Selective logging shows no impact on the dietary breadth of the fawn leaf-nosed bat (*Hipposideros cervinus*)

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Abstract

Logging activities degrade forest habitats across large areas of the tropics, but the impacts on trophic interactions that underpin forest ecosystems are poorly understood. DNA metabarcoding provides an invaluable tool to investigate such interactions, allowing analysis at a far greater scale and resolution than has previously been possible. We analysed the diet of the insectivorous fawn leaf-nosed bat *Hipposideros cervinus* across a forest disturbance gradient in Borneo, using a dataset of ecological interactions from an unprecedented number of bat-derived faecal samples. Bats predominantly consumed insects from the orders Lepidoptera, Blattodea, Diptera and Coleoptera, and the taxonomic composition of their diet remained relatively consistent across sites regardless of logging disturbance. There was little difference in the richness of prey consumed in each logging treatment, indicating potential resilience of this species to habitat degradation. In fact, bats consumed a high richness of prey items, and intensive sampling is needed to reliably compare feeding ecology over multiple sites regardless of the bioinformatic procedures used.

Introduction

Logging is a common form of anthropogenic disturbance in forests, with over 90% of those in the tropics logged to some degree (Asner et al., 2009). Most logging undertaken in tropical forests is selective, which tends to favour removal of the largest, and highest-quality trees. While this disturbance can have lasting effects on forest structure (Milodowski et al., 2021) selective logging tends to be much less destructive than clear-felling.

Forest modification through logging is especially pronounced on the island of Borneo, which has lost half of its forest area since 1940 (Gaveau et al., 2014) and 62% of the remaining forest is classified as 'degraded' or 'seriously degraded' (Gaveau et al., 2016). Most studies of the impact this has on biodiversity have focussed on species composition (e.g. Edwards et al., 2011; Slade et al., 2011; Kitching et al., 2013; Struebig et al., 2013; Deere et al., 2018; Hayward et al., 2021). These often subtle changes to ecological communities can result in changes to ecosystem functioning (Ewers et al., 2015) and the structure of trophic networks (Hemprich-Bennett et al., 2020), indicating that selective logging may alter resilience to future perturbations. Understanding the ecological shifts that take place in degraded forest is of great importance for conservation, especially given the vast scale at which forest is managed for timber extraction globally.

Animal diet can differ between individuals of a species depending on numerous intrinsic and environmental factors. In insectivorous bats for example, inter-individual variation in diet appears to correlate with multiple factors, including wing morphology (Oliveira et al., 2020), sex (Burgar et al., 2014), reproductive condition (Czenze et al., 2018), season (Andriollo et al., 2019; Kolkert et al., 2020), geographic location (Czenze et al., 2018;

Vallejo et al., 2019), and habitat (Aizpurua et al., 2018; Hemprich-Bennett et al., 2020; Tournayre et al., 2021). Such variation is of interest when because intraspecific differences in the feeding behaviour of consumers can alter the abundance, community composition and ecological functioning of their prey (Des Roches et al., 2018).

Intraspecific variation in diet is also an important consideration for research design. The analysis of diet in a highly generalist species requires many observations to obtain a representative sample. This can be especially true when studying the dietary ecology of insectivorous bats through metabarcoding, as the technique gives an unprecedented level of taxonomic resolution (Clare et al., 2009), highlighting variation which would not have been apparent with morphological study. Inter-individual variation in bat diet is however often obscured by the use of samples collected from underneath roosts, where numerous bats are defecating (hereafter 'roost-sourced' samples) (Clare et al., 2014; Andriollo et al., 2019) and samples cannot be linked to an individual. Obtaining faecal samples from individually identifiable animals (hereafter 'individual-sourced' samples) is labour-intensive due to the large trapping effort required, and so while many studies have used individual-sourced samples (e.g. Czenze et al., 2018; Oliveira et al., 2020), their sample sizes tend to be small. Mata et al (2018) used a dataset of individual-sourced samples to analyse the importance of technical and biological replication on the dietary completeness of *Tadarida teniotis* and reiterated the common rule of thumb that 20-50 such samples per species is preferable, but stressed that higher sample sizes may be required for bat species with greater dietary richness or intraspecific variation. The issue of sample size is further complicated in networks generated from metabarcoding data because of methodological considerations such as PCR primer bias and stochasticity (Alberdi et al.,

2018), and the influence of bioinformatic choices on the final data analysed (Hemprich-Bennett et al., 2021).

Here we use an unprecedented number of individually-sourced insectivorous bat faecal samples to test the hypothesis that selective logging alters the taxonomic composition and species richness of bats' diet. We also assess how sample size and bioinformatic parameters affect our inferences of insectivorous diet when using data derived from metabarcoding. Our evaluation focuses on the fawn leaf-nosed bat, *Hipposideros cervinus* - a cave-roosting insectivorous bat found throughout much of maritime Southeast Asia to northeastern Australia. Using high-duty cycle (HDC) echolocation, it is thought to use Doppler-shift compensation to detect the wingbeats of fluttering of prey such as moths (Bell and Fenton, 1984) against a cluttered backdrop (Schnitzler and Kalko, 2001; Lazure and Fenton, 2011). Although some bat species are negatively affected by logging, *H. cervinus* remains a dominant species in both old growth and logged forest in Borneo (Struebig et al., 2013; Hemprich-Bennett et al., 2020). It is not known whether bats such as *H. cervinus* respond to forest degradation by modifying their diets, or are able to maintain stable diets through prey selection or behavioural changes in foraging. We address three main predictions:

1) Taxonomic composition of the diet of *H. cervinus* is altered by rainforest degradation.

