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RESEARCH ARTICLE

# Comparative Analysis of Diet Composition and Gut Parasite Diversity in Bar-Headed Geese and Ruddy Shelducks Using Environmental DNA Metabarcoding

Yonggang DONG [1,2] Yuliang HOU [2] Xuelian WANG [3] Fang YANG [4] Zhuoma LANCUO [1] Wen WANG [1 (*)]

[1] State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University, Xining, 810016, Qinghai Province, CHINA
[2] College of Eco-Environmental Engineering, Qinghai University, Xining, 810016, Qinghai Province, CHINA
[3] Qinghai Forest Engineering Consulting Co., Ltd., Xining, 810012, Qinghai Province, CHINA
[4] Qinghai Duomei Ecological Environmental Protection Technology Co., Ltd., Xining, 810000, Qinghai Province, CHINA

(*) **Corresponding author:**
Wen WANG
Phone: +86-971-5201533
E-mail: 007cell@163.com

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## Abstract

We comprehensively analyzed the dietary preferences and intestinal parasite diversity of two waterbirds, the Ruddy shelduck *(Tadorna ferruginea)* and the Bar-headed goose (*Anser indicus*), using environmental DNA metabarcoding with the 18S rDNA V9 (for detecting plankton and parasites) and trnLg - trnLh (for detecting terrestrial plants) amplicon primer pairs. Our results showed that both species fed on multiple types of phytoplankton and terrestrial plants, but with different abundances. The ruddy shelduck mainly consumed *Chlorophyta*, *Bacillariophyta*, and *Poa*, while the bar-headed goose preferred *Chlorophyta*, *Xanthophyta*, *Pyrrophyta*, and *Potentilla*. Alpha - and beta-diversity analyses revealed significant niche differences in their food choices, enabling coexistence through different food selection or different consumption levels of the same food. Moreover, we detected the main intestinal parasites in both species: *Eimeria sp.* and *Tetratrichomonas sp*. in the bar-headed goose, and *Eimeria sp.* and some endogenous protozoan parasites in the ruddy shelduck. The dominance, species, and genetic variation range of this host-parasite system require further study and attention in future work. Our findings enhance the understanding of the ecological roles and dietary preferences of these two waterbirds in the Tibetan Plateau wetland ecosystem of China, and are significant for wetland environmental protection and species conservation.

**Keywords:** Bar-headed goose, Dietary analysis, Environmental DNA, Metabarcoding, Parasites, Ruddy shelduck

## INTRODUCTION

Dietary analysis plays a pivotal role in animal ecology, constituting an essential component of nutritional ecology. It is used to analyze the survival conditions, habitat preferences, and ecosystem functions of individuals or animal populations. Additionally, it reflects the interrelationships between species (including predator-prey relationships and food web interactions) and elucidates the ecological roles of various organisms within communities [1,2]. Furthermore, analyzing animal diets serves as a critical tool for tracing the origins and transmission pathways of zoonotic diseases [3], Consequently, studies on animal diets hold immense research value for wildlife conservation from a dietary perspective and ensuring human health. Birds, with their large populations, wide distribution, and sensitivity to environmental changes and human disturbances, act as sensitive indicators of ecological conditions [4]. Thus, research on avian feeding habits has long been a central focus in ornithology [5]. However, traditional methods have limitations in accurately and comprehensively analyzing avian diets [6]. Traditional methods for studying bird diets, including photographing [7], feces collection, stomach content analysis [8], and chick neck-tie sampling [9], have several limitations. These methods struggle with precise identification and quantification of food components, especially when dealing with small, quickly digested, or fragmented food items [10-13]. Additionally, these techniques can impact species by altering behaviors and even causing death [14]. Environmental DNA (eDNA) metabarcoding has revolutionized avian diet research by detecting prey

DNA in feces, vomit, or other environmental samples. This technique enables identification of consumed prey, quantification of dietary proportions, and detection of parasites [15]. Compared with traditional research methods, the advantage of environmental DNA sequencing and analysis lies in its capability to provide large-scale data. This approach facilitates a more comprehensive analysis of the spatial distribution and dynamic changes within animal populations [16].

