enabled dietary analysis of DNA food remains in gut or faecal samples while precluding the need for prey items to be taxonomically described (e.g., Brown et al. 2013). One technique, high-throughput sequencing (HTS), increases the breadth of prey items identified, as HTS involves
sequencing many pooled amplicons in parallel often using universal primers, such as
sequencing the preys’ mitochondrial DNA (mtDNA) cytochrome c oxidase I (COI) gene,
or DNA barcode (Hebert et al. 2003a; Mitchell 2008). Apart from negating the need for
physically dissecting individual prey remains, HTS allows complex, heterogeneous DNA
mixtures to be analysed, thus examining the prey base in its entirety and at a finer
taxonomic resolution than morphological methods, without adding to the cost of
analysis (Boyer et al. 2012; Pompanon et al. 2012; Razgour et al. 2011; Shokralla et al.
2012). Molecular studies, such as HTS, can still be limited in that prey DNA sequences
from genetically uncharacterised areas may not be confidently matched to reference
databases (e.g., Brown et al. 2013). However, DNA sequences derived from molecular
studies can be phylogenetically grouped into molecular operational taxonomic units
(MOTUs; Floyd et al. 2002), which, irrespective of taxonomic assignment, can then be
used to compare diets within, and between, predatory species (Caporaso et al. 2010),
elucidating complex predator-prey relationships in highly biodiverse ecosystems.

Insectivorous bats are important, yet often overlooked, top predators that consume a
variety of prey (Kalka & Kalko 2006; Morrison & Lindell 2012). Differences in
manoeuvrability, size, and foraging strategy influence the prey base of individual bat
species (Fenton 1990; Fullard et al. 1991). In addition, prey availability and accessibility
may vary sexually and seasonally, leading to both intra and interspecific differentiation
in bat diets (Andreas et al. 2012; Clare et al. 2011). While bats can minimise energy
expenditure behaviourally (e.g. torpor and hibernation) when prey are limited (Dietz &
Horig 2011; Hope & Jones 2012), forced fasting can cause metabolic deterioration
within relatively short timeframes (Freitas et al. 2010). Thus, regular prey consumption
is necessary, with bats often consuming over a quarter of their body weight in invertebrates each night and even greater amounts during energetically demanding periods (Kunz et al. 2011); the maternity season for females and mating season for males (Dietz & Kalko 2007). During these times, bats may forage less selectively to ensure adequate energetic intake (Whitaker 2004). Overall prey consumption by bats is typically diverse and even bat species conventionally considered specialists consume many prey species within a single order (Clare et al. 2011). Being nocturnal, cryptic and typically generalist predators, bat diet studies embody some of the most challenging aspects of studying predator-prey interactions (Andrew et al. 2013). However, the fine taxonomic resolution of molecular technologies, such as HTS, is enabling factors that influence dietary variation to be elucidated, leading to an improved understanding of predator-prey relationships and resource partitioning between sympatric bat species (Bohmann et al. 2011; Razgour et al. 2011).

Previous studies have compared MOTU diversity in bat diets (e.g., Alberdi et al. 2012; Clare et al. 2011; Clare et al. 2009; Zeale et al. 2011), two utilising HTS (Bohmann et al. 2011; Razgour et al. 2011), but this is the first study to use HTS approaches to investigate both intra and interspecific dietary differentiation in multiple bat species. Significantly, the target species are sampled within the biodiversity (Myers et al. 2000) and invertebrate diversity (Cooper et al. 2011) hotspot of south-western Australia, a region with high levels of habitat loss (Bradshaw 2012) and a rapidly drying climate that both pose a threat to biodiversity (Klausmeyer & Shaw 2009; Wardell-Johnson et al. 2011). Within this hotspot, the jarrah (Eucalyptus marginata) forest supports nine species of insectivorous bat and a highly speciose invertebrate fauna, estimated between
15,000 and 20,000 species, of which only 10% have been formally described (Abbott 1995). We examined intra and interspecific dietary differentiation between three sympatric jarrah forest insectivorous bat species to identify sexual and seasonal variations in diets and to determine if diets converge during times of resource limitation.