2) Individual bats are more specialised in logged forest sites than in primary forest.

3) Estimates of sampling completeness are heavily influenced by MOTU clustering threshold, quality-control methods used and the number of samples.

Methods

We sampled bats using six harp traps per night at four lowland tropical rainforest sites in Sabah, Malaysia, each <500m above sea level and limited seasonality. Two sites comprise mostly old growth rainforest (Danum Valley and Maliau Basin), and two sites have been subject to substantial logging disturbance (the Sabah Biodiversity Experiment and the Stability of Altered Forest Ecosystems Project) (Supplementary Table 1).

- Old growth rainforest:
    - The Danum Valley Conservation Area (hereafter 'Danum') is a 438 km^2 region protected area of old growth rainforest in Sabah (Reynolds et al., 2011). Traps were erected in 2016 for ten nights in a 21-night period and 2017 for ten nights in a 12-night period.
    - The Maliau Basin Conservation Area (hereafter 'Maliau') is a 588 km^2 protected forest made up of lowland and hill forest, most of which has neither been logged nor inhabited in historical times. Traps were erected in 2016 and 2017 for ten nights in a 16-night period.
- Logged forest:
    - The Stability of Altered Forest Ecosystems Project (hereafter 'SAFE') is a large area of degraded forest being converted to oil palm plantation, with fragments of forest retained for scientific study (Ewers et al., 2011). We sampled in the blocks 'LFE', 'B' and 'C', within the Ulu Segama Forest Reserve and Kalabakan area, during 2015, 2016 and 2017. Each block was sampled for a 5-night period, and then resampled at least 5 weeks later.

- The Sabah Biodiversity Experiment (Hector et al., 2011) (hereafter 'SBE') is an area of forest which was logged once in the 1950s and once in the 2000s, and during the sampling period was in the early stages of enrichment replanting (Hector et al., 2011). Sampling took place over a total of 10 nights in a 20-night period in 2016.

Fieldwork, laboratory work and bioinformatics took place as previously described (Hemprich-Bennett et al., 2020). Briefly, bats were captured using harp traps erected along linear features such as streams and trails to target bat flyways. Sampling effort is summarised in Table 1. Faecal samples were processed by DNA extraction, PCR amplification of the CO1 gene using the primers described by Zeale et al (2011), and sequenced on an Illumina MiSeq. For complete methods see (Hemprich-Bennett et al., 2020).

Bioinformatics pipeline

Sequences were assembled into contigs using mothur (Schloss et al., 2009), and forward and reverse primers were removed using the galaxy web platform on the public server at usegalaxy.org (Afgan et al., 2016) sequence falling outside of a length of 155-159bp (2bp outside of the expected amplicon length) were excluded from analysis.

When processing the sequence data it is common to cluster sequences into MOTUs (Molecular Operational Taxonomic Units) (Floyd et al., 2002), on the basis of a given threshold of similarity, but the appropriate MOTU clustering thresholds required to best-represent the taxonomic diversity within metabarcoding samples are currently poorly

understood (Hemprich-Bennett et al., 2021). At high clustering thresholds routine sequencing errors may be falsely designated as distinct MOTU, artificially inflating the measured diversity and richness within a sample (Clare et al., 2016). Algorithms implemented using software such as LULU (Frøslev et al., 2017) have been proposed as a method of mitigating this, by combining probable duplicate MOTUs based on patterns of sequence similarity and cooccurrence.

To assess the impact of clustering threshold on the datasets analysed (Hemprich-Bennett et al., 2021) we generated datasets using MOTU clustering thresholds at ranges 91-98% similarity, using the Uclust algorithm (Edgar, 2010) as implemented in the QIIME platform (Caporaso et al., 2010). Representative sequences for each MOTU per clustering level were then compared to one another using BLAST+ (Camacho et al., 2009), with the resulting data being reduced in LULU (Frøslev et al., 2017) for quality control. All resulting bat-MOTU adjacency lists were then transformed into adjacency matrices using a custom perl script. These matrices were then split into multiple binary adjacency matrices by site. Networks were created by pooling samples from multiple years. To test prediction 2, separate analyses took place on networks both generated as composites of multiple years, and as separate networks for each site and year (see Table 1). All bioinformatic and statistical steps are recorded at

https://github.com/hemprichbennett/hice.

