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Endozoochorous seed dispersal by free ranging herbivores

Endozoochorní šíření rostlin volně žijícími herbivory

Doctoral thesis

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Declaration / Prohlášení autora:

I hereby declare that I made this thesis independently, using only the mentioned references. I did not submit this thesis nor its part for any other degree or diploma.

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tuto práci ani její podstatnou část jsem nepředložila k získání jiného nebo stejného akademického titulu.

V Praze dne Barbora Lepková

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Abstract

Endozoochorous dispersal of seeds is a very common phenomenon which may occur anywhere some animals feed on plants bearing mature seeds. Endozoochory has been identified as a potential driver for long-distance migration but there is a strong discrepancy between endozoochory by frugivorous animals and herbivores. Despite the fact that the latter has been observed as early as a century ago, our understanding of the herbivorous endozoochory is still limited, even more so when it comes to the free-ranging wild species of herbivores. Furthermore, the endozoochorous dispersal shows significant differences between study areas and between herbivore species suggesting we need information about the process from various study areas to draw any conclusions.

This thesis aimed to: (i) research the species composition found in dung samples of wild boars and deer, (ii) quantify the effect of deer dung deposition on vegetation of dry grasslands, (iii) establish the adaptation of plant species to the passage through digestive tract, and (iv) disentangle the drivers of species composition dispersed in dung.

The results of my research indicates: (i) the species composition in dung samples differs up to a point between deer and wild boars: some species are dispersed by both dispersers while other only by one of them. (ii) The deposition of deer dung has a negligible effect on the vegetation of dry grassland as measured during a six year experiment. (iii) The species survival in the digestive system is connected to the seed shape and nitrogen content. However, the results differ between plant families. (iv) No clear driver influencing the species composition in deer and boar dung was found.

The results of my research suggested that seed dispersal by free-ranging wild herbivores (measured by a greenhouse experiment) may be a strong driver potentially able to change the species composition of habitats under endozoochorous pressure. However, the realized effect of endozoochory on vegetation is very small. Furthermore, the endozoochory by the most common ungulates - deer and wild boars - is accidental and not driven by any of the tested drivers.

Keywords herbivory, seed dispersal, endozoochory, abandoned landscape, dry grassland, dung seed bank, disturbance, plant traits, Ellenberg Indicator Values, seed traits, seed nutrient content, plant frequency in the landscape, leaf traits, leaf nutrient content

Abstrakt

Endozoochorní disperze semen je velmi častý fenomén, který můžeme pozorovat kdekoli, kde se zvířata živí rostlinami, které nesou zralá semena. Endozoochorie byla popsána jako potenciální mechanismus pro migraci na dlouhou vzdálenost, nicméně je zde velký rozdíl mezi endozoochorií frugivorními zvířaty a herbivory. Navzdory tomu, že herbivorní endozoochorie je známa již více než století, naše znalost tohoto fenoménu je stále omezena, o to více v případě volně žijících, divokých druhů herbivorů. Mimoto se ukazují velké rozdíly v endozoochorní disperzi mezi studovanými oblastmi i mezi studovanými herbivory. To naznačuje, že potřebujeme detailní znalost procesu z různých oblastí, abychom mohli dojít k jakýmkoli závěrům.

Předkládaná práce si dala za cíl: (i) popsat druhové složení nalezené v trusu divokých prasat a jelenů, (ii) kvantifikovat efekt jeleního trusu na vegetaci suchých trávníků, (iii) změřit míru adaptace na průchod trávicím traktem u vybraných druhů rostlin, a (iv) rozplést jednotlivé mechanismy ovlivňující druhové složení šířené v trusu.

Výsledky mého výzkumu naznačují: (i) druhové složení v trusu se do určité míry liší mezi jeleny a divokými prasaty: některé druhy jsou šířené oběma zvířaty, některé jen jedním z nich. (ii) Depozice jeleního trusu má zanedbatelný efekt na vegetaci suchých trávníků, jak jsem změřila během šestiletého experimentu. (iii) Přežití semen v trávicím traktu je ovlivněno tvarem semen a jejich obsahem dusíku. Výsledky se však liší mezi čeleděmi rostlin. (iv) Nebyl nalezen jednoznačný mechanismus řídící druhové složení v trusu jelenů a divokých prasat.

Výsledky mého výzkumu naznačily, že disperze semen volně žijícími herbivory (měřena pomocí skleníkového experimentu) může být mechanismus potenciálně schopný změny druhového složení v habitatech pod endozoochorním tlakem. Nicméně změřitelný efekt endozoochorie na vegetaci je velmi malý. Endozoochorie za pomoci nejběžnějších kopytníků - jelena a divokého prasete - je náhodná a není ovlivněna žádným z testovaných mechanismů.

