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Recovery and germination of seeds after passage through the gut of Kazakh sheep on the north slope of the Tianshan Mountains

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Abstract

Endozoochorous dispersal of seeds by livestock has long attracted the attention of grassland scientists. However, little is known about seed dispersal after ingestion by Kazakh sheep on dry grasslands in the Tianshan Mountains. The objective of this experiment was to learn more about the recovery and germinability of seeds from 17 plant species after either actual or simulated ingestion (i.e. insertion through a rumen fistula) by Kazakh sheep. The passage time of seeds through the sheep gut ranged from 12 to 96 h. More than 80% of all recovered seeds were defecated 24–48 h after ingestion. The mean retention time of seeds in the gut ranged from 27.3 to 42.2 h. Seed recovery percentage ranged between 12.6 and 17.6% for leguminous species and between 0.8 and 3.2% for gramineous species. Seed recovery percentage was positively correlated with seed mass, but negatively correlated with seed shape. The germination percentages of the gramineous species were greater in the non-ingested treatment (66–98%) than in the simulated ingestion treatment (3–10%). In contrast, for leguminous species, seed germination percentages were greater in the simulated ingestion treatment (23–70%) than in the non-ingested one (5–12%). Seed germination percentage after simulated ingestion was positively correlated with seed mass, but negatively correlated with seed shape. In conclusion, leguminous seeds were more likely than gramineous ones to pass through the gut of Kazakh sheep and then germinate. Free-ranging Kazakh sheep can contribute to the spread of plant species, especially leguminous species, in the Tianshan Mountains.

Keywords: dry grassland, gut retention time, seed recovery, seed germination, seed dispersal

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Introduction

Grazing livestock are one of the most important endozoochorous seed dispersal vectors in grasslands (Archer and Pyke, 1991; Gökbulak and Call, 2009). Seeds can be retained for long periods in the gut of livestock, thus allowing rapid seed dispersal as the animals travel (Pakeman, 2001; Mouissie *et al.*, 2005b; Cosyns and Hoffmann, 2005). Several reports indicate that seedling emergence and growth are promoted by organic matter and nutrients in livestock dung (Woldu and Saleem, 2000; Traveset *et al.*, 2001; Nchanji and Plumptre, 2003). However, other studies indicate that dung can suppress seedling development, especially during early growth stages (Uytvanck *et al.*, 2010; Milotić and Hoffman, 2016). Overall, seed ingestion by livestock can increase species richness and affect large-scale spatial community composition in grazed systems by intensifying intercommunity seed flow (Malo *et al.*, 2000; Cosyns *et al.*, 2005a).

Significant attention has been paid to the role of grazing mammals as endozoochorous dispersers of dry-fruited seeds after Janzen (1984) proposed the 'foliage is the fruit' hypothesis. Researchers have investigated the role of many ruminants in seed dispersal, including cattle (Doucette and Mccaughey, 2001; Gökbulak and Call, 2009), goat (Baraza and Valiente-Banuet, 2008; Mancilla-Leytón *et al.*, 2011), sheep (Manzano *et al.*, 2005), sika deer (Ishikawa, 2010), yak (Yu *et al.*, 2012), and Tibetan sheep (Yu *et al.*, 2012). However, little is known about the dispersal of seeds after ingestion by Kazakh sheep grazing on dry grasslands. Compared with other breeds, Kazakh sheep have evolved special adaptations to the harsh environments in which they live (Adeli and Chen, 2008). Kazakh sheep traditionally graze on dry grasslands on the north slope of the Tianshan Mountains in autumn and winter when plant seeds are mature. There are currently 5 million Kazakh sheep on the north slope of the Tianshan Mountains (Jia and Wang, 2013).