Both the Bar-headed Goose and the Ruddy Shelduck are waterfowl belonging to the *Anatidae* family of the *Anseriformes* order. They have wide distribution ranges and possess edible value, contributing to economic benefits [17]. Unfortunately, habitat destruction on a global scale and hunting pressures have led to a sharp decline in their population numbers [18]. As two representative species widely distributed and abundant within the wetland wildlife resources of the Tibetan Plateau, both the Bar-headed Goose and the Ruddy Shelduck hold considerable economic and ecological value. Therefore, dietary and parasitological studies of these two bird species could provide valuable insights into their habitat preferences, resource consumption behaviors, and physiological characteristics such as dietary niche differentiation, contributing to the conservation of wetland bird diversity and effective management of wetland ecosystems. Despite recent research focusing on aspects such as avian migration [19] and gut microbiota [20]. for both the Ruddy Shelduck and the Bar-headed Goose, issues related to their feeding habits and parasites have been less explored. This study aims to investigate the dietary preferences and differences between the Bar-headed Goose and the Ruddy Shelduck using environmental DNA metabarcoding with two primer pairs (18s-V9F and trnLg-trnLh) to determine whether there is dietary niche differentiation between the two species and to analyze their internal parasites.

## Material and Methods

### Ethical Approval

This study conformed to the guidelines for the care and use of experimental animals established by the Ministry of Science and Technology of the People's Republic of China (Approval number: 2006-398). The research protocol was reviewed and approved by the Ethical Committee of Qinghai University. This study did not involve capture or any direct manipulation or disturbance of Bar-headed goose and the Ruddy shelduck.

### Sample Collection and Processing

In this study, environmental DNA samples were collected from feces of five Ruddy shelducks *(Tadorna ferruginea*, abbreviated as RSD group) and five Bar-headed geese *(Anser indicus*, abbreviated as BHG group), resulting in a total of ten fecal samples (five from each species). The sampling location was within the province of Qinghai, China *(Fig. 1)*. Fresh fecal samples were collected and stored in sterile tubes. All freshly collected fecal samples were transported to the laboratory using liquid nitrogen and subsequently stored in a -80°C freezer until further processing.

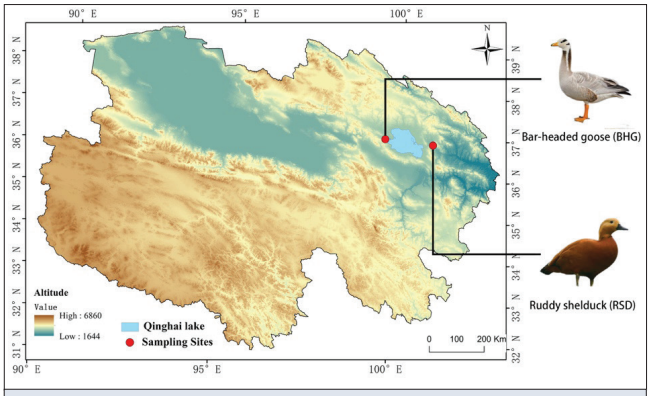


**Fig 1.** Map of the Qinghai-Tibetan Plateau with sampling sites

### DNA Extraction from Feces and PCR Amplification

DNA was extracted from fecal samples using the Qiagen QIAamp DNA Stool Mini Kit (Qiagen, Germany) following the manufacturer's instructions. Genomic DNA integrity was assessed via 1% agarose gel electrophoresis. Metabarcoding analysis was performed by amplifying food DNA present in fecal samples using two sets of universal primers targeting different regions. The first primer set targeted the V9 region of the eukaryotic 18S rRNA gene using the primer pair 18Sv9F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The second primer set targeted the P6-trnLh region of the terrestrial plant trnL gene using the primer pair g: 5'-GGGCAATCCTGAGCCAA-3' and h: 5'-CCATT GAGTCTCTGCACCTATC-3'. PCR amplification was conducted using an ABI GeneAmp® 9700 PCR System. Custom barcoded primers were synthesized for the designated sequencing regions. Each 20 µL reaction mixture contained 4 µL of 5x FastPfu Buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each forward and reverse primer (5 µM), 0.4 µL of FastPfu Polymerase, 10 ng of template DNA, and $ddH_2O$ to make up the total volume. The PCR program started with an initial denaturation at 95°C for 5 min, followed by 30 cycles of amplification (95°C for 30 sec, 58°C for 30 sec, and 72°C for 45 sec), and ended with a final extension at 72°C for 10 min, cooling down to 4°C. Based on preliminary quantification results obtained by agarose gel electrophoresis, the PCR products were accurately quantified using the QuantiFluor™ -ST Fluorometer (Promega) system. According to the DNA

concentration of each sample and the sequencing requirements, the PCR products were pooled in appropriate ratios.