Of the three species we studied Gould’s wattled bat (*Chalinolobus gouldii*, Gray 1841) is the largest and is capable of fast, agile flight (Bullen 2001). Compared to the other two species *C. gouldii* has a high aspect ratio and wing loading and low echolocation call frequency (Table S1, Supplementary Info); in the jarrah forest *C. gouldii* likely forages in open habitat adjacent to the forest edge (Bullen 2001; Fullard et al. 1991). The southern forest bat (*Vespadelus regulus*, Thomas 1906) and Gould’s long-eared bat (*Nyctophilus gouldi*, Tomes 1858) have similar, comparatively low, aspect ratio and wing loading (Fullard et al. 1991). While both are agile, *V. regulus* is capable of medium to fast flying, in contrast to *N. gouldi* which flies at slower speeds but is more manoeuvrable (Brigham et al. 1997; Bullen 2001; O’Neill & Taylor 1986). In the jarrah forest, *N. gouldi* and *V. regulus* are likely to exploit vegetated and edge habitat (Fullard et al. 1991). *N. gouldi* employs both aerial hawking and gleaning, in contrast to the other two bat species who primarily take prey aerially (Brigham et al. 1997; Fullard et al. 1991). We hypothesized that dietary partitioning would occur between species and that dietary divergence would be related to ecomorphological divergence. Specifically, we predicted that the most ecomorphologically divergent species, *C. gouldii* and *N. gouldi*, would have the most divergent diets. As *N. gouldi* exhibits multiple foraging strategies and is capable of exploiting multiple microhabitats, we also predicted *N. gouldi* would have the most
diverse diet whilst *C. gouldii*, would have the least diverse diet, with *V. regulus* having an intermediate level of dietary diversity. Lastly, we predicted that there would be dietary differentiation, both intra and interspecifically, based on the individual and combined influences of season and sex. Intraspecifically, we expected convergent diets during the mating season when females were not as constrained by roosting requirements. Interspecifically, we expected diets to converge when energy demands were high; i.e., during the maternity season for females and mating season for males.

**Materials & Methods**

**Study site**

The study was conducted at Huntly minesite (32°36’S, 116°07’E), operated by Alcoa of Australia, located 10 km N of Dwellingup in the northern jarrah forest of south-western Australia. The area has a Mediterranean climate with cool, wet winters and warm, dry summers. Rainfall at Dwellingup averages 1222 mm annually, with >75% falling between May and September. The minesite is a mosaic of unmined and restored forest, both with a canopy dominated by two eucalypt species, jarrah and marri (*Corymbia calophylla*). All bat faecal sample collection locations occurred adjacent to waterholes within unmined forest, although bats are known to forage in both forest types (J. Burgar, unpublished data).

**Sample collection**

Bats were trapped at eight locations over 14 nights between October 2010 and March 2011, in both the maternity (15 October to 1 December) and mating (3 February to 30 March) seasons. All bats were captured in harp traps (Two-Bank 4.2 square metres;
Ausbat Research Equipment, Victoria), removed almost immediately, placed in individual, clean bags and held for ~30 to 60 mins to obtain faecal samples. We collected 209 faecal samples from three species (24 from *C. gouldii*, 50 from *N. gouldi*, and 135 from *V. regulus*), which were placed in labelled sterile vials and frozen as soon after collection as possible.

**DNA extraction and amplification**

DNA was extracted from all *C. gouldii* faecal samples (24), and from subsets of *N. gouldi* (30) and *V. regulus* (27) faecal samples, randomly stratified by site, date and sex. Faecal samples remained frozen until processed for DNA extraction, which occurred in four batches alongside extraction controls. For the first two batches of DNA extraction, each pellet per faecal sample was cut in half using a sterile scalpel blade. On average, faecal samples contained 6.2 pellets (range: 1 to 19) per individual bat, with DNA extracted from approximately half of each pellet (average 230 mg extraction⁻¹). For the remaining batches, pellets were ground together prior to obtaining 100 mg from each sample, which was then placed into a 2 ml tube. Extractions were performed using QIAamp DNA Stool Mini Kit (QIAGEN) according to manufacturer’s instructions with the modifications noted in the supporting information (Appendix S1). A short section (157 bp) of the mtDNA COI gene was amplified via qPCR using generic arthropod primers (ZBJ-ArtF1c and ZBJ-ArtR2c; Zeale et al. 2011). All extracts deemed successful in yielding DNA, free of inhibition as determined via qPCR curves across dilutions, were selected for HTS library preparation.

**HTS library preparation and sequencing**
The generic arthropod forward primers were modified into fusion primers with the addition of Roche Genome Sequencer (GS) Junior FLX compatible A and B primers and a series of 30 unique DNA-based Multiplex Identifiers (MID). Each successful extract was assigned a unique MID tag and subsequent fusion tagged qPCR was carried out. See supporting information for detailed HTS sequencing methods (Appendix S1). HTS was carried out on the Roche GS Junior FLX system at Murdoch University, Australia, following the Lib-A amplicon sequencing protocols.