The retention time of seeds in the digestive tract varies, depending on seed traits as well as on the type of animal (Gökbulak, 2003). Blackshaw and Rode (1991) reported that small seeds passed through the digestive tract of cattle faster than large seeds; however, this relationship was not observed by either Simao and Jones (1987) or Gökbulak and Call (2009). Digestion can enhance or reduce germination depending on seed traits (Simao and Jones, 1987). Passage through the gut of domestic goats greatly increased the germination of leguminous seeds but reduced the germination of gramineous seeds (Baraza and Valiente-Banuet, 2008).

Kazakh sheep preferentially consume gramineous and leguminous plants in the dry grasslands of the Tianshan Mountains. However, there is no information about the endozoochorous dispersal of ingested seeds in the natural grasslands of Xinjiang Province. The objectives of this study were (i) to measure the dimensions (i.e. mass, length, width, thickness and shape) of seeds of 17 wild plant species (13 gramineous and 4 leguminous species) that are common on the north slope of the Tianshan Mountains, (ii) to determine temporal patterns in the defecation of seeds after ingestion by Kazakh sheep, and (iii) to determine the germinability of seeds after simulated ingestion. The latter objective was accomplished using seeds that had been placed in the rumen of fistulated sheep for 22–46 h.

Materials and methods

Study site

Seed samples were collected in the dry grassland of the Ziniquan sheep breeding farm, which is located on the north slope of the Tianshan Mountains (43°56′–44°03′ N, 85°40′–85°59′ E) in Xinjiang Province. The region has a temperate continental climate. The mean annual temperature is 7.2°C. The maximum average monthly temperature is 26.6°C in July. The minimum average monthly temperature is –18.5°C in January. The mean annual precipitation is 231 mm, with most precipitation falling between June and August. This area is important as autumn and winter pasture under the region's traditional grazing system. The vegetation consists predominantly of gramineous and leguminous plants. The soil types are typical grassland chernozem, chestnut soil and calcic brown soil. The grassland types are temperate desert steppe and temperate steppe.

Seed collection and seed attributes

Mature seeds were collected from over 100 individual plants of 17 species between August and October in 2013 (Table 1). The plant species are all perennials. Most of the species are common in temperate grasslands and were previously observed germinating in herbivore dung. The seeds were taken to the laboratory, air dried, and then stored in brown paper envelopes at –4°C to maintain vigor. The seed mass of each species was determined by weighing three subsamples of air-dried seeds (100 seeds per subsample; 0.01 mg precision). The dimensions (length, width and height) of 10 random seeds were measured using a stereoscopic microscope (25 μm precision). Seed length was defined as the longest of the three dimensions. Seed shape (i.e. divergence from sphericity) was expressed as the variance in seed dimension after dividing each dimension by the seed length (Thompson *et al.*, 1993) (Table 1).

Table 1. Selected characteristics (mass, length, width, thickness, shape) of the seeds of the perennial plant species used

Family	*Species name*	Seed mass (g/100 seeds)	Length (mm)	Width (mm)	Thickness (mm)	Shape
Gramineae	*Achnatherum inebrians*	0.30	6.92	0.89	0.88	0.17
	Bromus inermis	0.25	9.14	1.72	0.33	0.18
	Melica transsilvanica	0.07	1.91	0.55	0.80	0.10
	Agropyron desertorum	0.25	6.89	0.82	1.26	0.16
	Agropyron cristatum	0.24	9.33	0.68	1.04	0.18
	Elytrigia repens	0.79	12.56	1.67	0.87	0.18
	Roegneria mutabilis	0.34	8.06	1.23	0.70	0.17
	Roegneria sinkiangensis	0.33	8.24	1.20	0.89	0.17
	Dactylis glomerata	0.07	5.35	0.78	0.52	0.17
	Hordeum bogdanii	0.16	13.96	0.64	1.07	0.20
	Leymus tianschanicus	0.54	14.03	0.94	1.54	0.19
	Agrostis turkestanica	0.01	1.04	0.32	0.42	0.09
	Puccinellia tenuiflora	0.01	2.02	0.36	0.53	0.14
Leguminosae	*Sophora alopecuroides*	2.56	4.22	3.16	2.39	0.03
	Vicia tenuifolia	1.19	2.56	2.31	1.74	0.02
	Lathyrus pratensis	1.02	3.05	2.33	1.55	0.04
	Glycyrrhiza glabrae	0.57	2.31	1.99	2.03	0.004