Prediction 1: Taxonomic composition of the diet of *H. cervinus* is altered by rainforest degradation

To analyse the prey taxa consumed by each bat, we used BLAST+ (Camacho et al., 2009) to compare all MOTUs to a library of all arthropod CO1 genes identified to species level

using the Barcode of Life Database on 28/03/2018 (BOLD) (Ratnasingham and Hebert, 2007) (3,319,062 sequences), and assigned them taxonomy in MEGAN 6 (Huson et al., 2016) using the parameters in Salinas-Ramos *et al.* (2015). We then assigned MOTUs to order and family level where possible, importing the resulting data into R for analysis, and calculating the proportion of *H. cervinus* individuals per site consuming each taxonomic order. To test the hypothesis that habitat type alters the order-level taxonomic composition of the species' diet, we analysed the resulting values with a Chi-squared test. The hypothesis was further tested using a permutational multivariate analysis of variance test using distance matrices, and a non-metric multidimensional scaling ordination with 200 permutations using Bray-Curtis dissimilarity, both using the vegan package (Oksanen et al., 2017) on datasets of the order-level diets of each individual bat. We also used a similarity percentages analysis to identify the contribution of each taxonomic order to the observed dissimilarity between sites and years, using Bray-Curtis dissimilarity.

We calculated correlations between the presence/absence of prey orders in faecal samples, using the r package 'corrplot' (Wei and Simko, 2017), to identify both potential significant correlations of prey consumption (e.g. bats that feed on Coleoptera may be more likely to feed on Blattodea), and any potential taxonomic bias in PCR.

Prediction 2: Individual bats are more specialised in logged forest sites than in old growth forest

We created binary bipartite networks for each sampling site and year at 95% similarity clustering and quality control using LULU. In the networks each individual bat and MOTU was classed as a distinct node. A criterion of 95% similarity was chosen for this and all following analyses because it provided a balance between over and under-splitting

MOTUs (Hemprich-Bennett et al., 2021). Using the R package 'bipartite' (Dormann, 2011) in R 3.4.4 (R Core Team, 2017) these networks were then analysed using the functions 'specieslevel', to calculate the degree of each bat ('degree' = the number of prey nodes a bat consumes). Differences between the degree of individuals were compared among sites using an ANOVA with Tukey's HSD test.

Prediction 3: Estimates of sampling completeness are heavily influenced by MOTU clustering threshold and quality-control used

Using networks generated at each clustering threshold between 91 and 98% similarity, both with and without quality-control using LULU (Frøslev et al., 2017), we estimated total MOTU richness and sampling completeness of the diet of *H. cervinus* at each site and year using iNEXT (Hsieh et al., 2016), an R package for the interpolation and extrapolation of species diversity using Hill numbers (Chao et al., 2014).

To assess how sample size affects assessments of bat diet, we generated multiple datasets of *n* bats from each site, where *n* was a value of 10-100, increasing in increments of 10 (10, 20, 30, etc), with *n bats* taken at random from each site and the number of MOTUs consumed in that sub-dataset calculated. This was repeated 100,000 times per site and value of *n,* with the resulting data plotted in a violinplot.

Results

For the full sequencing run of multiple bat species (see Hemprich-Bennett et al., 2020) 18,737,930 contiguous reads were output when assembling the paired-end files. After removing adapters and primers this was reduced to 10,064,815 sequences, which was then further reduced to 932,459 haplotypes after collapsing to haplotype, removing singletons and discarding sequences outside of 2bp of the expected read-length. For full counts of MOTUs before and after clustering with LULU, see Supplementary information 2. Of these, 2,957,444 reads and187,800 haplotypes were derived from *H. cervinus* samples and included in this study.

Prediction 1: Taxonomic composition of the diet of *H. cervinus* is altered by rainforest degradation

The diet of the bat communities was dominated by insects from the orders Blattodea (especially family Ectobiidae), Diptera (especially family Cecidomyiidae) and Lepidoptera (Figure 1). The chi-squared test showed a non-significant effect of network identity on the order-level composition of a bat populations' diet ($\chi 2 = 0.16$, df = 48, $p > 0.05$). The NMDS showed almost total overlap between the sites (Figure 2) with a stress of 0.21, showing poor convergence. The permutational multivariate analysis of variance test gave an R^2 of 0.014 for the explanatory power of site on bat diet. A total of 23 arthropod orders were eaten based on the combined diets of all bats, with Blattodea, Coleoptera, Diptera and Lepidoptera collectively making up at least 79% of all MOTUs identified at each site. Positive correlations were observed between the occurrences of several taxa, with only Araneae and Hymenoptera being negatively correlated with the presence of one another (Supplementary information 3). Blattodea was the only taxon consistently observed to contribute significantly to inter-site dissimilarity scores (SAFE-

Maliau p<0.01, SAFE-SBE p = 0.014, Maliau-SBE p = 0.014, SBE-Danum p <0.01, see Supplementary information 4). There was almost complete overlap between the different years sampled at each site (Figure 2) and each site in 2016 (Figure 3).

Prediction 2: Individual bats will be more specialised in logged forest sites than in old growth forest

When comparing networks with all years pooled together, significant differences (p<0.05) were only observed between Danum (old-growth) and SAFE (logged), and between SAFE (logged) and SBE (logged).

Prediction 3: Estimates of sampling completeness will be heavily influenced by MOTU clustering threshold and quality-control used

None of the networks were estimated as near to fully sampled, with all estimates placing completeness at under 54% (Figure 4), with completeness estimates varying between both sites and years. The number of MOTUs expected increased markedly with clustering threshold when not using LULU for quality control, but this effect was dramatically reduced when using LULU. This algorithm increased estimated sampling completeness by reducing observed and estimated MOTU richness, and lowered the estimated number of samples required to sample the community. Full counts can be found in Supplementary information 2.