**MOTU selection**

Amplicon sequences obtained from the GS Junior FLX were separated into sample batches based on MID tags; tags and sequencing adapters were subsequently trimmed using Geneious v.5.6.5 (Drummond et al. 2012). In each case, an exact match in base composition and length was required. Sequences not meeting these criteria were discarded, as were sequences of short length that resulted from primer dimer. Each set of batched sequences was then compared against the National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) database through YABI, a bioinformatics workflow software system (Hunter et al. 2012). Sequences were searched without a low complexity filter, with a gap penalties existence of five and extension of two, expected alignment value <0.1 and a word count of seven. BLAST output files obtained from YABI were then imported into MEtaGenome ANalyzer (MEGAN) version 4.70.4 (Huson 2011) with the following Lowest Common Ancestor (LCA) assignment algorithm parameters: minimum support 1, minimum score 35, top percent 5, win-score 0 and no minimum complexity filter. For each sample, sequences assigned by MEGAN to the Arthropod phylum were extracted for further analysis.
Extracted Arthropod sequences were processed in Quantitative Insights in Microbial Ecology (QIIME) version 1.5.0 (Caporaso et al. 2010). All sequences were checked to ensure they were >95 bp in length, then grouped into MOTUs using the USEARCH method, with a specified 98% sequence similarity threshold. Potential chimeric sequences were removed, as were singleton sequences. MOTUs were aligned using the MUSCLE alignment (Edgar 2004). Representative MOTUs selected for taxonomic alignment and assignment were ~157 bp in length. Phylogenetic trees were constructed using the fasttree method.

MOTU sequences were queried through the Biodiversity of Life Database (BOLD) version 3 (Ratnasingham & Hebert 2007) on 29 May 2013, as the online BOLD engine enables sequence identification using both private and public records. Examination of intra versus interspecific variation of the COI gene suggests that arthropod sequence divergence ranges from 6% in Lepidoptera to over 11% in Coleoptera and Hymenoptera (Hebert et al. 2003b; Waugh 2007). Bat dietary studies have used a variety of percentage similarity cut-off criteria, for 157 bp fragments, to obtain taxonomic level thresholds, ranging from 99.3% (Zeale et al. 2011) to 98.5% (Razgour et al. 2011) for species and 98% (Razgour et al. 2011) to 94.9% (Zeale et al. 2011) for genus, with some researchers suggesting assignments below 97.4% are inaccurate and potentially erroneous (Alberdi et al. 2012). Intraspecific variation is known to increase with geographic distance (Bergsten et al. 2012), which may have implications for low matching success in areas with limited genetic reference databases. This is particularly relevant due to the paucity of Australian records and as <6% of Australian invertebrate
genetic records come from Western Australia. When species matches are unavailable, the accuracy of higher taxon assignment is questionable, particularly where reference libraries are incomplete (Wilson et al. 2011). Thus, a conservative matching system was employed where sequences were filtered to ensure those examined had a minimum 98% sequence similarity to a potential taxonomic assignment. Taxonomy was assigned to MOTUs matched against the BOLD database using the online batch identification engine, following slightly modified ‘strict’ and ‘best match’ methods (Ross et al. 2008). The ‘strict’ method refers to matching based on phylogenetic tree placement where the query sequence must be nested within a clade comprising members of a single taxon to be considered a match (Ross et al. 2008). The ‘best match’ method simply assigns taxonomy based on percent similarity. While this method may have similar true positive identification rates as the ‘strict’ method, it also has much higher false positive rates. Thus, only when the ‘best match’ was for a taxon sampled within Australia was the MOTU considered a match. MOTUs were considered a “species match” if sequences had ≥99% similarity to a single species and were considered a “genus match” if sequences had ≥98% similarity to one or more species within the same genus. The matching method was recorded for each MOTU taxonomic assignment.

