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High potential of sub-Mediterranean dry grasslands for sheep epizoochory

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Abstract: There is a general decline of grasslands across Europe due to habitat loss and degradation. Ensuring plant dispersal thus becomes a key process for preserving grassland patches in all scales. We examined diaspore dispersal by sheep epizoochory in the pastures of the North Adriatic Karst (NW Slovenia) and determined the qualitative and quantitative features of diaspores in fur. We recorded 25,650 diaspores of 141 plant taxa (with 107 taxa and 23,350 diaspores determined to species level), using three different methods: (i) the “whole-coat method”, (ii) the “part-of-the-coat method” and (iii) a “seedling emergence method”. A comparison of these techniques revealed that the “whole-coat method” provided the highest number of diaspores and plant species. All diaspores were clustered into five emergent groups based on seven functional traits (diaspore weight, length, width, height, volume, specific weight and the diaspore surface structure). Our research revealed that sheep represent an important dispersal vector, since about half of the plant species recorded in the pastures were found as diaspores in fur. This study contributes to knowledge about the modes of seed dispersal in semi-natural grasslands. Taking into account that livestock play a key role in vegetation dynamics, understanding their effects on seed dispersal is essential for conservation and restoration of these species-rich grassland communities.

Keywords: Community assembly, diaspore traits, plant dispersal, seedling emergence method, transhumance.

1 Introduction

Many threatened European ecosystems have resulted from low-intensity human activity over the centuries [1-5]. Huge areas in the Mediterranean Basin and its edges were severely deforested and converted to extensive dry and semi-dry grasslands in the early times of human settlement. Deforestation reached its peak in the Middle Ages and lasted until the beginning of the 20th century when abandonment started [6,7]. Today we can see the remains of large grasslands already affected by fragmentation processes (i.e., increased isolation and reduced habitat size) in all three large peninsulas of the Mediterranean: the Pyrenean, Apennine and Balkan peninsulas. One effect of the fragmentation of these grassland habitats is the reduction or inhibition of immigration which may result in decreasing population fitness and size [8-12]. Hence, knowledge about the intensity and mode of plant species dispersal is crucial both for our understanding of species assemblages in these species rich semi-natural grasslands, as well as to ensure their lasting and effective conservation.

Seed dispersal by adhesion to animal coats (i.e. epizoochory) is an important process in grazed ecosystems [13]. It determines, among other processes, colonization dynamics and the spatial structure of the vegetation [14,15].

Long-distance dispersal, although rare [16-21], has been consistently identified as an important process determining many aspects in the life history of plant species (i.e. seed mass and seed morphology) [22-25]. In fact, epizoochory may have acted as a selection pressure for the evolution of plant traits that increase the probability of dispersal by animals [26]. Different diaspore traits are characteristic for fur-epizoochory and hoof-epizoochory [27]. Diaspores (mainly seeds) with appendages, particularly elongated or hooked ones, are more likely dispersed via fur-epizoochory, whereas light and round diaspore without hooked appendages are associated with hoof-epizoochory [27].

In our study we focused on the dispersal potential of diaspores driven by sheep fur-epizoochory within

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the species-rich pastures of the North Adriatic Karst. During the twentieth century this area became more or less completely abandoned (and hence ungrazed) for socio-economic reasons. Therefore the typical large semi-natural grasslands, maintained mainly by sheep grazing in the past are now on the path to extinction [7].

We investigated a flock of sheep in extensive grazing conditions to answer the following questions: Which species and numbers of diaspores are dispersed by sheep fur-epizoochory in the montane Karst pastures?; and Which traits are typical for diaspores attached to sheep fur? We also aimed to compare three different methods for assessing the composition and richness of diaspores in the sheep fur. We aimed (i) to test to what degree examination of a smaller part of the fur could satisfactorily replace sampling of the entire fur of a sheep, and (ii) to test and assess an innovative “seedling emergence technique”, which was developed within the present study.

2 Methods

2.1 Study area

The study was carried out in the mountainous Vremščica area in the North Adriatic Karst, Slovenia, (45°41'N, 14°12'E, 820 m above sea level). The study area covers an area of 20 km² with an overall flat topography. This area, being at the edge of the Mediterranean basin, represents the most north-western part of the Balkan Peninsula [5]. The climate is sub-Mediterranean [28]. The mean annual rainfall is about 1834 mm. The mean annual temperature is 5.6°C. The climate is transitional between Mediterranean and continental pre-Alpine, with rainy cool winters and long, dry summers [29]. The geological bedrock is formed mainly of calcareous limestone, covered with chromic cambisol and rendzina, with a reasonably unstable soil depth [30].

