

Efficiency of endozoochorous seed dispersal in six dry-fruited species (Cistaceae): from seed ingestion to early seedling establishment

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Abstract

We combined laboratory and nursery experiments to analyse the effectiveness of sheep as endozoochorous seed dispersers of six native shrubby Cistaceae species collected in SE Spain (*Helianthemum apenninum* (L.) Mill., *H. violaceum* (Cav.) Pers., *Fumana ericoides* (Cav.) Grand., *F. thymifolia* (L.) Spach, *Cistus monspeliensis* L. and *C. laurifolius* L.), considering the main stages after seed ingestion, i.e. seed recovery, seed germination, seedling emergence and early seedling establishment. Seed recovery after gut passage was high (around 40%) for all the species, except *F. thymifolia* (12%). Most seeds (ca. 90%) were recovered within 48 h after ingestion for all the species, although seeds were still recovered up to 96 h after ingestion. Gut passage increased germination up to seven-fold compared to non-ingested seeds. Furthermore, seedling emergence from seeds contained in pellets was overall similar (intact pellets) to or higher (crumbled pellets) than emergence from seeds without dung. Survival of emerged seedlings and mass of seedlings after 20 days were not reduced by dung. Sheep act therefore as effective dispersers of these Cistaceae species by scattering seeds and promoting germination, while faeces do not hamper seedling establishment. We conclude that the interaction between herbivorous ungulates and these dry-fruited species may be considered a mutualism qualitatively similar to the mutualism between frugivorous vertebrates and fleshy-fruited plants.

Introduction

Seed dispersal has profound importance for providing favourable sites for germination and seedling establishment as well as for the colonization of new habitats (Willson 1992; Herrera 2002; Wang and Smith 2002). Animals are a main vector for the dispersal of many plant species (Regal 1977; Stiles 1992; Traveset 1995; García 2001), and in particular endozoochory (i.e. the dispersal of seeds after passage through the vertebrate gut; van der Pijl 1982) is common for many families (Stiles 1992; Traveset 1998). Endozoochory may imply the establishment of a mutualistic relationship

between plants and animals in which plants trade food for displacement (Herrera 2002). The effectiveness of endozoochory as a dispersal mechanism is a function of quantitative and qualitative traits (Herrera and Jordano 1981). The quantity of dispersal depends on the amount of seeds ingested, while the quality of dispersal depends on the percentage of undamaged seeds that are defecated, and on the movement patterns and deposition patterns of the animal (Schupp 1993; Fleming and Sosa 1994).

Animals may scatter seeds, but dispersal efficiency also depends on other processes affecting the fate of seeds (e.g. germination, establishment

from dung). Germination may be enhanced by the softening of the coats during the digestive process (van der Pijl 1982; Baskin and Baskin 1998; Traveset and Verdú 2002), but destruction of seeds or inhibition of germination can also occur (Traveset 1998; Graae et al. 2004). Deposition of seeds with faecal material may provide nutrients that promote seedling establishment (Stiles 1992; Woldu and Saleem 2000; Nchanji and Plumtre 2003), but seed germination and seedling establishment could also be inhibited due to the toxicity and hydrophobic nature of dung (Malo and Suárez 1995, 1998; Traveset et al. 2001). Thus, the determination of whether dispersal after animal consumption is advantageous for a plant requires an analysis of all the stages involved from seed ingestion to seedling establishment (Traveset 1998).

Most work related to endozoochory has focused on fleshy fruits, as they are a clear source of food for animals, but there are also many plants that produce seeds inside dry fruits which are dispersed via endozoochory by herbivores, the leaves and/or the dry fruits probably attracting the animal (i.e. 'Foliage is the fruit' hypothesis, Janzen 1984; Stiles 1992; Quinn et al. 1994). Moreover, numerous authors agree that 'unassisted' diaspores (i.e. diaspores with no obvious adaptations for dispersal; Myers et al. 2004) are often dispersed via endozoochory (Willson 1993; Pakeman et al. 2002; Myers et al. 2004), mainly by mammalian herbivores.

