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Regular research paper

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## STABLE CARBON AND NITROGEN ISOTOPES REVEAL THE FOOD DIET OF TIBETAN ANTELOPE, *PANTHOLOPS HODGSONI* (ABEL 1826) IN KEKEXILI NATURE RESERVE (CHINA)

**ABSTRACT:** The stable isotope technique has been widely used to infer the dietary ecology of a range of animal species. The  $\delta^{13}\text{C}$  technique provides a valuable tool for researchers when designing pastures for dual environmental and production purposes. Tibetan antelope, *Pantholops hodgsoni* (chiru), is endemic species to the high-altitude Qinghai-Tibet Plateau of western China – Kekexili Nature Reserve area. The aim of this study was to identify the food diet of Tibetan antelope among plants with  $\text{C}_3$  and  $\text{C}_4$  photosynthetic pathways. Faeces and plant samples were collected at the Kekexili Nature Reserve (KNR,  $34^\circ 19' \sim 36^\circ 16' \text{N}$ ,  $89^\circ 25' \sim 94^\circ 05' \text{E}$ ) in Qinghai Province, China. Stable isotope values of carbon and nitrogen of faeces and plant samples were measured under EAMS (element-analysis meter and spectrometer) conditions. Enrichment and food content ratio were calculated according to previous research methods to determine the relative importance of plant sources in the food diet of Tibetan antelopes. The results indicate that faecal samples provided the most convenient and uninjurious sources to predict the food diet and that  $\text{C}_3$  plants were selected as the food by chiru. Dual-isotope multiple-source mixing model suggested that the food content of antelope is including Gramineae, Cyperaceae, Compositae, Leguminosae, and Cruciferae. An understanding of what chiru selects allows for development of appropriate grazing and protecting strategies, especially in fragile ecosystem. According to our knowledge,

this is the first essay to reveal the food diet of chiru with stable isotope analysis method.

**KEY WORDS:** Tibetan antelope, stable isotope technique, food diet, Kekexili area

### 1. INTRODUCTION

Although direct observations of feeding behaviour and rumen content analyses can gain valuable dietary data, these techniques are often practically difficult and provide only limited information on cumulative patterns over long time periods (McNabb *et al.* 2001). A gut content analysis is not applicable to species which occurs on the verge of extinct, such as Tibetan antelopes. So, ecologists and paleontologist have shown increasing interest in stable isotope analysis, both to trace pathways of organic matter (particularly C and N) in food webs, and to determine the contributions of various food items to organisms' diets (Hesslein *et al.* 1993, Fry 2006). Early laboratory studies have shown that C and N isotope ratios in tissues of organisms closely resemble those in their diets (De Niro and Epstein 1981). So, analysis of stable isotope values from the tissues of mammals has proven to be a useful method for inferring the dietary ecology of a range of farmed, wild and extinct animal species (Norman *et al.* 2009).

Using of stable isotope analysis method, faecal and rumen samples were the best samples to reflect short-term dietary changes in sheep consuming different proportions of C<sub>3</sub> and C<sub>4</sub> plants. In practical terms, faecal samples were easier to obtain, more practically to carry out and less stressful to the extinctive animal than collecting rumen samples. The most accurate back-calculation method for faecal samples accounted for diet-tissue discrimination and differences in the indigestibility between the C<sub>3</sub> and C<sub>4</sub> forage (Norman *et al.* 2009).

Tibetan antelope, *Pantholops hodgsoni*, is the species endemic to the high-altitude Qinghai-Tibet Plateau of western China (Schaller 1998). Historical accounts indicate that very large populations of Tibetan antelope ranged across an area of 2,500,000 km<sup>2</sup> in the early 20<sup>th</sup> century. Illegal hunting for both meat and wool resulted in sharp declines in abundance and distribution. Current population estimates of Tibetan antelope are ~75,000 individuals and they are classified as endangered by IUCN (2001) and listed in Appendix I of CITES. The distribution of Tibetan antelope is now restricted to remote areas in the Tibetan autonomous region (TAR), Qinghai province and the Xinjiang autonomous region and the largest remaining populations are found in the Chang Tang reserve of northern TAR and the adjacent Kekexili nature reserve (KNR) in Qinghai (Schaller 1998).