The Vremščica area was chosen because of its long tradition of transhumant sheep farming in the past. The present landscape is characterized by a mosaic of temperate, deciduous beech forests and semi-natural grasslands. The proportion of semi-natural grasslands in the area has declined in recent decades, owing to the forest encroachment that followed the decline of traditional low-intensity grazing management [31]. The experiment was carried out in 2006 on pastures and with the sheep flock belonging to the “Vremščica Centre for Sustainable Recultivation”. A flock of about 450 sheep of the indigenous Slovenian breed Istrian pramenka grazed daily in the pastures. Pastures that were grazed within the

study period were species-rich, Mediterranean-montane Karst pastures (the alliance *Satureion subspicatae*, class *Festuco-Brometea*). They were characterized by many sclerophyllous and basiphilous sub-Mediterranean-Ilyrian species, known as “karstic species”, such as, *Plantago argentea* subsp. *liburnica* Chaix. & Vill., *Satureja montana* subsp. *variegata* (Host) P. W. Ball, *Satureja subspicata* subsp. *liburnica* (Šilić) Šilić, *Scorzonera villosa* Scop., *Polygala nicaeensis* Koch, *Koeleria lobata* (M.Bieb.) Roem. & Schult., *Thesium divaricatum* Jan ex Mert. & Koch, *Knautia illyrica* Beck and *Leucanthemum liburnicum* (Fiori) Horvatić [31]. On deeper soil with more humus and moisture, there also occurred species from mesic grasslands for example, *Arrhenatherum elatius* (L.) P.Beauv. ex J.Presl & C.Presl., *Dactylis glomerata* L., *Festuca pratensis* Huds., *F. rubra* L., *Poa pratensis* L., *Phleum pratense* L., *Bromus hordeaceus* L., *Daucus carota* L., *Taraxacum officinale* F.H.Wigg, *Achillea millefolium* L., *Plantago major* L. and *Trifolium pratense* L.

Owing to frequent drought in summer the vegetation season is short and grazing is limited to the period from June to September [30].

2.2 Diaspores in the fur

Investigations of fur were carried out in September 2006, at the end of the grazing season. We used three different methods to sample the diaspores in the sheep fur: the “whole-coat method”, the “part-of-the-coat method” and a “seedling emergence method”. In the “whole-coat method,” we randomly chose 10 adult sheep from a flock which were then shorn entirely (except tail and legs). In the laboratory, the entire fur (total area approximately 8.5 m²) was systematically investigated for diaspores. The examination was conducted by hand for each of the 10 samples. Collected diaspores and other unidentified particles were photographed and stored dry in glass containers. They were identified primarily using the seed reference collection of the SRC SASA (Scientific Research Centre, Slovenian Academy of Sciences and Arts, Ljubljana) and taxonomic literature for the identification of fruits, seeds, and vegetative plant fragments [32-40].

In the “part-of-the-coat method”, another 5 randomly chosen adult sheep were used. The fur from two selected body-parts – breast and flank (because these two body parts are the area where the vast majority of diaspores become attached [44]) – was shorn and sampled. To sample the flanks both the left and right sides of the body were used and treated as one sample. The fur area sampled each flank was ~0.25 m² and for each breast

~0.4 m². We examined and collected diaspores from the 10 samples following the same procedure described above. In this way it was possible to compare the importance of both body parts – breast and flank – to the transport of diaspores and also to test to what degree examination of a smaller part of the fur could replace sampling of the entire fur of a sheep.

For the third method we followed a “seedling emergence method” as described by Gross [41] and used for determination of the soil seed bank. Fur from 6 randomly chosen sheep were used (whole fur without legs and tails) in this experiment. To reach seed imbibition all 6 fur samples were soaked in water overnight. Then we set each of the soaked wool samples on agricultural fur (i.e. Covertan crop cover, designed to provide climate protection for vegetables and crops) layered with 1 cm of sterilized soil and covered the wool with another 1 cm of sterilized soil. The samples were then placed in an unheated glasshouse. Five control trays containing only sterilized soil were also prepared to check for contamination from the glasshouse environment. Samples were regularly saturated by tap water. As seedlings emerged, they were identified,

counted and removed every two weeks. We potted the undetermined seedlings until they developed to a stage where they could be identified. The experiment ran for 3 months and was finalised when emergence had stopped (December 2006). The temperature in the glasshouse ranged from 2°C to a maximum 31°C and lighting was from natural light that penetrated the glass.