Herbivores are key dispersal agents for plants that produce dry-fruits in several ecosystems (e.g. Myers et al. 2004), and in particular are the main dispersers in dry environments, including Mediterranean-type ecosystems (e.g. Russi et al. 1992; Malo et al. 2000). Most studies in these environments focus on the dispersal of herbaceous pastures, but almost no work investigates endozoochorous seed dispersal of shrubs or trees (but see Malo and Suárez 1998; Manzano et al. 2005). Shrubs represent the most characteristic plant form in Mediterranean-type ecosystems, especially in grazed areas (Le Houérou 1980; Kummerow 1989). These plants play a vital role in the feeding of herbivorous (Papanastasis 1993), and have developed a variety of structural, morphological and physiological characters which constitute adaptations to the climate, but also to browsing (Kummerow 1989; Barroso et al. 2001; Pignatti et al. 2002).

Cistaceae is an important family of dry-fruit-producing plants in Mediterranean shrublands and pastures which represent a high percentage of the vegetation cover. Most of these species are pioneers that require open habitats for establishment. Many are consumed and dispersed by domestic and wild ungulates (Fernández García et al. 1992; Malo and Suárez 1998; Barroso et al. 2001). In addition, Cistaceae and ungulates are known to coexist in this area since at least 1.8–1.9 million years ago (Eriksson and Bremer 1992; Arribas et al. 2004). All the members of this plant family produce seeds with traits presumably adapted to endozoochorous dispersal: high yields of small seeds (0.4–2.5 mm diameter) with hard coats and a low germination percentage (Thanos et al. 1992; Pakeman et al. 2002; Castro et al. 2005). Germination is therefore boosted by treatments that soften seed coats, simulating for example the physical or chemical damage that herbivores cause to the coat (Thanos et al. 1992; Castro and Romero-García 1999; Robles and Castro 2002). In this study, we seek to analyse the effectiveness of endozoochorous seed dispersal by herbivores in six shrubby Cistaceae species. For this, we consider the effect of gut passage during the most critical stages of endozoochorous dispersal, examining seed recovery after gut passage, germination, seedling emergence and early seedling establishment. Five specific questions were posed: (1) What is the percentage of seed recovery after seed ingestion? (2) What is the temporal pattern of seed recovery after ingestion? (3) What is the impact of gut passage on seed germination? (4) What is the capability of seeds to germinate from dung pellets? (5) What is the effect of pellets on early seedling establishment?

Methods

Seeds of *Helianthemum apenninum* (L.) Mill., *H. violaceum* (Cav.) Pers., *Fumana ericoides* (Cav.) Grand., *F. thymifolia* (L.) Spach, *Cistus monspeliensis* L. and *C. laurifolius* L. were collected from ripe fruits ($n \geq 30$ plants per species) in summer 2001 in Granada province, SE Spain. The number of seeds per fruit ranged from 10 (*Helianthemum* species) to 100 (*Cistus* species). The localities of collection were Sierra de los Guájares (36°54' N 3°34' W; *H. apenninum*, *C. monspeliensis*, *F. ericoides* and

F. thymifolia; mean annual temperature (MAT) = 15.1 °C; annual rainfall (AR) = 507 l m⁻²), Guadix-Baza Basin (37°26' N 3°05' W, *H. violaceum*; MAT = 16.3 °C; AR = 297 l m⁻²) and Sierra de Huétor (37°15' N 3°29' W, *C. laurifolius*; MAT = 13.3 °C; AR = 838 l m⁻²). All areas were shrublands dominated by Cistaceae, Labiatae and Leguminosae. Local soils are of limestone origin in the Sierra de los Guájares and Guadix-Baza localities while soils in Sierra de Huétor site are siliceous, bedrock formed by micaschists (Sierra et al. 1986; Delgado et al. 1989; Pérez Pujalte 1987). After collection, seeds were manually removed from the fruits and stored in paper bags at room temperature until the beginning of the experiments. Three types of experiments were performed to test the effect of ungulate consumption on: (1) seed recovery after gut passage, (2) seed germination after gut passage, and (3) seedling establishment from dung.