Tibetan antelopes are migratory across much of their range (Schaller 1998, Lian *et al.* 2005). In late June, adult and yearling females gather into migratory groups of up to 600 individuals and cross the Qinghai-Tibet highway to calving grounds near Zhuonai Lake and then return with lambs in early August (Lian *et al.* 2005). This period is critical for the antelope population because of the vulnerability of lambs to predators, the severe weather and the high energetic demands of migration, in addition to continuing problems of illegal hunting (Wong 1998, Xi and Wang 2004, Schaller *et al.* 2006).

We measured stable isotope ratios of carbon and nitrogen of plant species and faeces of Tibetan antelopes in KNR to determine the relative importance of plant sources in the food web. The sites also provided a key-point region to other ungulates. We included

samples of the flora, which were used by other ungulates, integrating the food sources in this region.

## 2. STUDY AREA

The study area is adjacent to the Qinghai-Tibet railway which forms the south-eastern boundary of the Kekexili Nature Reserve – KNR (34°19' ~ 36°16'N, 89°25' ~ 94°05'E) in Qinghai Province, China. The KNR, uninhabited by humans because of its remoteness, high elevation and harsh climate, occupies about 45,000 km<sup>2</sup> in western Qinghai Province on the border of TAR and the Xinjiang autonomous region. The average elevation is 4,600 m with a range from 4200 to 6860 m. The average annual temperature is -5.6°C and 69% of total precipitation (262.2 mm) falls during June-August (Zhang 1996).

Wild ungulates in KNR include Tibetan antelope, Tibetan gazelle *Procapra picticaudata* Hodgson, kiang *Equus kiang* Moorcroft, and wild yak *Bos grunniens* Linnaeus and are protected from hunting by national law (Schaller *et al.* 1991, Zheng 1994).

Domestic yak and sheep *Ovis aries* Linnaeus are herded by nomadic pastoralists in the study area. The most significant mammalian predator of Tibetan antelope within KNR is the wolf *Canis lupus* Linnaeus, which is relatively common, and the lynx *Felis lynx* Linnaeus and the brown bear *Ursus arctos* Linnaeus, which are much rarer. Large raptors including upland buzzard *Buteo hemilasius* Temminck, cinereous vulture *Aegypius monachus* Linnaeus and lammergeier *Gypaetus barbatus* Linnaeus are common in KNR and are frequent scavengers of dead antelope and other carrion.

## 3. METHODS

### 3.1. Plant sample collection

A sample line method was used to collect the plant samples. Five 2-m<sup>2</sup> in wide and 50-m in long cingulum were sampled randomly within the study area. The plant material was dugged integrally and sampled then, and they were sorted by species separately after 48 h of oven drying at 60°C. The samples were thoroughly cleaned of any soil contamination

before rubbing. Plant samples were sifted out by 25, 60, 100, and 200-mesh in turn. Then, the samples were collected airproofed in hermetic bags.

### 3.2. Analysis of stable carbon and nitrogen isotopes

Faeces samples were collected from individual animal species for isotope analysis within the KNR. Fresh faeces samples were collected only and were hermetically saved separately at  $-20^{\circ}\text{C}$ . Samples contained in tin boat were dispatched to isotope ratio mass online spectrometer under EAMS (element-analysis meter and spectrometer) conditions. Operation conditions: oxidizing furnace temperature is  $900^{\circ}\text{C}$ ; reducing furnace is  $680^{\circ}\text{C}$ ; pillar temperature is  $40^{\circ}\text{C}$ . The resulting  $\text{CO}_2$  was purified in a vacuum line and injected in a Finnigan Delta plus XL mass spectrometer (Finnigan Mat, Bremen, Germany) fitted with double inlet and collector systems respectively. Standards consisted of the Peedee Belemnite (PDB) formation from South Carolina, USA. Five state classed standards (black carbon) were intervened between every ten samples to make the precision  $<0.2\%$  and meet international standards. Ratios of  $^{13}\text{C}/^{12}\text{C}$  and  $^{15}\text{N}/^{14}\text{N}$  were expressed as the relative difference ( $\%$ ) between the sample and conventional standards as:

$$X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000 \quad (1)$$

where:  $X = \delta^{13}\text{C}$  or  $\delta^{15}\text{N}$  and  $R = ^{13}\text{C}/^{12}\text{C}$ ,  $^{15}\text{N}/^{14}\text{N}$ . All isotope values reported here are relative to the V-PDB standard. The overall (sample preparation plus analysis) analytical precision is  $\pm 0.2\%$ .