### Construction of High-Throughput Sequencing (NGS) Libraries

Library preparation for NGS was outsourced to Shanghai BioZeron Biotechnology Co., Ltd. The libraries were constructed for Illumina PE250 sequencing. Initially, 'Y'-shaped adaptors were ligated to both ends of the DNA fragments. Subsequently, non-specific fragments, including adaptor dimers, were removed using magnetic bead selection technology. PCR amplification was then performed to increase the library template quantity, effectively enriching the library. Upon completion of library construction, sodium hydroxide treatment was applied to denature the double-stranded DNA within the library into single-stranded DNA fragments, preparing them for subsequent bridge PCR and Illumina PE sequencing.

### Data Accessibility

Based on the effective sequences from all samples, the Trimmomatic software was utilized to perform quality control filtering of the reads. Reads with quality scores below 20 at their tails were trimmed using a sliding window of 10 base pairs; if the average quality within the window fell below 20, the end of the read was removed starting from that point. After quality control, reads shorter than 50 base pairs were discarded. Paired-end (PE) reads were merged into a single sequence based on their overlap, with a minimum overlap length of 10 base pairs and an allowable maximum mismatch rate not exceeding 20%; sequences that did not meet these criteria were excluded. The orientation of sequences was corrected according to the forward and reverse barcodes and primers, and chimeric sequences were removed. The high-quality sequences were then separated by barcode and primer sequence to obtain the high-quality sequences for each sample. Subsequently, duplicate sequences were removed, and the remaining reads were processed through the DADA2 algorithm in QIIME 2 (version 2020.11) for quality filtering, denoising, merging, and chimera removal. Sequences with 100% similarity were grouped into amplicon sequence variants (ASVs), and representative sequences were generated. Taxonomic annotation of the representative sequences was performed using the uclust algorithm (with a confidence threshold of 0.8) to assign classification information at various taxonomic levels including domain, kingdom, phylum, class, order, family, genus, and species. Comparative analysis was conducted using databases such as Silva for plankton, PR2 for protists, and NT for general nucleotide sequences. The community composition and phylogenetic structure were further analyzed and visualized using the vegan package in R software (version 3.6.3). Bar charts were created using the ggplot2 package. And significant differences between sample groups were identified using the Analysis of Similarities (ANOSIM). Statistical comparisons were carried out using the non-parametric Wilcoxon test, and multiple testing corrections were made using the Bonferroni method. For all statistical tests, p-values less than 0.05 were considered statistically significant. Additionally, rarefaction curves were constructed using Mothur (version 1.21.1) to evaluate diversity indices such as Chao1, Pielou J, and Shannon. Beta diversity analysis was performed using principal component analysis (PCoA) plots were generated using the Vegan 2.0 package available on R-Forge.

## Results

### Sequencing Outcome Statistics

Following quality control, filtering, and merging procedures, 923.360 valid sequences were obtained from amplification using the 18S-V9 region primers. Of these, 923.356 sequences had lengths between 101-200 bp, with an average length of 138 bp. Detailed statistics regarding the optimized sequence counts, base pairs, and average sequence lengths for each sample were presented in *Table 1*. Additionally, 943,910 valid sequences were acquired through amplification using the trnLg-trnLh region primers. Among these sequences, 943.863 were found to have lengths within the 1-100 bp range, averaging 64 bp. Comprehensive sequence information for each sample was tabulated in *Table 2*.

### Phytoplankton Plant Species Composition and Abundance Statistics

In the field of phytoplankton, a total of 213 ASVs were detected. At the phylum and family levels, all ASVs were

**Table 1.** *Quality control results of sequencing data (18SV9F).*

| Primer | Samples | Sequences | Bases (bp) | Average Length (bp) |
|---|---|---|---|---|
| 18SV9F-18SV9FR | BHG1 | 63.178 | 8.429.955 | 133.43 |
| 18SV9F-18SV9FR | BHG2 | 62.028 | 8.600.322 | 138.65 |
| 18SV9F-18SV9FR | BHG3 | 56.152 | 7.756.751 | 138.14 |
| 18SV9F-18SV9FR | BHG4 | 67.496 | 9.422.336 | 139.6 |
| 18SV9F-18SV9FR | BHG5 | 65.172 | 8.976.968 | 137.74 |
| 18SV9F-18SV9FR | RSD1 | 129.737 | 18.017.519 | 138.88 |
| 18SV9F-18SV9FR | RSD2 | 121.234 | 16.859.068 | 139.06 |
| 18SV9F-18SV9FR | RSD3 | 126.258 | 17.554.538 | 139.04 |
| 18SV9F-18SV9FR | RSD4 | 112.763 | 15.675.603 | 139.01 |
| 18SV9F-18SV9FR | RSD5 | 119.342 | 16.575.467 | 138.89 |