Seed recovery after gut passage

On November 2002, seeds were provided to adult sheep (*Ovis aries*, Segureña race) kept in metabolic pens with a collector system for faeces. Seeds of each species were offered to two sheep (2000 seeds per animal; 4000 seeds per species), seeds enveloped in small balls of moistened salted bread to facilitate the intake. At the beginning of each week, seeds of a given species were offered to the animals for roughly 1/2 h, changing the seed species at weekly intervals. Animals had *ad libitum* access to their usual diet (*Medicago sativa* L. hay) and water. Dung pellets were collected at 24, 48, 72 and 96 h after ingestion, dried at room temperature, and stored in the laboratory. Collecting intervals are according to the seed retention time obtained for sheep (Olson and Wallander 2002; Manzano et al. 2005). For each time interval and for each sheep, 100 or 200 pellets (N) were individually crumbled, recording the number of seeds found per pellet (s_i). A subsample of 100 pellets for each time interval was weighed to calculate the mean pellet weight (p). The total mass of faeces at each interval (m) was also determined. The percentage of seeds recovered (PSR) at each

interval for one animal was estimated by the following expression:

$$\text{PSR} = \frac{m \sum s_i}{SeNp} \times 100$$

where m is the total mass of faeces by time interval, s_i is the number of seeds found per pellet, Se is the number of seeds ingested by sheep, N is the number of pellets and p is the mean pellet weight.

Seed germination after gut passage

Dung collected in the previous experiment was crushed manually to obtain seeds for the germination experiment. We concentrated on the two sampling days with the highest seed recovery (24 and 48 h after ingestion), as on the other days we could not get enough seeds due to low recovery (see Results). This resulted in three treatments per species: (1) Control, intact seeds that were not ingested; (2) 24 h, seeds recovered 24 h after ingestion; and (3) 48 h, seeds recovered 48 h after ingestion. Additionally, sandpaper-scarified seeds were sown for germination to determine seed viability. Seeds of *F. thymifolia* showed a very low recovery percentage due to an experimental accident (see Results), and thus seeds from 24 to 48 h treatment were mixed and constituted a different treatment (24 + 48 h) only for this species. Seeds from all treatments were cleaned in tap water, then disinfected by immersion in a 1% sodium hypochlorite solution for 10 min, and thoroughly rinsed with sterile distilled water. Germination experiments started in January 2004 using a growth chamber with a photoperiod of 16 h day/8 h night, at 22/16 °C. This temperature is in the range of optimal temperature used for germination experiments with Cistaceae species (e.g. Thanos et al. 1992; Ferrandis et al. 1999). Seeds were placed in sterile, plastic Petri dishes (5 cm diameter) containing filter paper disks resting on a single layer of 5 mm glass beads, all materials having previously been sterilised. Dishes were initially moistened with 7 ml of a suspension of Benomyl fungicide at 0.1%, being thereafter moistened as needed (adding 2 ml per week) with sterile water. Each Petri dish contained 25 seeds, and there were

8 replicates per treatment. Dishes were randomly repositioned within the chamber every 5 days. Germination, identified as visible radicle protrusion, was recorded at 2-day intervals for 30 days.

Early seedling establishment

The ability of seeds encased in dung to emerge and become an established seedling was evaluated by nursery experiments simulating natural conditions at the Estación Experimental del Zaidín (CSIC-Granada) facilities. For each Cistaceae species, a mixture (1:1) of native soil and autoclaved river sand was placed in 40-ml pots. Soil was sieved through a 1-mm mesh to discard any Cistaceae seed in the soil without eliminating microbes (e.g. possible mycorrhiza). Seeds removed from the fruits were lightly sanded to erode the seed coat and thereby ensure imbibition. Next, seeds were distributed among three treatments: (1) Control, seeds placed on the soil surface; (2) Crumbled pellet, seeds placed on the soil surface together with manually crumbled pellets, simulating crumbling of pellets under natural conditions (i.e. from rainfall or from animals trampling the pellets); (3) Intact pellets, seeds placed inside an intact pellet. For this, using a needle, we made a hole 3 mm deep in the pellet, inserted the seed, and sealed the opening of the hole by gently pressing the pellet. This simulates the situation of an intact pellet that contains seeds inside. A subsample of sandpaper-scarified seeds was germinated in a growth chamber to test seed viability. For unknown reasons, *C. laurifolius* seeds lost their viability and were thus discarded from this experiment (2% germination and heavy fungal attack). In the other species, germination in the growth chamber was around 95% in all cases, and thus differences among treatments were ascribed to experimental factors.