Test animals and treatments

The six male Kazakh sheep in the study were similar in weight (weight 42 ± 1.25 kg) and in age (2 years old). The sheep were kept in individual metabolic crates (1.4 m × 0.6 m) with a faeces collection system (Fig. 1). The sheep were fed a seed-free diet for 7 days. On day 8, three sheep were fed 3000 seeds of each plant species in a single meal. The seeds were mixed with 300 g of feed concentrate to facilitate intake. The dung pellets of these sheep were collected 6, 12, 24, 36, 48, 72 and 96 h after ingestion. The pellets were dried at room temperature and then stored in the laboratory. The seeds from the feeding experiment were used to determine seed recovery percentage and mean retention time (MRT).

The seed recovery percentages were low in the feeding experiment. Therefore, a simulated ingestion experiment was conducted so that there were enough seeds to accurately determine seed germination percentage (Peco *et al.*, 2006). A permanent rumen fistula was made in the three sheep that were not used in the feeding experiment. Heat-sealed nylon bags (11 cm × 7 cm, 40 μm pore size) containing 100 seeds of each plant species were introduced through the fistula. Most seeds are retained in the rumen for 22–46 h and in a heavily acid part of the gut (abomasum and duodenum) for 2–4 h (Warner, 1981). To simulate these conditions, the bags were incubated for 22, 34 or 46 h inside the sheep rumen. After removal from the rumen, each bag and its contents were rinsed with tap water and placed in a 0.1 N pepsin-hydrochloric acid solution for 2 h in an oven at 40°C. The solution was produced by dissolving 2 g of pepsin (Merck reference 1.07190.1000 with activity 2000FIP-U/g) in 1 litre of 0.1 N HCl. The seeds from the simulated ingestion experiment were used to determine germination percentage.

Recovery, mean retention time and germination

The total mass of dung pellets was weighed for each sheep at each time interval. A 100 g subsample of the pellets was manually crushed and then the number of seeds of each plant species was determined. The recovery percentage of the seeds (RPS) was estimated for each plant species using the following equation:

$$RPS = m_f s_r / 100s$$

where m_f is the total mass of dung defecated within each time interval, s_r is the average number of seeds found in 100 g of pellet, and s is the number of seeds ingested by the sheep. The MRT of seeds in the digestive tract was calculated using the following equation:

$$MRT = \sum_{i=1}^{n} m_i t_i / \sum_{i=1}^{n} m_i$$

where m_i is the number of seeds defecated at time t_i after ingestion by the sheep.

The germination percentage of seeds from the simulated ingestion study was compared with that of non-ingested seeds. All seeds were disinfected by immersion in a 1% sodium hypochlorite solution for 2 min and then rinsed with sterile distilled water for 10 min. The seeds were placed on moist filter paper in 5-cm Petri dishes. Each Petri dish contained 25 seeds. There were four replicates per treatment. The incubations were conducted in controlled environment chambers with 16 h light at 25°C and 8 h of darkness at 15°C. The filter paper was remoistened with distilled water as necessary. The incubation conditions allow for the germination of a large range of plant species (Picard *et al.*, 2015). The dishes were examined daily. Seeds were considered to have germinated when the root was 1–2 mm long. Seeds that had germinated were counted and then removed from the dishes. The germination percentages in this paper are the average of the three incubation times (i.e. 22, 34 and 46 h).

Figure 1. The individual metabolic crates with a faeces collection system.