There was a positive correlation between the number of bats included in a dataset and the number of MOTUs detected (figure 5)

Discussion

Logging is widespread in tropical forests, yet the consequences of this structural disturbance for trophic interactions are poorly understood. Here we set out to assess how the diet of a generalist insectivorous bat differs between old-growth and degraded forest habitats. We observed broadly similar feeding habits in fawn leaf-nosed bats across forest type with bats consuming many arthropod orders, particularly Blattodea, Coleoptera, Diptera and Lepidoptera. Fawn leaf-nosed bats have extremely high dietary richness, with many hundreds of samples being required to fully capture their diet.

We observed very little alteration in the taxonomic composition of the diet of *H. cervinus*. We saw no significant difference between the consumption of prey at the order-level between sites or years. This suggests that while northeast Borneo may possess high beta-diversity of some insect species (Kitching et al., 2013), at coarse taxonomic levels there is little spatial difference in the prey consumed by *H. cervinus*. Previous findings suggested that, as high-duty cycle echolocators, *H. cervinus* primarily consumed flying insects (Bell and Fenton, 1984; Link et al., 1986; Schnitzler and Kalko, 2001; Lazure and Fenton, 2011), in particular Lepidoptera, Blattodea, Diptera and Coleoptera. The regular presence of diverse families of spiders indicates a dietary contribution of these taxa previously unknown in the Hipposideridae family of bats. Hipposiderids have been observed gleaning stationary targets with fluttering wings (Bell and Fenton, 1984), but the consumption of spiders would either suggest they are gleaning non-fluttering animals, or taking them when ballooning as juveniles. Alternatively, the consumption of spiders could be due to secondary predation: where the bat consumes a primary prey item which has ingested a spider. This seems an unlikely explanation for our dataset, since predatory

283 arthropods other than Araneae are poorly represented in the MOTU dataset. In this study
284 we used one of the most reliable primer sets for amplification of a wide range of digested
285 arthropods (Zeale et al., 2011; Alberdi et al., 2018), but they are also reported to have
286 taxonomic biases towards Diptera and Lepidoptera. However, we found no significant
287 negative correlations between detecting Dipteran or Lepidopteran DNA in a sample, and
288 the detection of any other prey order. This indicates that amplification of dipteran or
289 lepidopteran DNA did not consistently inhibit the amplification of another taxonomic
290 order during PCR, and that sequencing depth is sufficient.

291

292 There was no clear pattern of degree differing between logged and old growth habitats.
293 This is in contrast to our previous findings in these study sites (Hemprich-Bennett et al.,
294 2020), that the overall assemblage of bat species in these sites consistently had reduced
295 degree in logged forest than old growth. The diversity of the overall bats' diet is likely
296 due to the high diversity of prey available to them, and the lack of observed differences
297 in diet between sites may indicate highly flexible foraging, with low impact of land-use
298 change on their diets. Being able to forage adaptively, or fly long distances to viable
299 feeding sites (Struebig et al., 2009) may enable them to remain abundant despite selective
300 logging, while conspecific species experience population declines (Struebig et al., 2013).
301 This species may, as a result, provide ecological redundancy and continue to contribute
302 insectivory when more sensitive bat species have become locally extinct.

303

304 A crucial concern in network ecology is the minimum number of samples or observations
305 required to characterise reliably the structure and identity of the interactions within a
306 network (Nielsen and Bascompte, 2007; Rivera-Hutinel et al., 2012). This requirement is

complicated in studies utilising DNA metabarcoding as the number of nodes generated is dependent on the bioinformatic choices used to generate them. While MOTU approaches frequently apply a standard resolution to all nodes which helps control for variation in identification, altering MOTU clustering threshold will change the number of nodes and estimates of completeness, analogous to lumping taxonomy-based identifications to higher levels, but without a biological equivalent. We tested MOTU clustering and the use of LULU for quality-control and demonstrated that it was possible to alter estimates of sampling completeness greatly (Figure 4). However, when generating networks with a range of bioinformatics combinations, we observed that none exceeded an estimate of 50% completeness and thus regardless of parameters used, obtaining the full estimate of *H. cervinus* diet would require several hundred samples per site, with the same likely true of many ecologically similar species. Altering MOTU clustering parameters has previously been shown to cause great variation in MOTU counts (Clare et al., 2016) and changes in numerous measures of network-level architecture (Hemprich-Bennett et al., 2021). The reduction in number of estimated MOTUs provided by LULU (Frøslev et al., 2017) is expected to be of great use in future metabarcoding-based studies to reduce spurious MOTU generation.

The dietary richness found here echoes previous studies (Clare et al., 2009; McCracken et al., 2012) but raises question about the capacity of bats to distinguish between prey types in detail (Neuweiler, 1990) and if this has implications for prey-choice. At the same time, our results highlight the substantial challenge of characterising the diets of this and other insectivorous bat species, especially in hyperdiverse ecosystems such as tropical rainforests. Their large dietary breadth is further highlighted by the fact that DNA extractions performed here were for pooled faecal samples from each individual bat, a

technique which Mata *et al.* (Mata et al., 2018) found underestimated the total richness of the diet per bat. Previous intensive studies of arthropod diversity in lowland tropical rainforest have failed to reach an asymptote (Novotný and Basset, 2000; Basset et al., 2012), and if bats are foraging opportunistically it is perhaps unsurprising that the taxonomic breadth of their diet is extremely large and nearly impossible to sample completely.