### 3.3. Estimation of enrichment factor and food content ratio

Enrichment factor of C, N isotope was calculated by formula as follows:

$$C_{\text{enrichment}} = (C_{\text{top}} - C_{\text{producer}}) / (TP_{\text{top}} - 1) \quad (2)$$

$$N_{\text{enrichment}} = (N_{\text{top}} - N_{\text{producer}}) / (TP_{\text{top}} - 1) \quad (3)$$

where:  $C_{\text{enrichment}}$ ,  $N_{\text{enrichment}}$  indicates the

enrichment / fractionation factor of stable isotope C and N in ecosystem, separately.  $C_{\text{top}}$  and  $C_{\text{producer}}$  denote the Carbon stable isotope ratio of top consumer and primary producer.  $N_{\text{top}}$  and  $N_{\text{producer}}$  denote the nitrogen stable isotope ratio of top consumer and primary producer.  $TP_{\text{top}}$  denote the trophic position of top consumer.

Dual-isotope multiple-source mixing model was used to estimate the food content ratio (FCR) of A in animal food.

$$\text{FCR}_i = (Z_{\text{Ai}} - 1 / \sum Z_{\text{Ai}} - 1) \times 100 \quad (4)$$

where:  $Z_{\text{Ai}}$  indicates the ratio between  $A_i$  and animal.  $Z$  value was calculated by the formula as follow:

$$Z = \sqrt{x^2 + y^2} \quad (5)$$

where:  $Z$  indicates the O's distance between revised animal stable isotope value and stable isotope values of each food content.  $X$  indicates the margin  $\delta^{13}\text{C}$  value of each food content minus by  $\delta^{13}\text{C}$  value of animal.  $Y$  indicates the margin  $\delta^{15}\text{N}$  value of each food content minus by  $\delta^{15}\text{N}$  value of animal. The above formulas were used by Yi and Zhang (2005).

### 3.4. Data analysis

All the data were analyzed using SPSS (Statistical Package for Social Scientists) for windows 15.0. Data were expressed as mean  $\pm$  SD. Stable carbon isotope ratios were determined whether there were significant differences between animal species by independent-samples t-test or one-sample t-test. All tests were two-tailed.  $P < 0.05$  was the significant level.

## 4. RESULTS

### 4.1. Stable isotope value of plants in Kekexili protected area

Thirty-eight kinds of plant species, which belonged to 5 families and 15 genera, were collected. Carbon and nitrogen stable isotopes values were measured. The result showed that all plant species were remained with  $C_3$  plant, *i.e.*, no  $C_4$  plants were found at Chumaer River area (Table 1). One-sample

T test was used to analyze the stable isotopes value among different plant species, there was significantly inter-specific difference of  $\delta^{13}\text{C}$  value ( $t = -161.145$ ,  $df = 37$ ,  $P < 0.001$ ) and insignificantly inter-specific difference of  $\delta^{15}\text{N}$  value ( $t = 0.607$ ,  $df = 37$ ,  $P > 0.05$ ).

The difference of stable isotopes among plants in the same family, which has 3 kinds of species at least, was analyzed at the same time. There were very significant differences of  $\delta^{13}\text{C}$  in Gramineae ( $t = -76.721$ ,  $P = 0.000$ ), Cyperaceae ( $t = -84.661$ ,  $P = 0.000$ ), Compositae ( $t = -42.375$ ,  $P = 0.000$ ), Cruciferae ( $t = -102.993$ ,  $P = 0.000$ ), Leguminosae ( $t = -96.191$ ,  $P = 0.000$ ). Except the Leguminosae ( $t = -4.082$ ,  $P = 0.027$ ), there was no difference in other four families (Gramineae:  $t = -0.31$ ,  $P > 0.05$ ; Cyperaceae:  $t = 0.107$ ,  $P > 0.05$ ; Compositae:  $t = -0.122$ ,  $P > 0.05$ ; Cruciferae:  $t = 0.998$ ,  $P > 0.05$ ) in  $\delta^{15}\text{N}$  value. The average  $\delta^{13}\text{C}$  value of Gramineae was the highest ( $-24.935 \pm 0.7961$ ), however, the average  $\delta^{13}\text{C}$  value of Leguminosae was the lowest

( $-25.985 \pm 0.5403$ ). The average  $\delta^{15}\text{N}$  value of Cruciferae was the highest ( $1.4933 \pm 3.6651$ ), however, the average  $\delta^{15}\text{N}$  value of Leguminosae was the lowest ( $-1.045 \pm 0.512$ ) (Fig. 1).