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Data analysis

Analysis of variance was conducted to evaluate differences among the plant species in seed recovery percentage and MRT. The data were tested for normality with the Kolmogorov–Smirnov test. The Tukey test was used to verify significant differences among species. Pearson's correlation was used to test how seed recovery and seed germination percentage after ingestion were related to seed mass and seed shape. The statistical analyses were conducted using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA). A phylogenetic tree of the plant species was drawn with Phylomatic software version 3.0.

Results

The mass and dimensions of the rather elongated gramineous seeds were as follows (means of 13 species with ranges in parentheses): 100-seed mass, 0.26 g (0.01–0.79 g); length, 7.65 mm (1.04–14.03 mm); width, 0.91 mm (0.32–1.72 mm); thickness, 0.83 mm (0.33–1.54 mm); divergence from sphericity, 0.16 (0.09–0.19) (Table 1). In comparison, the rather rounded leguminous seeds had the following characteristics (means of four species with ranges in parentheses): 100-seed mass, 1.34 g (0.57–2.56 g); length, 3.04 mm (2.31–4.22 mm); width, 2.45 mm (1.99–3.16 mm); thickness, 1.93 mm (1.55–2.39 mm); divergence from sphericity, 0.02 (0.004–0.03) (Table 1).

There was a clear peak in the recovery of seeds of all plant species between 24 and 48 h after ingestion (Fig. 2). More than 80% of all recovered seeds were defecated during this period. No seeds were recovered from dung during the first 6 h after feeding. Seeds from three plant species (i.e. *Dactylis glomerata, Agrostisis turkestanica* and *Melica transsilvanica*) were still being recovered in dung 96 h after feeding.

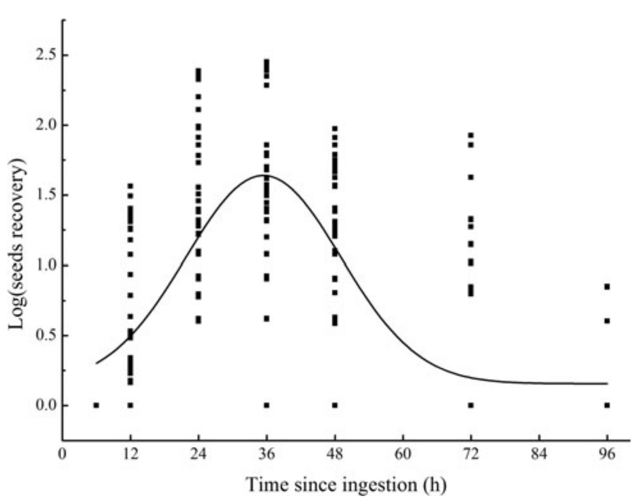

Figure 2. Temporal changes in seed recovery after ingestion by Kazakh sheep. The data were fitted to a Gaussian model: $y = 0.16 + 1.48e^{-2((x-35.42)/27.27)2}$, $r^2 = 0.55$, $F(df_1 = 16, df_2 = 34) = 311.16$, $P < 0.0$.

There were significant differences among plant species in the number of seeds recovered from sheep dung [$F(df_1 = 16,\ df_2 = 34) = 1924.07$, $P < 0.01$]. The recovery percentages of the four leguminous species (12.7–17.5%) were significantly greater than those of the 13 gramineous species (0.8–3.2%) (Table 2).

The MRT of most species was between 30 and 39 h. There were significant differences in MRT among species [$F(df_1 = 16,\ df_2 = 34) = 7.511$, $P < 0.05$]. *Achnatherum inebrians* had the longest MRT (42.2 h), followed by *D. glomerata* (41.9 h) and *B. inermis* (40.7 h). *Agropyron cristatum* had the lowest MRT (27.3 h) (Table 2).