**Table 2.** *Quality control results of sequencing data (trnLg).*

| Primer | Samples | Sequences | Bases (bp) | Average length (bp) |
|---|---|---|---|---|
| trnLg-trnLh | BHG1 | 62,468 | 3,988,623 | 63.85 |
| trnLg-trnLh | BHG2 | 62,343 | 4,120,298 | 66.09 |
| trnLg-trnLh | BHG3 | 56,125 | 3,630,090 | 64.68 |
| trnLg-trnLh | BHG4 | 57,426 | 3,852,546 | 67.09 |
| trnLg-trnLh | BHG5 | 61,904 | 4,140,291 | 66.88 |
| trnLg-trnLh | RSD1 | 125,894 | 8,042,020 | 63.88 |
| trnLg-trnLh | RSD2 | 118,573 | 7,509,705 | 63.33 |
| trnLg-trnLh | RSD3 | 137,940 | 8,819,061 | 63.93 |
| trnLg-trnLh | RSD4 | 126,661 | 8,132,803 | 64.21 |
| trnLg-trnLh | RSD5 | 134,576 | 8,633,477 | 64.15 |

**Table 3:** *Statistical information on the annotation of each sample to the taxonomic levels of phytoplankton (phylum, family, genus, species)*

| Taxonomy | BHG1 | BHG2 | BHG3 | BHG4 | BHG5 | RSD1 | RSD2 | RSD3 | RSD4 | RSD5 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Phylum | 4 | 3 | 1 | 6 | 5 | 7 | 4 | 3 | 7 | 6 | 8 |
| Family | 6 | 4 | 1 | 6 | 13 | 21 | 9 | 6 | 14 | 13 | 32 |
| Genus | 6 | 3 | 1 | 5 | 18 | 25 | 12 | 7 | 15 | 15 | 52 |
| Species | 3 | 0 | 0 | 2 | 2 | 2 | 1 | 0 | 0 | 1 | 7 |

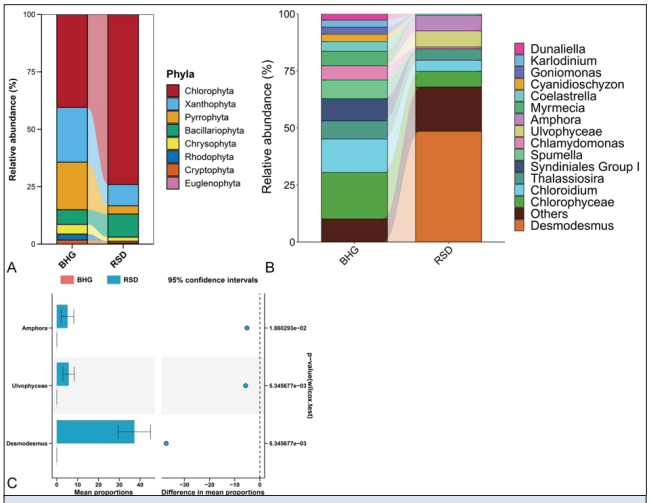


**Fig 2.** Phytoplankton composition in bar-headed goose (BHG) group and ruddy shelduck (RSD) group. **A**- Relative abundance of the dominant phytoplankton phyla in each group, **B**- Relative abundance of the dominant phytoplankton genera in each group, **C**- Phytoplankton composition at the genus level showing inter-group differences

annotated. At the genus level, 62.91% of ASVs were successfully annotated, while at the species level, only a modest 3.76% of ASVs received annotations. The results indicated that the phytoplankton consumed by the BHG and the RSD covered 8 phyla, 32 families, 52 genera, and 7 species. The number of distinct taxonomic levels annotated for each sample was detailed in *Table 3*. At the phylum level *(Fig. 2-A)*, the dominant phytoplankton groups for BHG were *Chlorophyta* (40.48%), *Xanthophyta* (23.86%), and *Pyrrophyta* (20.67%). For RSD, the major phytoplankton groups were *Chlorophyta* (74.1%), *Bacillariophyta* (10.08%), and *Xanthophyta* (9.23%). Furthermore, the comparative analysis at the phylum level revealed that there were no significant differences in the relative abundances of these phyla between the two groups. At the genus level *(Fig. 2-B)*, the dominant phytoplankton groups for BHG were *Chlorophyceae* (20.38%), *Chloroidium* (14.66%), and *Syndiniales Group*

*I* (9.70%). For RSD, the major phytoplankton groups were *Desmodesmus* (48.58%), *Ulvophyceae* (7.00%), and *Amphora* (6.93%). Furthermore, the comparative analysis at the genus level revealed that a total of three genera showed significant differences between the two groups *(Fig. 2-C)*.