Klíčová slova herbivorie, šíření semen, endozoochorie, opuštěná krajina, suché trávníky, semenná banka v trusu, disturbance, vlastnosti rostlin, Ellenbergovy indikační hodnoty, vlastnosti semen, obsah živin v semenech, frekvence rostlin v krajině, vlastnosti listů, obsah živin v listech

Contents

Introduction

0.1 Seed dispersal

Seed dispersal is a process enabling the connection of habitat patches, increasing genetic diversity (Lozada-Gobilard et al. 2021), and colonizing new areas including long-distance dispersal and migration (Cain et al. 2000). Many vectors have been shown as effective dispersers, either abiotic (wind, water) or biotic (animals, humans). In databases, plant species are assigned all potential dispersal vectors (Pérez-Harguindeguy et al. 2013) but when using this information in analyses, the most frequent or important is used (i.e, the type of dispersal providing the longest-distance dispersal, Pérez-Harguindeguy et al. 2013). However, most plants are polychorous — they are dispersed via multiple vectors (Sádlo et al. 2018). The dispersal mode approach is based on morphological features of the dispersal propagule (be it seed, fruit, infructescence, or even the whole plant): for example, plants producing seeds with hair or wings are automatically considered as wind-dispersed (anemochorous). Similarly, only plants with colourful fleshy fruits are considered as dispersed by internal animal transport (endozoochory) despite the growing body of evidence that seeds dispersed zoochorously often lack any morphological adaptations (Pérez-Harguindeguy et al. 2013).

However, expert knowledge (Sádlo et al. 2018), as well as experimental testing (Almeida et al. 2022), suggest this morphological approach to dispersal modes is not sufficient. According to Sádlo et al. (2018), diaspore morphology is important, but just as important is the species' ecology. The diaspore morphology can easily be misleading: *Taraxacum* spp. seeds are equipped with pappuses which are considered an adaptation to wind dispersal. However, the seeds are also equipped with small hooks which allow them to be easily attached to the fur (epizoochory) of grazing animals. Just as well, dandelions can be dispersed by water (hydrochory), ants (myrmecochory), and in the digestive system of herbivores which consume the seeds when grazing (endozoochory). All of the above-mentioned suggest that seed or diaspore morphology and morphological adaptations to dispersal vectors play much smaller roles than previously anticipated (Sádlo et al. 2018).

From an ecological point of view, seed dispersal is an important mechanism which allows the survival and spread of plant populations (Cain et al. 2000). Dispersal by animals — zoochory — is an important vector in many cases even though it does not play a major role in the dispersal of individual species (Sádlo et al. 2018). Zoochory provided the movement of propagules necessary for island colonization (Cain et al. 2000) and Holocene migration (Pakeman 2001). Nowadays, it plays an important role when plants need to escape the global change (González-Varo et al. 2017) or in maintaining dispersal between habitat fragments (Matías et al. 2010) in fragmented landscape (Cazetta & Fahrig 2022).

0.2 Zoochory

One of the reasons why animals are useful vectors for seed dispersal is because they visit similar habitats allowing the dispersed seeds to be deposited in a favourable environment (Janzen 1984). Different types of dispersal are facilitated by various animals. Endozoochory is a dispersal of seeds or fruits after consumption. Epizoochory is a dispersal of propagules attached to the fur and the propagules may include seeds, infructescence or even entire plants. Both endozoochory and epizoochory may be facilitated by a number of animals but large mammals are the most efficient dispersers (Chen & Moles 2015).

It is not possible to cover all types of zoochorous dispersal and associated processes within one thesis. Thus, the presented thesis deals only with endozoochorous dispersal by large herbivorous animals. Furthermore, it is possible to study endozoochory in a non-invasive way or using experiments which cause the animals minimal stress and discomfort. These include a collection of dung samples (e.g., Malo & Suárez 1995c) or feeding experiments (e.g., D'hondt & Hoffmann 2011).

Definitions of terms used in this thesis

Throughout the thesis, specific terms are frequently used. See the definitions for the precise meaning used in this thesis.

Seed dispersal: sensu lato. The term is used to describe the general process of dispersal without recognizing if it is only potential dispersal or realized dispersal.

Endozoochory: seed dispersal after consumption and passage through the digestive tract of animals feeding on fruits or plants with mature seeds.

Epizoochory: seed dispersal after the dispersing propagule is attached to the exterior of the animal. Epizoochory includes not only dispersal in fur but often also dispersal in soil attached to the hooves.

Potential seed dispersal: our assumption that the seeds are dispersed without direct knowledge of consequent processes like germination and establishment under field conditions. Most studies on endozoochory report the potential seed dispersal based on the germination of dung samples under greenhouse conditions but it does not say anything about possible germination under field conditions (both biotic and abiotic).

Realized seed dispersal: dispersal becomes realized when seeds are not only dispersed in dung, but are able to germinate and establish until they reach maturity and produce their own seeds.

Species adapted to dispersal: species showing a higher germination rate after direct passage through the digestive system than the average germination rate of all tested species.

Dispersing propagule: based on the type of dispersal, the dispersing propagule may be the fruit, infructescence, or entire plant (particularly in epizoochory). In endozoochory, the dispersing propagule is always referred to as 'seed' or 'diaspore' and has the meaning of one seed from which one seedling emerges.