We demonstrate the vast richness of prey consumed by insectivorous bats in tropical rainforest and show that although quality-control steps in metabarcoding can reduce our estimates of the number of distinct prey items in a site, many hundreds of samples are required to collect a representative description of total diet. Although we focussed our sampling on a single species of insectivorous bat, some inferences likely also apply to similar species, and to other studies that use metabarcoding. The number of sites analysed in this study was low, but it has been shown here that this Hipposiderid species has a highly diverse diet; relying on cockroaches more than previously thought and potentially having a strategy of gleaning non-fluttering prey previously unknown in the family. This bat species is thus thought to exhibit low levels of dietary response to habitat degradation, potentially indicating reasons for their known versatility in the face of landscape modification.

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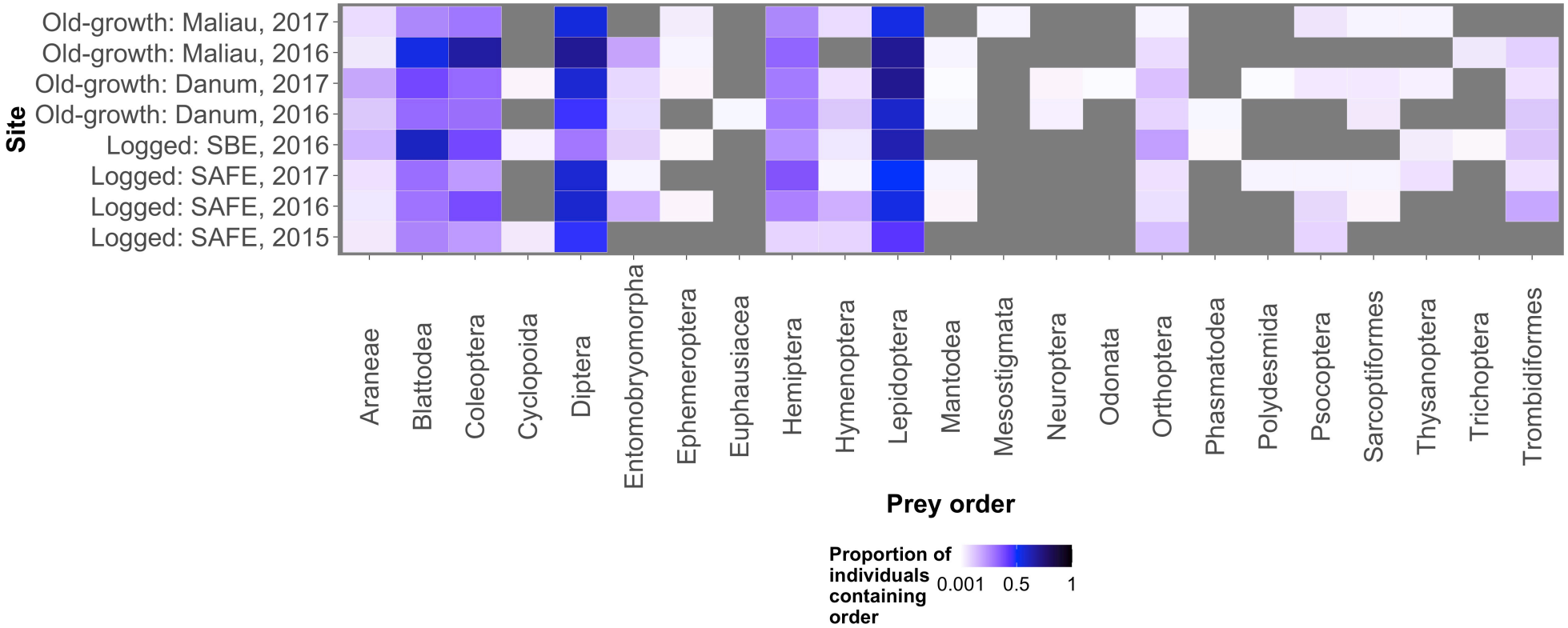
570

Tables

Table 1. Trapping effort per site, in harp trap nights. One harp trap night is a harp trap erected for a single night. Six harp traps were used per night, so a single night's trapping was equal to six harp trap nights.