Simulated ingestion significantly affected seed germination percentage [Fig. 3; $F(df_1 = 16,\ df_2 = 34) = 60403.38$, $P < 0.01$]. The germination percentage of all 13 gramineous species was significantly less in the simulated ingestion treatment (3.18–10.12%) than in the non-ingested treatment (66.67–97.67%) [$F(df_1 = 16,\ df_2 = 34) = 1052.1$, $P < 0.01$]. Among the gramineous species, *P. tenuiflora* and *H. bogdanii* had the highest germination percentage after simulated ingestion, whereas *A. cristatum* had the lowest germination percentage. The germination percentage of all four leguminous species was significantly greater in the simulated ingestion treatment (22.55–70.22%) than in the non-ingested treatment (5.33–12.33%) [$F(df_1 = 16,\ df_2 = 34) = 1595.16$, $P < 0.01$]. The germination percentage of the leguminous species after simulated

Table 2. Total recovery percentages and mean retention times of ingested seeds

Species	Total recovery percentages (%)	Mean retention time (h)
Achnatherum inebrians	2.64 ± 0.51^{cde}	42.20 ± 1.57^{a}
Bromus inermis	3.02 ± 0.12^{cd}	41.89 ± 3.92^{ab}
Melica transsilvanica	0.78 ± 0.09^{e}	40.74 ± 4.17^{abc}
Agropyron desertorum	1.09 ± 0.48^{e}	37.50 ± 2.35^{abcd}
Agropyron cristatum	2.38 ± 0.32^{cde}	37.50 ± 4.46^{abcd}
Elytrigia repens	1.27 ± 0.16^{de}	37.17 ± 1.11^{abcde}
Roegneria mutabilis	1.29 ± 0.11^{de}	27.30 ± 2.34^{f}
Roegneria sinkiangensis	2.11 ± 0.31^{cde}	34.45 ± 1.29^{abcdef}
Dactylis glomerata	2.00 ± 0.43^{cde}	32.83 ± 2.98^{cdef}
Hordeum bogdanii	1.61 ± 0.38^{cde}	34.00 ± 3.00^{bcdef}
Leymus tianschanicus	3.40 ± 0.61^{c}	38.04 ± 2.14^{abcd}
Agrostis turkestanica	3.22 ± 0.35^{c}	39.41 ± 2.90^{abcd}
Puccinellia tenuiflora	1.90 ± 0.19^{cde}	35.93 ± 2.86^{abcde}
Sophora alopecuroides	17.46 ± 1.64^{a}	32.51 ± 0.87^{def}
Vicia tenuifolia	13.33 ± 0.86^{b}	35.67 ± 1.17^{abcde}
Lathyrus pratensis	12.64 ± 0.48^{b}	29.38 ± 1.22^{ef}
Glycyrrhiza glabrae	15.98 ± 1.07^{a}	39.49 ± 2.38^{abcd}

Values are presented as means ± standard error ($n = 3$). Values within a column followed by a different letter are significantly different at $P < 0.05$.