## Terrestrial Plant Species Composition and Abundance Statistics

Analysis of the sequencing data for terrestrial plants revealed that the terrestrial food groups consumed by the BHG and the RSD encompassed 1 domain, 1 phylum, 1 class, 19 orders, 34 families, 172 genera, and 261 species *(Table 4)*. The terrestrial food groups for BHG consisted of 17 orders, 26 families, 150 genera, and 223 species, while those for RSD consisted of 15 orders, 21 families, 62 genera, and 91 species. From the statistical results at the taxonomic levels, BHG had higher totals of species at the order, family, genus, and species levels compared to RSD. The analysis at the phylum level revealed that the terrestrial plant composition of both BHG and RSD groups was dominated by *Streptophyta*, with no significant difference in relative abundance observed between the two groups *(Fig. 3-A)*. In the analysis of the BHG group, it was found that the genus exhibiting the highest abundance was *Potentilla* (46.99%), followed by *Elymus* (8.86%) and *Avena* (6.02%), amongst others *(Fig. 3-B)*. In the analysis of the RSD group, it was identified that the genus exhibiting the highest abundance was *Poa* (60.92%), followed by *Potentilla* (18.03%) and *Puccinellia* (13.81%), among others *(Fig. 3-B)*. A total of 31 genera exhibited significant differences in abundance between the two groups. Furthermore, Linear discriminant analysis effect size (LEfSe) analysis was employed to identify taxa exhibiting significantly different abundances between groups. The LDA score plot illustrated that several taxa showed marked distinctions across the compared groups *(Fig. 3-C)*. Specifically, in the BHG group, 18 taxa, including *Symphyllocarpus*, *Barnadesia*, and *Hordeum*, demonstrated statistically significant differences in abundance ($P<0.05$). Similarly, for the RSD group, 18 taxa such as *Poa*, *Poales*, and *Puccinellia* also exhibited statistically significant differences in their abundance levels ($P<0.05$).

## Analysis of Alpha Diversity

The dilution curves and species accumulation curves derived from the sequencing of phytoplankton and terrestrial plant communities indicated that the current sequencing depth is sufficient for the dietary diversity

**Table 4:** *Statistical information on the annotation of each sample to the taxonomic levels of terrestrial plants (phylum, class, order, family, genus, species)*

| Taxonomy | BHG1 | BHG2 | BHG3 | BHG4 | BHG5 | RSD1 | RSD2 | RSD3 | RSD4 | RSD5 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Phylum | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Class | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Order | 10 | 13 | 13 | 9 | 11 | 11 | 12 | 8 | 10 | 13 | 19 |
| Family | 13 | 17 | 17 | 11 | 19 | 14 | 14 | 10 | 12 | 14 | 34 |
| Genus | 59 | 73 | 93 | 32 | 81 | 46 | 41 | 32 | 30 | 35 | 172 |
| Species | 88 | 97 | 127 | 51 | 105 | 63 | 56 | 41 | 42 | 49 | 261 |

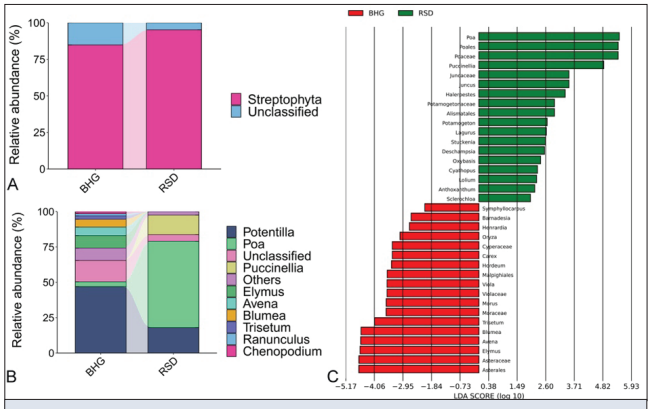


**Fig 3.** Terrestrial plants composition in bar-headed goose (BHG) group and ruddy shelduck (RSD) group. **A**- Relative abundance of the dominant terrestrial plants phyla in each group, **B**- Relative abundance of the dominant terrestrial plants genera in each group, **C**- A plot displaying the LDA scores obtained through LDA analysis (linear discriminant analysis) for taxa that have a significant role in the two groups