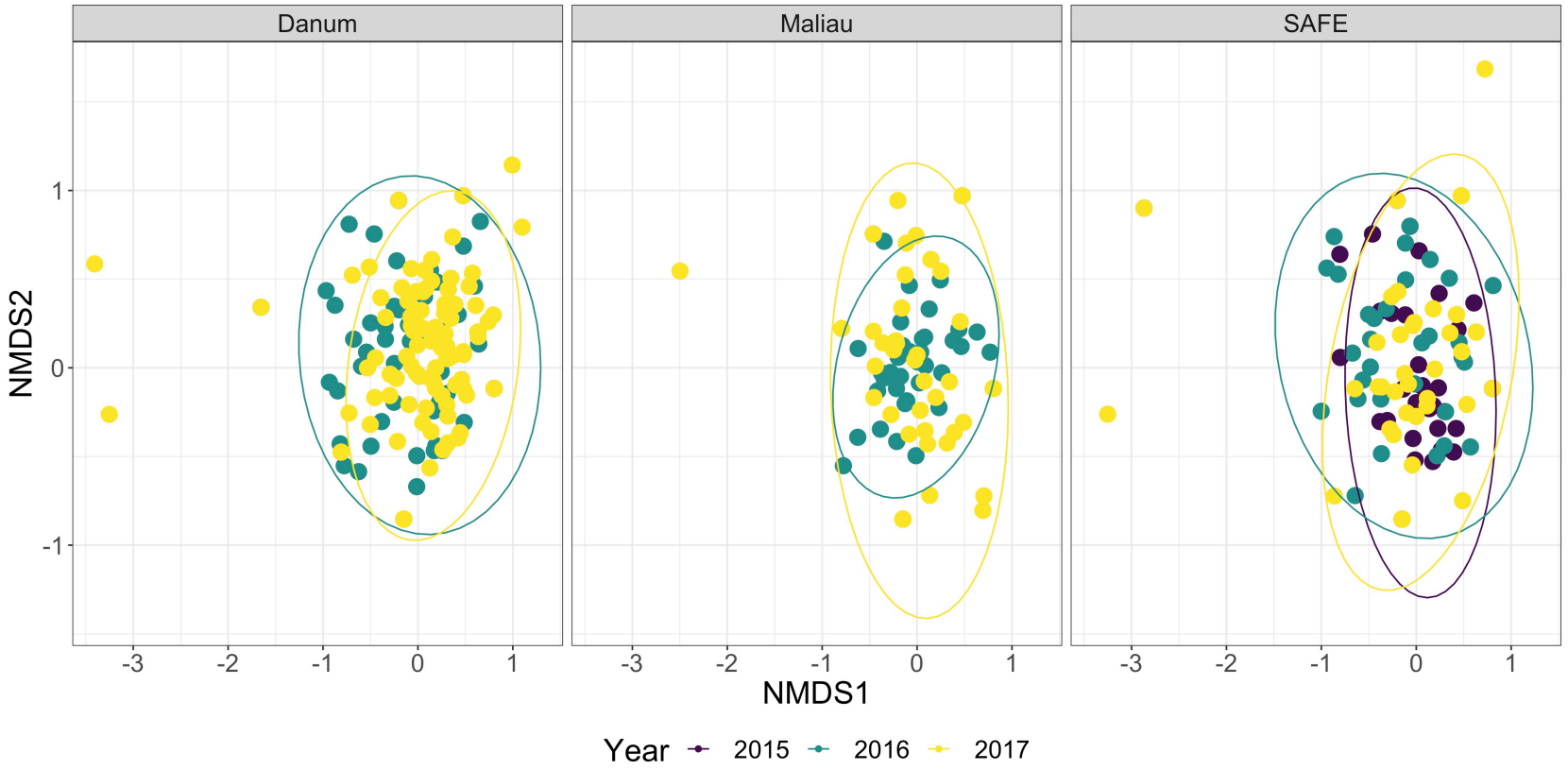
Sample Site	**2015**	**2016**	**2017**
SAFE	216	180	180
Danum	0	60	60
Maliau	0	60	60
SBE	**0**	**60**	**0**

Figures



578

579 Figure 1. The proportion of all individual bats within a sampling event found to consume each potential prey order. Diptera, Lepidoptera and Blattodea
580 were the commonest prey items, with other prey orders being consumed rarely. The grey background shows locations in the plot where no arthropods of
581 that order were detected in any bats.

