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Foraging Niche Partitioning of Three *Myotis* Bat Species and Marine Fish Consumption by *Myotis pilosus* in a Subtropical East Asian Region

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ABSTRACT

Most bats are insectivorous, but some species have evolved the ability to prey on fish. Although piscivory has been confirmed in the Rickett's big-footed myotis (*Myotis pilosus*), the extent of piscivory of other cohabiting *Myotis* species is uncertain. This study aims to explore the dietary niches and fish consumption of three *Myotis* species in a subtropical East Asian region, and specifically the fish diet of *M. pilosus*. Our findings reveal, for the first time, that *M. pilosus* consumes marine fishes, in contrast to previous research conducted in inland regions that suggested year-round consumption of cyprinids in freshwater habitats. We also observed seasonal variation in the diets of *M. pilosus*. It predominately hunted wide-banded hardyhead silverside [31% relative read abundance (RRA) of all 12S reads], sailfin flying fish, and shorthead anchovy during the wet season, while mainly preying upon mullets (31%) during the dry months. In more inland areas, *M. pilosus* was found to primarily feed on invasive freshwater poeciliids (13%). Furthermore, *M. pilosus* consumed more fish during the dry season, while there was a greater consumption of insects during the wet months. Most notably among our findings is the consumption of fish by two individuals of Horsfield's myotis (*M. horsfieldii*), indicating that the species is potentially piscivorous. We revealed that both *M. horsfieldii* and *M. pilosus* consumed water striders, suggesting that foraging of aquatic insects could be driving the evolution of fishing behavior. Our findings have also shed light on the flexibility of foraging behavior in piscivorous bats.

1 | Introduction

There are over 1400 known bat species worldwide, forming a diverse group that has adapted to a wide range of diets (Taylor 2019). The majority of bats are insectivorous, feeding primarily on insects, while others are frugivorous and nectarivorous bats, which consume fruit and nectar, respectively (Ramírez-Fráncel et al. 2022). In addition to insects, certain bat species have adapted to feed on other animals; for instance, sanguivorous vampire bats consume blood (Riskin and Carter 2023), and piscivorous bats prey on fish (Aizpurua and Alberdi 2018). Piscivorous bats can be found among two distinct genera: *Noctilio* (bulldog bats) and *Myotis* (mouse-eared bats). Among these, only the *N. leporinus* (greater bulldog bat) in Latin America has been confirmed as mainly piscivorous (Brooke 1994). Three other species display 'limited' fishing behaviors, including *M. vivesi* (fish-eating myotis) in the Gulf of California (Otálora-Ardila et al. 2013), *M. pilosus* (Rickett's big-footed myotis) in southern and eastern China, Vietnam, and Laos (Ma et al. 2003), and *M. capaccinii* (long-fingered myotis) in the Mediterranean Sea (Aihartza et al. 2003). Additionally, several species have 'unconfirmed' fishing behaviors, such as *N. albiventris* (lesser bulldog bat), *M. daubentonii* (Daubenton's myotis), and *M. macropus* (large-footed myotis).

In Asia, *M. pilosus* is relatively large for its genus, exhibiting a body length ranging from 51 to 65 mm (Wilson and Mittermeier 2019). They possess remarkably enormous feet with enlarged, laterally compressed claws, resembling those of *N. leporinus* (Fish et al. 1991; Ospina-Garcés et al. 2016). However, it was not until 2003 that the piscivorous nature of *M. pilosus* was confirmed (Ma et al. 2003). The bats capture insects in mid-air and use their hind limbs to employ a trawling method for catching fish (Jiang et al. 2003). While fish remains have been discovered in their diets in some regions, regular fish consumption has only been verified in Beijing in northern China (Ma et al. 2006). Studies conducted in Beijing, Shandong, and Guizhou have shed light on the dietary habits of *M. pilosus* (Ma et al. 2006, 2003; Chang et al. 2019; Wang et al. 2024). These studies reported on the piscivorous and insectivorous feeding behaviors of *M. pilosus*, with species from the order Cypriniformes (cyprinids) being their primary fish prey. In Beijing, they found that *M. pilosus* consumed grass carp (*Ctenopharyngodon idella*), Eurasian carp (*Cyprinus carpio*), pale chub (*Zacco* spp.), and Amur minnow (*Rhynchocypris* spp.) (Ma et al. 2006; Chang et al. 2019). In Shandong, in addition to grass carp and Eurasian carp, they also found bitterlings (*Rhodeus* spp.) to be their primary prey (Chang et al. 2019). These fish species are similarly found in freshwater habitats within inland areas (Froese and Pauly 2024). Additionally, *M. pilosus* was found to consume at least seven orders of insects, such as Coleoptera (beetles), Lepidoptera (moths and butterflies), Hemiptera, Diptera, and more (Ma et al. 2006, Chang et al. 2019).

However, discrepancies in the extent of fishing incidence have been observed among these studies. One possible factor contributing to these discrepancies could be the variations in spatiotemporal prey availability across different sampling locations and seasons. Further research on their fish and insect consumption is crucial to gain a better understanding of how resource variation or resource partitioning is associated with their adaptations to the environment through foraging strategy. Thus, it is necessary to investigate the dietary compositions of *M. pilosus* in various locations or habitats across seasons to provide further insights into their fishing behavior. However, beyond the few populations studied, the dietary composition of *M. pilosus* remains largely unknown.

There have been speculations that several other Asiatic *Myotis* species, such as *M. macrotarsus* (pallid large-footed myotis), *M. horsfieldii* (Horsfield's myotis), *M. hasseltii* (lesser large-footed myotis), *M. stalkeri* (Kei myotis), *M. adversus* (large-footed bat), *M. macrodactylus* (big-footed myotis), and *M. macropus*, might be piscivorous due to their large hind feet and claws (Aizpurua and Alberdi 2018). Notably, *M. horsfieldii* is a native bat species in South Asia. Although it is smaller compared to *M. pilosus*, with a body length ranging from 44 to 51 mm, its capability to directly capture insects from water surfaces (Wilson and Mittermeier 2019) suggests a potential for piscivory. It is widely believed that the fishing behavior in bats evolved from their insectivorous habits, with the hypothesis that capturing insects from water represents an intermediate stage in this behavior (Aizpurua et al. 2013). Despite the relatively large hind feet of *M. horsfieldii*, exceeding half the length of the tibia, and their observed circular flight patterns above open water surfaces at a close distance of a few centimeters to search for insects, there is currently a lack of evidence for fishing behavior in *M. horsfieldii* (Aizpurua and Alberdi 2018). Furthermore, their dietary composition has never been reported to date, leaving unanswered questions regarding whether fish forms part of its diet and what insect species they prey upon.

Hong Kong, situated at the southern coast of China, is the distribution range of *Myotis* species (Shek 2006). It is a semi-island known for its diverse habitats, including various types of forests, coastal areas, wetlands, marine, and freshwater habitats, each hosting a wide array of arthropod and fish species (Dudgeon and Corlett 2004). In this region, four *Myotis* species coexist, including *M. pilosus*, *M. horsfieldii*, *M. chinensis* (Chinese myotis), and *M. muricola* (whiskered myotis, locally rare). *Myotis chinensis* is bigger in size than *M. pilosus*, with a body length ranging from 91 to 97 mm (Wilson and Mittermeier 2019), and it possesses relatively large hind feet compared to some other bat species. However, there are only a few studies conducted in China reporting its diets. Despite its large size, it is believed that *M. chinensis* exclusively feeds on insects. They are capable of preying on larger insects and capturing ground-dwelling insects through picking behavior, primarily consuming species in Coleoptera (e.g., carabid beetles), Orthoptera (grasshoppers), and Diptera (flies), according to an earlier study (Ma et al. 2008). Given the uncertainties surrounding the extent of piscivory by different *Myotis* species in the region, this study seeks to investigate the diets of *M. pilosus*, *M. horsfieldii*, and *M. chinensis* in Hong Kong by analyzing their fecal compositions using DNA metabarcoding (de Sousa et al. 2019; Monterroso et al. 2019).

By analyzing the prey identities and relative quantities of the three *Myotis* species, we can gain insights into the spectra of habitats that bat individuals forage and the prey they target in a habitat-diverse environment. Notably, both *M. chinensis* and *M. pilosus* are listed as Near Threatened species in the Red List of China's Vertebrates (Jiang et al. 2016), with *M. pilosus* also being classified as a Vulnerable species in the IUCN Red List of Threatened Species (IUCN 2023; Jiang et al. 2019). Hence, it would be important to further explore the dietary niche partitioning of these sympatric species. The findings would inform us of their trophic relationships and what types of food resources and habitats are critical to their population sustainability and significantly contribute to the conservation efforts for each of these species in the region.