**Dietary diversity**

Dietary diversity for each bat species was assessed using MOTUs, irrespective of taxonomic assignment. Two types of diversity were assessed: α-diversity for diversity within each individual bat and β-diversity for diversity within each bat species. To determine α-diversity independent of sample size, 10 rarefactions were performed at a minimum depth of five and maximum depth of 95 sequences per sample, with a step...
increase of 10. Rarefaction plots were derived from collated \( \alpha \)-diversity metrics generated from two diversity indices: Chao1 (Chao 1984) and Faith's phylogenetic diversity (PD) (Faith 1992). Chao1 provides a relatively unbiased and conservative estimate of species richness (Bunge & Fitzpatrick 1993) while Faith's PD reflects evolutionary history with higher values indicating greater taxonomic distinctiveness (Faith & Baker 2006). Rarefaction curves not only provide information on \( \alpha \)-diversity, irrespective of sample size, but also act as a check to ensure sufficient sequence sample depth in subsequent analyses. Examination of \( \alpha \)-diversity values was set at an even depth of 65 sequences per sample, which was selected based on the relative levelling off of rarefaction curves at this depth while considering sample sizes (\( C. gouldii \) \( n = 21 \); \( N. gouldii \) \( n = 15 \); \( V. regulus \) \( n = 19 \)) for further analyses.

\( \beta \)-diversity was computed as a function of jackknifed \( \beta \)-diversity using the previously created phylogenetic tree and rarefaction was set at an even depth of 60 sequences per sample. A rarefaction level of 60 ensured \( \beta \)-diversity analyses were not influenced by sequencing effort, but instead reflected the underlying biology; bat samples with fewer than 60 sequences were removed from \( \beta \)-diversity analyses. \( \beta \)-diversity was not limited by the size of the step increase (as were \( \alpha \)-diversity values), so the slight difference in rarefaction depths was to ensure similar depths while maximising the number of sequences per sample. Distance matrices, generated from unweighted Unifrac statistics, formed the basis of the principal coordinate analysis (PCoA) plots and further analyses. Unifrac is an ecological distance measure based on phylogenetic information and is able to deal with undersampled environments (Lozupone & Knight 2005). As HTS is frequency based, treating each prey item equally, HTS can overestimate the presence of
rare prey while underestimating common prey, potentially biasing HTS towards the
detection of resource partitioning. Consequently we analysed dietary diversity twice,
once including all MOTUs (in 64 samples: \textit{C. gouldii} \(n = 23\); \textit{N. gouldi} \(n = 19\); \textit{V. regulus} \(n = 22\)) and again after removing MOTUs only present in one bat sample (e.g., Bohmann \textit{et al.} 2011; Brown \textit{et al.} 2013). Diversity values and subsequent distance matrices were
generated in QIIME, using the default parameters unless otherwise stated.

MOTU diversity was compared using unweighted Unifrac distance matrices, permuted
9999 times, in the R Vegan package – function Adonis (Oksanen \textit{et al.} 2012). Diversity
was compared between bat species, seasons (maternity and mating) and sexes, as well
as combinations of these factors depending on sample sizes. We also examined the
interactive effects of season and sex both intra and interspecifically. Bat species, season
and sex were considered fixed factors while site was included as a random factor to
account for any spatial-variation in invertebrate communities. Tukey's HSD post-hoc
tests were run to determine homogeneity of group variances, pooled across sites, using
the R Vegan package – function betadisper (Oksanen \textit{et al.} 2012). These analyses were
performed in R version 2.15.0 (R Development Core Team 2012).

**Results**

Of 81 bat faecal samples processed, 64 yielded DNA of sufficient quality for deep
sequencing, resulting in 14,673 amplicon sequences representing 579 MOTUs
(deposited in DRYAD doi:10.5061/dryad.0gq63). Bat samples contained between one
and 44 MOTUs (median of 11 and mean of 15 MOTUs sample\(^{-1}\)), with 23 \textit{C. gouldii}
samples yielding 193 MOTUs, 218 MOTUs in 19 \textit{N. gouldi} samples and 267 MOTUs in 22
V. regulus samples. Removing MOTUs found in only one bat sample resulted in 190 MOTUs (33%), excluding one V. regulus sample for a total of 63 samples: 95 MOTUs in C. gouldii samples; 83 MOTUs in N. gouldi samples; and 111 MOTUs in V. regulus samples. The significance of tests did not differ when dietary diversity was analysed with all MOTUs, or the subset, so we only present results derived from all MOTUs to ensure potentially important prey items were not removed, considering MOTUs occurring in low abundance were removed earlier in the analysis. Refer to Table S2 (Supplementary Info) for the results derived from analyses with the subset of MOTUs.

MOTU taxonomic assignment

Matching all MOTUs against BOLD databases resulted in MOTUs being assigned taxonomically in ~4% (19 of 579) of cases using the ‘strict’ method and ~20% (121 of 579) of cases using the ‘best match’ method (Table 1). Using the ‘strict’ method, prey DNA was detected solely from Lepidoptera, comprising seven families and 11 genera. The ‘best match’ method detected prey DNA from five insect orders: Diptera, Hemiptera, Lepidoptera, Mantodea and Neuroptera, with most (51 genera within 19 families) assigned to Lepidoptera. C. gouldii consumed the most assigned MOTUs (43), compared to V. regulus (32) and N. gouldi (17). More assigned MOTUs were consumed during the maternity season (54) than the mating season (18), with only eight taxa consumed during both seasons.