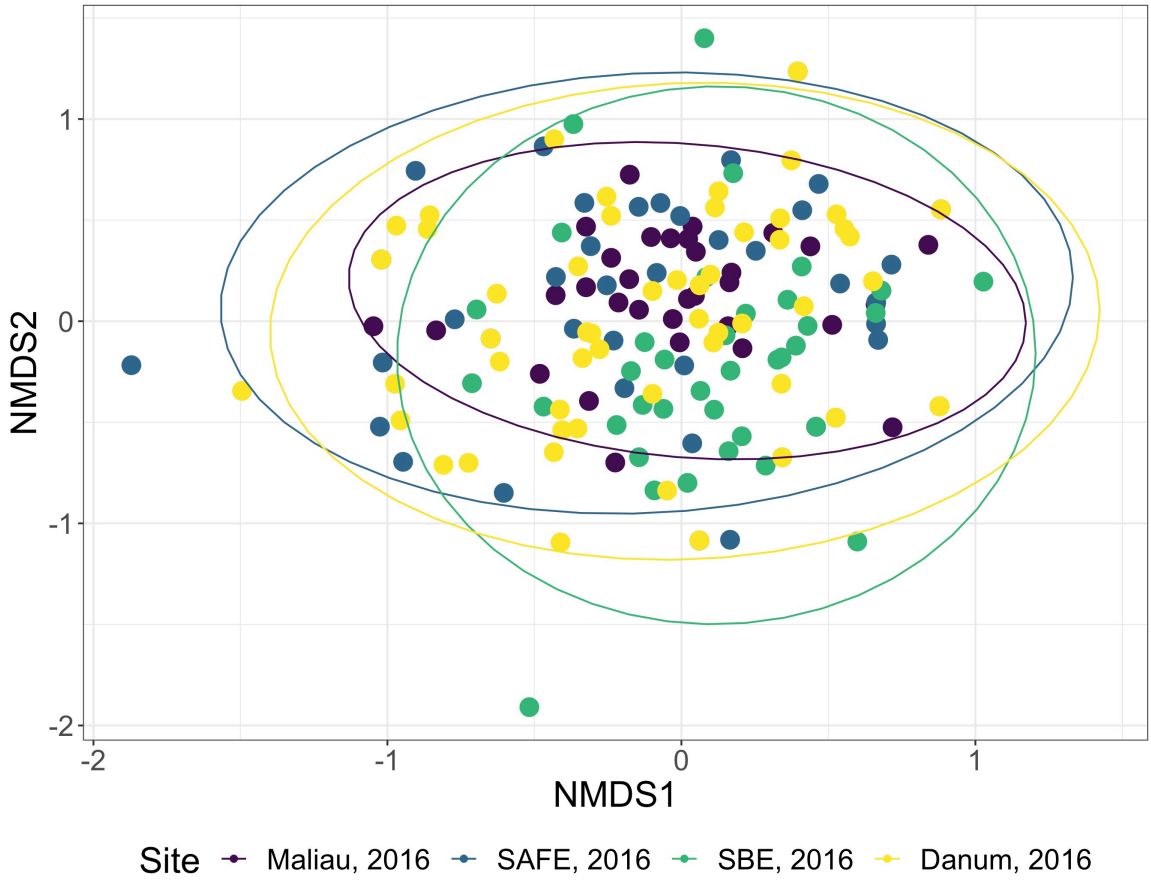


582

583 Figure 2. Non-Metric Multidimensional Scaling ordination of the order-level consumption of individual bats across multiple years. The ellipses of each

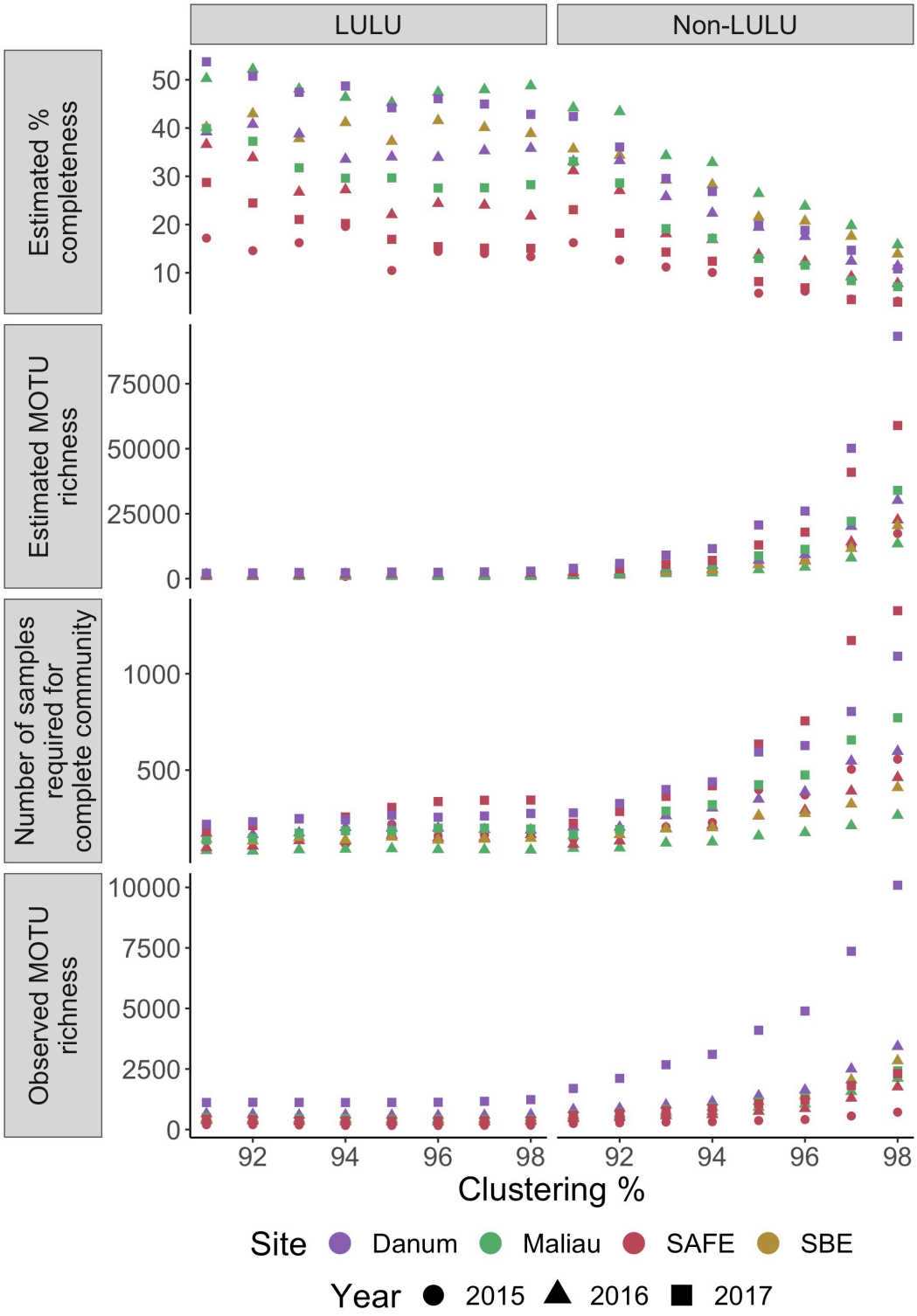
584 site show almost complete overlap. Stress was 0.21, indicating poor convergence. Danum and Maliau are old-growth sites, SAFE is a logged forest site.