We used 72 replicates (pots) per treatment and species (226 seeds per species, i.e. 72 replicates \times 3 treatments). Pots were randomly repositioned within the nursery every 3 days. Each pot had only one seed. In the Crumbled and Intact treatments, one additional pellet with no seeds was added to the pot in order to simulate the content of seeds in sheep excrements under natural conditions (not all pellets contained seeds, see Results), which might also have implications for early seedling establishment as it provides more manure for the establishing seedling.

In addition to the experimental pots, we used 24 pots containing soil and pellets with no seeds to control for the presence of seeds either of Cistaceae species or other species. No Cistaceae species appeared except those used in the experiment. A few seedlings of other plant species (*Portulaca oleracea* L., *Amaranthus* spp., *Chenopodium* spp., and possibly Compositae species) appeared during the experiment, but in all cases were easily identifiable and differentiated from sown Cistaceae.

The experiments, started on the 20 May 2004, were conducted in a nursery with pots protected from rain and direct sun by a translucent roof. Mean temperature during the days of experiment was 22.8 °C, with a maximum value of 38 °C and a minimum of 11.2 °C; while relative humidity was 31.2% (data from La Cartuja meteorological station [University of Granada], which is located close to the site of the experiment). Pots were watered by imbibition as needed (around two-days interval, depending on weather conditions) with tap water, providing the same amount of water to all the replicates. Because visualization of the moment of radical protrusion was not possible in seeds from Crumbled and Intact treatments, emergence was used as the criterion for analysis, considering a seedling to be emerged by the appearance of cotyledons. Each emerged seedling was allowed to grow for 20 days. In order to test seedling growth, we harvested surviving seedlings after this period, gently washed their roots, and dried the whole seedling for 48 h at 60 °C to determine the dry weight.

Data analysis

Comparisons of seed recovery among days and of seedling emergence from pellets were performed with a χ^2 -test. Seed germination after gut passage was analysed using a one-way ANOVA. The survival of the emerged seedlings was analysed with a failure-time analysis, which measures the time to failure (death) of each individual (Fox 2001). *C. monspeliensis* was excluded from this analysis due to low number of sample in some treatments. We used the Cox's Proportional Hazards semi-parametric model, which produces estimates of regression models with censored survival data using maximum partial likelihood as the estimation method (Allison 1995; Fox 2001). The mass of

the seedlings was compared among treatments with a one-way ANOVA; in some species (*F. ericoides* and *C. monspeliensis*) the number of seedlings available was low to perform the ANOVA, and were discarded from this analysis. We did not seek to analyse differences among species, and thus all the analyses were performed separately for each species. ANOVAs were performed with either arc-sin transformed data (proportions) or log-transformed data (rest of variables) and figures are presented with raw data. Analyses were performed using JMP 5.0 software. Means \pm SE are shown throughout the paper. Seeds from the two sheep used for each species were pooled for all the experiments and analyses.

Results

Seed recovery after gut passage

Seed recovery was around 40% in most cases and even up to 50.3% in *C. monspeliensis* (Figure 1). Only *F. thymifolia* showed a lower percentage (11.9%); this was probably due to an experimental accident as we found *F. thymifolia* seeds in a sheep where its ingestion was not prescribed. We discarded these seeds from data analysis, and therefore *F. thymifolia* recovery was underestimated given that the number of seeds ingested by

prescribed sheep was lower than 4000. In any case, the pattern of recovery among days was not affected and was properly identified (Figure 1). There were significant differences in the number of seeds recovered per day for all species (χ^2 -test, $p < 0.05$ in every case), most seeds being dispersed between 0–24 h and 24–48 h after ingestion (Figure 1).