Interspecific dietary diversity

Our results show that while sequencing breadth was not sufficient to capture the entire prey base within any of the species (Figure 1), sequencing depth was sufficient to
capture the prey base within an individual bat (Figure 2). *V. regulus* consumed the most MOTUs, although prey accumulation curves did not approach asymptotes for either all species combined, or individual species. There were no differences in prey α-diversity levels, for either Chao1 or Faith’s PD, between bat species (Figure 3). Seasonal differences were apparent, though, with bat species having similar α-diversity values during the maternity season, but significant differences during the mating season (Chao1: $F_{2,18} = 4.20, P = 0.032$; Faith’s PD: $F_{2,18} = 5.20, P = 0.017$); post-hoc tests indicated *N. gouldi* had higher α-diversity than *C. gouldii* in the mating season (Chao1: $P = 0.025$; Faith’s PD: $P = 0.014$).

β-diversity differed significantly between species overall ($F_{2,52} = 2.40; P < 0.001$) and between pairs of species (*C. gouldii* – *N. gouldi*, $F_{1,41} = 2.37, P < 0.001$; *C. gouldii* – *V. regulus*, $F_{1,44} = 1.78, P < 0.001$; *N. gouldi* – *V. regulus*, $F_{1,40} = 2.94, P < 0.001$). Multivariate dispersion was heterogeneous across species ($F_{2,61} = 4.44, P = 0.014$), being significantly different between *C. gouldii* and *N. gouldi* ($P = 0.014$) but not between *V. regulus* and either *C. gouldii* ($P = 0.086$) or *N. gouldi* ($P = 0.113$).

Examination of interspecific β-diversity revealed an interactive effect between species and season on bat species’ diets ($F_{3,52} = 1.93, P < 0.001$), with differences between species in both the maternity ($F_{2,31} = 2.03, P < 0.001$) and mating ($F_{2,21} = 2.40, P < 0.001$) seasons. In the maternity season, β-diversity differed between all species (*C. gouldii* – *N. gouldi*, $F_{1,22} = 1.69, P = 0.012$; *C. gouldii* – *V. regulus*, $F_{1,25} = 2.12, P = 0.123$; *N. gouldi* – *V. regulus*, $F_{1,21} = 2.29, P = 0.004$). In the mating season, β-diversity differed between *N. gouldi* and both *C. gouldii* ($F_{1,16} = 2.81, P = 0.002$) and *V. regulus* ($F_{1,16} = 2.76, P = 0.014$).
but was marginally non-significant between *C. gouldii* and *V. regulus* ($F_{1,16} = 1.70, P = 0.066$). Multivariate dispersion was homogeneous across all three species in both seasons (maternity, $F_{2,34} = 1.51, P = 0.237$; mating, $F_{2,24} = 1.78, P = 0.191$).

Interspecific β-diversity differed for females during both the maternity ($F_{2,23} = 1.66, P = 0.004$) and mating ($F_{2,14} = 1.79, P = 0.003$) seasons. Female *C. gouldii* and *N. gouldi* had significantly different β-diversity in both seasons (maternity, $F_{1,17} = 1.63, P = 0.019$; mating, $F_{1,9} = 2.04, P = 0.028$) whilst there were no differences in β-diversity between female *C. gouldii* and *V. regulus* in either season (maternity, $F_{1,15} = 1.50, P = 0.116$; mating, $F_{1,8} = 1.88, P = 0.331$). Female *N. gouldi* had similar β-diversity to *V. regulus* in the mating ($F_{1,10} = 2.06, P = 0.167$) but not the maternity ($F_{1,14} = 1.88, P = 0.006$) season. There was no difference in β-diversity between male bat species’ diets overall ($F_{2,15} = 1.27, P = 0.166$) or when analysed by season ($F_{2,15} = 1.32, P = 0.310$). Examination of interspecific β-diversity revealed no effect of sex ($F_{3,52} = 0.84, P = 0.866$) or an interactive effect of sex and season ($F_{3,52} = 0.85, P = 0.903$) on bat species’ diets.

