

Metabarcoding of fecal DNA reveals the broad and flexible diet of a globally endangered bird

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Abstract

Knowing the diet of endangered wild animals is a prerequisite for species-specific conservation and habitat management. The Sichuan partridge *Arborophila rufipectus* is a globally endangered Galliformes species endemic to the mountains of southwest China. Existing information on the diet of this species is biased and fragmented owing to traditional observation methods. Little is known about their dietary composition or how they respond to temporal variations in food resources throughout the year. In this study, a dietary analysis was performed on 60 fecal samples using DNA Metabarcoding of invertebrates and plants to determine the primary animal and plant components of the diet across 3 critical periods of adult life history (breeding, postbreeding wandering, and overwintering). Preys from the dipteran order, followed by the lepidopteran and araneae spp., were the predominant, animal-derived foods. *Symplocos*, *Rubus*, *Celastrus*, *Holboellia*, and *Actinidia* spp. supply a large abundance of fruits and seeds for this omnivorous bird. Substantial temporal dietary changes among the 3 periods and a general shift toward lower dietary diversity during the breeding season were observed, suggesting that the Sichuan partridge can adjust their diet according to the availability of food resources and their own needs. Characterizing the composition and seasonal changes in Sichuan partridge diets informs the habitat management of native flora (the plant taxa that can generate berries and seeds, such as *Symplocos*, *Rubus*, *Celastrus*, and *Holboellia*, which are likely of conservation interest) to achieve full life-cycle conservation.

Key words: *Arborophila rufipectus*, dietary composition, endemic endangered species, molecular diet analysis, seasonal dietary variation, Sichuan partridge.

Diet represents a fundamental aspect of ecological, evolutionary adaptations, and trophic interactions in animal species (Kissling et al. 2014; Gainsbury et al. 2018). Elucidating the composition of diet can provide basic information on the ecology and evolution of key species (Symondson 2002), as well as define different feeding strategies. In particular, advancing the understanding of the diets of endangered or threatened wildlife is a cornerstone for drafting effective species-specific conservation strategies (Oehm et al. 2011; Liu et al. 2018; Sullins et al. 2018; Ghosh-Harihar et al. 2021; Hacker et al. 2021).

The Sichuan partridge *Arborophila rufipectus* is a globally endangered Galliformes species endemic to the mountains of southwest China (Dai et al. 1998, 2009; Fu et al. 2020). It is classified as “Endangered” (EN) on the IUCN Red List (<http://www.redlist.org>) because of its small population and severely fragmented habitat (BirdLife International, 2022). It is also listed as a nationally protected animal in China (Liu et al. 2012). This endemic species inhabits subtropical evergreen and deciduous broadleaf forests at 1,100–2,250 m above sea level in southwest China (Johnsgard 1988; Fu et al. 2017). Prior investigations have shown that this bird undergoes seasonal altitudinal movement (Fu and Chen 2017), spending the breeding season (from March to July) at elevations of 1,400–2,250 m. The chicks gradually move far away from the birthplace with their parents in the following 2–3

months (postbreeding wandering, from August to October). In mid or late autumn, chicks are separated from their parents and start an independent life. They then migrate to lower elevations (generally <1,400 m) during the winter (from December to February of the next year). Accordingly, the Sichuan partridge uses various habitats in different seasons. Minimizing the degradation of the remaining available habitat will require a comprehensive understanding of the Sichuan partridge ecology, including dietary needs (Liao et al. 2008a, 2008b). Effective conservation management strategies for endangered wildlife require a better understanding of how foraging habitats and food resources are used.

Previous studies often focused on diet composition at particular life-history period, such as breeding (Shutt et al. 2020) or wintering (Sherry et al. 2016; Lourenço et al. 2017). Generating full-life dietary information and food selection can inform comprehensive conservation of this threatened bird at all life-history periods. Variability and availability of foods are significant drivers of bird population fluctuations (Young et al. 2020), because they influence breeding (Kadin et al. 2012; Fayet et al. 2021), juvenile survival (Trevelline et al. 2018), migration (Sherry et al. 2016; Liu et al. 2018), and adult overwintering survival (Lourenço et al. 2017; Li et al. 2021). Sichuan partridges often forage on the withered leaves of the understory surface layer (Zheng 2015; Fu and Chen

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2017). The Sichuan partridge is an omnivorous bird, and its main feeds include invertebrates, fruits, seeds, and grassroots (Li et al. 1974; Xu et al. 1994; Lei & Lu 2006; Zheng 2015). The existing information on the diet of the Sichuan partridge is fragmented because of the limits on the microhistological analysis of digested food items. In particular, little is known about their dietary composition with high accuracy and resolution during different critical periods of adult life history (e.g., during breeding and over winter). Elucidating the dietary composition and food preferences of the Sichuan partridge is essential to understanding their ecology, population dynamics, and conservation.

For natural bird populations, it is challenging to accurately and efficiently characterize the complex composition of diets (Gong et al. 2017; Nota et al. 2019). Owing to their elusive predatory behavior and versatile feeding habits, direct field observations of prey or food capture are not possible (Jedlicka et al. 2017; Trevelline et al. 2018). The traditional method for estimating food sources and dietary composition is microhistological identification based on crop content or gastrointestinal dissection (Rayé et al. 2011; Pompanon et al. 2012). Conventional dietary identification depends significantly on the presence of prey remnants. Thus, relatively undigested food items may be overrepresented, whereas highly digested foods may be missed (Pompanon et al. 2012; Hou et al. 2021). Ornithologists have historically studied bird diets using various destructive or invasive methods (Garfinkel et al. 2021). Nevertheless, for rare and endangered species, this is not feasible and it is difficult to obtain crop or stomach content samples. Furthermore, traditional observation methods based on fecal dissection may not be effective and are taxonomically challenging for detecting lepidopteran larvae (e.g., butterfly and moth caterpillars), which occur frequently in avian diets (Sullins et al. 2018). Soft foods such as insect larvae tend to be more digestible, resulting in the underestimation of food item identification in content samples (Sullins et al. 2018; Rytönen et al. 2019; Hou et al. 2021). Another method of dietary analysis, stable isotope analysis, is unable to identify prey items at a fine enough taxonomic resolution (Bohmann et al. 2018). Therefore, a cost-effective and nondestructive method with a higher taxonomic resolution is necessary for the dietary study of generalist birds.

The high-throughput sequencing DNA metabarcoding approach has been widely used with varying success in the field of ornithology (Jedlicka et al. 2017; McClenaghan et al. 2019; da Silva et al. 2020; Shutt et al. 2020; Bourbour et al. 2021; Garfinkel et al. 2021). The DNA-based approach is especially advantageous because samples can be obtained in ways that minimize interaction with and cause harm to animals (Pompanon et al. 2012; Kartzinel and Pringle 2015), with little disturbance of endangered birds (Young et al. 2020). In addition, DNA metabarcoding is particularly useful when dealing with arthropods, which can be identified to the genus or lower taxonomic level (Garfinkel et al. 2021). This facilitates dietary characterization of the Sichuan partridge, which is a generalist, in the presence of mixed prey DNA in the gastrointestinal tract and feces. Therefore, DNA metabarcoding of fecal samples might be the best option for providing valuable dietary information at critical periods of the annual cycle in the Sichuan partridge because it can identify prey items for conserved species when the collection of individuals is not practical (Pompanon et al. 2012; Sullins et al. 2018).

For Sichuan partridges, reproduction takes a lot of energy. While the energy intake during the postbreeding wandering is

significant for the development and survival of chicks, and winter is the season when food is scarce. Therefore, these 3 periods are the critical periods in the life history of the adult Sichuan partridge. In this study, we aimed to characterize the dietary profile of the Sichuan partridge and advance our understanding of the foraging ecology of this endangered Galliformes species. We also attempted to investigate the dietary variation of the Sichuan partridge during 3 crucial periods of adult life history and examined the temporal patterns in consumption of the main prey taxa. Based on the opportunistic foraging characteristics of the Sichuan partridge, we hypothesized that the composition and diversity of diets would change throughout the study period. Thus, this study provides detailed information on the foraging strategies of this endangered species at different periods of adult life history, and may have implications for the development of effective conservation management at critical phases.