In this study, we aim (1) to investigate the dietary compositions of the three *Myotis* species at both individual and species levels, (2) to estimate the dietary diversity within individuals and populations, (3) to assess the effect of environmental and host factors on the dietary compositions of these species, and (4) to determine the patterns of dietary niche partitioning among the *Myotis* species. We hypothesize that the three species might demonstrate dietary niche partitioning. We predict distinct dietary compositions for each species, resulting in minimal dietary overlap between species. Specifically, we predict that *M. pilosus* might include diverse fish taxa in its diet due to the diverse aquatic habitats close to the roosting sites. We also hypothesize that there is seasonal variation in the dietary compositions of the bat species. We predict a shift in food consumption patterns across different seasons.

2 | Materials and Methods

2.1 | Sample Collection

Between 2018 and 2021, we visited water tunnels or caves in Hong Kong to capture *Myotis* bats during the wet (April to September) and dry (October to March) seasons. We refrained from visiting roosting sites during the months when bats were overwintering or breeding/nursing, thus minimising any potential disturbance. The locations include Lin Ma Hang (LMH01), Tai Lam Chung (TLC01 and TLC02), Pak Tam Chung (PTC), and Sai Kung (SK0, SK02, and SK03) (Figure S1 and Table S1a–c) (Shek 2004). We captured the bats using hand-held hoop nets and placed them in breathable sterilized bags. To prevent cross-contamination, each individual bat was kept in a separate sterilized bag. We identified the species and sex of each bat based on its morphology (Poon et al. 2023; Shek 2006). The fresh feces from each bat were collected and placed in individual 2 mL tubes. They were preserved in 100% ethanol and then kept at −80°C until DNA extraction. In total, we have collected fecal samples from 62 *M. pilosus*, 51 *M. horsfieldii*, and 43 *M. chinensis*. Physical parameters of each bat, such as body weight and forearm length, were measured. After collecting samples and recording their physical parameters, all bats were released immediately back to the wild. Approvals for animal experiments were granted by the Department of Health (ref. 19-177 in DH/SHS/8/2/3 Pt. 30), the Committee on the Use of Live Animals (ref. 4963-19), and the Agriculture, Fisheries, and Conservation Department (AFCD; ref. 35 in AF GR CON 09/51 Pt.8).

2.2 | DNA Extraction and Metabarcoding

The QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used to extract the fecal DNA. We included negative controls and mock communities (Table S2a) during DNA extraction to later check for contamination during PCR. We quantified the fecal DNA using the Qubit dsDNA high-sensitivity (HS) assays on an Invitrogen Qubit 4 Fluorometer (Thermo Fisher Scientific, Massachusetts, US) (Huang et al. 2021).

All fecal DNA samples, mock communities (Table S2a), and negative controls were used for library preparation using three genetic markers through two-step PCR (Huang et al. 2022, 2021) (Supporting Materials and Methods: Appendix S1). The first pair of markers used was a universal pair (18s_SSU3_F: 5′GGTCTGTGATGCCCTTAGATG3′ and 18s_SSU3_R: 5′GGTGTGTACAAAGGGCAGGG3′), which targets the V7 region of 18S small subunit ribosomal DNA (rDNA; ca. 174 bp) (McInnes et al. 2017). This pair of markers provides an overview of the dietary compositions of *Myotis* bats. To investigate whether fish was consumed by the three *Myotis* species, we used the mitochondrial 12S rDNA specific primers (12S_AcMDB07_HK_F: 5′GCCTATATACCRCCGTCG3′ and 12S_AcMDB07_ R: 5′GTACACTTACCATGTTACGACTT3′; ca. 283 bp) to amplify fish DNA (Bylemans et al. 2018). Considering the important prey of *Myotis* bats, which consists of arthropods, we used a third pair of markers which amplifies the mitochondrial cytochrome c oxidase subunit I gene (COI; COI_Fwh2_F: 5′ GGDACWGGWTGAACWGTWTAYCCHCC3′ and COI_Fwh2_R: 5′ GTRATWGCHCCDGCTARWACWGG3′; ca. 219 bp) (Vamos et al. 2017). This pair of markers offers a higher resolution in identifying the macroinvertebrate species consumed by the bats. For each marker, we generated a library multiplex by pooling libraries from different samples in an equimolar ratio. Subsequently, all three libraries were sequenced on an Illumina NovaSeq (PE 150 bp) by Novogene (Hong Kong).

2.3 | Sequencing Data Preprocessing

Raw paired-end DNA reads were merged by using the -fastq_mergepairs function in USEARCH v11.0.667 (Edgar 2010). Primer sequences were removed with CUTADAPT v2.5 (Martin 2011). The assessment of trimmed reads quality was completed with FastQC v0.11.9 (Wingett and Andrews 2018) and VSEARCH v2.18.0 (Rognes et al. 2016). Only high-quality trimmed reads within the target lengths (18S: 130–180 bp; COI: 130–210 bp; 12S: 140–290 bp) were retained for later analysis. These pre-processed reads were then dereplicated by using the -derep_fulllength command in VSEARCH. Chimeras and singletons were removed from the dereplicated reads by using USEARCH. Using the -usearch_global function in VSEARCH, all pre-processed reads were clustered into amplicon sequence variants (ASVs) based on 99% similarity.

Using the SINTAX algorithm in USEARCH, each ASV was assigned to the lowest identifiable taxonomic level with a confidence cutoff of 0.7 (Edgar 2016). The ribosomal RNA database SILVA (Glöckner et al. 2017), the mitochondrial database MIDORI for eukaryotes (Leray et al. 2022), and MitoFish for fish (Zhu et al. 2023) were used as taxonomic classification reference databases for 18S, COI, and 12S sequences, respectively. We also assigned ASVs against the NCBI non-redundant nucleotide sequences database to obtain the best 1000 blast hits with a similarity higher than 99% and an e-value less than 1e-50. The BASTA with the lowest common ancestor (LCA) algorithm (Kahlke and Ralph 2019) helped us assign the lowest common taxonomic level shared by 80% of blast hits. To obtain a high taxonomic classification resolution, the results from SINTAX and LCA were combined by assigning ASVs with lower ranks of taxonomies.

Potential false-positive and contaminant ASVs were eliminated by comparing them with mock communities and negative controls (Table S2b–d). Non-diet ASVs (e.g., Fungi, Bacteria, and Algae) and unclassifiable ASVs (e.g., ASVs that could not be identified beyond the domain level) were discarded from the analysis. After ASV cleaning, samples with reads number lower than 100 were subsequently excluded. To better present our taxonomic data in figures, we classified each identified taxon as high and low abundance taxa according to whether it occupies over 0.1% of the number of reads. High abundance taxa were grouped to the lowest taxonomic level, while low abundance taxa were grouped into higher taxonomic levels. In this way, the taxa were classified into taxonomic categories (Table S3a–c) (Huang et al. 2022).

2.4 | Data Analysis

After data preprocessing, we used 18S data of 144 samples, 12S data of 48 samples, and COI data of 100 samples for downstream data analysis. In the 18S dataset, a total of 11 taxonomic categories were classified from 91 ASVs. The identifiable taxonomic levels categorized included three classes, eight orders, two families, and one genus (Table S3a). In the 12S dataset, 22 taxonomic categories were classified from 24 ASVs. The taxonomic levels included nine families and 12 species (Table S3b). In the COI dataset, 29 taxonomic categories were classified from 654 ASVs. The taxonomic levels included two classes, 18 orders, 13 families, 19 genera, and 18 species (Table S3c). In the statistical analysis, we calculated (1) the percentage of read count for each taxon in a sample (relative read abundance, RRA), (2) the percentage of occurrence for each taxon in a sample (weighted percentage of occurrence, wPOO), and (3) the proportion of samples in which a taxon is detected (frequency of occurrence, FOO). The RRA or wPOO at the population level is presented as the mean of RRA or wPOO of all individual samples of a bat species (Lee et al. 2021). We conducted this analysis in R v4.2.1 and visualized it with the R package ggplot2 v3.4.2 (Hadley 2016).

2.4.1 | Diet Diversity Analysis

To determine the relationships between sample numbers and dietary species Chao2 diversity, we generated rarefaction curves using hill numbers from ASVs or taxa ($q=0$) via the R package iNext (Hsieh et al. 2016) (Figure S2). Using hill numbers based on variant q values (the order of diversity), we estimated the diet diversity of three *Myotis* species at both individual (alpha diversity) and population/species (gamma diversity) levels. Hill number only considers the occurrence of each ASV when $q=0$. The weight of species abundance in hill number increases when the q value increases. The hill number is equivalent to the exponential of Shannon's diversity index and inversion of Simpson's dominance at $q=1$ and $q=2$, respectively (Alberdi and Gilbert 2019). Pairwise diversity comparisons at the individual level (alpha) between *Myotis* species were carried out using the Kruskal–Wallis test, followed by Benjamini-Hochberg correction ($p<0.05$).