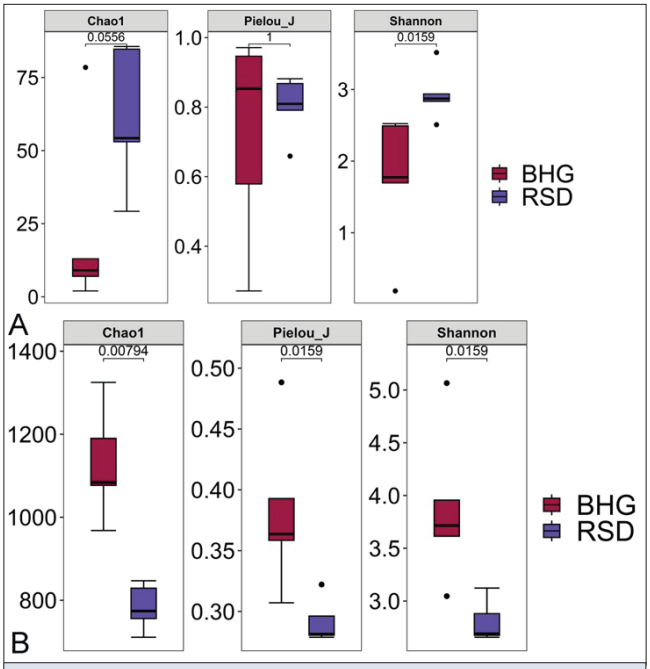


**Fig 4.** Comparison of alpha diversity indices. **A**- A boxplot representing the alpha diversity indices for phytoplankton, **B**- A boxplot representing the alpha diversity indices for terrestrial Plant(s)

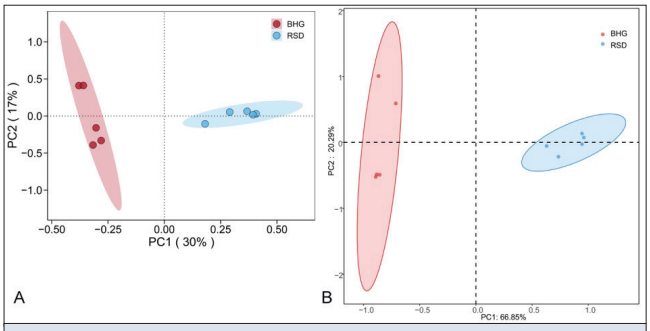


**Fig 5. A**- the Principal Component Analysis (PCoA) plots for phytoplankton, **B**- the Principal Component Analysis (PCoA) plots for terrestrial plants

analysis of the BHG and the RSD. The sample size is adequate for estimating species richness. Box plots of various alpha diversity indices, including the Chao1, Pielou_J, and Shannon indices, were compared between the phytoplankton communities of two groups *(Fig. 4-A)*. These analyses indicated that the phytoplankton communities consumed by RSD had higher community diversity compared to those of BHG. Similarly, alpha diversity indices of terrestrial plant communities were compared between the two groups *(Fig. 4-B)*. These results indicated that the terrestrial plant communities consumed by BHG had a higher number of species, greater community diversity, and more even species distribution, without any single species dominating the community.

## Beta Diversity Analysis

PCoA was performed on the phytoplankton and terrestrial plant communities of BHG and RSD. In the

**Table 5:** *Statistical information on the annotation of each sample to the taxonomic levels of zooplankton (phylum, family, genus, species).*

| Taxonomy | BHG1 | BHG2 | BHG3 | BHG4 | BHG5 | RSD1 | RSD2 | RSD3 | RSD4 | RSD5 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Phylum | 3 | 5 | 4 | 5 | 5 | 7 | 4 | 3 | 4 | 6 | 9 |
| Family | 6 | 11 | 7 | 8 | 15 | 16 | 7 | 7 | 8 | 14 | 31 |
| Genus | 7 | 8 | 6 | 5 | 14 | 15 | 6 | 7 | 8 | 10 | 35 |
| Species | 2 | 1 | 1 | 1 | 1 | 3 | 3 | 2 | 2 | 2 | 8 |

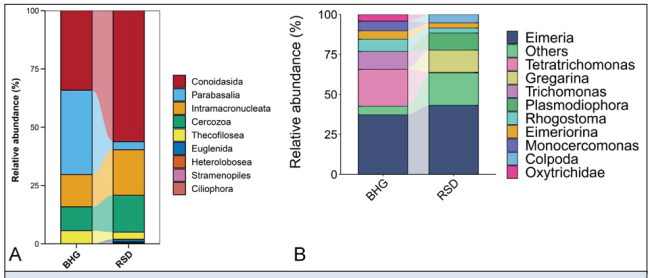


**Fig 6. A**- Relative abundance of the dominant zooplankton phyla, B- relative abundance of the dominant genera in each group