The percentage of pellets containing at least one seed was 23.1% for the first day, 28.3% for the second day, 6.5% for the third day and 2.3% for the fourth day (all species together). As a whole, pellets containing seeds had a single seed in 83.3% of the cases, two seeds in the 15.0% of the cases, and three or four in the rest of the cases, with significant differences between them ($\chi^2 = 707.72$, d.f. = 2, $p < 0.001$; all species pooled).

Seed germination after gut passage

Seed viability of control seeds was around 95% for all the species once the seed coat was eroded with sandpaper. Gut passage boosted seed germination in all the species (F -value from 6.06 to 63.80, d.f. = 2, 21, $p \leq 0.0084$; F -value = 14.73, d.f. = 1, 14, $p = 0.0018$ for *F. thymifolia*; Figure 2), with values up to seven-fold higher than in non-ingested seeds (e.g. 24 h vs. Control treatment in *C. laurifolius*). In most cases, germination was higher for seeds

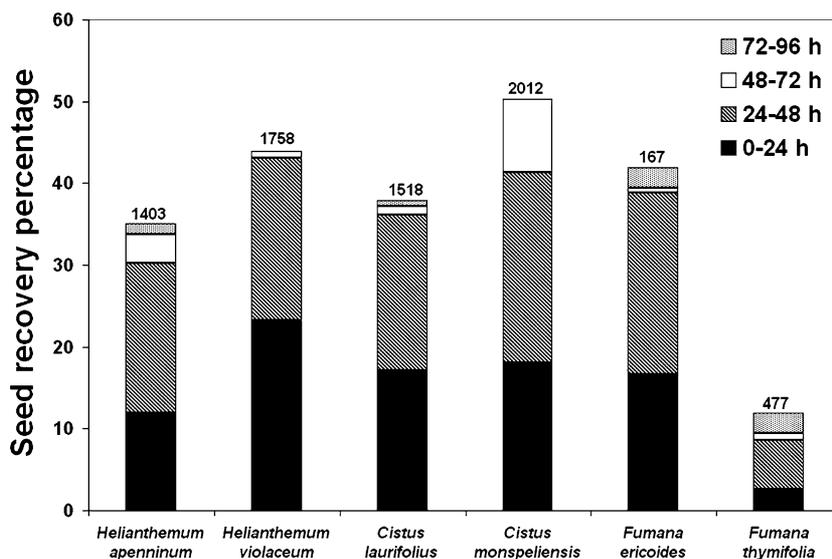


Figure 1. Daily percentage of seed recovery of six Cistaceae species after seed consumption by sheep. Numbers above bars are the estimate of total seeds recovered.