Beta diversity refers to the differences in dietary taxa compositions between individual samples. To analyze beta diversity, we calculated pairwise binary Jaccard dissimilarity distances and pairwise Bray–Curtis dissimilarity distances using the occurrence of each ASV and the fourth root transformed RRA of each ASV, respectively. We visualized the dissimilarities with principal coordinates analysis (PCoA) with the plot_ordination function in R package phyloseq v1.42.0 (McMurdie and Holmes 2019), and fitted the effect of each taxonomic category into the PCoA result using the envfit and ordiArrowMul functions in R package vegan v2.6.4 (Oksanen et al. 2022). Pairwise permutational multivariate analysis of variance (PERMANOVA) tests were performed to investigate the separation of dietary compositions between the *Myotis* species. Significant interspecific composition variants observed in PERMANOVA tests are premised on the intraspecific homogeneity, which was tested by the betadisper function. We also carried out similarity percentage (SIMPER) analyses to assess the contribution of each taxon to the difference between *Myotis* species by using R scripts simper_pretty.R (Steinberger et al. 2016), which were then checked by the Kruskal-Wallis test by using R scripts R_krusk.R (Steinberger 2020).

Within each species, we performed multiple analyses to determine the contribution of host variables (i.e., sex) and environmental variables (i.e., sampling seasons and sampling locations) to the diversity variation. Data from SK01-03 and PTC were combined for analyses due to their close geographic proximity. Generalized linear models were performed to evaluate the effect of variables on alpha dietary diversity by using the logarithmic hill number of order $q=1$ as the dependent variable. We visualized the beta diversity dissimilarities of variable groups using PCoA based on Bray-Curtis and Jaccard dissimilarity distances with fitted taxonomic categories. PERMANOVA and corresponding beta-dispersion tests were used to assess the beta diversity variations among variable groups. We identified taxa that significantly contributed to the composition variation among variable groups using the SIMPER test, which was checked by the Kruskal–Wallis test. The dietary composition overlap between bat individuals was measured by Pianka's niche overlap index using the R package "spaa" v0.2.2 (Zhang et al. 2016). A network was generated to visualize individual-level diet overlap by using the "qgraph" package v1.9.5 (Pedersen et al. 2017).

3 | Results

3.1 | Dietary Compositions of the Three Myotis Bat Species

Based on 18S data, the three *Myotis* species primarily preyed upon insects, arachnids, and/or ray-finned fish (Actinopteri) (Figure 1 and Figure S3). Specifically, flies (dipterans), moths and/or butterflies (lepidopterans), orthopterans, and true bugs (hemipterans) were the most commonly consumed insect groups. Arachnids such as spiders (Araneae) and mites (Mesostigmata), as well as fish, also formed part of their diet.

The three *Myotis* species exhibited noticeable interspecific variations in their primary food sources (Figure 1b–d). *Myotis chinensis* (18S, $n=40$) primarily consumed insects (72% RRA), particularly bush crickets (*Sasima* spp. in order Orthoptera at 39%) and moths and/or butterflies (22%). Additionally, spiders accounted for 27% of their diet (Figure 1b and Table S4). *Myotis horsfieldii* (18S, $n=49$) mainly hunted smaller-sized insects (91%), with flies (55%) and true bugs (16%) being the primary prey. They consumed arachnids (7%) but in smaller proportions (Figure 1c; Table S4).

Fish was mainly found in the diets of *M. pilosus* ($n=55$; Figure 1d; Table S4). The 18S data also revealed the presence of fish contents in two samples of *M. horsfieldii*, which were not detected by the 12S (Figure 1a,c; Table S5a,b). To validate the presence of

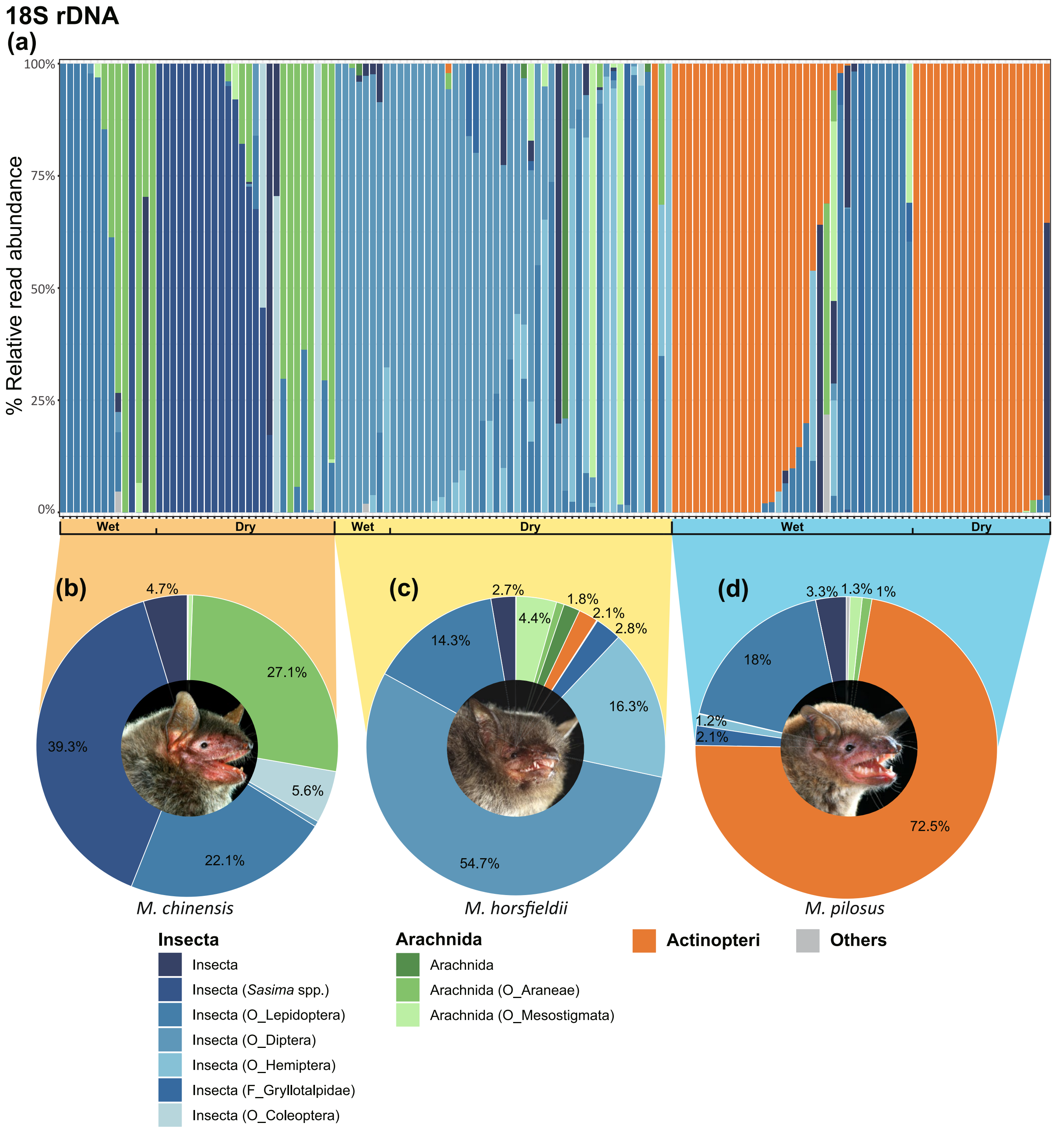


FIGURE 1 | Dietary composition of *Myotis* bat species based on taxa detected in fecal samples using the 18S rDNA marker. The relative read abundance of each taxon was visualized at both (a) individual level ($n = 144$) and (b–d) species level for (b) *M. chinensis* ($n = 40$), (c) *M. horsfieldii* ($n = 49$), and (d) *M. pilosus* ($n = 55$). Prey in classes with read abundance lower than 0.1% of all taxa detected were grouped into "Others". Only taxa with abundance higher than 1% in relative read abundance are shown. F, Family. O, Order. Bat photos Agriculture, Fisheries and Conservation Department.

fish in the samples of *M. horsfieldii*, we first confirmed the bat species identity by DNA barcoding using primers SFF_145f and SFF_351r (Walker et al. 2016), and then we confirmed the fish contents of these samples by performing PCR using the 18S and 12S primers (Section 2.2) and Sanger sequencing. We concluded that the results were consistent with the DNA metabarcoding finding that fish was identified in these two samples.