**Intraspecific dietary diversity**

Examination of intraspecific gender and seasonal differences found no gender differences in dietary β-diversity for any bat species (*C. gouldii*, $F_{1,21} = 0.98, P = 0.624$; *N. gouldi*, $F_{1,17} = 0.74, P = 0.861$; *V. regulus*, $F_{1,20} = 0.82, P = 0.617$) and this effect was independent of season (*C. gouldii*, $F_{1,19} = 1.88, P = 0.014$; *N. gouldi*, $F_{1,15} = 2.04, P = 0.641$; *V. regulus*, $F_{1,18} = 1.91, P = 0.737$). However, even our limited sample sizes revealed seasonal dietary differences for *C. gouldii* ($F_{1,21} = 1.90, P = 0.002$) and *N. gouldi* ($F_{1,41} = 2.08, P = 0.025$), but not *V. regulus* ($F_{1,20} = 1.95, P = 0.127$) (Figure 3). Only *C. gouldii*
showed seasonal intraspecific dietary differences in prey α-diversity, with higher Chao1 values during the maternity than mating season ($t_{14} = 3.27, P = 0.006$). We were unable to compare dietary diversity between sexes within a season due to inadequate samples sizes.

**Discussion**

We were successful in identifying bat dietary differences and niche partitioning in three sympatric bat species in a biodiversity hotspot where prey is largely undescribed. As predicted, dietary divergence was positively related to ecomorphological divergence but dietary convergence did not occur when resources were limited. Our study demonstrates that the fine resolution of HTS, and MOTU analysis, provides important insight into complex predator-prey relationships; we elucidated seasonal intra and interspecific differences in prey consumption in a genetically uncharacterised area that is increasingly fragmented and experiencing a drying climate (Batini 2007; Klausmeyer & Shaw 2009).

**MOTU taxonomic assignment**

Using the BOLD reference database we detected between one and five prey orders (Diptera, Hemiptera, Lepidoptera, Mantodea and Neuroptera) for each bat species, depending on the assignment method. As the ‘best match’ method has both higher true and false positive rates than the ‘strict’ method (Ross *et al.* 2008), our ‘best match’ assignments should be considered cautiously, as a working list of prey items. As we only accepted ‘best match’ assignments within Australian sampled taxa, often from south-western Australian, we are fairly confident in these assignments. Based on this, our
study shows similar results to morphological studies that identified between three and six prey orders for the same species, although prey orders differed. A morphological study elsewhere in the jarrah forest detected three prey orders (Coleoptera, Hymenoptera and Lepidoptera) for these three bat species (Fullard et al. 1991). In Tasmania *C. gouldii* foraged primarily on caterpillars, as well as adult Lepidoptera and Coleoptera while *V. regulus* primarily consumed Lepidoptera, in addition to Coleoptera, Hemiptera, Isoptera, Neuroptera and Trichoptera (O’Neill & Taylor 1989). In south-eastern Australia, *C. gouldii* consumed Hemiptera and Lepidoptera, *N. gouldi* consumed Coleoptera and Lepidoptera and *V. regulus* consumed Coleoptera, Diptera and Lepidoptera (Lumsden & Bennett 2005). The lack of Coleoptera, Hymenoptera, Isoptera and Trichoptera in any of our bat samples is most likely a reflection of their poor taxonomic representation in the genetic reference database, as all orders occur within the jarrah forest (Bunn 1983; Farr et al. 2011).

The majority of assigned MOTUs were Lepidoptera and most were consumed by *C. gouldii*. As predicted, species richness estimates suggest individual *N. gouldi* preyed on more species than *C. gouldii* individuals and prey accumulation curves suggest *C. gouldii* also had the least diverse prey base for all three bat species. These somewhat contradictory results may be indicative of *C. gouldii* consuming larger and/or more ubiquitous prey species that are also invertebrate species most often sampled (Farr et al. 2011) and represented within global reference databases (Dodd et al. 2012), as opposed to an actual greater α-diversity of Lepidoptera prey within *C. gouldii*’s diet.

While one-third of known Lepidoptera species DNA sequences are available in global genetic reference databases, other taxa have substantially lower proportions of
described species represented by DNA sequences, e.g., 6% for Formicidae and 18% for Trichoptera (Jinbo et al. 2011). Whilst our study only assigned between 4 and 20% of MOTUs taxonomically, depending on the method, dietary HTS studies in other biodiverse regions did not yield any matches of invertebrate prey to online reference databases (Brown et al. 2013). These results reinforce how limiting molecular approaches can be in identifying specific prey items in areas lacking a comprehensive genetic reference database and underscore the necessity of examining dietary diversity irrespective of taxonomy. Even in regions where taxonomy is relatively well described, prey can be highly speciose and/or underrepresented in genetic reference databases, limiting taxonomic assignment (Clare et al. 2011).