PCoA analysis of phytoplankton communities *(Fig. 5-A)*, the first principal component explained 30% of the variation, and the second principal component explained 17%. The samples from RSD and BHG were distantly located, indicating low similarity between the community compositions. Further ANOSIM analysis indicated that the differences between RSD and BHG were greater than the differences within each group for phytoplankton communities (R=0.556, P=0.015) and that these differences were statistically significant.In the PCA analysis of terrestrial plant communities, the first principal component explained 66.85% of the variation, and the second principal component explained 20.29% *(Fig. 5-B)*. The samples from RSD and BHG were again distantly located, indicating low similarity between the community compositions. For terrestrial plant communities, ANOSIM analysis also showed that the differences between RSD and BHG were greater than the differences within each group (R=0.596, P=0.007) and were statistically significant.

### Parasite and Protozoan Composition Analysis

A total of 401 ASVs were annotated to zooplankton. The sequencing results indicated that the protozoans and parasites in BHG and RSD covered 9 phyla, 31 families, 35 genera, and 8 species *(Table 5)*. At the phylum level *(Fig. 6-A)*, the dominant phyla for BHG were *Parabasalia* (36.2%), *Conoidasida* (34.12%), and *Intramacronucleata* (13.81%). For RSD, the dominant phyla were *Conoidasida* (56.19%), *Intramacronucleata* (19.58%), and *Cercozoa* (15.77%). The top 10 genera *(Fig. 6-B)* revealed that the parasites in BHG were predominantly by *Eimeria sp.* and *Tetratrichomonas sp.*, which are common endoparasites in vertebrates. In RSD, the parasites were mainly *Eimeria* sp., along with some endoparasitic protozoa genera that infect animals *(Gregarina* sp.) and plants *(Plasmodiophora* sp.).

## Discussion

This study used an environmental DNA (eDNA) metabarcoding method for the first time. With two primer pairs, it determined the phytoplankton and terrestrial plant diet compositions of BHG and RSD on the Qinghai-Tibet Plateau in China. The results revealed that the BHG primarily fed on *Chlorophyta*, *Xanthophyta*, and *Pyrrophyta* in phytoplankton, while it showed a preference for *Potentilla* within *Streptophyta* in its terrestrial diet. In contrast, the Ruddy Shelduck predominantly consumed *Chlorophyta* and *Bacillariophyta* in phytoplankton, and mainly fed on *Poa* within *Streptophyta* in its terrestrial diet. Although the experimental results revealed the presence of zooplankton group sequences, the annotations were primarily dominated by protists. These included several single-celled eukaryotes *(Heterolobosea, Cercozoa, Ciliophora, Euglenida)* and parasitic organisms *(Parabasalia, Conoidasida)*. However, this did not necessarily indicate that these organisms were directly consumed by the two waterbird species. Both the BHG, an omnivorous waterbird that feeds primarily on seeds of herbaceous plants -including grasses, leaves, roots, tubers, grains, and nuts- but also consumes small fish and aquatic macro-invertebrates when seed availability is limited [21], and the RSD, another omnivorous waterbird that adjusts its diet according to seasonal and habitat-specific food resources [18], are unlikely to directly prey on protists. Instead, they are more likely to indirectly ingest these microorganisms through intermediate hosts such as small fish, aquatic invertebrates, mollusks, or other planktonic organisms While these protozoa cannot serve as direct food sources for these waterfowl, they play a critical role in aquatic ecosystems as primary producers, supporting the base of the food chain [22,23]. Based on the current findings, future studies should employ stable isotope analysis and behavioral observations [24] to directly verify the types of zooplankton consumed by these two waterbird species and their utilization of resources at different trophic levels. In addition to the dominant eukaryotic protist group *Cercozoa* found in free-living aquatic environments [25], the identification of these zooplankton groups provided additional information about parasitic taxa. *Parabasalia* and *Conoidasida* were most abundant in BHG, while *Conoidasida* and *Intramacronucleata* were most abundant in RSD. Among these, *Eimeria sp.*, a common endoparasite in vertebrates [26], had relatively high abundance. Additionally, the composition of parasites was found to be highly correlated with diet composition [27]. Therefore, further research was suggested to investigate

the relationship between the diet composition and parasite load in the BHG and RSD, which could provide insights into their health status.