Materials and Methods

Study site and sample collection

In total, 60 fecal samples (20 samples for each period) were collected from the Laojunshan National Nature Reserve (28°39'36"–28°43'38"N, 103°57'36"–104°04'12"E) (Figure 1) in southwest China during postbreeding wandering (September–October 2020), overwintering (December 2020–January 2021), and breeding (June–July 2021) periods. The climate at the study site is temperate with high precipitation (>1,500 mm per year) and relative humidity (>85%). The air temperatures of the 3 periods in this study were ~12.0 °C (postbreeding wandering), ~0.9 °C (overwintering), and ~16.9 °C (breeding), respectively. Abundant seeds and fruits are available in autumn, typically with alternating sunny and rainy periods throughout the season. The reserve has the world's largest wild population of the Sichuan partridge, with approximately 300–400 individuals (Fu et al. 2020). Prior research on the habitat of the Sichuan partridge demonstrated that the natural vegetation in this area is an evergreen broadleaf forest (Liao et al. 2008a, 2008b), characterized by, among others, species in the families Fagaceae (*Castanopsis* spp.), Theaceae (i.e., *Schima* spp., *Camellia* spp., *Eurya* spp.), Lauraceae (*Cinnamomum* spp.), Alangiaceae (i.e., *Alangium* spp.), Ericaceae (i.e., *Rhododendron* spp.), and Rosaceae (Liao et al. 2008a, b; Dai et al. 2009). In addition, some non-native coniferous forests and tea plantations exist at lower elevations (<1,450 m above sea level) (Fu et al. 2017).

The foraging Sichuan partridges create characteristic scrapes that can be easily distinguished from those created by other sympatric Galliformes (e.g., *Tragopan temminckii* and *Lophura nycthemera*). Scrapes of the Sichuan partridges are typically approximately 2–5 m long and 1–3 m wide, with several or more than 10 shallow pits (4.3–6.5 cm deep) (Liao et al. 2007). During the fieldwork, we collected fresh fecal samples mainly at different foraging sites of the Sichuan partridges, which can be easily distinguished from those excreted by other sympatric Galliformes with experience because of significant differences in both size and shape of their feces (Liao et al. 2008a, 2008b). To avoid fecal samples from the same individual, we sampled in different areas (at intervals of more than 500 m), and only 1 sample was collected at the same site and time. In addition, the feces of chicks or juveniles can be excluded based on the smaller size of the feces. We minimized sample contamination using a disposable plastic spoon, scraping feces off the ground, or vegetation with a

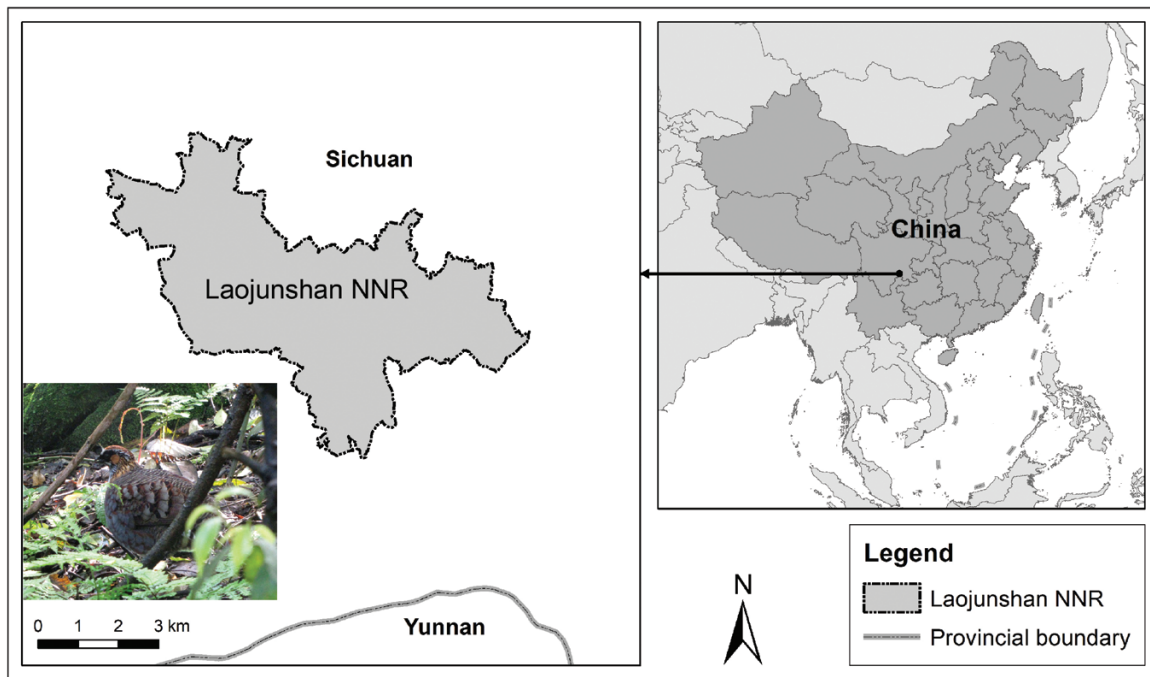


Figure 1 Location of Laojunshan Natural Reserve (Sichuan Province, China) and a photograph of the male Sichuan partridge (the China map is downloaded from Resource and Environment Science and Data Center, Institute of Geographic Sciences and Natural Research, CAS at the website <https://www.resdc.cn/data.aspx?DATAID=200>).

sterile EP tube. Fresh feces from foraging Sichuan partridges left on the ground within 2–3 h before collection were collected in the morning. The collected fecal samples were immediately stored at 0–4 °C in an incubator with ice bags for transportation. The inner pellet of each fecal drop was used for subsequent laboratory procedures to minimize possible contamination from the ground (including the underlying soil, leaves, and sand). Fecal samples were stored at –80 °C in the laboratory for DNA extraction. All fecal samples were collected according to the guidelines and approval of the Animal Ethics Committee of Sichuan Normal University.

DNA extraction and PCR amplification

DNA was extracted from fecal samples using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany; cat. no. 51604), according to the manufacturer's introduction and a protocol optimized for avian feces (Trevelline et al. 2016). The extracted DNA was further concentrated by evaporating samples in vacuum and then was stored at –40 °C prior to library preparation and sequencing. In addition to our samples, we extracted 1 “blank” water sample per extraction kit.

When the diet is mainly composed of invertebrates, mitochondrial gene cytochrome c oxidase subunit 1 (*COI*) has become the standard and allows identification to genus level in many cases (Shutt et al. 2020). In this study, a short fragment (177 bp) of the mtDNA *COI* gene was amplified via PCR using arthropod-specific primers (ZBJ-ArtF1c and ZBJ-ArtR2c) (Zeale et al. 2011; Burgar et al. 2014) for animal prey identification. Universal arthropod-specific primers preferential detect Lepidoptera, Diptera, and Coleoptera (Alberdi et al. 2018; Browett et al. 2021). The main prey groups of interest in the Sichuan partridge diets are Diptera and Lepidoptera, which are amplified well with the chosen primers (Alberdi et al. 2018). The arthropod-specific primers also allowed for high taxonomic resolution of certain arthropod taxa from fecal samples,

which is not necessarily the case with other primers (Clarke et al. 2014; Garfinkel et al. 2021). The used primers were also tested against the Sichuan partridge sequences to ensure no significant amplification of host DNA (Browett et al. 2021). To determine the contribution of plants to diets, the chloroplast *rbcL* genes (ribulose-bisphosphate carboxylase gene) primers (h1aF and h2aR primers) were used to identify the plant taxa referred to previous study (Poinar et al. 1998; McClenaghan et al. 2015; Khanam et al. 2016). The primer sequences used in this study were listed in Supplementary Table 1.