**Interspecific dietary diversity**

As expected, our study found significant differences in $\beta$-diversity, suggesting niche partitioning, between the three bat species over the entire sampling period, likely influenced by ecomorphology and foraging strategy. The relatively large *C. gouldii* forages in edge and open habitat whereas both *N. gouldi* and *V. regulus* exploit closed and edge habitat, navigating through small openings in vegetation (Fullard et al. 1991; O’Neill & Taylor 1986). Differences between *N. gouldi* and *V. regulus* are likely explained by *N. gouldi* employing two foraging strategies, aerial hawking and gleaning from vegetation, increasing accessibility to a diversity of prey. Unsurprisingly, the more ecomorphologically divergent species, *C. gouldii* and *N. gouldi*, showed the greatest divergence in diet, suggesting niche partitioning is greatest between these two species.
Interspecific dietary differentiation also occurred seasonally for \( \alpha \) and \( \beta \)-diversity. \( N. gouldi \) individuals consumed more prey taxa than \( C. gouldii \) individuals during the mating season. Marginal non-significance of \( \beta \)-diversity between \( C. gouldii \) and \( V. regulus \) during the mating season suggests an overall greater degree of dietary overlap between these two aerial hawkers than between either species and the facultative gleaner, \( N. gouldi \). During the maternity season, dietary composition differed between all three bat species but not the number of prey taxa consumed per individual bat. Contrary to predictions, we found \( \beta \)-diversity dietary differentiation between bat species depended on season for females, but not for males, and within females \( N. gouldi \) and \( V. regulus \) diets converged during the mating, rather than the maternity, season. Our results, instead, suggested that ecomorphologically distinctiveness was the best predictor of \( \beta \)-diversity dietary differentiation in females. Females of the more ecomorphologically divergent, \( C. gouldii \) and \( N. gouldi \), had divergent diets regardless of season whilst females of the more ecomorphologically similar species, \( C. gouldii \) and \( V. regulus \), had similar diets in both seasons. Our findings may reflect how constrained female bats are by roosting sites during the maternity season (Lumsden et al. 2002b; Taylor & Savva 1988) and, while able to commute relatively large distances between roosting and foraging sites (e.g., Lumsden et al. 2002a), the associated energetic costs may influence selective foraging, or niche partitioning, during the maternity season. As energetic requirements and dietary diversity vary for lactating and pregnant females (Dietz & Kalko 2007; Leelapaibul et al. 2005), pooling females of various reproductive stages may be masking dietary differentiation. Alternatively, some bat species contend with increased energetic demands by employing metabolic compensation, rather than increasing prey consumption (Becker et al. 2013), which may explain the inconsistent
patterns between bat studies examining dietary differences between sexes (e.g., Carter et al. 1998; Whitaker 2004). Our study occurred during a very dry year (rainfall ~50% of long-term average) and further research into bat diets would benefit from longitudinal surveys, as determining clear mechanistic processes is best achieved by collecting multi-year data to disentangle seasonal and environmental influences.

**Intraspecific dietary diversity**

Our study corroborated other bat dietary studies that have shown intraspecific seasonal differences in some species (e.g., Andreas et al. 2012) but not others (e.g., Johnson et al. 2012). *C. gouldii* individuals consumed fewer prey taxa during the maternity season than the mating season. In addition, *C. gouldii* and *N. gouldi* showed intraspecific differences in dietary β-diversity between seasons whilst there was no difference for *V. regulus*. This suggests that *C. gouldii* and *N. gouldi* are more opportunistic foragers, consuming available prey, in comparison to *V. regulus*, who appears to be tracking the same prey species over time. This is in contrast to a Tasmanian study where *C. gouldii* was considered a specialist, and *V. regulus* a generalist, forager (O’Neill & Taylor 1989). The difference between these two studies may reflect a difference in foraging ecology of two geographically distinct populations or be reflective of the taxonomic resolution of each study as the Tasmanian study relied on ordinal level analyses.

We expected dietary differentiation for male and female bats in both the maternity season, when energetic requirements for females are higher (Kurta et al. 1989), and the mating season, when energetic demands for males are higher (Dietz & Kalko 2007). However, small sample sizes limited our ability to meaningfully compare intraspecific
sexual dietary differences within a season. Studies with increased sample sizes are required to address sexual dietary differentiation of jarrah forest bats between seasons.