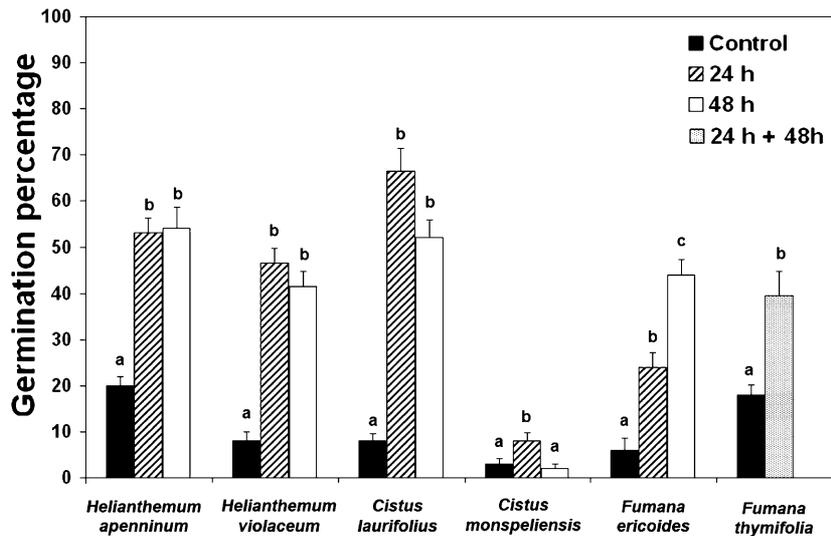


Figure 2. Percentage of seed germination of six Cistaceae species recovered from dung pellets of sheep (24 and 48 h after ingestion). Different letters above bars indicate significant differences among treatments according to Bonferroni–Dunn test ($\alpha=0.05$). *F. thymifolia* seeds from 24 to 48 h were pooled due to lack of sample (see text for explanation).

recovered both at 24 and 48 h after ingestion (Figure 2). *C. monspeliensis* showed an exceptionally low germination percentage even after gut passage, but germination was still promoted for seeds recovered 24 h after ingestion (Figure 2).

Early seedling establishment

Seedling emergence spanned day 4 to day 19 after the start of the experiment. Emergence differed significantly among treatments except for *F. thymifolia* (Table 1). The highest values were obtained in the Crumbled treatment (up to 26%), where emergence consistently exceeded Control values. Furthermore, emergence in Control and Intact treatments overall had similar values (Table 1). Survival of seedlings 20 days after emergence ranged from 40 to 70% of emerged seedlings (Figure 3), and did not differ among treatments ($L-R \chi^2 \leq 1.76$, d.f.=2, $p \geq 0.4147$); nor did the mass of the seedlings that survived 20 days differ among treatments ($F \leq 2.35$, treatment d.f.=2, residual d.f. from 17 to 19, $p \geq 0.12$).

Discussion

Effective endozoochorous dispersal implies that seeds need not only to be transported from the maternal plant, but also the probabilities of

recruitment should be, overall, at least as high as those of non-dispersed seeds. In fact, whether seed ingestion by dispersers is really advantageous to a plant can be assessed only if the probability of the ingested seeds to become an established plant is compared to the probability of seeds that have not been ingested (e.g. Traveset 1998). In this sense, our results show that seed ingestion increase seedling success through consecutive stages of establishment, supporting that sheep disperse effectively Cistaceae seeds.

Seed recovery after gut passage was considerably high (up to 50%) for five Cistaceae species, and most probably could also reach high values for *F. thymifolia*, if no experimental accident had occurred. This recovery rate is even higher than reported for other hard-seeded species after passage through the ungulate gut (Russi et al. 1992; Fredrickson et al. 1997; Razanamandranto et al. 2004; Mouissie et al. 2005) and is likely to be due to the small size and hard coats of Cistaceae seeds, which protect them from the damage during mastication and rumination (Pakeman et al. 2002; Castro et al. 2005; Mouissie et al. 2005). The dispersal of these Cistaceae after sheep consumption is thus guaranteed, and in fact ungulates are known to defecate a large amount of hard seeds under natural conditions, (e.g. between 8000 and 24,000 seeds day⁻¹ for *Cistus ladanifer*; Malo and Suárez 1996, 1998). Furthermore, seeds appeared

Table 1. Percentage of seedling emergence of five Cistaceae species in native soil from each locality of seed collection, without dung (control), with crumbled pellets and with intact pellets.

Species	<i>n</i>	Control	Crumbled pellets	Intact pellets	χ^2	<i>p</i> -value
<i>Helianthemum apenninum</i>	45	10.8	15.8	5.8	8.25	0.0161
<i>Helianthemum violaceum</i>	38	7.2	13.7	6.5	5.81	0.0547
<i>Cistus monspeliensis</i>	5	0.0	6.9	0.0	–	–
<i>Fumana ericoides</i>	28	4.2	26.4	8.3	17.81	0.0001
<i>Fumana thymifolia</i>	40	16.7	23.2	18.1	1.17	0.5582

n: number of emerged seedlings.