Myotis pilosus (18S, $n = 55$) displayed significant intraspecific differences in diets (Figures 1 and 2a; Table 6a,b). Approximately one-fifth (9/55) of the individuals were found to exclusively feed on macroinvertebrates, with a majority of these individuals found in the wet season. These individuals primarily prey on moths and/or butterflies. In contrast, the remaining individuals preyed on fish in varying degrees,

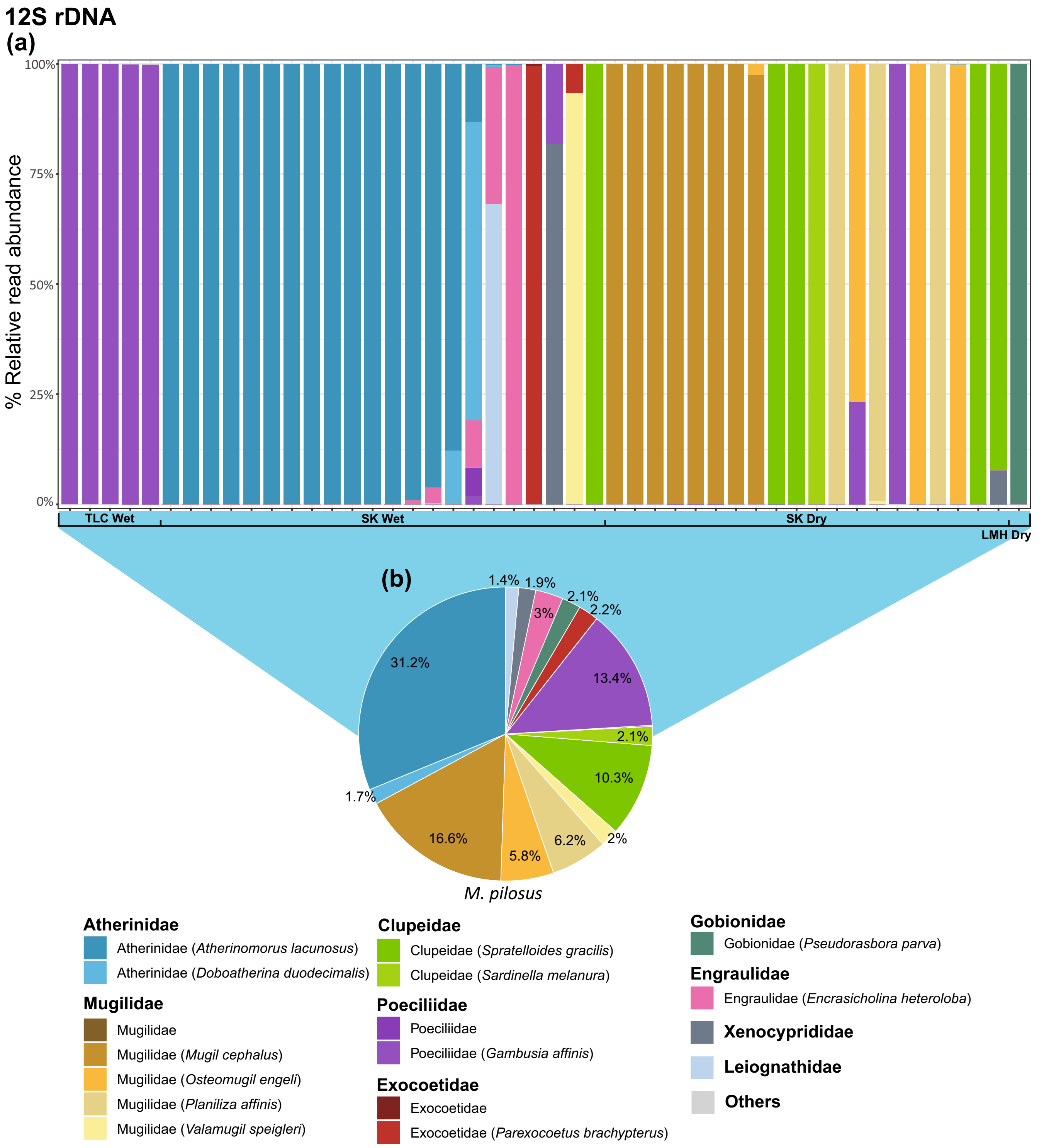


FIGURE 2 | Dietary composition of *Myotis pilosus* based on taxa detected in fecal samples using the 12S rDNA marker. The relative read abundance of each taxon was visualized at both (a) individual ($n = 48$) and (b) species levels. Individual bars were grouped by sampling locations and seasons. Prey in classes with read abundance lower than 0.1% of all taxa detected were grouped into "Others". Only taxa with abundance higher than 1% in relative read abundance are shown. Refer to Figure S1 for locations. SK, Sai Kung (including Pak Tam Chung); TLC, Tai Lam Chung; LMH, Lin Ma Hang.

constituting 6%–100% of their individual diets (Figure 1a,d; Table S5a,b).

Based on the 12S metabarcoding data, all individuals that consumed fish were *M. pilosus* (Figure 2a and Figure S4; Table S6a–c). Both freshwater, marine, and brackish fish were found in the diet of *M. pilosus*; at least 12 fish species were detected. *Myotis pilosus* primarily hunted marine fish, with Old World silversides (Atherinidae) making up 33% of their diet, including wide-banded hardyhead silverside (*Atherinomorus lacunosus*, max. size < 25 cm) at 31%. Mullets (Mugilidae) comprised 31% of their diet, with flathead gray mullet (*Mugil cephalus*, common max. size < 50 cm) making up 17%, and eastern keelback mullet (*Planiliza affinis*, max. size < 40 cm) and kanda mullet (*Osteomugil engeli*, max. size < 48 cm) each contributing about 6%. Herrings and sprats (Clupeidae) accounted for 12% of their diet, with silver-stripe round herring (*Spratelloides gracilis*, max. size < 11 cm) being commonly consumed at 10%. Mosquitofish (*Gambusia affinis*, max. size < 5.1 cm, from the family Poeciliidae) was the dominant freshwater fish at 13% (Froese and Pauly 2024). Notably, mosquitofish was mainly consumed by individuals from Tai Lam Chung (Figure 2; Table S6a,b).

The COI data offered a more detailed revelation of the macroinvertebrate prey consumed by the *Myotis* species (Figure 3 and Figure S5; Table S7a–c). Overall, the majority of prey consisted of dipterans (about 32%), such as lake flies in the family Chironomidae (e.g., *Procladius culiciformis*, *Glyptotendipes tokunagai*, *Chironomus flaviplumus*, etc.); lepidopterans (14%), including grass moths like *Syntonarcha iriastis* and *Cirrhochrista brizoalis*; orthopterans (13%), including crickets like *Mecopoda* spp. (bush crickets) and *Gryllotalpa* spp. (mole cricket); and spiders (12%), such as the northern golden orb weaver (*Nephila pilipes*) (Figure 3; Table S7a).

The macroinvertebrate orders in diets revealed by COI were consistent with those unveiled by 18S. Notable variations were observed in the dietary compositions among *Myotis* species, as revealed by COI. *Myotis chinensis* (COI, $n = 34$) primarily preyed on arthropods in several orders, such as bush cricket *Mecopoda elongata* (23%) from Orthoptera (30%), *S. iriastis* (8%) from Lepidoptera (16%), and *N. pilipes* (7.5%) from Araneae (25%). Spiders were found in more than 50% of the *M. chinensis* samples (Figure S5b; Table S7a). In *M. horsfieldii* (COI, $n = 44$), smaller-sized insects like dipterans accounted for more than 66% of their total diet, with *P. culiciformis* (24%) and *G. tokunagai* (15%) being the main contributors. More than 50% of the *M. horsfieldii* samples contained various lake fly species. Lepidopterans and hemipterans, on the other hand, made up 9% and 10%, respectively (Figure 3c; Table S7a). Although a larger proportion of 30% of insects remained unidentified compared to the other two bat species, the primary prey of *M. pilosus* (COI, $n = 22$) consisted of arthropods from various orders, including Lepidoptera (20%), Araneae (15%), Orthoptera (7%), Hemiptera (8%), and Trichoptera (caddisflies, 7%) (Figure 3d; Table S7a).

3.2 | Alpha and Gamma Diversity of Consumed Taxa

The alpha diversity of individual diets indicated that the diets of *M. horsfieldii* exhibit the highest diversity compared to the other two *Myotis* species, as shown by 18S and COI data (Figures 4 and 5; Table S8a,b). Notably, there is a distinct decrease in hill numbers as the q value increases, particularly observed in the macroinvertebrate (COI) composition of *M. horsfieldii* and *M. chinensis* diets (Figure 5; Table S8b). This suggests that although these bats consume a greater variety of macroinvertebrates compared to *M. pilosus*, their prey compositions are highly uneven, with certain taxa dominating their diets. Furthermore, the macroinvertebrate (COI) compositions of *M. chinensis* were more unevenly distributed compared to *M. horsfieldii* (Figure 5; Table S8b). In the case of individuals of *M. pilosus* that consumed fish, mostly two to three fish species were detected in their diets (12S data; Figures 2a and 5). A similar pattern of prey consumption based on 18S data was also observed at the population level as reflected by gamma diversity, with the dietary composition of *M. horsfieldii* being the most diverse (Figure 5; Table S8a). Despite having the lowest macroinvertebrate (COI) diversity in their diets, *M. pilosus* displayed comparable diversity of overall (18S) diets to *M. chinensis* at the population level (Figure 5; Table S8a,b).