585



586

587 Figure 3. Non-Metric Multidimensional Scaling ordination of the order-level
588 consumption of individual bats in 2016. The ellipses of each site show almost complete
589 overlap. Stress was 0.22, indicating poor convergence. Danum and Maliau are old-growth
590 sites, SAFE and SBE are logged forest sites.



591

592 Figure 4. Completeness and richness for each network over a range of MOTU clustering
593 thresholds, with and without use of LULU for post-clustering quality-control. Number of
594 MOTUs is strongly positively correlated with clustering level when not using LULU for
595 quality-control, reducing the estimated completeness of each network.

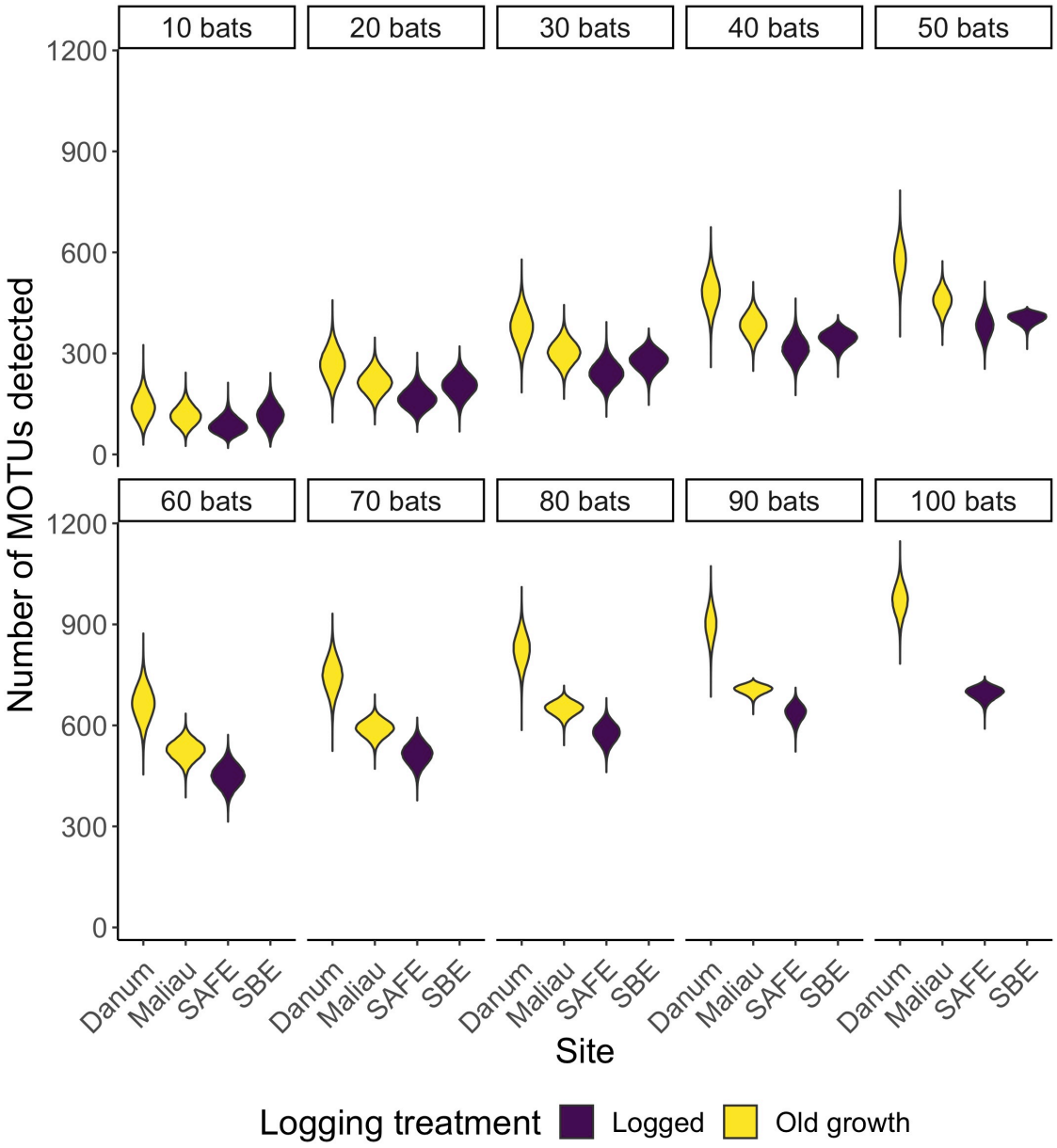


Figure 5: Violinplots showing the distribution of the number of MOTUs consumed when reducing a dataset to *n* bats. With small datasets, sites appear to be rather similar in MOTU richness, but differences emerge as sample sizes increase.