0.2.1 Herbivorous vs. frugivorous endozoochory

Endozoochory can be provided by any animal consuming fruits, seeds or entire plants with seeds at maturity. For a long time, plants were considered endozoochorous if their seeds were enclosed in fleshy, often colourful fruit (Pérez-Harguindeguy et al. 2013). The fleshy fruit was considered a reward for the disperser, without the reward dispersal might have happened only accidentally and did not play a major ecological role. This endozoochory was provided by frugivorous animals: in the context of Europe, this means frugivorous birds and some omnivorous mammals like martens and bears (D'hondt et al. 2011).

However, another type of endozoochorous dispersal has been observed as long as a century ago: the dispersal of pasture plants by large herbivorous mammals (Ridley 1930). Contrary to plants dispersed by frugivorous endozoochory, these species usually lack not only fleshy fruit, but any other dispersal adaptation whatsoever (Pakeman et al. 2002). This herbivorous endozoochory has been observed in any large herbivore studied (Malo & Suárez 1995b in cattle; Eycott et al. 2007; Campos-Arceiz et al. 2008 in elephants; Jaroszewicz et al. 2013b in free-ranging wild animals; Wang & Hou 2021 in yaks). The theoretical background ensuring the effectiveness of herbivorous endozoochory has been described by Daniel Janzen (1984). In his 'foliage-is-the-fruit' hypothesis, he specified a list of traits he expected in plants dispersed by herbivores. These traits or characteristics included plant edibility (at least when it has mature seeds) and nutrient rich foliage, the mature seeds remain on the plant and are adapted to survive the passage through the digestive tract (small size, strong seed coat) (Janzen 1984). However, not all plants dispersed by herbivorous endozoochory need to comply with all of Janzen's predicted traits.

In general, we can state that there is a strong dichotomy between endozoochory provided by frugivores (consuming only or in majority fleshy fruits) and by herbivores (consuming primarily foliage) (Almeida et al. 2022). The division, however, is not complete, herbivorous animals can also forage on plants bearing fleshy fruits (Picard et al. 2016). Using seed morphology to predict herbivorous endozoochory (as is used in frugivorous endozoochory or other types of dispersal) is thus very difficult. However, herbivorous endozoochory can be predicted using a combination of plant traits and habitat characteristics (Albert et al. 2015b), namely seed releasing height and habitat openness.

This thesis focuses on large herbivorous ungulates as possible vectors for herbivorous endozoochory. Their forage consists of graminoids, legumes, or foliage in various ratios based on their feeding preferences (Hofmann 1989). Animals closer to the grazing end of the continuum (grass/roughage eaters according to Hofmann 1989) graze on the vegetation available and we can assume they consume the seeds accidentally. These include cattle from domestic animals or bison from wild species. On the other hand, animals belonging to browsers (concentrate selectors) are highly selective using olfactory organs (Hofmann 1989) suggesting they can pick out fleshy fruits if they are interested in them. Roe deer is a typical concentrate selector and from domestic animals, the goat is the closest to this type of feeding type. According to this division, even in large ungulates we can see a potential for the herbivore-frugivore dichotomy. Furthermore, there are species of large ungulates which are not included in the grazing/browsing continuum because it involves only ruminants. Specifically, omnivorous animals can play a big role in dispersal of species with fleshy fruit (D'hondt et al. 2011) as well as plants without adaptation to frugivorous endozoochory. Wild boars present an important example of omnivorous animal which frequently feeds on vegetation (Genov 1981).

0.3 Effect of endozoochory on vegetation

Endozoochory affects vegetation through two processes: the dispersal of seeds but also by the deposition of dung samples in which the seeds are enclosed. Furthermore, there is a number of other processes associated with endozoochory which are mainly driven by the presence of animals. However, most of the field experiments monitoring the effect of endozoochory on vegetation have been done on pastures. Here, the effect is more pronounced since the animals have limited possibility of movement outside the fenced area. Contrary to this, wild animals are not limited by fenced enclosures and may move (more or less) freely. The effect of wild and free-ranging animals on vegetation is thus weaker and more spatially spread out. This includes not only the direct effect of endozoochory, but also the effects of other associated processes (see 0.3.1 Associated effects of endozoochory).

The presence of dung affects species richness at the site: increases alpha diversity but decreases beta diversity (Malo & Suárez 1995b). Welch (1985) also recorded an increase in alpha diversity but not all newly observed species were transported in dung. The dung deposition caused a change in abiotic conditions (deposition of nutrients, Aarons et al. 2009; or creation of gaps, Cosyns et al. 2005a) which promoted germination of species from the soil seed bank (Welch 1985).