3.3 | Effects of Environmental and Host Factors on the Dietary Compositions

According to the PERMANOVA tests conducted using Bray–Curtis and Jaccard dissimilarity distances, the diets of *M. pilosus* were found to differ significantly between seasons and locations based on 12S (Table S9a,b) and 18S (Table S10a,b) data. Specifically, during the wet season, there was a higher consumption of macroinvertebrates by *M. pilosus*, mainly lepidopterans as evidenced by the 18S data, while in the dry season more fish was consumed. Moreover, the fish compositions of *M. pilosus* from SK, where a majority of samples were collected from, exhibited notable variations between the wet and dry seasons (Figure 2a). The SIMPER analysis results further identified the main contributors to location difference. Old World silverside (Atherinidae) were consumed more at SK, while mosquitofish were consumed more at TLC (Figure 6a,b and Table S11a,b) (Froese and Pauly 2024).

Similarly, the diets of *M. chinensis* also differed significantly between the two seasons based on both 18S (Figure 1a and Table S10a,b) and COI (Figure 3a and Table S12a,b) data. The 18S data showed that during the wet season, *M. chinensis* mainly consumed moths and/or butterflies (Lepidoptera), while in the dry season, they consumed a large proportion of *Sasima* bush crickets (Orthoptera) (Figure 1a). Although both GLM and PERMANOVA analyses consistently demonstrated a significant divergence in the seasonal diets of *M. horsfieldii* (Tables S12a,b and S13), it's important to note that the small sample size ($n = 4$) from the wet season in the COI dataset may limit the accuracy of these findings. Further investigations are required in the future to confirm this result. Furthermore, the PERMANOVA analysis revealed an additional noteworthy disparity in the diets of the two sexes of *M. horsfieldii* (Table S12a,b), which was mainly attributed to the distinct consumption of dipterans, lepidopterans, and hemipterans by each sex (Table S14a–d).

3.4 | Dietary Niche Partitioning between the Three Myotis Species

Based on the PCoA analysis, 18S and COI data revealed highly similar patterns of dietary niche partitioning among the three *Myotis* species (Figure 6c–f). The PCoA analysis using Bray–Curtis and Jaccard distances within each of the 18S and COI datasets also revealed consistent patterns. The overall (18S) and macroinvertebrate (COI) diets of *M. horsfieldii* were distinctly differentiated from those of *M. pilosus* and *M. chinensis*, while the dietary compositions of *M. pilosus* and *M. chinensis* were more overlapping with each other (Figure 6c–f). The SIMPER

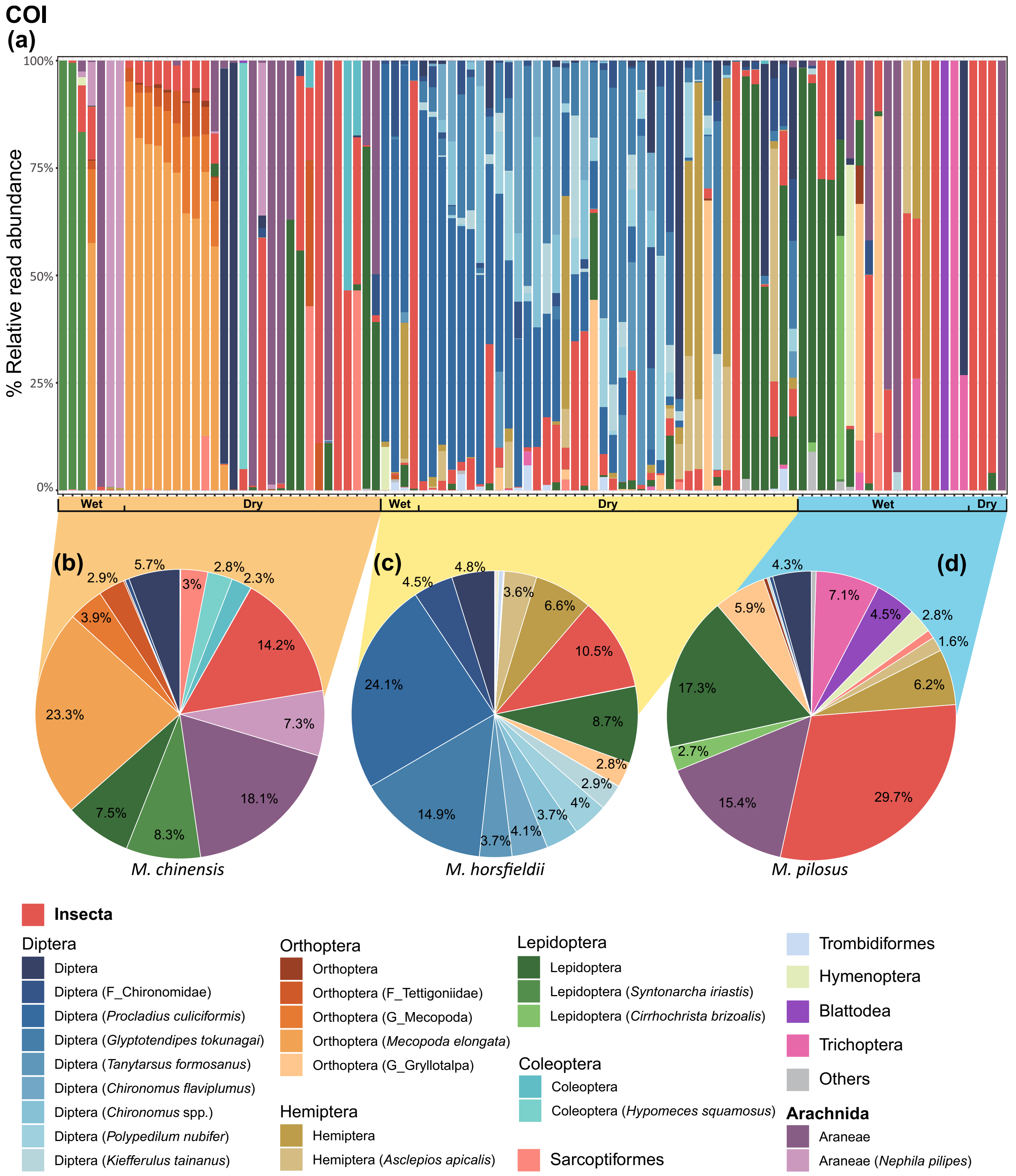


FIGURE 3 | Dietary composition of *Myotis* bat species based on taxa detected in fecal samples using the COI marker. The relative read abundance of each taxon was visualized at both (a) individual level ($n=102$) and (b–d) species level for (b) *M. chinensis* ($n=34$), (c) *M. horsfieldii* ($n=44$), and (d) *M. pilosus* ($n=22$). Prey in classes with read abundance lower than 0.1% of all taxa detected were grouped into "Others". Only taxa with abundance higher than 1% in relative read abundance are shown. G, Genus. F, Family. O, Order.

analysis results indicated the main contributors to this differentiation in *M. horsfieldii* were predominantly dipterans and hemipterans, which constituted a significant portion of their diet (Figure 6c,d; Table S15a,b). Specifically, the true bugs included water striders (e.g., *Asclepios apicalis*) while dipterans included a group of lake flies (e.g., *P. culiciformis*, *G. tokunagai*, *Kiefferulus tainanus*, *Polypedilum nubifer*, *Chironomus* spp. etc.) (Figure 6e,f; Table S16a,b).