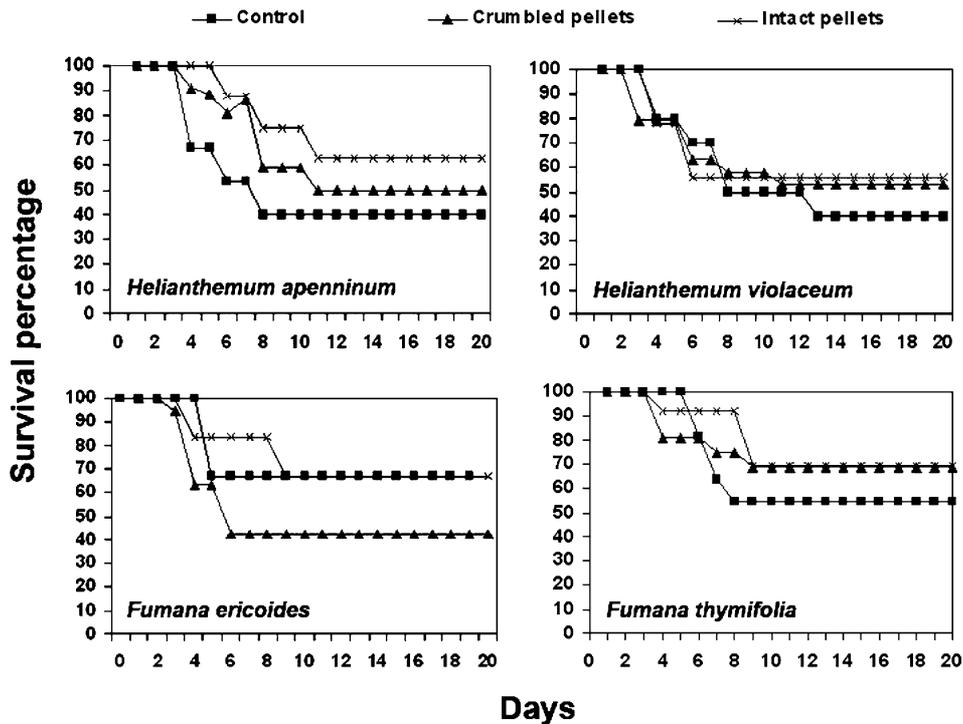


Figure 3. Survival curves for seedlings of four Cistaceae species placed in native soil from each locality of seed collection, without pellets (control), with crumbled pellets, and inside intact pellets.

in dung in a high proportion during the first 48 h, coinciding with the characteristic food-retention time of ungulates (Olson and Wallander 2002) and continue appearing even during the third and fourth day. In southern Spain, free-ranging sheep usually graze in open shrublands where they can walk up to 6.1 km per day (Cory 1972) and, eventually, more than 25 km per day (transhumant livestock; Klein 1981). Therefore, seeds ingested during grazing can be spread over a large area. Moreover, the scattered deposition of faeces and the homogenous distribution of seeds among pellets (see Results) diminish the risk of seed-clumping and, thus, of seedling competition.

In addition to dispersal, gut passage boosted germination of Cistaceae seeds (see also Malo and Suárez 1996 for *C. ladanifer*, and Manzano et al. 2005 for *Halimium umbellatum*). Germination increased up to seven-fold in relation to the Control treatment (e.g. 24 h treatment vs. Control in *C. laurifolius*). Cistaceae seeds are characterised by a primary seed dormancy (i.e. physical dormancy) imposed by hard coats (Thanos et al. 1992; Baskin et al. 2000) and thus any factor softening the coats without damaging the embryo may promote germination. Ruminal liquid contains proteolytic and cellulolytic enzymes (Prins and van der Vorstenbosch 1975) that may soften the seed coat.