Polymerase chain reactions (PCR) were carried out with PCR Using Q5® High-Fidelity DNA Polymerase (M0491, NEB) as recommended by the manufacturer. And then PCR reactions were performed primarily according to Trevelline et al. (2016). Each sample was amplified with both *COI* and *rbcL* primers in separate reactions. Blank extraction controls were included on each PCR plate and for each different primer set. PCR products were visualized by electrophoresis on 1.5% agarose gels and then purified using an AxyPrep PCR purification kit (AXYGEN, Hangzhou, China). Taking the purified PCR product as the template, quantitative real-time PCR was performed on a Microplate reader (BioTek, FLx800) using Quant-iT PicoGreen dsDNA Assay Kit. The amplicons for each sample were pooled and purified according to our previous study (Tang et al. 2021).

Library preparation and sequencing

Amplicons were combined in approximately equimolar concentrations to produce a DNA library of all extracts for sequencing. Ampliconic libraries for sequencing were built using the TruSeq Nano DNA LT Library Prep Kit (Illumina, San Diego, CA, USA) following manufacturer's protocols. Unique combinations of specific tags were assigned to each individual DNA extract to allow for the assignment of sequences to a sample. Prior to sequencing, the library needs

to be inspected on Agilent Bioanalyzer using Agilent high-sensitivity DNA kit. Libraries were sequenced on NovaSeq 6000 platform (2 × 250 bp paired-end reads) using NovaSeq 6000 SP Reagent Kit by Personalbio Bioinformatics Technology Corporation (Shanghai, China).

Bioinformatic processing and statistical analyses

The raw sequence underwent quality trimming prior to the taxonomic assignment using the QIIME2 (Caporaso et al. 2010). Sequences were assigned to samples on the basis of their tags and barcode; the barcode sequence and potential chimeras were removed using USEARCH v9.2 (Edgar 2013). We clustered sequences at 97% similarity into Molecular Operational Taxonomic Units (MOTUs) following the standard setting in USEARCH v9.2 (Edgar 2013). We calculated rarefaction curves that act as a check to ensure sufficient sequence sample depth in subsequent analyses. Clean sequences that passed quality filtering were queried against the National Center for Biotechnology Information's (NCBI) database using BLASTn (Basic Local Alignment Search Tool) according to the previous study (Berry et al. 2017).

MOTUs were resolved to species, genus, or higher level following sequencing similarity threshold: sequences with identity $\geq 99\%$ to a single species were considered as a “species match,” and sequences were identified to genus when reads had $\geq 98\%$ similarity to one or more species within the same genus. If taxonomy classification could not be resolved at the species level, taxonomy was assigned at the genus level instead (identity $\geq 98\%$). We performed subsequent analyses at the genus or higher level.

The percentages of all sequences assigned to a given MOTU for each sample were calculated as RA (relative abundance) (Kartzinel et al. 2015). Frequency of occurrence (FO; the number of pellets containing that foods divided by the total number of pellets in the species sample) of diets was calculated as the proportion of samples in which each prey item occurred. We compared preys community dissimilarity inferred from the FO and RA data.

Alpha diversity (i.e., Chao1, Shannon, Simpson, Observed_species and Good_coverage) matrices were performed using QIIME2 and visualized using the “ggplot2” package of R software. We calculated Jaccard distance for beta diversity using QIIME2, to evaluate the pattern of dispersion of samples within 3 periods of Sichuan partridge's annual life history. Beta diversity was visualized using 2-dimensional Principal Coordinate Analysis (PCoA) and Non-metric Multidimensional Scaling (NMDS) based on Jaccard distance matrices in R software. Analysis of similarity (ANOSIM) was performed based on the Jaccard distance matrix using the R “vegan” package. We also compared the RA of diets at various taxonomic levels in different periods based on the linear discriminatory analysis (LDA) effect size (LEfSe) method using LEfSe package. To test the difference in diet composition between 3 periods, we conducted permutational multivariate analysis of variance (PERMANOVA) implemented in QIIME2 using Jaccard distance matrix and 999 permutations. Kruskal–Wallis test was used to assess the difference in alpha diversity index between different periods. We also adopted Mann–Whitney *U* test to measure significance in nonparametric relative abundance profiles between different groups by SPSS 20.0 software. Sankey plots, boxplots, and taxa summary bar charts were generated using the “networkD3” and “ggplot2” packages of R software.

Results

Sequencing summary and dietary richness/taxonomic assignment

A total of 60 fecal samples were collected in this study; 60 *COI* and 59 *Rbcl* barcode amplicons were successfully sequenced using Illumina high-throughput sequencing with only 1 failed sequencing of *Rbcl* amplicons during postbreeding wandering. The libraries generated 6,732,295 and 6,080,685 raw sequences based on *COI* and *Rbcl* amplification, respectively, which were reduced to 6,470,593 and 5,339,263 reads by quality filtering, with average clean read counts of 107,843 (*COI*) and 90,495 (*Rbcl*) per sample (Supplementary Table 2). The rarefaction curves showed that the sequencing depth was sufficient to capture the diversity of the entire animal and plant content in the diets of the Sichuan partridges (Supplementary Figure 1).

The animal-derived diets of the Sichuan partridges included a taxonomically broad variety of arthropods. A total of 60 orders were identified with *COI*, including 34 orders of arthropods, 5 orders of mollusks, 4 orders of nematodes, 3 orders of annelids, and the remaining 14 orders were from 6 other phyla. Arthropods were identified in all samples spanning the 3 periods, accounting for more than 85% of the reads (postbreeding wandering, 91%; overwintering, 86%; and breeding, 95%) (Table 1). The breadth of arthropod taxa detected across all samples was substantial. At the lower taxonomic level, invertebrate sequences among the 60 fecal samples were assigned to 220 animal families and 339 genera. The relative abundance of 17 orders and 39 genera accounted for more than 1% of their annual feeding time. With respect to the *Rbcl* metabarcoding assay, 70 orders were obtained from 59 fecal samples, consisting of 150 plant families and 270 genera. Among them, 28 plant genera accounted for $\geq 1\%$ of their annual feeding time. The top 10 plant genera contributed the majority (62.5–76.1%) of the plant-derived diet (Table 2). The top 10 taxa at different taxonomic levels accounted for the vast majority of all sequences based on *COI* or *Rbcl* metabarcoding assays (Tables 1 and 2).

Dietary diversity and composition

Overall, alpha diversity indices (Chao1, Shannon, Simpson, observed species, and Good's coverage) indicated intergroup differences in dietary diversity between different periods of adult life history (Figure 2). The Sichuan partridges had a greater diversity of animal and plant prey species during postbreeding wandering than during overwintering and breeding. The lowest alpha diversity was detected during breeding. For animal-derived food items, the number of orders and families identified during breeding was significantly lower than during overwintering ($P = 0.015$ and 0.007 , respectively) and postbreeding wandering ($P < 0.001$) (Supplementary Figure 2). There were consistent trends in the dietary shift in the plant-derived food items, whether in alpha diversity or the number of identified taxa (Figure 2B and Supplementary Figure 2). The postbreeding wandering period exhibited higher alpha diversity of plant-derived diet compared to overwintering and breeding periods (Figure 2B).

The animal-derived diet of the Sichuan partridge included a taxonomically broad variety of arthropods (RA: 85–95%), including flies, butterflies, spiders, very few earthworms, and centipedes (Figure 3A, Table 1, and Supplementary Figure 3). Using order-level taxonomy only, Diptera (flies), Araneae

Table 1 The FO and average RA in the top 10 animal food items at the order level in the diet of the Sichuan partridge

Order Level	Postbreeding wandering		Overwintering		Breeding		Total FO (N = 60)
	FO (N = 20)	Average RA	FO (N = 20)	Average RA	FO (N = 20)	Average RA	
Diptera	100%	0.3760	100%	0.4242	100%	0.6409	100%
Araneae	100%	0.1455	100%	0.0495	95%	0.0305	98%
Lepidoptera	80%	0.0040	100%	0.0413	100%	0.1401	93%
Mesostigmata	30%	0.0008	90%	0.0350	95%	0.0474	72%
Coleoptera	95%	0.0135	100%	0.0594	80%	0.0042	92%
Adinetida	45%	0.0001	75%	0.0424	60%	0.0127	60%
Rhabditida	100%	0.0477	25%	0.0003	10%	<0.0001	45%
Orthoptera	90%	0.0325	80%	0.0079	30%	0.0004	67%
Philodinida	55%	0.0005	65%	0.0158	95%	0.0178	72%
Polydesmida	20%	0.0009	75%	0.0130	95%	0.0177	63%
Total		0.6216		0.6888		0.9119	

Numbers in bold denote average relative abundance >10%.