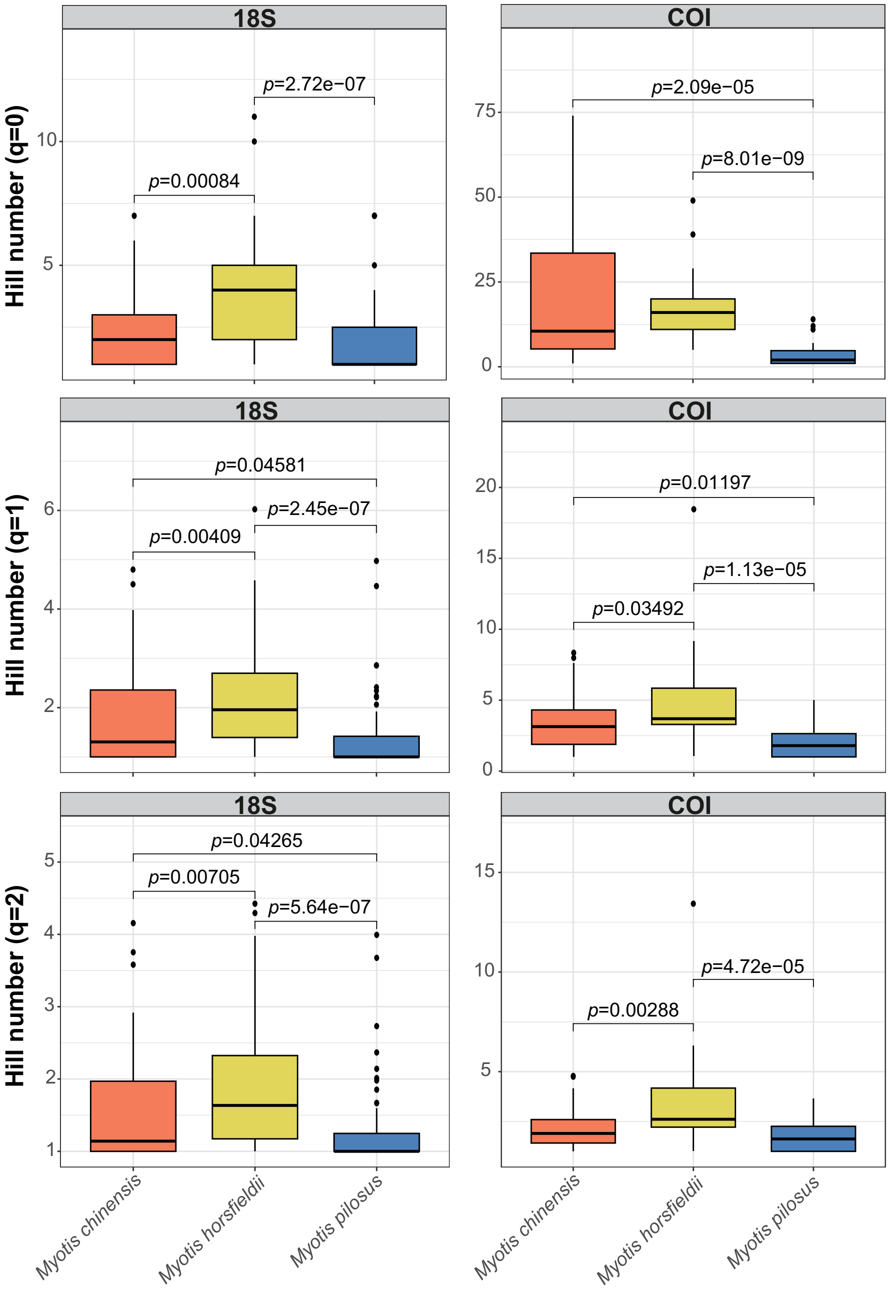


FIGURE 4 | Pairwise alpha diversity comparison between *Myotis* species. Alpha diversity is represented by using the hill numbers of order $q=0$ (upper panel), $q=1$ (middle panel), and $q=2$ (lower panel), which were calculated based on the ASVs abundance detected by18S rDNA (left panel) and COI (right panel) marker at the individual level. Only the p-values of species pairs that have significantly different alpha diversity are shown ($p<0.05$).

Myotis chinensis also differentiated from *M. horsfieldii* and *M. pilosus* by consuming a considerable proportion of bush crickets (Orthoptera), such as *Sasima* spp. and *M. elongata*, as well as spiders (Araneae, including *N. pilipes*) (Figure 6c–f; Tables S15a,b and S16a,b). On the other hand, the dietary niche of *M. pilosus* differed from the other two species by including a large portion of fish (Actinoptera) in its diet (Figure 6c,d and Table S15a,b). *M. pilosus* also differentiated from *M. chinensis* by consuming caddisflies (Trichoptera) (Figure 6e,f and Table S16a,b).

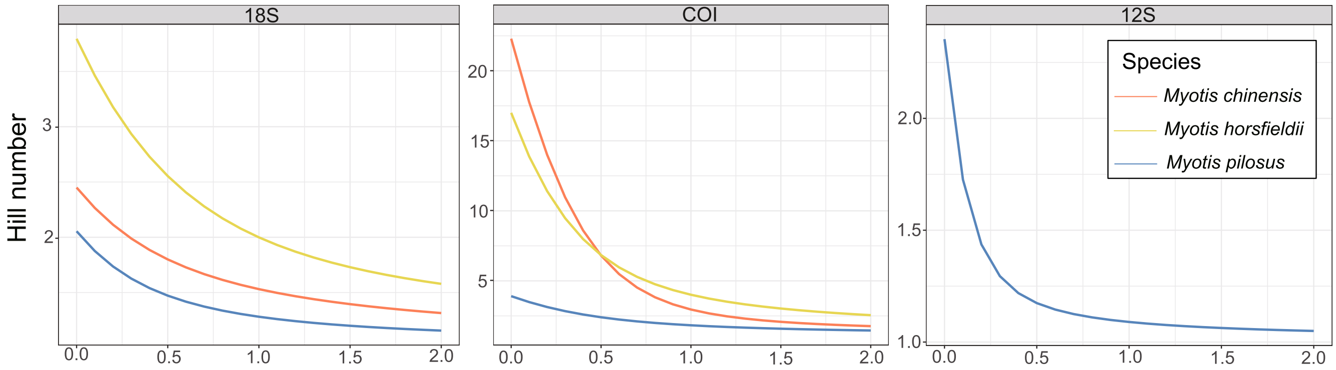
At the individual level, *M. horsfieldii* showed greater intraspecific overlap of individual diets compared to those of *M. chinensis* and *M. pilosus* (Figure S6). This suggests the dietary compositions among *M. horsfieldii* individuals were more similar, and the dietary compositions among individuals of *M. chinensis* and *M. pilosus* were more variable.

4 | Discussion

4.1 | Fish Eating Behavior of *Myotis*

This study provides new insights into the dietary compositions of *M. pilosus* and *M. chinensis* in coastal habitats, expanding on previous research that primarily focused on inland regions of China. Additionally, our research contributes to the

(a) Alpha diversity



(b) Gamma diversity

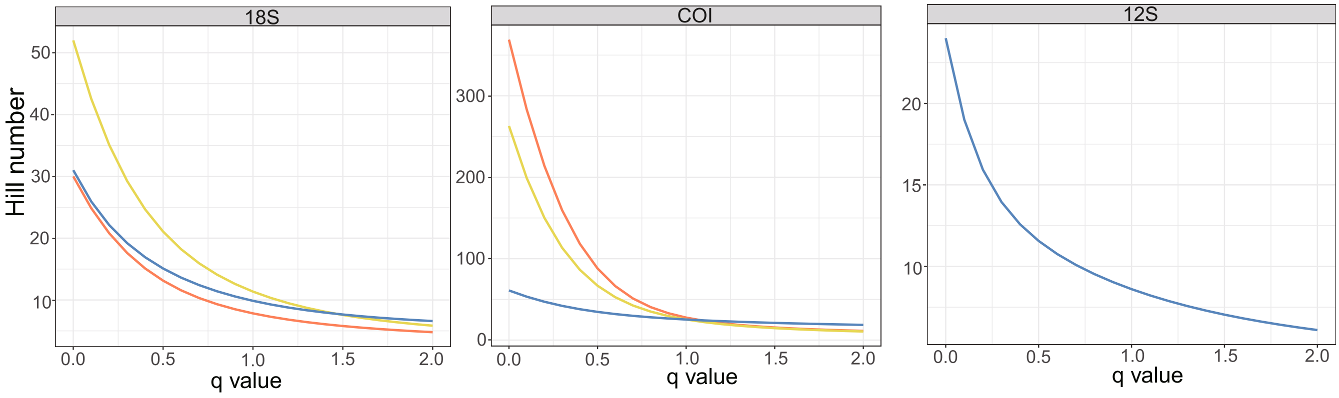


FIGURE 5 | Dietary diversity for each *Myotis* represented in the hill numbers of variant order *q*. The hill numbers were calculated at the individual level and species level to estimate the (a) alpha diversity and (b) gamma diversity, respectively. The ASVs abundance of each sample was detected by using 18S rDNA (left panel), COI (middle panel), and 12S rDNA (right panel) marker gene.

understanding of the dietary compositions of *M. horsfieldii*, a species found in Southeast and South Asia that has not been previously studied in terms of its foraging ecology. Contrary to earlier studies conducted in inland regions of China, which suggested that *M. pilosus* primarily fed on cyprinids in freshwater habitats year-round (Ma et al. 2006; Chang et al. 2019), our research in Hong Kong unveiled a different dietary pattern. Our findings show that *M. pilosus* in Hong Kong did not prey on cyprinids but instead exhibited a diverse diet, targeting a wide variety of fish prey species from nine different families. These prey species encompassed marine, brackish, and freshwater fish, highlighting the versatility of *M. pilosus* in its feeding habits. Moreover, we observed variations in the species compositions consumed by *M. pilosus* across different seasons. For instance, marine species such as *A. lacunosus*, *Doboatherina duodecimalis* (tropical silverside, max. size 11 cm), *Parexocoetus brachypterus* (sailfin flying fish, max. size 13 cm), and *E. heteroloba* were only fished by *M. pilosus* during the wet season at Sai Kung. This was observed specifically at sampling site SK01 but not at the other sites. Worth noting is that SK01 is located 1.7 km or less away from the nearest shore at the southwest, making it a likely site for *M. pilosus* to fish rather than flying north- or eastward to reach the shore, which is located over 3 km away.