#### 4.2. Food diet of Tibetan antelope at species and family level

According to the analysis of SOURCE combined with FCR calculation (formula (4)), there were 3 kinds of plant species whose FCR value exceeding 10%. They were *Festuca rubra* Linn., *Leontopodium pusillum* Hand.-Mazz., and *Carex ivanovae* Egorova. There were 3 kinds of plant species whose FCR value exceeding 5%. They were *Festuca coelestis* St.-Yves, *Kobresia humilis* Serg., and *Oxytropis glacialis* Benth. ex Bunge. Other plants' FRC values were relatively lower (Table 2). There was very significant inter-species difference in FCR value ( $t = 5.39$ ,  $P < 0.01$ ).

In family level, food content of antelope includes Gramineae, Cyperaceae, Composi-

Table 1. The stable isotope value of plant species in Kekexili area.

Sample size	Species	$\Delta^{13}\text{C}$	$\Delta^{15}\text{N}$
1	<i>Parrya pulvinata</i> M. Popov	-25.40	-3.52
1	<i>Saussurea gossypiphora</i> D. Don	-27.65	-3.34
4	<i>Festuca ovina</i> Linn	$-25.98 \pm 0.02$	$-3.28 \pm 0.07$
3	<i>Koeleria cristata</i> Linn	$-24.60 \pm 0.01$	$-2.55 \pm 0.03$
2	<i>Artemisia frigida</i> Willd	$-26.02 \pm 0.04$	$-2.30 \pm 0.03$
2	<i>Festuca coelestis</i> Krecz. et Bobr.	$-25.18 \pm 0.01$	$-2.10 \pm 0.01$
3	<i>Festuca rubra</i> Linn	$-25.63 \pm 0.01$	$-1.97 \pm 0.02$
1	<i>Leontopodium pusillum</i> Hand.-Mazz.	-25.83	-1.97
1	<i>Oxytropis glacialis</i> Benth. ex Bunge	-26.27	-1.50
1	<i>Oxytropis falcate</i> Bunge	-26.32	-1.33
3	<i>Carex ivanovae</i> Egorova	$-25.74 \pm 0.03$	$-1.32 \pm 0.01$
2	<i>Thermopsis lanceolata</i> R.Br	$-25.18 \pm 0.02$	$-1.01 \pm 0.03$
4	<i>Kobresia humilis</i> Serg.	$-25.93 \pm 0.02$	$-1.01 \pm 0.01$
2	<i>Ajania khartensis</i> Dunn	$-27.93 \pm 0.06$	$-0.77 \pm 0.03$
2	<i>Saussurea melanotrica</i> Hand.-Mazz.	$-25.57 \pm 0.05$	$-0.45 \pm 0.02$
1	<i>Astragalus polycladus</i> Bur.et Franch.	-26.17	-0.34
3	<i>Poa psiolepis</i> Keng	$-24.47 \pm 0.04$	$-0.29 \pm 0.02$
2	<i>Blysmus compressus</i> Linn	$-24.84 \pm 0.02$	$0.08 \pm 0.01$
2	<i>Poa malaca</i> Keng	$-24.46 \pm 0.02$	$0.30 \pm 0.01$
1	<i>Torulularia humilis</i> C. A. Mey.	-26.83	0.38
1	<i>Neslia paniculata</i> Linn	-25.53	0.48
1	<i>Arctogeron gramineum</i> Linn	-23.26	3.24
1	<i>Saussurea arenaria</i> Maxim	-24.73	4.63
4	<i>Poa indattenuata</i> Keng ex L.Liou.	$-23.89 \pm 0.02$	$5.08 \pm 0.08$
3	<i>Carex moorcroftii</i> Falc.ex Boott	$-24.51 \pm 0.04$	$5.56 \pm 0.06$
1	<i>Phaeonychium parryoides</i> Kurz ex Hook. f. & T. Anderson	-25.37	7.68

Table 2. Food plant composition ratio (FCR) (formula (4) of Tibetan antelope in Kekexili area.