2.3 Diaspore traits

To distinguish diaspores according to their morphological properties, we selected seven seed-traits (Table 1). Trait information was compiled from our own field and laboratory measurements. To assess diaspore weight, length, width, height, volume and density (specific gravity), protocols for the standardised measurement of plant functional traits [43,44] were followed. The diaspore surface structures were described in five categories [22,45-47] (Table 1): diaspores with (1) hairs, (2) with awns, (3) with hooks, with (4) remains of flowers – petals, sepals and (5) diaspores without any of these appendages.

Table 1. Seed-traits recorded on 107 vascular plant species found in the fur of sheep

Diaspore traits	Attribute classes		Variable type
Diaspore weight	Low	≤ 0.5 mg	Quantitative
	Medium-weight	0.5-2.0 mg	
	High	≥ 2.0 mg	
Diaspore length	Low	≤ 2.2 mm	Quantitative
	Medium-length	2.2-4.8 mm	
	High	≥ 4.8 mm	
Diaspore width	Low	≤ 1.0 mm	Quantitative
	Medium-width	1.0-2.0 mm	
	High	≥ 2.0 mm	
Diaspore height	Low	≤ 0.5 mm	Quantitative
	Medium-height	0.5-2.0 mm	
	High	≥ 2.0 mm	
Diaspore volume	Low	≤ 1.5 mm ³	Quantitative
	Medium	1.5-11.7 mm ³	
	High	≥ 11.7 mm ³	
Diaspore density	Low	≤ 0.2 mg mm ⁻³	Quantitative
	Medium	0.2-0.4 mg mm ⁻³	
	High	≥ 0.4 mg mm ⁻³	
Adhesive structures	(1) Hairs, (2) awns, (3) hooks, (4) remains of flowers - petals, sepals; (5) no appendages (smooth surface)		Qualitative

2.4 Data analysis

Identified plant species were classified into five classes according to their frequency on the investigated Karst pastures: I, rare on the Karst pastures; II, moderately frequent; III, very frequent; ab, absent in Karst pastures: species of the surrounding area; and † also common in the surrounding area. To determine frequency of the plant species on the Karst pastures, two published studies about dry grassland vegetation of Vremščica area were used [30,42]. Taxonomic nomenclature of all plant species follows Flora of Slovenia [40].

The delineation of species groups according to the morphological traits of their diaspores was based on a similarity matrix between individual species using Syn-Tax [48]. To calculate the matrix the Gower's similarity coefficient [49] was applied, since this coefficient can deal with missing values as well as with mixed data, i.e. qualitative, ordinal and quantitative [50,51]. The cut-level for end groups (i.e. emergent groups, EGs) was determined graphically from visual screening of the dendrogram. To determine which traits defined the EGs, the relationships between individual traits and groups were investigated by means of Pearson's χ^2 tests (when the trait was qualitative) and a Kruskal-Wallis statistic (when the trait was quantitative or ordinal). The post hoc comparison of mean ranks for all pairs of groups was computed using a Mann-Whitney U test.

We used a one-way ANOVA, with subsequent post-hoc Least Significant Difference (LSD) test, to test differences in number of diaspores among defined EGs. We only included data recorded in the experiment with the "whole-coat method". To detect relationship between the length, width, height, and volume of diaspores we used Spearman rank correlation.

To examine differences in the number of diaspores found on the breast and flanks, we used the Student's t-test.

All tests were performed with the statistical package SPSS Base® for Windows 21.0 (Released in 2012, IBM, New York).

3 Results

3.1 Overall dispersal spectra

A total of 25,650 diaspores of 141 taxa of vascular plants were recorded by means of the three sampling techniques. One hundred and seven taxa (23,350 diaspores) were determined to the species level (Table S1). A further 34

taxa were determined to the genus only and were not included in the seed-trait analyses.