Table 2 The FO and average RA in the top 12 plant food items at the genus level in the diet of the Sichuan partridge

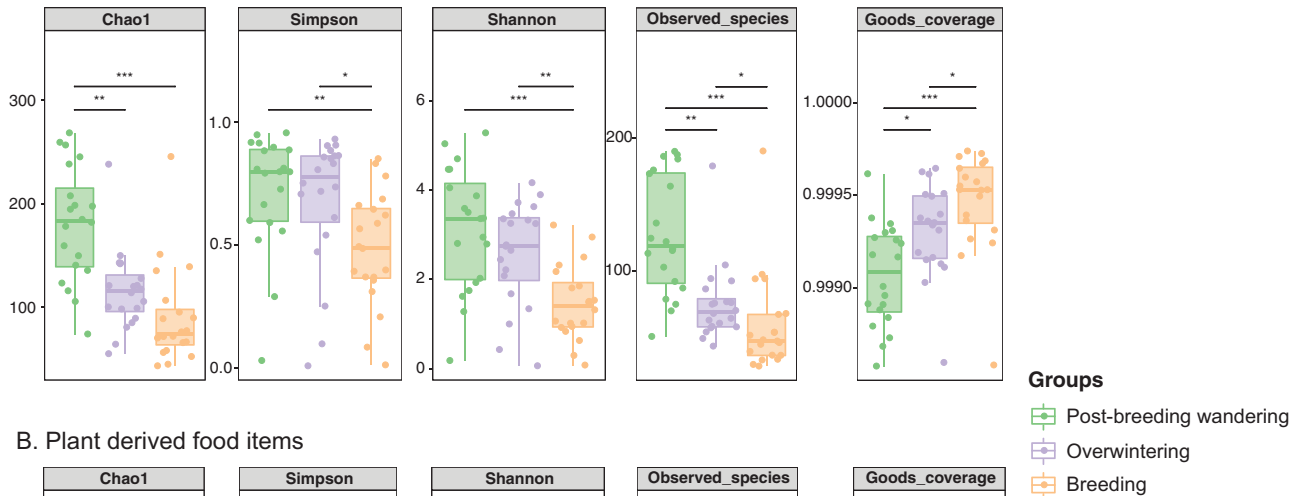
Order Level	Family Level	Genus Level	Postbreeding wandering		Overwintering		Breeding	
			FO (N = 19)	Average RA	FO (N = 20)	Average RA	FO (N = 20)	Average RA
Ericales	Symplocaceae	<i>Symplocos</i>	100%	0.2815	100%	0.4072	100%	0.1863
Rosales	Rosaceae	<i>Rubus</i>	78%	0.0349	100%	0.1347	95%	0.1161
Celastrales	Celastraceae	<i>Celastrus</i>	63%	0.0066	95%	0.0276	100%	0.1863
Ranunculales	Lardizabalaceae	<i>Holboellia</i>	100%	0.0912	100%	0.0484	80%	0.0155
Ericales	Actinidiaceae	<i>Actinidia</i>	100%	0.0457	100%	0.0453	80%	0.0291
Cornales	Cornaceae	<i>Alangium</i>	84%	0.0239	90%	0.0360	95%	0.0575
Fabales	Fabaceae	<i>Pueraria</i>	57%	0.0041	95%	0.0159	75%	0.0487
Cucurbitales	Cucurbitaceae	<i>Thladiantha</i>	78%	0.0443	85%	0.0048	55%	0.0145
Rosales	Urticaceae	<i>Elatostema</i>	36%	0.0008	85%	0.0017	50%	0.0563
Fagales	Betulaceae	<i>Carpinus</i>	63%	0.0575	5%	0.0001	5%	<0.0001
Apiales	Apiaceae	<i>Angelica</i>	10%	<0.0001	60%	0.0019	40%	0.0502
Vitales	Vitaceae	<i>Vitis</i>	89%	0.0342	70%	0.0085	15%	0.0002
Total				0.6247		0.7321		0.7607

Numbers in bold denote average relative abundance >5%.

(spiders), and Lepidoptera (butterflies or moths) spp. dominated the animal-derived diet of the Sichuan partridges (Table 1 and Supplementary Figure 3). Their combined relative abundances accounted for 52.6% of animal-derived diets during postbreeding wandering, 51.5% during overwintering, and 81.2% during breeding. Diptera (annotated to 44 families and 93 genera) were always observed at 100% frequency in all samples (Figure 4A), and had the largest relative abundance of invertebrate prey (RA: ~37–64%) throughout the year. Araneae (annotated to 23 families and 35 genera) and Lepidoptera (annotated to 19 families and 73 genera) were frequently detected and contributed to the majority of animal-derived sources during the postbreeding wandering and the breeding seasons, respectively (Table 1, Figures 3A and 4A). The rest (including Mesostigmata, Coleoptera, Orthoptera, Polydesmida orders) of the top 10 orders were consistently observed at high frequencies (FO: 63–92%) (Figure 4A), but contributed to a low relative abundance (RA: 5–15% in total) in the Sichuan partridge's diet (Table 1). At

the family level, the dominant preys in terms of both the FO and RA were the top 5 taxa (Supplementary Tables 3 and 4), for example, the Chironomidae, Psychodidae, Anthomyiidae, Hepialidae, and Theridiidae families. At the lower taxonomic level, the genus *Conchapelopia* of the family Conchapelopi, *Psychoda* genus of the family Psychodidae, *Delia* genus of the family Anthomyiidae, and *Korscheltellus* genus of the family Hepialidae were the main preys accounting for 22.1–57% of the animal-derived diet. A few uncommon prey items (e.g., Noctuidae, Rhabdiphoridae, Megascolecidae, Carabidae, and Annulariidae families) were detected with relative high RA (>10%) in some fecal samples (red numbers in Supplementary Table 4). On an individual scale, some of the heterogeneity observed between individuals in the relative abundance of invertebrate prey (Supplementary Table 4) showed a moderate level of variation among individuals. At the species level, a total of 86 species of arthropods were identified in the present study, 9 of which each accounted for >1% of animal-derived diets. For instance, *Psychoda ermine*, which belongs to

A. Animal derived food items



B. Plant derived food items

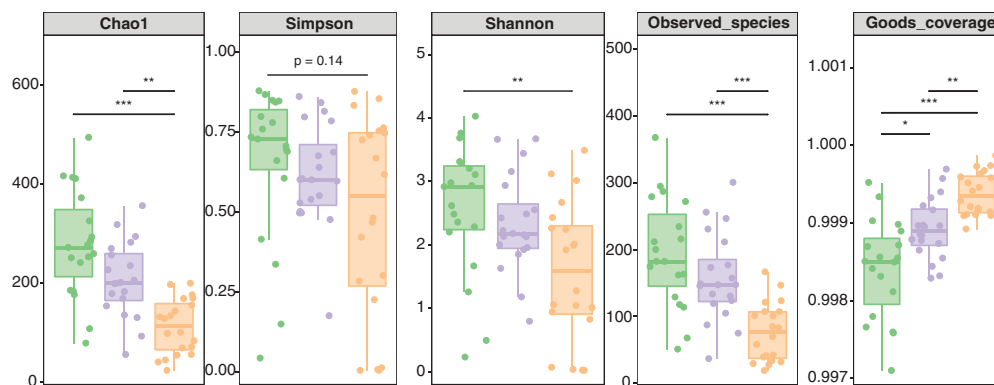


Figure 2 Box-and-whisker plots for alpha diversity in (A) animal- and (B) plant-derived food item estimators (Chao1, Shannon, Simpson indices, observed species, and Good's coverage) in the Sichuan partridge; * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$.

the Diptera order, was the most common and abundant prey, particularly during breeding (RA: 0.22, FO: 100%). The second abundant species was *Korscheltellus lupulinus*, a member of the order Lepidoptera, with a higher FO (95%) and RA (0.07) during breeding compared to the other 2 periods.