The selection of fecal samples from BHGs and RSDs for dietary analysis and interspecific comparison was methodologically justified, as both species are omnivorous waterbirds exhibiting highly congruent habitat preferences [20]. However, two species with identical niches cannot stably coexist in the same environment for a long time [28]. To achieve stable coexistence, species had to differentiate their niches in aspects like time, space, or food. They could reduce resource competition and realize trophic niche segregation by selecting different food, staggering activity times, or occupying different locations [29]. At the taxonomic level, zooplankton exhibited significantly more ASVs than phytoplankton (401 vs. 213), suggesting that both waterfowl species preferred zooplankton when feeding on plankton. This preference likely resulted from the ingestion of zooplankton while drinking water [30], or due to trophic interactions within the food chain. Alpha-diversity analysis revealed that RSD had a higher number of species and a more even distribution in their phytoplankton food sources, indicating efficient utilization of diverse phytoplankton resources. In contrast, BHG exhibited richer species numbers and higher community diversity in terrestrial plant food sources. These findings suggest that BHG and RSD reduce resource competition and achieve trophic niche segregation by selecting different food sources or varying the intake amounts of the same food source. This allows them to coexist under limited resources and maintain ecosystem diversity and balance.

Birds' food choices mirror their habitat use. Food resources greatly impact birds' reproduction and habitat selection, and are closely linked to birds' energy needs and habitat resource abundance [31]. According to the optimal foraging theory, foragers seek food options that offer the highest energy returns [32]. RSD's preference for zooplankton may stem from these foods' high protein and energy content, meeting the species' needs during rapid growth or reproduction. In contrast, BHG's preference for terrestrial plants could be due to the stable supply of these foods, supporting their nutritional requirements across seasons and reproductive cycles. These dietary differences reflect divergent habitat resource use and niche partitioning, thereby reducing interspecific competition and promoting coexistence. The specific reasons for these differences remained unclear. However, PCoA analyses indicated significant dietary differences between the two waterfowl species, showing that they occupied distinct ecological niches. The niche differentiation hypothesis posited that coexisting species in the same geographic area could reduce interspecific competition and promote coexistence and ecosystem stability through niche differentiation, despite similarities in morphology, behavior, or resource use [33].

Limitations within this study warrant acknowledgment. Firstly, the restricted sample size may compromise the precision of certain conclusions. Secondly, no primer set can perfectly amplify DNA from every species, despite some primer combinations showing high efficacy under specific conditions. Even with the design of two primer sets for amplification in this study, the ideal species results for zooplankton consumed by the two waterbird species were not obtained. Furthermore, since these waterbirds are not obligate specialists, they incidentally ingest non-target items such as plants or zooplankton during feeding, which might be taken up with water. The presence of these species in fecal samples and their detection could influence the interpretation of the final results.

In summary, this study employed environmental DNA (eDNA) metabarcoding using two primer pairs to comprehensively investigate the dietary composition and feeding preferences of the BHG and the RSD, as well as to analyze the parasite species present in both species. Through comparative analysis of their diets, we have uncovered differences in their utilization of trophic niches. Although the precise causes of these distinctions remain to be fully elucidated, these findings significantly enhance understanding of avian dietary adaptability and ecological strategies. They also showcase a novel perspective and methodology for studying wildlife diets using modern molecular biology techniques. The application of environmental DNA metabarcoding has not only improved the accuracy and efficiency of dietary analysis but also provided a powerful tool for monitoring biodiversity, assessing ecosystem health, and informing conservation management strategies. Moving forward, interdisciplinary research combining behavioral ecology, physiological ecology, genetics, and other fields will further unravel the ecological and evolutionary mechanisms underlying avian feeding choices.

## Declarations

**Availability of Data and Materials:** The data given in this study may be obtained from the corresponding auther (W W) on reasonable request.

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**Ethical Approval:** This study conformed to the guidelines for the care and use of experimental animals established by the Ministry of Science and Technology of the People's Republic of China (Approval number: 2006-398).

**Competing Interests:** The authors declared that there is no conflict of interest.

**Declaration of Generative Artificial Intelligence (AI):** The authors declare that the article, tables and figures were not written/created by AI and AI-assisted Technologies.

**Author Contributions:** WW conceived and designed the study. WW and YGD drafted the manuscript and provided critical revisions. YGD, YL H, FY, XLW, and ZMLC performed the experiments and analyzed the data. All authors read and approved the final manuscript.

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