Among 22 families, Poaceae had the highest number with 58%, followed by Fabaceae with 29% of all diaspores. The rest of the families share 13% of the total number of diaspores. The most frequent diaspores belonged to crimson clover (*Trifolium incarnatum* L.) (5036 diaspores) (Fabaceae) (Table S1). These diaspores as well as those of barley (*Hordeum vulgare* L.) (113) and corn (*Zea mays* L.) (12), which were also found in the coats, did not originate from the grasslands in the study area, but from winter forage transported from elsewhere. The most frequent grass species in the fur was orchardgrass (*Dactylis glomerata*) (3105 diaspores) (Table S1). Other grass species that were represented by a high number of diaspores were characteristic species of the calcareous Karst pastures: upright brome (*Bromopsis erecta* (Huds.) Fourr.) (2959) and *Festuca rupicola* Heuff. (1432). The typical Fabaceae species of the pastures on Vremščica, hop clover (*Medicago lupulina* L.) (915) and red clover (*Trifolium pratense*) (711), were also often found in the sheep wool. Approximately one third of the species in the fur were found only in small number of diaspores (< 10 diaspores) (Table S1).

3.2 Diaspore traits

On the basis of seven selected morphological seed-traits (Table 1), the 107 detected species were clustered (dendrogram not shown) into five emergent groups (EGs) (Table 2). This revealed five functional types of the diaspores, which could also be interpreted as five diaspore dispersal strategies: (i) smooth, structureless diaspores of medium weight and size, e.g., horseshoe-vetch (*Hippocrepis comosa* L.), fairy flax (*Linum catharticum* L.), birds-foot trefoil (*Lotus corniculatus* L.), common sage (*Salvia pratensis* L.) (EG1 = 17 species); (ii) large, heavy diaspores with greater volume and distinctive surface structures (with hairs, awns, hooks, remains of flowers), e.g., burdock (*Arctium lappa* L.), heath false brome (*Brachypodium pinnatum* (L.) P.Beauv), *Bupleurum exaltatum* M.Bieb., *Leontodon hispidus* L., common sainfoin (*Onobrychis viciifolia* Scop.) (EG2 = 18 species); (iii) diaspores with the remains of flowers (petals, sepals), e.g., narrowleaf plantain (*Plantago lanceolata* L.), oakforest woodrush (*Luzula luzuloides* (Lam.) Dandy & E.Willm.) and red clover (EG3 = 23 species); (iv) small setaceous diaspores, mostly grasses, e.g., soft brome, crested dog's tail grass, orchardgrass, meadow fescue (EG4 = 30 species) and (v) diaspores with hooked appendages and/or of high specific weight, e.g., wild carrot, cleavers (*Gallium aparine*

Table 2. Comparison of the morphological trait values of the diaspores between the five emergent groups (EG1-EG5), defined for 107 plant species found in the fur of sheep.

	<i>P</i>	EG1	EG2	EG3	EG4	EG5
Description		smooth, structureless; medium weight and size	large, heavy; greater volume; distinctive surface structures	with the remains of flowers (petals, sepals)	small setaceous	with hooked appendages and/or of high specific weight
Number _{sp}		17	18	23	30	19
Percentage _{dia} (%)		3.4	7.5	30.7	51.5	6.9
Surface structure†	***	-2.3/-3.8/-3.0/-2.7/11.8	-2.4/2.5/1.2/1.1/-2.4	-0.2/-4.2/-0.6/7.3/-2.3	4.6/9.8/-5.0/-4.8/-4.6	0.4/-4.2/7.4/-1.0/-2.6
Diaspore weight‡	**	2 ^{ac}	3 ^{bc}	2 ^{ac}	2 ^c	2 ^{ab}
Diaspore length‡	***	2 ^{ac}	3 ^b	2 ^{ac}	2 ^c	2 ^a
Diaspore width‡	***	2 ^{ac}	3 ^a	1 ^b	1 ^b	2 ^c
Diaspore height‡	***	3 ^a	3 ^a	1 ^{bc}	1 ^c	3 ^a
Diaspore volume‡	***	2 ^a	3 ^b	1 ^c	2 ^c	2 ^a
Diaspore specific weight‡	***	2 ^a	1 ^a	3 ^b	2.5 ^{bc}	3 ^{bc}

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

† Pearson χ^2 test, values are differences between observed and expected frequencies for the different categories defined in Table 1.