As for the plant-derived components of the diet, this threatened Galliforme bird predominantly consumed plant taxa that generate berries and seeds (Figure 3B). *Symplocos*, *Rubus*, *Celastrus*, *Holboellia*, and *Actinidia* were the 5 most commonly detected genera of plants consumed by the Sichuan partridges over the full year (Table 2, Figures 3B and 4B). The most common plant genus was *Symplocos*, which belongs to the Symplocaceae family, based on both FO (100%, and RA (peak value >40%) over the course of the year (Table 2, Figure 4B, and Supplementary Table 5). *Rubus* (belonging to Rosaceae) was the second most abundant plant food, followed by *Celastrus* (FO: 63–100%), based on both FO and RA (peak value >18%) (Table 2 and Figure 4B, Supplementary Table 5). At the higher taxonomic level, the orders Ericales, Rosales, and Celastrales constituted 40–64% of plant-derived diets (Supplementary Table 6), and the order Ericales was primarily represented by the genera *Symplocos* and *Actinidia*. The genera *Rubus*, *Elatostema*, and *Prunus* made up the largest proportion of the order Rosales; the order Celastrales was mainly represented by the genus *Celastru*, showing that these berry and seed plants were the most commonly and predominantly consumed groups in the Sichuan partridge's diet (Figures 3B and 4B and Supplementary Tables 5 and 6)

Temporal variation in dietary composition

Substantial temporal variation in the dietary composition of this omnivorous pheasant species was observed at the 3 different periods of adult life history. The invertebrate prey taxa in the postbreeding wandering period clustered together and were separated from those in the other 2 periods (Figure 5A). Animal food items formed a cluster during the breeding period, whereas invertebrate food items during overwintering had a higher dispersion (Figure 5A). As for plant-derived diets, there was more dispersion of all samples within the 3 critical periods of adult life history, but there was still an apparent separation between the postbreeding wandering and breeding periods (Figure 5B). Plant-derived food items during the overwintering periods indicated a substantially higher dispersion, suggesting a high degree of intragroup variability. A similar temporal variation pattern in the Sichuan partridge's diet was further supported by NMDS based on Jaccard distance (Supplementary Figure 4). The temporal variations in dietary composition were corroborated by the ANOSIM results, especially in the animal-derived diets (R value: 0.278–0.605, $P = 0.001$) (Supplementary Table 7). Using multifactorial PERMANOVA to test for group differences, differences ($P < 0.01$) between the 3 critical periods were found using the Jaccard distance metric (Supplementary Table 8).

The Sichuan partridge foraged on the invertebrate prey assigned to the dipteran order at a higher proportion during breeding (average RA: 64.1%, $P = 0.038$) than during the other 2 periods (Figure 6A and Table 1). Consumption

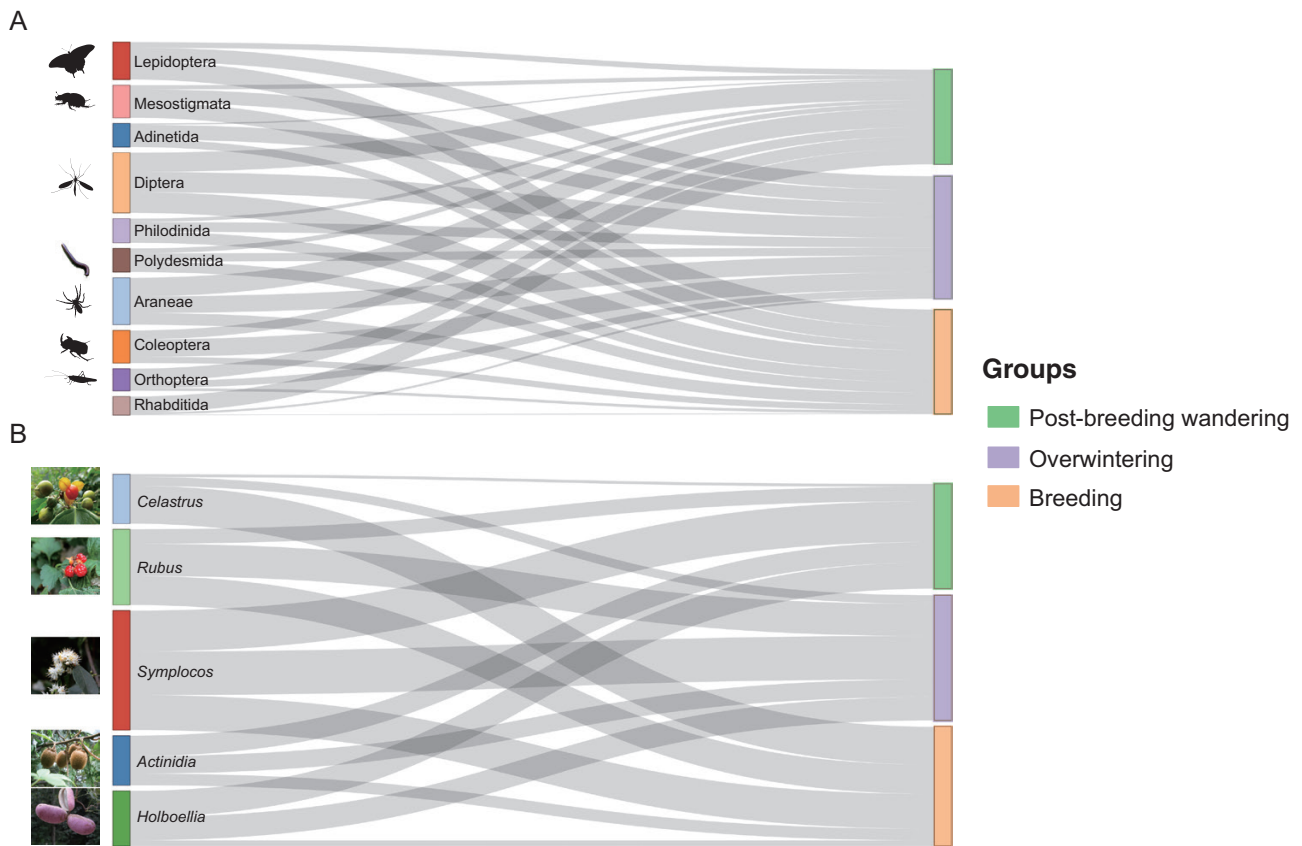
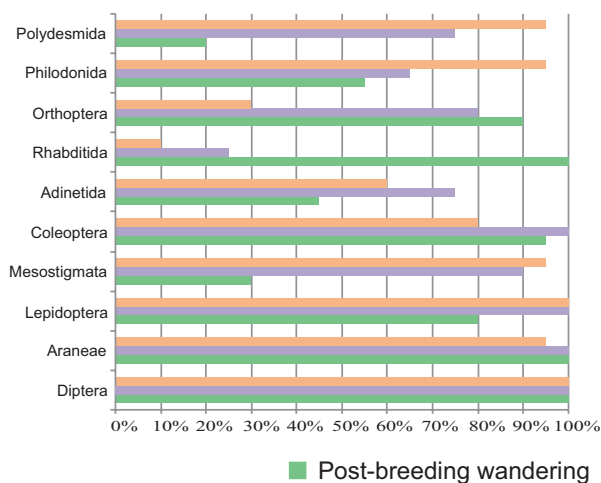


Figure 3 Sankey plots showing associations between 3 periods of adult life history (right) and invertebrate prey (A) at the level of taxonomic order and plant-derived food items (B) summarized at the level of taxonomic genus (left) in the Sichuan partridge. Connecting bar width represents the relative abundance of dietary items among periods and dietary items.