The dung pat contains high amounts of nutrients (Aarons et al. 2009). Thus, the vegetation surrounding the dung pat can grow faster and taller, shadowing the seedlings germinating from dung pat (Bakker & Olff 2003) which are under decreased grazing pressure because the animals tend to avoid vegetation in the direct vicinity of dung pats (Castle & MacDaid 1972; Gillet et al. 2010). However, such findings are applicable only in the case of cattle pastures where these experiments were performed. Not only cattle are usually confined in fenced areas, but also because bovids create large and thick dung piles which are able to suffocate vegetation lying underneath them (MacDiarmid & Watkin 1971; Welch 1985). This creates a gap in the vegetation which increases the chance of seedling germination (Oesterheld & Sala 1990; Cosyns et al. 2006). However, this is in direct contrast to other domestic species (e.g., sheep) and most free-ranging animals of Central Europe (family *Cervidae*). All these animals create dung droppings of much smaller scale compared to bovids (Anděra & Horáček 2005) which lack the ability to suffocate underlying vegetation.

Yet, research studying the effect of endozoochory by free-ranging herbivores on vegetation is scarce. There are some exceptions, e.g., from the Białowieża Forest where the European bison increased the build-up of soil seed bank via endozoochorous dispersal but seeds of non-forest species were dispersed on the forest sites where they remained viable in the soil seed bank (Jaroszewicz 2013). The dispersal of non-forest species in forest stands has been observed also indirectly by Eycott et al. (2007): the non-forest species germinated from dung samples collected in forest in the greenhouse. The question remains whether the species dispersed to non-favourable habitats stay viable in the soil seed bank long enough to wait for the conditions to change. The dispersal by large animals has been hypothesized to be effective precisely because the animals prefer to stay in the same habitat (Janzen 1984). However, the free-ranging animals show different habitat preferences for feeding (Anděra & Gaisler 2012) and resting sites (Gallina et al. 2010; Yokoyama et al. 2020) (open vegetation and closed canopy, respectively). This may be the cause for the dispersal of plant species outside their preferred habitats.

0.3.1 Associated effects of endozoochory

Large ungulates provide a number of ecological functions besides seed dispersal. These include the removal and distribution of biomass via grazing, redistribution of nutrients via defecation and urination, toughening of soil on trails via trampling, and creation of small-scale disturbances via rooting.

These effects differ in strength based on the fact whether or not the animals are enclosed in fenced pastures. In general terms, this also means the difference between domestic herbivores (cattle, horses) and wild species (*Cervidae*, wild boar). Under free-ranging circumstances, the animals might choose to have much larger home ranges (i.e., the territory where the animal moves, feeds and reproduces) compared to the fenced pasture, whereas animals living in enclosures are limited in their movement and forage choice. All effects the animals have on the vegetation and individual plants are thus much stronger and more concentrated. Often, the animals are also confined in limited habitat selection.

0.3.1.1 Grazing Grazing and browsing are the main paths of biomass relocation via herbivorous ungulates with a significant difference between the two (via Hofmann 1989). Grazers (or grass/roughage eaters, e.g., cattle) are best adapted to consume and digest forage high in cellulose (grasses). On the other hand, browsers (or concentrate selectors, e.g., roe deer *Capreolus capreolus*) are less optimized to digest high amounts of cellulose. They use olfactory signals to search for easily digestible and high-quality forage. There is a third group of ruminant herbivores which do not fall into either of the categories: intermediate feeders. They try to avoid fibrous foods and are able to switch between forage quality based on its availability, e.g., during the winter/summer growing season. Intermediate feeders include both domestic (e.g. goat, *Capra hircus*) and free-ranging animals (e.g. red deer, *Cervus elaphus*).

According to their feeding strategy, the herbivores choose different forage. The ratio of grazers, browsers, and intermediates in the landscape can thus affect the way how different plant groups are consumed (Donaldson et al. 2022) which may lead to changes in vegetation composition (McNaughton 1984).

0.3.1.2 Nutrients relocation and creation of disturbances As an effect of grazing, nutrients consumed during feeding periods are later redistributed during defecation and urination. Often, animals are given extra forage which brings extra nutrients into the system. In the case of intensively grazed pastures, the vegetation is under heavy grazing pressure causing changes in species composition and the eutrophication can fasten or increase these changes. This can lead to a state of the pasture when the species composition is dominated by unpalatable plants.

Animals are also responsible for creating a number of disturbances which have a direct effect on plants and vegetation composition. These include wild boar rooting, burrowing by small mammals, or digging by hooves. The disturbances create gaps with bare soil where seeds from soil or dung germinate more easily compared to germination in undisturbed vegetation (Oesterheld & Sala 1990). A specific case is trampling, i.e., a frequent movement of individuals which causes the creation of more or less permanent trails. The trails have a higher density of soil and different thermal, hydrological, and chemical conditions. These processes, together with grazing and browsing pressure from passing animals, cause changes in plant fitness and species composition in the vicinity of trampling trails, e.g., reduce the plant cover, plant height and species density (Kissling et al. 2009).