‡ Kruskal-Wallis test, values are medians for the different categories defined in Table 1; numbers with different letters are significantly different at the 0.05 level.

Number_{sp}, number of species per group; Percentage_{dia}, number of diaspores (in percentage) per group.

L.), meadow buttercup (*Ranunculus acris* L.) and creeping buttercup (*R. repens* L.) (EG5 = 19 species).

The traits of diaspore length, width, height and volume were all significantly correlated with diaspore weight (Spearman's $\rho \leq 0.01$ for all correlations).

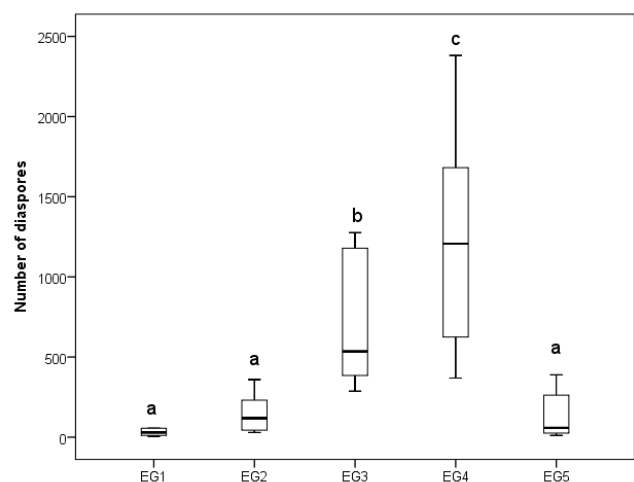
Comparing the number of diaspores using different dispersal strategies yielded high differences among EGs (Table 2). We found significant differences in number of diaspores between EGs ($P < 0.001$), with the highest values in EG4 (mean = 1190 ± 657 s.d., $N = 10$) and EG3 (mean = 715 ± 394 s.d., $N = 10$). About 50% of all diaspores belonged to small setaceous diaspores (EG4) and 30.7% to the diaspores with the remains of flowers (EG3). However, while many species without any adhesive appendages were found on the sheep the number of transported diaspores was small (3.4%) (Table 2). The same results were obtained when testing for differences in the number of diaspores between the five EGs and including only diaspores recorded in the fur of ten sheep (the “whole-coat method”) (Figure 1).

3.3 Comparison of the sampling methods

About 98% of all diaspores (22,910 diaspores) and 97% (104) of all plant species were recorded with the “whole-coat method” (mean = 53 ± 10.6 s.d., $N = 10$). Only 253 diaspores were counted in the “part-of-the-coat method”

(see Table S1), where 24 plant species in total were found. Diaspores from the following vascular species were recorded in the highest numbers: upright brome (99), *Gladiolus illyricus* W.D.J.Koch (24), perennial yellow-woundwort (*Stachys recta* L.) (16) and herb bennet (*Geum urbanum* L.) (15). We found no differences in the number of diaspores between the two body parts (breast, flank).

A total of 187 diaspores from 15 species were recorded in the experiment with the “seedling emergence method”

**Figure 1.** Number of diaspores (mean $\pm$ s.d., $N = 10$) in five emergent groups (EG1-EG5, Table 2), defined for 104 plant species found in the fur of sheep (“the whole coat method”). Different letters (a, b, c) indicate significant differences (LSD post-hoc test) between the EGs.

(Table S1). 55% of the seedlings belonged to the grasses and 30% to the Fabaceae. The most common offspring belonged to crimson clover (*Trifolium incarnatum*) (47), couch grass (*Elytrigia repens* (L.) Nevski.) (22), crested dog's tail grass (*Cynosurus cristatus* L.) (16) and hop clover (*Medicago lupulina*) (15).

4 Discussion

Our study suggests that most of the plant species in Karst pastures can be transported by sheep if they come into contact with fur, since nearly 50% of the species found in the aboveground vegetation of the pastures on Mt. Vremščica recorded in previous studies [30,42], were also found in the fur. In many studies the ability of diaspores to move with sheep transhumance has been observed [1,10,13,20,27]. However, no single study has sampled the high number of species demonstrated in the present work. This observation confirms the anthro-po-zoogenic origin of these grassland habitats [52] which owe their existence to a very specific kind of land-use – mostly grazing by sheep [6].