Atherinomorus lacunosus, which forms large schools along sandy shorelines, serves as an important food source for *M. pilosus*. This fish species forages nocturnally to capture zooplankton that migrate vertically to the upper water column during nocturnal hours (Skibinski 2005). This feeding behavior creates a large amount of ripples that trawling bats like *M. pilosus* can detect. Another noteworthy prey species is *P. brachypterus*. The sailfin flying fish is commonly found in coastal waters in large shoals and possesses elongated, wing-like pectoral fins, which enable it to leap out of the water and glide rapidly for considerable distances above the surface, an adaptation to evade underwater predators. During the breeding season, spawning flying fish aggregate in abundance near the surface at night, with many vigorously jumping and flying out of the water to release ripe eggs and sperm (Stevens et al. 2003; Digo et al. 2015; Lewis 1961), exposing them to attack by aerial predators, such as *M. pilosus*.

During the dry season in Sai Kung, *M. pilosus* captured the marine fish species *Spratelloides gracilis* and *Sardinella melanura* (blacktip sardinella, common size 10 cm). Clupeids, such as *S. gracilis* and *S. melanura*, are primarily forage fish known for their high egg production. For instance, *S. gracilis* spawns near the water surface, releasing approximately 1600 eggs per unit body mass (Dalzell and Wankowski 1980). Although previous studies in China have reported crustaceans as prey for *M. pilosus* and other bat species (Wang et al. 2024), our study did not identify any crustaceans in the diet of *M. pilosus*. Based on our results, one notable target species for *M. pilosus* is *M. cephalus*, along with other mullet species, during dry months. *Mugil cephalus* is known for congregating in schools over sand or mud in coastal and brackish waters. In Hong Kong, this species exhibits two short spawning peaks in winter (dry) months, resulting in a

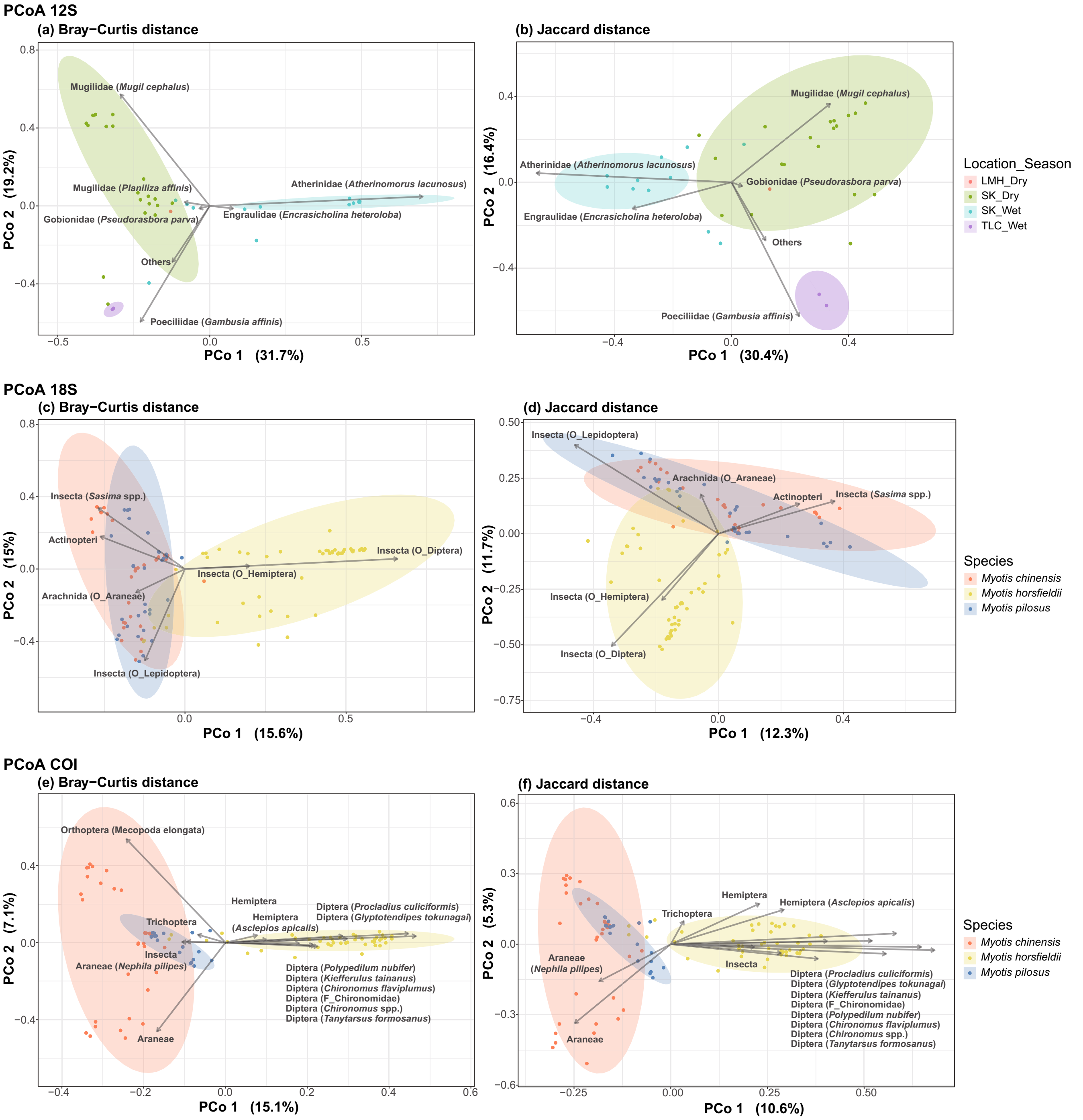


FIGURE 6 | Principal coordinate analysis (PCoA) of dietary compositions in (a, b) *Myotis pilosus* and (c–f) three *Myotis* species based on (a, c, e) the pairwise Bray–Curtis dissimilarity distances calculated from the fourth root transformed RRA of each ASV, and (b, d, f) the pairwise Jaccard dissimilarity distances calculated from the occurrence of each ASV. Each point corresponds to one sample, with the color indicating (a, b) sampling locations, seasons, and (c–f) *Myotis* species. The ASVs were identified by the (a, b) 12S rDNA, (c, d) 18S rDNA, and (e, f) COI marker gene. Taxonomic categories significantly contributing to the difference between groups were shown (see Tables S11, S15, and S16). Refer to Figure S1 for locations. SK, Sai Kung (including Pak Tam Chung); TLC, Tai Lam Chung; LMH, Lin Ma Hang.

large number of juveniles appearing inshore, especially in estuaries, during February and March (Sadovy and Cornish 2000).

In the Tai Lam Chung woodland area, which is inland, *M. pilosus* primarily preys on the exotic freshwater poeciliid, *G. affinis*. The southern shore is obstructed by highland with an elevation of at least 400 m, and it is at least 9 km away from the nearest shore in any direction (Figure S1). *Gambusia affinis* is a freshwater species and a widespread non-native fish in Hong Kong, originating from North America and released by the Hong Kong Government for mosquito control purposes around 1940 (Dudgeon and Corlett 2004). Surveys on the distribution of *G. affinis* in Hong Kong have reported its presence in the lower reaches of the closest streams within less than 1 km of TLC forest (Tsang and Dudgeon 2021a). Experiments conducted on the ecological effects of *G. affinis* have shown that it reduces the abundance and richness of invertebrates and alters assemblage compositions in Hong Kong wetlands (Tsang and Dudgeon 2021b). Our findings suggest that *M. pilosus* may play a potential role in controlling invasive poeciliids in the local ecosystem. Similar findings have been reported in *M. capaccinii* in northwest Israel and the eastern Iberian Peninsula, where *M. capaccinii* fed on exotic *Gambusia* spp. (Aizpurua et al. 2014; Levin et al. 2006). Mosquito fish frequently swim close to the water surface, using their upturned mouth to break the surface and capture floating insects. Furthermore, the decrease in oxygen during the night may compel these fish to come to the water surface for breathing (Aizpurua et al. 2014, Levin et al. 2006).

Our study has made an intriguing discovery regarding the feeding habits of *M. horsfieldii*. We reveal two individuals of *M. horsfieldii* from Sai Kung have included fish in their diet. Interestingly, we have found the first case of *M. horsfieldii* preying on *Asclepios apicalis*, a water strider species that is also targeted by *M. pilosus*. While most water striders are known for their capacity to glide effortlessly on the calm freshwater surface, *Asclepios* spp. are considered sea skaters, which are found primarily in brackish water along coasts (Poolprasert et al. 2022; Andersen and Foster 1992). *Myotis horsfieldii* might be drawn to this food source due to the consistent ripples produced by *Asclepios* as they maneuver across the water's surface. Reports on *M. horsfieldii*'s foraging behavior are limited, but one literature mentions that *M. horsfieldii* typically flies in circles around 10 cm above the water to skim insects, and they roost near water sources (Wilson and Mittermeier 2019). Our finding suggests that *M. horsfieldii* may capture fish while foraging for insects over waters. Despite this finding, it should be noted that fish was only detected in very few individuals of *M. horsfieldii*. Therefore, further investigation is needed to determine whether *M. horsfieldii* actively engages in fishing or if those individuals simply mistakenly catch live or dead fish on the water surface while foraging for insects. Our discovery that *M. horsfieldii*, which was not previously known to consume fish, does in fact include fish in their diets has significant implications for the evolution of fishing behavior. The active foraging of aquatic insects by bats may lead to occasional consumption of fish, which in turn creates the selective pressure that drives the evolution of fishing behavior.