0.4 Filters influencing endozoochorous seed dispersal

There is a number of factors which influence whether or not a species will be successfully dispersed via endozoochory. Each of the factors acts as a **filter** and only a portion of species passes through to the next filter. The filters can be classified into four categories:

(i) filters on the level of vegetation;

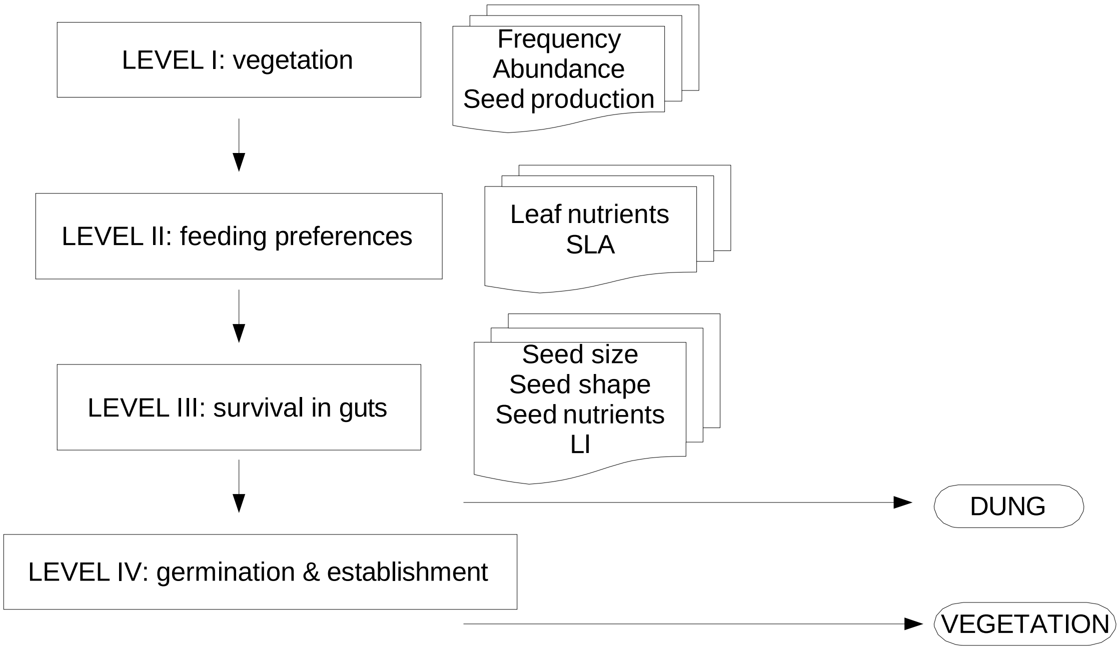
(ii) filters on the level of herbivore feeding preferences;

(iii) filters on the level of herbivore digestive system and the ability of seeds to survive in the digestive system;

(iv) filters on the level of dung sample: what allows the seeds in the dung to successfully germinate and establish in the vegetation.

See Fig. 1 for an illustration of these categories. However, the categories as well as specific filters are often difficult to disentangle as they may influence each other, are correlated, and are difficult to study within one experimental set-up.

Fig. 1 There are four levels of environmental filters influencing the success of endozoochorous dispersal. Each level acts as a bottleneck and only a portion of the diaspores passes to the next stage. Passage through levels I-III guarantees the seeds consumed by the herbivores safely survive the travel through the digestive system. The seeds can then be found in collected dung samples and germinate under favourable conditions. However, without successful germination and establishment in the field, the diaspores cannot be assigned as successfully dispersed.



0.4.1 Filters on the level of vegetation

As mentioned above, the available vegetation is one of the main drivers of species composition found in dung (Jaroszewicz et al. 2013a). As a result, the species composition in dung differs between habitats as well as between geographical regions (Oheimb et al. 2005). This suggests that animals grazing in species-rich habitats may disperse higher numbers of plant species compared to animals grazing in species-poor habitats. However, most dispersed species are represented by a very small number of individuals, often less than 1 % of all dispersed seeds (Jaroszewicz et al. 2013a). The majority of seedlings belong to only one or a few species (Eycott et al. 2007). Common species have a higher probability of being dispersed in dung because the animals can consume the seeds accidentally when grazing on other plant species.

On the other hand, species can be rare in the landscape but abundant locally due to specific environmental conditions. Such species may be frequently dispersed (i.e., found in the dung of herbivores in a greenhouse experiment) but do not establish in the vegetation because they are not deposited in a favourable environment. As a result, such species are considered as often dispersed and well adapted to endozoochory by the researchers even though the realised dispersal is poor. This may happen also when species are dispersed from one type of habitat to another, e.g., from open landscape to forest. The species may then enter the soil seed bank and wait for the environmental conditions to change (Malo et al. 1995; Jaroszewicz 2013).

Another factor playing on the landscape level is the production of seeds (Bruun & Poschlod 2006). Species with high seed production have a larger probability of dispersal for several reasons. First, high production of seeds also means seeds of smaller size. Smaller seeds have an increased probability of surviving the passage through the digestive system. Second, high number of seeds increases the probability that at least a portion of seeds survive when they are consumed. Third, the high seed number increases the probability that seeds are consumed accidentally when the animal grazes on other plant species.