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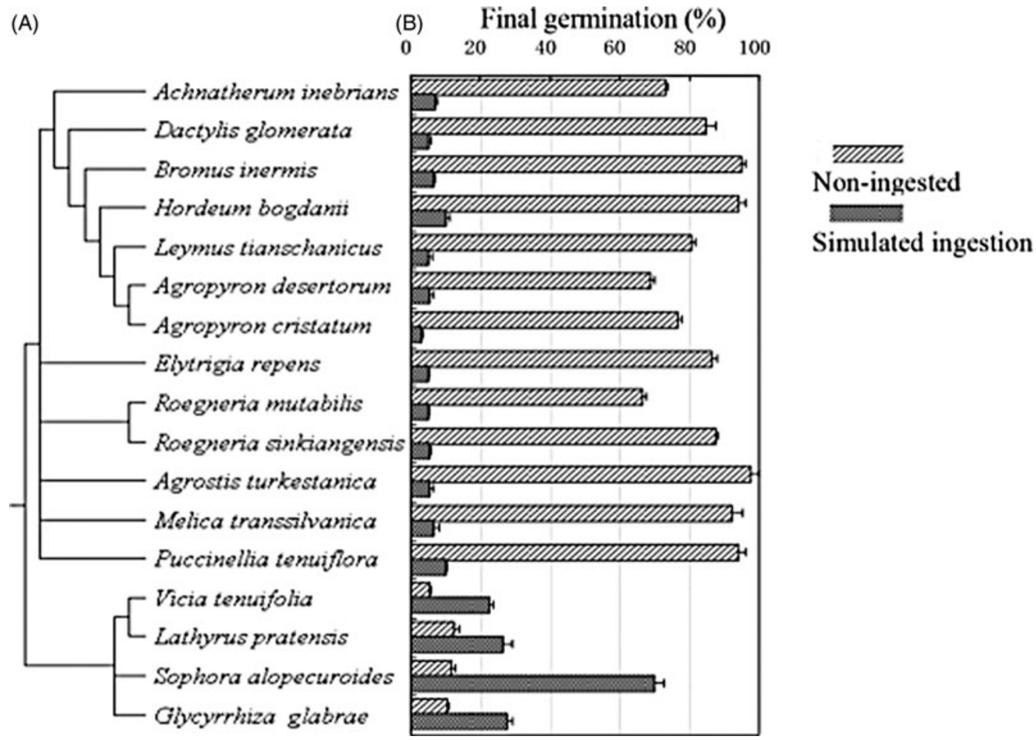

Figure 3. A: working phylogeny of the 17 plant species in this experiment. B: final seed germination percentage in the non-ingested and simulated ingestion treatments. Values are means ± SD.

ingestion decreased in the order *S. alopecuroides* > *L. pratensis* > *G. glabrae* > *V. tenuifolia* (Fig. 3).

Seed recovery percentage was positively and most significantly correlated with seed mass ($r = 0.91$, $P < 0.01$). Seed recovery percentage was significantly negatively correlated with seed shape ($r = –0.71$, $P < 0.05$). The germination percentages were negatively correlated with seed shape ($r = –0.93$, $P < 0.01$). Mean retention time was not significantly correlated with either seed mass ($r = 0.18$, $P > 0.05$) or seed shape ($r = –0.44$, $P > 0.05$).

Discussion

The distance and effectiveness of seed dispersal is determined by the combined effects of (i) seed retention time in the vector's digestive system, (ii) the spatial extent of its movements, and (iii) the ability of the seeds to germinate once released (Picard *et al*., 2015). The present study indicated a clear peak in seed defecation between 24 and 48 h after ingestion. Previous studies have shown a similar time span with sheep (Manzano *et al*., 2005). Seed recovery percentages after ingestion ranged from 0.8 to 17.5%. Those percentages agreed with other studies involving small ruminants. For example, seed recovery percentages after ingestion were 0–28% (Yu *et al*., 2012) and 10.4–23.0% (Manzano *et al*., 2005) in sheep, 7.4–17.4% in goat (Robles *et al*., 2005) and 0.5–42.0% in fallow deer (Mouissie *et al*., 2005a). A likely reason for the relatively low recovery percentage in these studies is that small digestive tracts increase the likelihood of seeds contacting the gut wall which damages the seed by abrasion (Razanamandranto *et al*., 2004).

The MRT of seeds in the digestive tract of Kazakh sheep ranged between 27 and 42 h. In comparison, other researchers have reported MRT values of 41 to 66 h in sheep (Illius and Gordon, 1992; Cosyns *et al*., 2005b). These values are greater than those of rabbit and equid species (30–31 h), roe deer (18–36 h), and red deer (3–36 h) (Picard *et al*., 2015). The MRT of ingested seeds in this study varied depending on plant species; however, the results indicate that the time span was long enough to result in seed dispersal in the grassland. Under the traditional grazing system in the Tianshan Mountains, Kazakh sheep move freely in the grassland. A previous report indicated that free-grazing Kazakh sheep move about 7–10 km per day (Wang *et al*., 2016). This distance is far less than the distance of 25–30 km per day that other authors have reported (Klein, 1981; Manzano *et al*., 2005). Obviously, these distances are affected by grazing management.