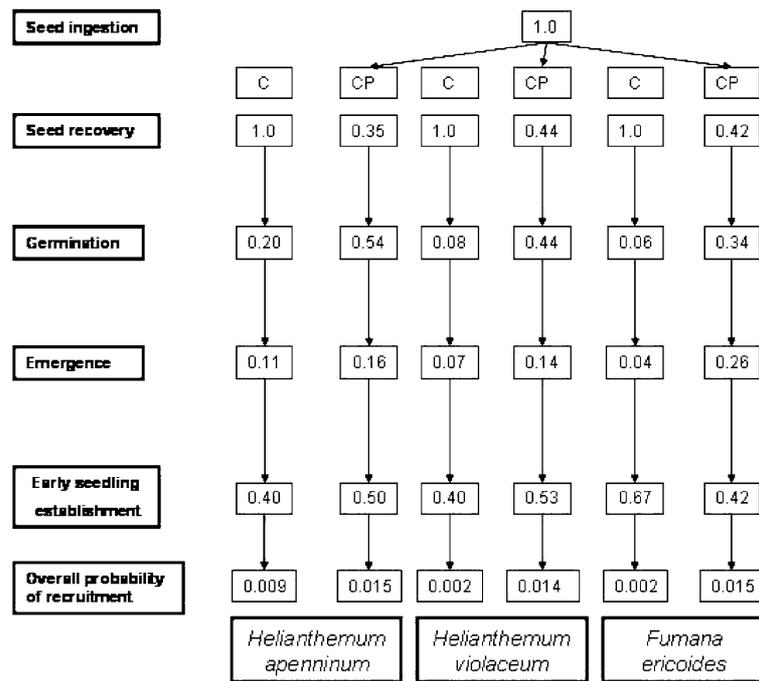


Figure 4. Diagram showing all the stages from seed ingestion to early seedling establishment for three Cistaceae species on native soil from each locality of seed collection. Transition probabilities are shown for every stage. C=control (non-ingested seeds); CP=crumbled pellets (ingested seeds contained in crumbled pellets).

In addition, seeds are exposed to an acidic medium and enzymatic activity into the abomasum and duodenum (Gardener et al. 1993), which could further erode seed coats. In this sense, Robles and Castro (2002) found that germination of *H. apenninum* was promoted when incubated in ruminal liquid. However, gut passage had a weak effect on germination of *C. monspeliensis* (8%, Figure 2), perhaps due to even harder coats that would need a more aggressive treatment (mechanical scarification registered 93% germination). The promotion of germination may increase the probability of seed germination over a longer period in Mediterranean environments (Izhaki and Safriel 1990), as a persistent soil seed bank is combined with a germinable fraction of seeds transported by the ungulates.

Emergence was lower than expected according to seed viability (95%). This was probably due to unusual high temperatures registered in the nursery during the time of the experiments (ca. 37% above the average; <http://www.ugr.es/%7Eiag/meteo/meteorologia.html>), which might have caused the death of germinated seeds before emergence. In any

case, emergence was higher or similar for seeds contained in faecal material simulating natural conditions compared to seeds not contained in dung. The highest values were reached for crumbled pellets, perhaps due to their higher water-retention capacity (Traveset et al. 2001; see also Malo and Suárez (1998) for similar results with *Cistus laurifolius*). In addition, seedling growth was not blocked by the presence of faecal material (see also Nchanji and Plumptre (2003) and Traba et al. (2003) for other species). Faecal material may in fact confer greater vigour to seedlings due to the release of nutrients (Traveset et al. 2001; Traveset and Verdú 2002), and we may have failed to detect differences because 20 days were insufficient time to decompose the manure into available nutrients for plants.

In summary, we may conclude that ungulate herbivores such as sheep are effective dispersers of Cistaceae seeds, as seed recovery after gut passage is high, seeds can be dispersed to distant places, germination is boosted, and seedling emergence, survival and growth are not depressed by manure (the contrary can occur, as in seedling emergence).

In fact, with the pooling of all the stages analysed, transition probabilities show that seed consumption may considerably increase the overall probability of recruitment (Figure 4), with values 1.7, 7.0 and 7.5 times higher (*H. violaceum*, *H. apeninnum* and *F. ericoides*, respectively) for seeds from crumbled pellets than for non-ingested seeds. The interaction among ungulate herbivores and Cistaceae species can therefore be considered a mutualism in which both partners derive a benefit, and this relationship does not differ qualitatively from the widely accepted mutualism between frugivorous vertebrates and fleshy-fruited plants.

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