0.4.2 Filters on the level of herbivore feeding preferences

Herbivorous animals prefer different types of vegetation for feeding based on their feeding behaviour and digestive system (Hofmann 1989). The most common herbivores in the landscape of Central Europe are deer (red deer, roe deer) and wild boars (Linnell et al. 2020). Even though deer are ruminants, they are very different from the most commonly studied herbivore — cattle (see above, e.g., the effect of cattle dung pats compared to deer droppings). Contrary to deer, wild boar is often omitted in studies about herbivores as it is an opportunistic omnivore able to feed on anything from larvae and roots to carcasses (Genov 1981). However, in the vegetation season, the majority of the wild boar diet is herbivorous (Genov 1981) and it acts as an effective seed disperser (Schmidt et al. 2004).

Both deer and wild boars show preferences for feeding in the open landscape (Genov 1981). This is of course driven by what type of open landscape is available: in the cultural landscape of Central Europe, this often means agricultural fields. However, in areas where agriculture is rare, the herbivores feed on meadows, semi-natural grasslands, or, when none of these are available (e.g., in areas of primeval forests like Białowieża), in forest clearings. As a result, plant species associated with open vegetation types have a higher probability of being dispersed in dung.

Finally, herbivores show preferences for certain species or groups of species. The most commonly grazed plants are from families *Poaceae* and *Fabaceae* (Potter et al. 2022), however, it remains a question why. Herbivores are able to distinguish levels of certain nutrients, either toxic or beneficial. For example, red deer preferred plants with low levels of sulphur, copper, strontium, and zinc but the strongest factor seemed to be the excess sulphur rather than any of the other minerals because of its toxicity (Ceacero et al. 2015). Furthermore, cervids avoid plants with low foliar nitrogen and prefer higher levels of phosphorus (Forsyth et al. 2002). Leaf nutrient content is thus another driver of herbivore preferences.

0.4.3 Filters on the level of herbivore digestive system

The probability with which seeds survive the passage through the digestive tract is driven by a number of variables, both on the side of the dispersing vector and the dispersed plant species. This is because the consumed seeds must overcome very harsh and unfavourable conditions in the digestive tract: both mechanical (avoid the molar mill, twice in the case of ruminants) and chemical (deal with the digestive enzymes and the acidic environment in the stomach).

Contrary to epizoochorous dispersal, where the seed size and shape clearly affect the probability seed is attached to the fur, in endozoochory the effect of seed traits is less clear, in some cases even ambiguous. The current literature agrees on one seed trait positively influencing the survival in the digestive tract — small seed size (Pakeman et al. 2002; Mouissie et al. 2005a; Albert et al. 2015a). Small seeds have a higher probability of escaping the molar mill. Furthermore, small seeds can be dispersed by both large and small herbivores suggesting a higher probability of dispersal in general (Chen & Moles 2015). However, plant species with small seeds also produce a larger number of seeds. In a study with cattle, Bruun & Poschlod (2006) found that plant species are primarily dispersed based on their seed production and when the abundance in dung was corrected for seed production, there was no effect of seed mass, seed shape, or seed coat thickness (Bruun & Poschlod 2006).

Another seed trait has been frequently tested — seed shape. However, there are conflicting results from various studies. Mouissie et al. (2005a) found a better survival of round seeds but Cosyns et al. (2005b) reported increased survival of elongated seeds. Furthermore, in some studies, there was no effect of seed shape at all (Bruun & Poschlod 2006; D'hondt & Hoffmann 2011). In some cases, the conflicting results could be caused by different methods, either statistical (e.g., including phylogeny, D'hondt & Hoffmann 2011) or experimental (feeding experiment in Mouissie et al. 2005a; and Cosyns et al. 2005b; versus comparison of species in dung and in available vegetation, Bruun & Poschlod 2006).

The third trait which often appears in analyses of seed traits adapted to endozoochorous dispersal is the seed longevity index (LI), i.e., the probability with which a seed survives for a certain time in the soil seed bank. There are three types of seed persistence in the soil bank: (i) transient seeds (T) stay viable in the soil for less than 1 year, (ii) short-term persistent seeds (SP) stay viable in the soil for more than 1 but less than 5 years, (iii) long-term persistent seeds (LP) stay viable in the soil for more than 5 years.

The index is calculated as a portion of records in individual classes following an equation by Bekker et al. (1998a). The longevity index has been positively correlated to species appearance in the dung of different herbivores: in cattle, sheep, horses, donkey, and rabbit dung after a feeding experiment (Cosyns et al. 2005b); in sheep and rabbit dung collected in the field (Pakeman et al. 2002), and in a meta-analysis including both domestic and wild species of herbivores (Albert et al. 2015a). The possible reason is that seed adaptations to long-term survival in the soil seed bank are the same or similar to adaptations needed for survival in the digestive tract (Pakeman et al. 2002). However, it is necessary to take into account the correlations between longevity index and other seed traits, e.g., seed size and shape, but these correlations are habitat-specific (Thompson et al. 1998). Furthermore, the persistence in seed bank differs based on some abiotic conditions making measurements difficult (Mašková et al. 2022). The positive effect of the longevity index suggests that digestion does work as a filter because all mentioned studies were done primarily on grassland species and grassland habitats have in general low number of plants with long-term persistent seed bank (Bekker et al. 1998a).