A. FO for top10 animal-derived foods at the order level



B. FO for top12 plant-derived foods at the genus level

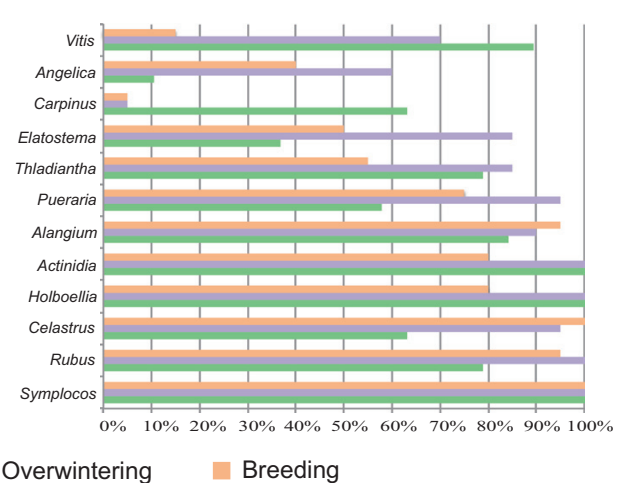


Figure 4 FO of the top 10 animal-derived food items at the order level (A) and top12 plant-derived food items at the genus level (B) in the Sichuan partridge.

of the Araneae order was at its peak (average RA: 14.5%) during postbreeding wandering, while the prey of the order Lepidoptera was consumed by the Sichuan partridge in the highest proportion (RA: 14.0%) during breeding periods (Figures 3A and 6A and Table 1). Spider and centipede species were found to substantially increase in relative abundance during postbreeding wandering (e.g., average RA of Araneae

>14.6%) compared to other periods based on LefSe analysis (Figure 7A and Table 1). During the breeding period, a diverse assemblage of moths (38 genera) was commonly detected in fecal samples from the Sichuan partridge. For example, species from the Hepialidae (FO: 95%; average RA: 7.8%) and Geometridae (FO: 90%; average RA: 3.2%) families were eaten by the Sichuan partridge in significantly higher

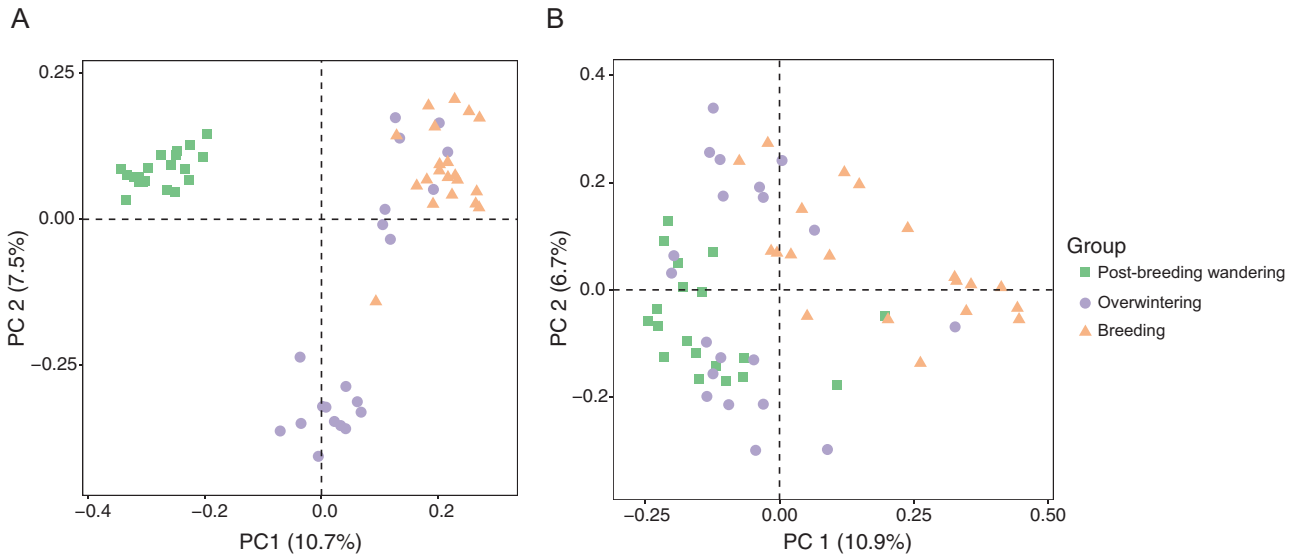
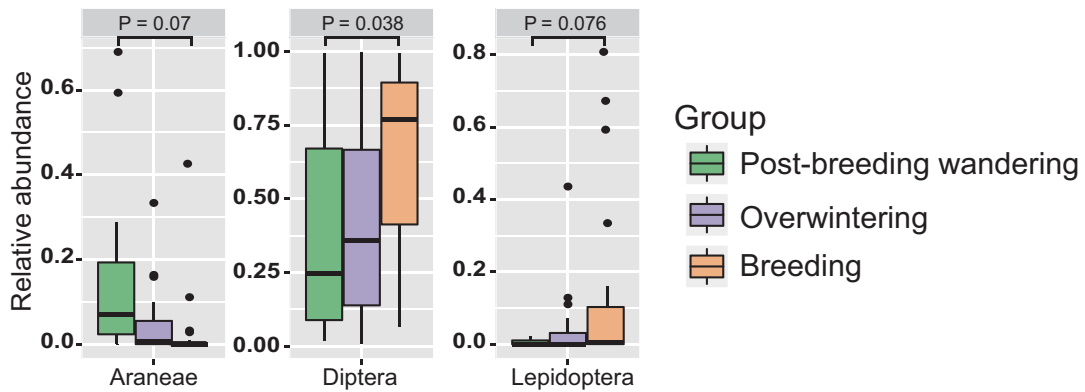


Figure 5 PCoA of MOTUs for the Sichuan partridge diet in 3 periods of adult life history. (A) represents animal food items and (B) represents plant food items. Each symbol corresponds to 1 fecal sample. The first 2 principal coordinate (PC) axes are shown.

A. Top3 animals order



B. Top5 plants genus

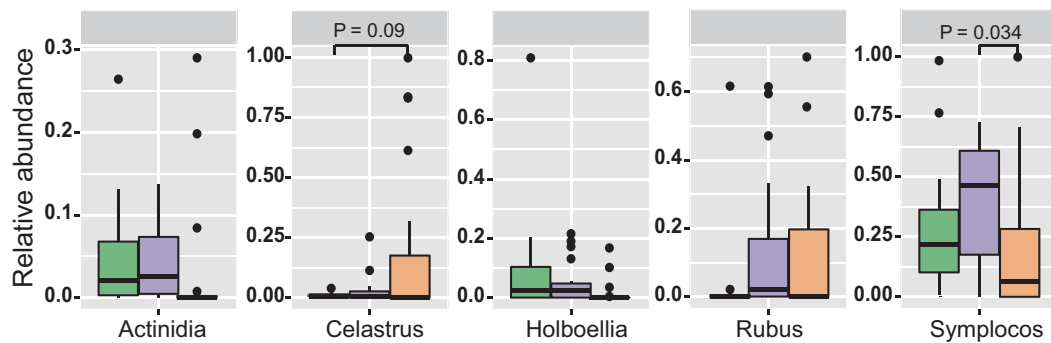


Figure 6 Box-and-whisker plots for differences in relative abundance of the top 3 invertebrate preys at the order level (A) and the top 5 plant taxa at the genus level (B) in the 3 critical ecological periods of adult life history in the Sichuan partridge.

proportions during breeding than during the other 2 periods (Figure 7A and Supplementary Table 4).