4.2 | Foraging Niches of the Three *Myotis* Species

We observed a higher proportion of fish in the diet of *M. pilosus* during the dry season compared to the wet season, with a significant increase in insect components during the wet season, which is consistent with Ma et al. (2006) but different from Chang et al. (2019). Our results showed that this increase in insect consumption was due to a 43% rise in the number of individuals preyed on macroinvertebrates during the wet season (22/35) compared to the dry season (4/20). Furthermore, while there were some variations in the insect dietary compositions compared to previous research, our study revealed a similar finding to Chang et al. (2019) in terms of *M. pilosus* primarily preying on lepidopterans in the wet season. Based on the nocturnal behavior of most moths and the fact that the lepidopterans identified in our study are all moth species, such as *S. iriastis* and *C. brizoalis* consumed by *M. pilosus*, we suggest that most of the lepidopterans preyed upon by *M. pilosus* are moths. Surveys on the abundance of forest invertebrates in Hong Kong have shown that local lepidopterans vary seasonally, with both number and biomass experiencing winter (dry season) lows and early summer (in May during wet season) maxima (Kai and Corlett 2002). During the wet season, local *M. pilosus* also targeted trichopterans. Trichopterans are moth-like and closely related to lepidopterans, which are nocturnal and commonly associated with freshwater bodies (de Moor and Ivanov 2008).

Here, we provide additional evidence for the specialization of *M. pilosus* in fishing, as we have consistently found fish in its diet throughout the year. It is worth noting that the abundance of lepidopterans in the local terrestrial habitat is higher during summer (Kai and Corlett 2002); this high abundance likely explains why some individuals of *M. pilosus* have shifted their target prey from fish to insects during this time. This shift may be driven by higher energetic profit associated with capturing a larger number of moths compared to capturing fish. Despite the higher nutritional value of a single fish compared to a single moth, catching fish likely incurs a greater energy cost (Aizpurua et al. 2013). This is due to the heavier body mass of fish and the increased effort required to accurately detect fish underwater and subsequently pull it out of the water (Aihartza et al. 2008). This results in lower capture efficiency when targeting fish (Altenbach 1989). Therefore, our study highlights the adaptive feeding behavior of *M. pilosus*, which adjusts its diet based on prey availability, underscoring the significance of this feeding strategy.

Our results show that there is a greater overlap in the macroinvertebrate diet between *M. pilosus* and *M. chinensis*, while the diet of *M. horsfieldii* is distinct from the other two species. We found that local *M. chinensis*, similar to *M. pilosus*, also hunted a higher proportion of lepidopterans, such as grass moth, *S. iriastis*, during the wet season. Another noteworthy foraging habit shared between *M. chinensis* and *M. pilosus* is their considerable consumption of non-flying nocturnal arthropods, specifically spiders like the northern golden orb weaver (*N. pilipes*). *Nephila pilipes* typically build intricate webs to capture prey on bushes and trees near water sources (Harvey et al. 2007). Spiders have been documented as part of the diet of several other *Myotis* species, such as *M. myotis*, *M. emarginatus*, *M. lucifugus*, *M. nattereri*, *M. evotis*, and *M. septentrionalis* (Goiti et al. 2011; Maucieri and Barclay 2021; Swift and Racey 2002; Kaupas and Barclay 2018). However, previous studies did not report spiders as a food source for *Myotis* bats in China (Ma et al. 2003, 2008, 2006). Moreover, *M. chinensis* mainly preyed on orthopterans, especially bush crickets, such as *Mecopoda* and *Sasima* spp., during the dry season. The consumption of spiders and orthopterans suggests that *M. chinensis* is capable of foraging in cluttered environments of woodlands and gleans arthropods from various substrate surfaces, such as spider webs and/or foliage in bushes. While there are some geographical variations in dietary composition, our findings agree with Ma et al. (2008), which remarked that conspecifics in Beijing prey on 'non-wing beating' insects such as coleopterans (e.g., ground beetles in Carabidae) and orthopterans while lacking hymenopterans (sawflies, wasps, bees, and ants) and lepidopterans (Ma et al. 2008).

Based on our results, we found that the diet of *M. horsfieldii* is distinctive, which prominently includes a high consumption of smaller flying insects, specifically dipterans. Notably, *M. horsfieldii* preys on a wide variety of lake fly species from the Chironomidae family, leading to a higher dietary diversity compared to *M. chinensis* and *M. pilosus*. Conversely, our observations indicate that spiders, orthopterans, and coleopterans were seldom found in their excrement. In the forests of Hong Kong, dipterans constitute the majority of the insect biomass, and their abundance did not exhibit seasonal variation (Kai and Corlett 2002). Numerous chironomid species bear a striking resemblance to mosquitoes, and adult swarms in terrestrial habitats serve as vital sources of food for bats (Puig-Montserrat et al. 2020; Beck 1995). Local *M. horsfieldii* also targets hemipterans and lepidopterans, though to a lesser extent compared to dipterans.

Our findings indicate that *M. pilosus* is primarily a fish-eating bat, while *M. chinensis* and *M. horsfieldii* primarily feed on insects. One plausible reason for this dietary differentiation pattern could be differences in their morphological characteristics. Previous studies suggested that the body size of aerial hunting bats limits their weight-carrying capacity, and fishing bat species tend to have relatively larger body sizes and longer hind feet that give them morphological advantages for fish hunting (Chang et al. 2019). Despite *M. chinensis* (91–97 mm body length, 25–30 g) (Wilson and Mittermeier 2019) and *M. pilosus* (51–65 mm, 11.7–32.5 g) exhibiting larger body sizes and heavier body masses, *M. pilosus* shares a similar hind foot to forearm length ratio (0.31 ± 0.014) to other fishing bats (0.31 ± 0.051) (Chang et al. 2019), while the ratio for *M. chinensis* (0.26) is more similar to those of other non-fishing bats (0.25 ± 0.038). *Myotis horsfieldii* (44–51 mm, 5.0–7.5 g) (Wilson and Mittermeier 2019) is smaller in size compared to the other two species. It has a low value ratio (0.23), which is also more akin to other non-fishing trawling bats (Chang et al. 2019). Apart from the influence of morphological adaptations on prey capturing ability, other factors contributing to the dietary differentiation pattern could include differences in prey detection ability through variations in echolocation call structures during foraging (Aizpurua and Alberdi 2018).

This dietary study uncovered the overlapping macroinvertebrate diets of *M. pilosus* and *M. chinensis*, as well as the distinct diet of *M. horsfieldii*, with the latter being documented for the first time. Both *M. pilosus* and *M. chinensis* are considered regionally threatened. The potential interspecific competition observed in our findings, particularly between *M. pilosus* and *M. chinensis* during the wet season, underscores the importance of protecting macroinvertebrate food sources in the local terrestrial ecosystem to prevent intensifying competition among the congenerics. To safeguard the sustainability of vulnerable *M. pilosus*, it is also crucial to preserve the integrity of marine, brackish, and freshwater ecosystems in Hong Kong, as these diverse environments serve as essential foraging grounds for *M. pilosus* to capture the diverse range of fish species.

Author Contributions

S.Y.W.S.: conceptualization; E.S.K.P. and S.Y.W.S.: methodology; E.S.K.P., J.C.T.C., D.T.C.C., C.T.S., W.C.T., and S.Y.W.S.: investigation; X.W.: formal analysis; C.T.S., W.C.T., and S.Y.W.S.: resources; X.W.: visualization; X.W. and E.S.K.P.: writing – original draft; E.S.K.P., S.Y.W.S. and all authors: writing – review and editing; S.Y.W.S.: supervision; S.Y.W.S.: project administration; S.Y.W.S.: funding acquisition.

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Ethics Statement

Approvals for animal experiments were granted by the Department of Health (ref. 19-177 in DH/SHS/8/2/3 Pt. 30), the University of Hong Kong (ref. 4963-19), and the Agriculture, Fisheries, and Conservation Department (AFCD; ref. 35 in AF GR CON 09/51 Pt.8).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Raw 18S rDNA, 12S rDNA, and COI sequences and sample information in this study are available in the NCBI Sequence Read Archive (SRA) under BioProject accession PRJNA1232591. Biological information for the samples can be found under BioSample accessions SAMN47430747-SAMN47431102. Raw sequences are available under SRA accessions SRR32751230-SRR32751585.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.