0.4.4 Filters on the level of dung sample: seedling establishment

Germination from the dung sample may be easier due to the increased amount of nutrients (Aarons et al. 2009) but the seeds need to protrude a hard crust which develops on the surface of dung pellets or droppings in the case of dry weather. This has been reported in the literature for the case of bovid dung piles (Jaroszewicz & Pirożnikow 2011) but it holds true for deer droppings as well (personal observation). Once the crust covering the dung is broken and seeds start germinating, several ecological factors influence their later development: (a) the amount of nutrients in the dung may be too high, the seedlings are burnt and die soon, (b) the seeds start germinating in very high numbers. Competition takes over and only individuals with a good starting point (e.g., earlier germination or higher seed reserves) survive.

The effect of dung on the germination rate of seeds differs between plant species. Generally, the effect is more or less negative: seeds germinate from dung worse compared to seeds not deposited in dung (Milotić & Hoffmann 2017). There are cases of plant species which show higher germination in dung (D'hondt & Hoffmann 2011) but it is not possible to compare the studies directly because of methodological differences. Milotić & Hoffmann (2017) put fresh seeds inside samples of cattle and horse dung and compared the germination with seeds on the garden substrate. On the other hand, D'hondt & Hoffmann (2011) compared the germination of seeds which passed through the digestive system with unpassed seeds. The treated seeds were thus not only influenced by the germination within the dung sample but also by the passage through the digestive system which may break dormancy.

After successful germination, seedlings must be able to establish in the vegetation so that they are able to reproduce themselves. Since animals may graze on different vegetation types during one grazing period and in the case of ruminants their forage is then regurgitated, ruminated, and thus mixed, it is possible that seeds excreted in dung are deposited in an unfavourable environment. Such seeds may germinate but do not survive until the next vegetation season or reach the reproductive stage. However, the presence of dung may help those seedlings which are in their favourable environment. Even though the seeds germinate from dung slower and have a lower germination rate, they benefit from the dung environment in the growing phase. The plants grow faster and flower more (Milotić & Hoffmann 2017) which may act as compensation for the high cost of this dispersal mode.

0.5 How to study individual filters?

The dung sample (pat, or pile of droppings) acts as a black box. The easiest way how to study the dispersal in dung is to look at the dispersed species composition without taking into account all the processes influencing it. However, each researcher has their own approach to data collection and as a result, standardization across multiple studies may become impossible. See Tab. 1 and Tab. 2 for a summary of published studies sorted by the dispersing animal. However, individual papers differ in the number of collected samples, the method of standardization, and were performed in different vegetation types.

If we want to look inside the black box, we need to disentangle the filters influencing the dispersal from the very beginning: what the animals may eat and what they choose from the available forage, how the processing of food in their digestive system affects the survival of seeds, and also, what happens after the seeds are deposited in dung. Each level of the filters brings different challenges when studying it and it is not possible to study all filters at once. Furthermore, it is necessary to point out that the study of endozoochorous dispersal is by nature multidisciplinary and requires knowledge of both plant ecology and animal ethology. Since the focus of this thesis is on plant ecology, no experiments on animal behaviour were performed. The required information was either taken from available literature or proxy variables were used.

Table 1 Summary of published papers studying endozoochorous dispersal by animals included in this thesis — red deer and wild boar. *No. of species* refers to the total number of plant species found during the entire experiment. *No. of samples* refers to how many samples were collected and germinated. In samples marked with an asterisk, the producing animal could not be determined exactly. *Two most common species* are based on the number of germinated seedlings with the exception of Picard et al. (2016) where the number of seedlings was not available and the species are based on their frequency in dung samples. The column *trait* refers to other characteristics included as explanatory variables in the analyses. *Relations to abundance in vegetation* states whether or not the species composition of the dung seed bank was connected to nearby vegetation. Only papers marked with asterisk included their own vegetation data (the study with double asterisk included information on regional species pool from other literature). Other papers used general information about surrounding vegetation, e.g. habitat species lists. The last column indicates whether more than one herbivore species were included in the study.