The top 5 plant genera accounted for two-thirds (average RA: 46–66%) of the plant-derived diet throughout the year, showing temporal differences in RA (Table 2, Figures 3B and 6B). *Symplocos*, which constituted the majority of the order Ericales, were observed at a higher proportion (average RA: 40.7%; $P = 0.034$) during overwintering than

during breeding periods (average RA: 18.6%) (Figures 6B and 7B and Supplementary Table 6). The species of the genus *Celastrus* apparently foraged more during breeding periods (FO: 100%; average RA: 18.6%) than during the other 2 periods (FO: 63% and 95%; average RA: 0.6% and 2.8%, for postbreeding wandering and overwintering, respectively) (Figures 4B and 6B, and Table 2). The genus *Rubus*, which comprised the largest proportion of the order Rosales, was

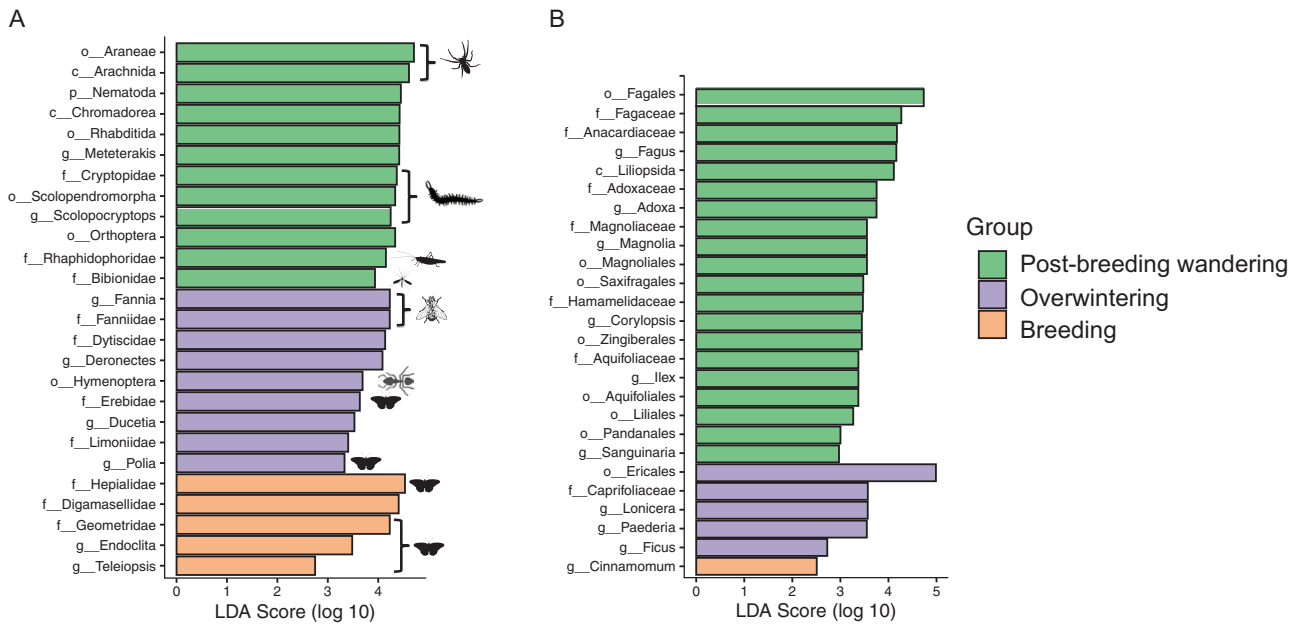


Figure 7 Animal (A) and plant (B) food items at various taxonomic levels differentiated between the 3 periods of the Sichuan partridge, as determined by LDA effect size (LEfSe). LDA scores were interpreted as the degree of difference in relative abundance.

detected at a higher RA during the breeding (average RA: 11.6%) and overwintering (average RA: 13.5%) periods than during the postbreeding wandering period (average RA: 3.5%) (Table 2 and Figure 6B). In addition, the food items of the genera *Actinidia* and *Holboellia* showed the lowest RA (Table 2) and FO (Figure 4B) during the breeding period. The species of *Holboellia* increased and reached their peak during the postbreeding wandering period (RA: 9.1%) (Figure 6B and Table 2). Furthermore, a diverse array of plant-derived taxa, whose proportions were very low, were also detected during postbreeding wandering periods based on LEfSe analysis (Figure 7B), indicating that the Sichuan partridge might feed on specific plant assemblages at this period.

Discussion

Fecal metabarcoding can provide profound insights into avian diets and achieved a greater taxonomic resolution in this study than in previous studies of the Sichuan partridge. This study showed that the Sichuan partridge is a dietary generalist that feeds on a broad range of invertebrates and plants. Arthropods and plants that produced berries or seeds were the main food items detected in feces during the 3 critical periods of adult life history, indicating a relatively broad dietary niche for the Sichuan partridge. We also demonstrated a strong temporal pattern in the taxonomic richness and composition of invertebrate prey and plant-derived food items. Seasonal dietary variability has implications for the optimal scheduling of ontogenic events, such as breeding and overwintering (Thorup et al. 2017; Shutt et al. 2020).

This endangered pheasant bird consumed various insects (including species from Diptera, Lepidoptera, Coleoptera, and Orthoptera orders), spiders (Araneae), and myriapods (Polydesmida and Cryptopidae), as well as some mites (Mesostigmata) throughout the year (Figures 3A and 7A, and Table 1). Other invertebrate preys, such as spiders, centipedes, and other myriapods, have a subterranean lifestyle, and direct field observations of prey captures are nigh impossible,

making molecular method very appealing. As a ground-dwelling bird with limited flight ability, the Sichuan partridge has adapted to capture leaf-litter dwellers (such as centipedes and spiders; Figure 7A and Supplementary Table 4), which are difficult to observe. We were successful in high-resolution taxon recovery of a broad diet using DNA metabarcoding in the present study. However, the universal primers employed in this experiment were previously indicated to be biased among particular arthropod orders (Browett et al. 2021; O'Rourke et al. 2021). They are known for preferential detection of Diptera, Lepidoptera, and Coleoptera (Alberdi et al. 2018; Browett et al. 2021), which may result in the overrepresentation of certain taxa. Thus, a relatively higher proportion of lepidopteran- and dipteran-classified (RA: 37.6–64.1%, Table 1) sequences in our results seem to be explained by the primer bias. This is in accordance with previous studies, demonstrating that primer bias may have contributed to the overrepresentation of Diptera (Alberdi et al. 2018; McClenaghan et al. 2019). Previous studies have shown that Hymenoptera species (e.g., ants) and mollusks (e.g., snails and slugs) were observed in the diets of the Sichuan partridge (Li et al. 1974; Zheng 2015). These common invertebrate preys may be potentially underrepresented or even omitted in our sequencing results due to primer bias. Despite potential shortcomings, various theoretically underrepresented groups (e.g., centipedes, millipedes, earthworms, and spiders) were detected frequently and in abundance, indicating that some invertebrate taxa within the groups were still recovered. Under-amplified taxa (Orthoptera, Hemiptera, and Hymenoptera) were frequently found but at low abundances (Supplementary table 4), suggesting a relatively minor impact of such primer bias on prey selection conclusions in the present study. Unexpectedly, Heterakidae, belonging to the order Rhabditida, a nonfood taxon, was also detected using this molecular approach (Table 1 and Supplementary Table 4). They are a class of parasites that commonly appear in the intestines of Galliformes, and have been reported many times in *Tragopan temminckii* (Zhang et al. 2008), *Phasianus*

colchicus (Shi et al. 2010), *Tragopan satyra* (Ushigome et al. 2010), and *Lagopus mutus japonicus* (Tomoo et al. 2008). Therefore, strengthening the research on the threat of these parasites to the health of partridges is essential.

DNA metabarcoding and sequencing also provide more detailed information on plant-derived diets than previous studies (Xu et al. 1994; Zheng 2015), confirming that the Sichuan partridge is an omnivorous and granivorous bird. This threatened bird inhabits subtropical evergreen and deciduous broadleaf forests (Johnsgard 1988; Liao et al. 2008a, b). A diverse assemblage of plant taxa can provide various fruits, seeds, and leaves for the Sichuan partridge throughout the year. For instance, the *Symplocos*, *Rubus*, *Celastrus*, *Holboellia*, and *Actinidia* genera were the dominant plant feed items in terms of both FO and RA, comprising a total of 46–66% of plant DNA sequences (Figure 3B and Table 2). Consistent with the present results, seeds of the *Rubus*, *Quercus*, and *Elaeagnus* genera, which are important food sources for the Sichuan partridge, were also observed using conventional methods in previous studies (Xu et al. 1994; Zheng 2015). The characteristic of seed eating is also consistent with the plant-derived diets of 2 other partridges, the Sand partridge (*Ammoperdix heyi*) (Kam and Nagy 1987) and the Gray partridge (*Perdix perdix*) (Chitty 1937). These fruits and seeds have a higher energy and nutritional content, making them an important food source for many granivorous or omnivorous birds (Ando et al. 2013; De Leon et al. 2014; da Silva et al. 2020; Hou et al. 2021). In addition, the rhizomes, roots, leaves (or buds), and flowers are also regarded as potentially important food sources for gray partridges *Perdix perdix* (Chitty 1937), red-headed wood pigeons *Columba janthinaitens* (Ando et al. 2013), and Siberian cranes *Leucogeranus leucogeranus* (Hou et al. 2021). However, the current study did not involve the detection of different parts of vegetation. The Sichuan partridge may also feed on a few rhizomes, leaves, or buds during the nonflowering and nonfruiting stages. Traditional dietary analysis methods cannot detect these digestible plant tissues.