Attachment of diaspores to sheep wool is also affected by seed density in the vegetation [53]. The Poaceae *Dactylis glomerata*, *Bromopsis erecta*, *Festuca rupicola* were attached in considerable numbers (Table S1). *D. glomerata* is a moderately frequent species of the Karst pastures, however it is very frequent along the sheep tracks and waysides and the surrounding areas. Other grass species that were represented by a high number of diaspores are dominant grass species of the calcareous Karst pastures [30].

Various strategies of fur-epizoochoric dispersal according to diaspore morphology have already been proposed [1,27]. Both seed mass and seed morphology were positively correlated (on 130 species tested) with attachment potential in a model [25]. The “retention potential” of seeds has also been experimentally tested in 100 species [44]. A positive effect of different morphological structures was proven, and species with a diaspore mass below 2 mg were found to be capable of dispersal over long distances. Our findings are consistent with the results from the above mentioned studies, confirming that diaspores with appendages (e.g. hooks, awns, spines, bristles, hairs and wings) are more likely to be dispersed in the fur [1,27,44]. However, the lack of these diaspore structures (e.g. smooth surface) does not mean that epizoochoric dispersal is impossible.

Comparison of three different sampling methods revealed that the “whole-coat method” provided the

highest number of diaspores and plant species. Of a total 107 species only 3 species were not recorded in the “whole-coat method”, and were instead identified by the “part-of-the-coat method”. We found that sampling of a smaller part of the fur cannot satisfactorily replace sampling of the entire fur of a sheep, nor do the “part-of-the-coat method” and “seedling emergence method” contribute to a more efficient sampling. Using all three methods is not practical and therefore we recommend the “whole-coat method” as the most efficacious method for detecting those plant species and diaspores that are dispersed by sheep fur-epizoochory in the montane Karst pastures. Particularly small seeds however are sometimes difficult to identify or even locate within the fur, and for these reasons the “seedling emergence method”, which is the most demanding in terms of time and tools and laboratory conditions, might be considered as an additional complementary method.

Several other methods of seed determination have been used in epizoochorous research, e.g. investigations of fur by hand or by brushing with a fine brush [1,54,55]. In contrast to these methods, our three different sampling methods are destructive methods (shearing) and can only be applied across larger time-intervals to the same animal. However, since we were able to identify such a high proportion of the Karst pasture flora with the “whole-coat method”, we consider this method effective for the investigation of epizoochorous seed dispersal.

Sheep epizoochory is an important dispersal factor that also has implications for grassland conservation and restoration [1,25,44,56]. The long-distance dispersal potential of species within the present-day communities is positively associated with the amount of grassland in the historical landscape and with the continuity of grazing management [57]. Thus, it is clear that dispersal potential is connected with the distribution of unfragmented, grazed grassland. But why not also vice-versa? The species-rich NW Balkan karstic dry grasslands are associated with epizoochoric dispersal [5,6]. This means that dispersal in space - the movement of dispersal units away from parent plants - enables species not only to re-colonize unoccupied sites, but also to colonize suitable new habitats [57]. Biogeographers have long considered the presence of organisms outside their normal distribution range to be important, since this will indicate where the tails of dispersal curves may lie and what the species migration potential may be [20]. It has already been shown that grazing by transhumant herds of domesticated herbivores during the Holocene contributed to further distribution and mixing of previously isolated taxa [58]. Furthermore, it has been established [59] that transhumance became an

important factor contributing to vegetation and landscape development over many centuries. However, it was likely not transhumance alone, any kind of human migration, including early migrations associated with the “transition to agriculture,” contributed to the vegetation patterns of secondary grasslands that are still evident today [60].

From the results presented in our study, we can conclude that the diaspores of plant species from the North Adriatic Karst (NW Balkan) are likely to be dispersed by sheep, at least in small numbers. Additionally there appears to exist a range of dispersal strategies both overt and covert (i.e. structureless diaspores). The present study contributes to the knowledge on epizoochorous dispersal of plant species in semi-natural dry grasslands. Understanding the contribution of moving livestock to dispersal dynamics and the long-term maintenance of ecosystems processes is essential for the conservation and restoration of these species-rich grassland communities on the edge of the Balkan Peninsula.

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Conflict of interest: The authors declare that they have nothing to disclose.

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