Conclusion

This study shows the value of HTS as a technique for determining dietary differentiation in three sympatric insectivorous bat species consuming a speciose prey base lacking representation in genetic reference databases. Niche partitioning likely facilitates the co-existence of bat species and while ecomorphologically divergent species show the strongest dietary divergence future studies are needed to determine causation between bat species co-existence and dietary overlap. Understanding the extent of niche partitioning is particularly important in disturbed systems where predator species evolved adaptations to minimise dietary overlap but changing environments now interfere with traditional predator-prey relationships and species co-existence. HTS-based approaches clearly have the power to elucidate complex predator-prey relationships, including dietary differentiation between sympatric predatory species, and will facilitate best-practice management and conservation of biodiversity in a rapidly changing environment.

Acknowledgements

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permits CE002999 and SF007648. We thank Susan Campbell, Sian Contarino, Tina Jorgensen, Rod McGregor, Rebecca Parsons, Paul Mitrovski and Jesse Young for assistance in the field, and Nicole White, Frances Brigg and Matthew Bellgard and the iVEC supercomputing resources for assistance and advice regarding data generation and analysis. MB is funded by the Australian Research Council (FT0991741).
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Data accessibility:

All additional supporting materials have been deposited to DRYAD and are accessible under doi:10.5061/dryad.km6ph.

Table 1: Table 1 with associated MOTUs.

Table S1: Table listing bat species traits.

Table S2: β-diversity results for all MOTUs and subset of MOTUs.

Table S3: Table with geographic coordinates of data collection locations.

Table S4: Input table used for analyses within R – unweighted unifrac distance matrix.

Table S5: Input table used for analyses within R – prey (species) accumulation data.

Table S6: Input table used for analyses within R – Chao1 and PD rarefaction.

Appendix S1: Molecular methods.

Appendix S2: Alignment of representative sequences generated for this study.

Appendix S3: Scripts used for analyses in R.

Appendix S4: Scripts used for analyses in QIIME.

Appendix S5: Mapping file for QIIME and R analyses.

Appendix S6: List of MOTUs found in only one sample.

Appendix S7: DNA Sequences.

Author Contributions Box:

J.M. Burgar, D.C. Murray, M.D., Craig, J. Haile, V. Stokes and M. Bunce designed and supervised the experiments; J.M. Burgar contributed samples; J. Houston, D.C. Murray and J.M. Burgar performed the experiments; J.M. Burgar and D.C. Murray analysed the results; J.M. Burgar and M.D. Craig drafted the article; all authors revised the article.
Figure Legends:

Figure 1: Prey accumulation curves for all species combined and individual bat species.

Figure 2: Rarefaction curves for three bat species using two diversity indices: (a) Chao1 and (b) Faith's phylogenetic diversity. Ten rarefactions were performed at a minimum sequence depth of five and maximum of 95, with a step of 10 between. Error bars denote standard error.

Figure 3: Diversity indices for three species of bat, by sex and season: species richness as estimated by Chao1 for each bat species by season (top left) and sex (top right); Faith’s phylogenetic diversity for each bat species by season (bottom left) and sex (bottom right). Statistical differences in $\alpha$-diversity within a species are indicated by * and between species by letters. Error bars denote standard error.

Figure 4: Principal coordinates analysis (PCoA) of MOTUs for three bat species, based on UniFrac distances unweighted by MOTU abundances. Each symbol corresponds to one faecal sample, i.e., individual bat. The first two principal coordinate (PC) axes are shown, explaining 15.4 % of total variation.
Table 1: Taxonomic assignment of MOTUs through the BOLD online identification engine using two different methods: the neighbour-joining hierarchical tree-based ‘strict’ (S) and the sequence similarity ‘best match’ (BM) approach (Ross et al. 2008). Only sequences with >98% similarity were considered as a possible match: >98% for a “genus” match and >99% for a “species” match. For the BM approach, only matches with sampling sites in Australia were considered; * indicates sampling sites in south-western WA while ** indicates sampling sites in WA but outside of the south-west. Species highlighted in grey are thought to use hearing based defences against the echolocation calls of bats.

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Number of OTUs vs Number of Samples

- + All Species
- ▲ C. gouldii
- ● N. gouldi
- ♦ V. regulus
a) 

Sequences per Sample

Chao1 Diversity Index

C. gouldii
N. gouldi
V. regulus
Sequences per Sample

Pylogenetic Diversity

- C. gouldii
- N. gouldi
- V. regulus

Sequences per Sample
PC1 - Percent variation explained 8.2%

PC2 - Percent variation explained 7.0%

- C. gouldii
- N. gouldi
- V. regulus