Species	FCR	Species	FCR
<i>Festuca rubra</i> Linn	13.11	<i>Neslia paniculata</i> Linn	2.30
<i>Leontopodium pusillum</i> Hand.-Mazz.	12.08	<i>Poa malaca</i> Keng	2.12
<i>Carex ivanovae</i> Egorova	10.73	<i>Torularia humilis</i> C. A. Mey	2.05
<i>Festuca coelestis</i> Krecz. et Bobr.	8.21	<i>Saussurea melanotrica</i> Hand. -Mazz	2.04
<i>Kobresia humilis</i> Serg	6.17	<i>Festuca ovina</i> Linn	1.94
<i>Oxytropis glacialis</i> Benth. ex Bunge	5.50	<i>Ajania khartensis</i> Dunn	1.46
<i>Oxytropis falcate</i> Bunge	4.95	<i>Saussurea gossypiphora</i> D. Don	1.29
<i>Koeleria cristata</i> Linn	4.19	<i>Poa psiolepis</i> Keng	1.28
<i>Artemisia frigida</i> Willd	3.95	<i>Arctogeron gramineum</i> Linn	0.81
<i>Thermopsis lanceolata</i> R.Br	3.25	<i>Saussurea arenaria</i> Maxim	0.69
<i>Parrya pulvinata</i> M. Popov	3.04	<i>Poa indattenuata</i> Keng ex L.Liou.	0.63
<i>Astragalus polycladus</i> Bur.et Franch.	2.75	<i>Carex moorcroftii</i> Falc.ex Boott	0.60
<i>Blysmus compressus</i> Linn	2.58	<i>Phaeonychium parryoides</i> Kurz ex Hook. f. & T. Anderson	0.46

Note: There was very significant inter-species difference among values of food composition ratio (FCR) in Kekexili area ( $t = 5.39$ ,  $P < 0.01$ ).

tae, Leguminosae, and Cruciferae, which account for 98.18% of total food sources, other plants occupied only a little part (Fig. 2). Simultaneously, the proportion of 5 families in the food was different as well as the difference in FCR among these families was significant ( $t = 5.708$ ,  $P < 0.01$ ).

## 5. DISCUSSION

The stable carbon isotope technique can be used to back-calculate accurately the relative proportion of food in a sheep's diet. For all of the samples collected in this study there was a positive relationship between  $\delta^{13}\text{C}$  values of the diet and  $\delta^{13}\text{C}$  of the sample ( $R^2 = 0.703$ ,  $P = 0.002$ ). To our knowledge this is the first time that Tibetan antelopes were used as experimental animals to predict food diet with stable isotope analysis. To estimate diet selection accurately and simply provides a tool for design of grazing systems and *ex situ* protection which is an appropriate combination of forages to optimize feeding value and endangered species protection.

The stable carbon isotope technique is reliant on differences in carbon isotope accumulation in plants with different photosynthetic pathways. The  $\text{C}_3$  of photosynthesis discriminates against  $^{13}\text{C}$  in favour of  $^{12}\text{C}$  considerably more than the  $\text{C}_4$  pathway. This leads to different isotope ratios in the plant biomass and influences isotope ratios throughout the food webs. Deviations

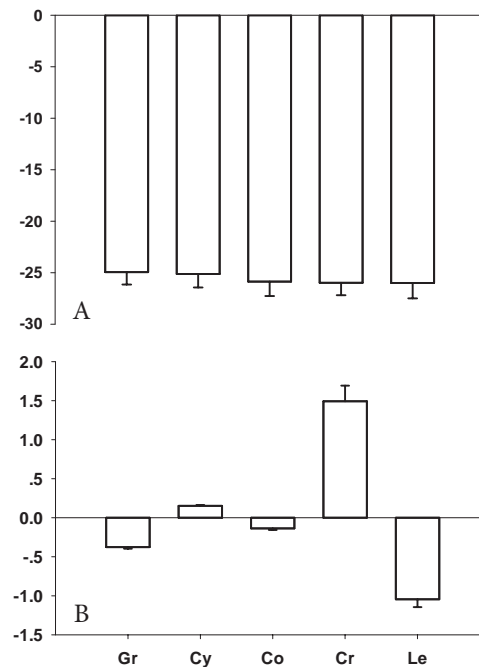


Fig. 1. Comparison (Mean  $\pm$  SEM) of carbon (A) and nitrogen (B) stable isotope value among families in Kekexili area. Gr – Gramineae; Cy – Cyperaceae; Co – Compositae; Cr – Cruciferae; Le – Leguminosae.