Diet and foraging ecology are highly consistent across time and space (Liu et al. 2018; Tiede et al. 2020; Young et al. 2020; Schmack et al. 2021). Temporal variation in resource availability has implications for the optimal scheduling of ontogenic events such as breeding (Charmantier et al. 2008; Trevelline et al. 2018), overwintering (Lourenço et al. 2017; Li et al. 2021), and seasonal movement (Bounas and Sotiropoulos 2017; Thorup et al. 2017). We found substantial temporal variability in both animal- and plant-derived diets in the Sichuan partridge (Figure 5, Supplementary Figures 2 and 4). Previous studies demonstrated that dietary variations were partly due to the availability of food resources in different seasons (Wray et al. 2021), and food availability fluctuated substantially over time (Hou et al. 2021). The seasonality of food resources caused by fluctuations in temperature and precipitation throughout the year in the reserve may explain such changes in the dietary composition of the Sichuan partridge.

Breeding productivity in birds is linked to pronounced temporal variation in the availability of prey items during the breeding season (Trevelline et al. 2018; Young et al. 2020). We observed lower animal- and plant-derived dietary diversity during the breeding season than during the other 2 periods (Figure 2 and Supplementary Figure 2). With an increase in food resource abundance during the breeding season, the dietary breadth of the Sichuan partridge narrows. The limited

range of movement and shortened foraging time of parent birds during breeding are also possible reasons for lower dietary diversity. During breeding, the Sichuan partridge fed largely on Diptera and Lepidoptera (Figures 6A and 7A, and Table 1) with high energy to meet the high-calorie demands of brooding or incubation. Preys from lepidopteran and dipteran orders provide higher concentrations of protein and are likely more digestible than those in plants. The life histories of lepidopterans may largely determine their importance as prey items (Sullins et al. 2018). The increased consumption of lepidopteran taxa during the breeding season may be attributed to the peak availability of lepidopteran larvae or pupae in late May and early June, which is consistent with previous studies (Shutt et al. 2019, 2020). The possible reasons for the high abundance of Diptera (RA: 37.6–64.1%) (Table 1) in the diet could be the species richness and continued availability. The climate in the research area is temperate, with high precipitation (>1,500 mm per year) and relative humidity (>85%) (Fu et al. 2020). The robust dipteran taxa available throughout the breeding season are likely due to high precipitation and humid habitats, especially during the rainy season, consistent with the diets of Indiana Bats, who also consume a large proportion of Diptera (O'Rourke et al. 2021).

The greater alpha diversity of diets (Figure 2) and the higher number of detected feed items (Supplementary Figure 2) during postbreeding wandering may be due to the expansion of the foraging range and time to benefit from a wide range of food resources. The small number of reads for some foods (Supplementary Tables 4 and 5) may result from secondary consumption (Pompanon et al. 2012; Gerwing et al. 2016; Ingala et al. 2021), such as plants eaten by invertebrate herbivores that are then eaten by the Sichuan partridge. Secondary predation is common, particularly in many insectivorous species (Ando et al. 2013; Ingala et al. 2021), but this bias is likely to be minimal (McClenaghan et al. 2019). With respect to plant food items, fruit and seed abundance and availability are subject to temporal change. The fruiting period of the *Holboellia* genus is from September to October. An increased abundance of *Holboellia* was observed during postbreeding wandering (Figure 6B and Table 2), indicating that the Sichuan partridge consumed these juicy fruits at maximum fruiting times.

Diet during the winter period is likely a crucial factor that influences the survival of Sichuan partridge adults and the growth of chicks. The availability of food resources during winter seems to be limiting for Galliformes species (Hagen et al. 2009; Sullins et al. 2018). Whether for adults or juveniles during cold winters, meeting high energy demands for thermoregulation with the available food items of limited nutrient content may be challenging (Olawsky 1987; Sedinger 1997; Sullins et al. 2018). A decreased consumption of spiders, butterflies, and moths during overwintering was observed in our study (Figures 6A and 7A, and Table 1), because the species from the Araneae and Lepidoptera orders may not be sufficiently abundant and available in winter. In response to animal-derived resource limitations in winter, the Sichuan partridge needs to expand its diet to compensate for the reduced availability of invertebrate prey taxa. We observed an increased use of plant feed items from *Symplocos* and *Rubus* genera during overwintering (Table 2), which might be an adaptive compensatory strategy. Most species of *Symplocos* are evergreen (Soejima and Nagamasu 2004), the fruits of which are rich in endosperm (Liu and Qin 2013). The consistent presence of *Symplocos* in our results may be explained

by their significant abundance (Symlocaceae species) (Liao et al. 2007) and the availability of edible parts at the study site. The fruits of the *Rubus* species are juicy berries, which are high in sugars (3.34–16.59%), amino acids, and vitamins (Zhong and Tian 1993; Xiang 2007). The fruiting periods of the *Rubus* genus are long and irregular, and *Rubus* spp. can be used by the Sichuan partridge even in winter (Figure 3B and Table 2). Therefore, plant matter becomes particularly important in Sichuan partridge diets during winter, which is congruent with the compensatory strategy of the Asian Great Bustards (Liu et al. 2018). Wintering birds move across relatively large winter home ranges to maintain a diverse diet (Kessler et al. 2013). It is also possible for the Sichuan partridge to exploit a wider variety of feeding habitats during winter, resulting in higher dietary diversity and more food items than are available during the breeding season (Figure 2 and Supplementary Figure 2).

In conclusion, DNA metabarcoding of fecal samples is a practical way of deciphering the composition of the diet of the Sichuan partridge and reveals how the species responds to temporal variety in feed resources. Detailed dietary descriptions at different critical periods in the annual cycle strengthen our understanding of the basic temporal strategies in the Sichuan partridge. Our results indicate that this threatened Galliforme is opportunistic with a diverse diet comprising invertebrates (such as orders Diptera, Araneae, and Lepidoptera) and plants (such as fruits and seeds from *Symplocos*, *Rubus*, *Celastrus*, *Holboellia*, and *Actinidia* genera). Temporal dietary variation in the 3 periods of adult life history and temporal patterns of consumption of the animal and plant-derived food taxa suggest that this generalist forager employs a flexible foraging strategy to adapt its diet to seasonal variations. This study provides fundamental dietary information and elucidates the foraging ecology of the Sichuan partridge, contributing to the development of effective conservation strategies.

Conflict of Interest statement

The authors declare that they have no conflict of interest.

Author contributions

K-Y Tang carried out DNA extraction, experimental procedures, data analysis, and drafted the manuscript. Y-F Wang and S-F Wang participated in the sample collection and experimental procedures. M-L Wu and C-K Fu participated in data analysis. Zh-W Zhang participated in drafting the manuscript, and study design. Y-Q Fu conceived of the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Data Accessibility

The raw sequence data in this study have been deposited in the Genome Sequence Archive (Genomics, Proteomics, and Bioinformatics) at the National Genomics Data Center, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences, under accession numbers CRA006486 (for animal-derived diets) and CRA006496 (for plant-derived diets), which are publicly accessible at <https://bigd.big.ac.cn/gsa>.

Supplementary Material

Supplementary material can be found at <https://academic.oup.com/cz>.

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