Many studies have indicated that ingestion reduces hard-seededness, with a greater proportion of seeds capable of germinating after ingestion (Russi and Roberts, 1992; Malo and Suárez, 1996; Milotić and Hoffmann, 2016). However, other researchers have reported that germination declined when soft-coated

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or non-dormant seeds were soaked in rumen fluid (Yu *et al*., 2012). Hard-seededness is a common feature and the main mechanism of seed dormancy in legumes. In our study, simulated ingestion increased the germinability of *S. alopecuroides, V. tenuifolia, L. pratensis* and *G. glabrae*. This indicated that digestion can break dormancy and promote germination of leguminous seed. In contrast, simulated ingestion reduced the germination percentage of seeds from all 13 gramineous species. One possibility is that these seeds were either soft-coated or non-dormant. It should be noted that the conditions in this experiment simulated the environment of one specific part of the digestive system (i.e. rumen). In fact, the chemical composition varies among different parts of the digestive systems, as enzyme activity and pH differ between the mouth, rumen, stomach and intestines. These factors could have significant effect on seed germinability.

The seed recovery percentages were too low to accurately determine the germination percentage of the ingested seeds in this study. Therefore, we had to simulate the effects of ingestion. The effects of mastication, however, should be overlooked. Some plant species have seeds with hard seed coats that cause physical dormancy. For those plant species (e.g. many species in the Leguminosae and Cistaceae families), physical damage caused by chewing might break the seed coat and enhance germination. In other cases chewing may damage seeds and reduce germinability (Milotić and Hoffmann, 2016). Ruminants generally only chew enough to get the proper mixture of food and saliva to form a bolus and facilitate swallowing (Church, 1976). It is unclear whether seeds are damaged more by mastication or by the harsh environment of the reticulorumen. Seed size is probably also a factor, because small seeds are more likely to escape mastication (Russi and Roberts, 1992; Gökbulak, 2006).

Bruun and Poschlod (2006) and D'Hondt and Hoffmann (2011) observed no relationship between endozoochorous dispersal potential and seed characteristics such as mass, shape (roundness) and thickness. Nevertheless, Janzen (1984) provided a good hypothesis of ecological interaction for seed dispersal through ingestion and defecation by large herbivores. According to Janzen (1984), large and round seeds of Leguminosae are best adapted to endozoochory. Our results also showed that seed recovery percentage and germination percentage were both related to seed mass and seed shape. Large and round seeds (e.g. those of *S. alopecuroides, V. tenuifolia, L. pratensis* and *G. glabrae*) had high recovery and germination percentages, whereas small and long seeds (e.g. those of *B. inermis* and *H. bogdanii*) had low recovery and germination percentages. Conflicting ideas about the relationship between seed characteristics and seed dispersal potential may be due to differences in plant species. The MRT was not significantly correlated with either seed mass or seed shape. Similarly, Cosyns *et al*. (2005b) reported that MRT was not significantly correlated with seed germination, seed recovery, or seed characteristic. This may be the result of complex interplay between animal and plant species.

The above results imply that the grazing activities of Kazakh sheep may contribute to the gathering of plant seeds under traditional seasonal grazing in Xinjiang Province. This is especially true in autumn and winter, when most plants still retain seeds. These seeds are available for consumption by moving livestock. Although the cost of gut passage for dry-fruited species is undoubtedly high (Traveset *et al*., 2002), ingestion can be advantageous for plant establishment due to the potential benefits of long distance dispersal. The results presented in Table 2 and Fig. 3 indicate that seed recovery and germination percentages of leguminous species were both relatively high after ingestion by Kazakh sheep. The MRT, recovery percentage and germination percentage indicate potential for long distance seed dispersal. This dispersal capacity could increase the heterogeneity among plant communities under free-range conditions.

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