in carbon isotope ratios are expressed as  $\delta^{13}\text{C}$  and measured as deviations in parts per thousand from the isotope ratio in a standard carbonate (Lerman 1975). Biomass of  $\text{C}_3$  plants has  $\delta^{13}\text{C}$  values of about  $-28\text{‰}$  (range of  $-20\text{‰}$  to  $-35\text{‰}$ ) while that of  $\text{C}_4$  plants has values of  $-12\text{‰}$  (range of  $-9\text{‰}$  to  $-16\text{‰}$ ) (Deines 1980, O'Leary 1988). In our study,



the  $\delta^{13}\text{C}$  value was ranged from  $-27.93$  to  $-23.26$  at Chumaer River area (Table 1). The results indicated that all plant species were  $\text{C}_3$  plants, that's to say, no  $\text{C}_4$  plants were found at Chumaer River area. The similar research carried out in Tibetan Plateau found that there was no  $\text{C}_4$  plant species in Haibei station (Yi and Zhang 2005).

Analysis of isotopic values in the scat of Tibetan antelopes from KRA revealed significant comparability with faecal micro-histological analysis (FMA) results. Similarities in the food diet obtained from stable isotope analysis and FMA were very high, the main food is including 4 plant families: Poaceae, Cyperaceae, Compositae, and Leguminosae (Cao *et al.* 2008), and disliking in Brassicaceae proportion. Based on the scat, the antelopes appear to have the wider range of resource use. Part of plant species, like *Thermopsis lanceolata* Linn and *Torularia humilis* Botsch, have occupied much higher proportion than others in Tibetan antelope's food diet (Table 2).

Our results are from a single point in time, in summer in the rainy season. We can expect that there could be seasonal variations in isotope signatures, because in northern Tibet,  $\delta^{13}\text{C}$  increases by 0.46% with an increase of 100 mm in precipitation, while in the southern plateau  $\delta^{13}\text{C}$  decreases by 1.82% with an increase of 100 mm in precipitation (Guo and Xie 2006). At the same time, Tibetan antelopes migrate across much of their range. Previous field surveys in western Qinghai have demonstrated that there is a traditional migratory route from the southern winter range in Qumalai county to northern calving grounds within the KNR near Zhuonai Lake

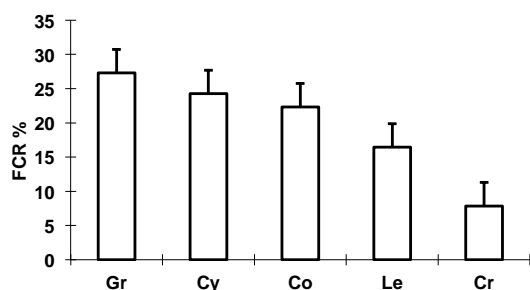


Fig. 2. Food composition (Mean  $\pm$  SEM) of Tibetan antelope at family level in Kekexili area. FCR: food composition ratio (formula (4)). Gr – Gramineae; Cy – Cyperaceae; Co – Compositae; Cr – Cruciferae; Le – Leguminosae.

(Schaller 1998, Lian *et al.* 2005). The Qinghai – Tibetan railway Chumaer River section was the unique migratory road of Tibetan antelope. There are lots of wild animal passages, which provide channels to wild animals, under the railway. And the wild animals are used to the railway (Yang and Xia 2008). Before traversing the Qinghai – Tibetan railway, antelopes would wait for other individuals for a long time and then they passed the railway. So, faecal and plant samples were collected on the other site of the railway. It can reflect the food content of chiru from this area.

Chemical analysis of food materials stored by the grass and large herbivorous studied here has confirmed that  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  status of termite faeces accurately reflects the composition of Tibetan antelope diet. However, care must be taken in inter-habitat comparisons due to difference in  $\delta^{15}\text{N}$  values among ungulates distributed in local environments. Relative researches about other ungulates would help to understand of the competition for sources, and then provide strategy for endangered species.

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