animal	no. of species	no. of samples	two most common species	trait	relation to abundance in vegetation	different vectors
red deer						
Eycott et al. (2007)	96	235*	*Agrostis stolonifera, Juncus effusus*	dispersal mechanism	yes	yes
Iravani et al. (2011)	47	180	*Minuartia verna, Cerastium caespitosum*	seed size	yes*	no
Jaroszewicz et al. (2013b)	137	80	*Urtica dioica, Lythrum salicaria*		yes	yes
Karimi et al. (2018)	79	182	*Portulaca oleracea, Cyperus fuscus*	growth forms, life history, rarity	yes*	yes
Karimi et al. (2020)	22	182	*Blitum virgatum, Polygonum lapathifolium*		no	yes
Lepková et al. (2018)	75	190	*Urtica dioica, Galium mollugo*	seed mass, longevity index, seed appendages	yes*	yes
Malo & Suarez (1995)	66	104	*Cistus ladanifer, Spergularia purpurea*		yes	yes
Panter & Dolman (2012)	33	127*	*Agrostis capillaris, Plantago major*		yes	yes
Picard et al. (2016)	34	60	*Juncus sp., Calluna vulgaris*	seed size, seed shape, longevity index	yes**	yes
Oheimb et al. (2005)	59	220	*Urtica dioica, Juncus effusus*		yes	no

animal	no. of species	no. of samples	two most common species	trait	relation to abundance in vegetation	different vectors
wild boar						
Dovrat et al. (2012)	31	136	*Morus sp., Amaranthus blitum*	exotic species	no	
Jaroszewicz et al. (2013b)	24	90	*Urtica dioica, Juncus effusus*		yes	yes
Karimi et al. (2018)	69	149	*Cyperus fuscus, Portulaca oleracea*	growth forms, life history, rarity	yes*	yes
Karimi et al. (2020)	22	149	*Urtica dioica, Cyperus fuscus*	seasonality	no	yes
Lepková et al. (2018)	33	87	*Fragaria vesca, Poa pratensis*	seed mass, longevity index, seed appendages	yes*	yes
Picard et al. (2016)	24	120	*Juncus sp., Digitaria sanguinalis*	seed size, seed shape, longevity index	yes**	yes
Schmidt et al. (2004)	51	245	*Juncus effusus, Chenopodium polyspermum*		yes	yes
Heinken et al. (2002)	21	2448 g	*Poa trivialis, Agrostis capillaris*	diaspore morphology	yes	yes

* pooled samples of red and fallow deer

* vegetation sampling included in the study

** regional species pools

Table 2 Summary of published papers studying endozoochorous dispersal by animals not included in this thesis — roe deer and moose. *No. of species* refers to the total number of plant species found during the entire experiment. *No. of samples* refers to how many samples were collected and germinated with the exception of Heinken et al. (2002) who summed all collected samples and provide only the total weight. *Two most common species* are based on the number of germinated seedlings with the exception of Picard et al. (2016) where the number of seedlings was not available and the species are based on their frequency in dung samples. The column *trait* refers to other characteristics included as explanatory variables in the analyses. *Relations to abundance in vegetation* states whether or not the species composition of the dung seed bank was connected to nearby vegetation. Only papers marked with asterisk included their own vegetation data (the study with double asterisk included information on regional species pool from other literature). Other papers used general information about surrounding vegetation, e.g. habitat species lists. The last column indicates whether more than one herbivore species were included in the study.

animal	no. of species	no. of samples	two most common species	trait	relation to abundance in vegetation	different vectors
roe deer						
Schmidt et al. (2004)	36	216	*Juncus effusus, Urtica dioica*		yes	yes
Jaroszewicz et al. (2013b)	64	33	*Urtica dioica, Lythrum salicaria*		yes	yes
Eycott et al. (2007)	40	225	*Urtica dioica, Juncus effusus*	dispersal mechanism	yes	yes
Picard et al. (2016)	10	120	*Calluna vulgaris, Juncus sp.*	seed size, seed shape, longevity index	yes**	yes
Karimi et al. (2020)	7	50	*Portulaca oleracea, Sonchus oleraceous*	seasonality	no	yes
Panter & Dolman (2012)	17	161	*Agrostis capilaris, Geranium molle*		yes	yes

animal	no. of species	no. of samples	two most common species	trait	relation to abundance in vegetation	different vectors
Karimi et al. (2018)	24	50	*Portulaca oleracea, Echinochloa crus-galli*	growth forms, life history, rarity	yes*	yes
Auffret & Plue (2014)	67	219	*Capsella bursa-pastoris, Stellaria media*		yes*	no
Heinken et al. (2002)	7	152 g	*Stellaria media, Cerastium semidecandrum*	diaspore morphology	yes	yes
moose						
Jaroszewicz et al. (2013b)	27	35	*Molinia caerulea, Lythrum salicaria*		yes	yes
Jaroszewicz et al. (2013a)	74	56	*Urtica dioica, Poa subcerulea*	seasonality	yes	no
					* vegetation sampling included in the study ** regional species pools	

0.5.1 Dung seed bank

The dung seed bank describes the composition of plant species which already survived the passage through the digestive system and are excreted in the dung. It is the easiest information to obtain in any study system as it requires only collection of a sufficient amount of dung and recognition of seeds inside (usually by germination experiment). In the temperate zone, it is necessary to stratify the dung seed content. The dung is then submitted to a germination experiment, all germinating seedlings are identified, recorded, and removed to allow other seeds to germinate. However, starting with the dung collection, there are important differences in the methodology of individual papers making comparison difficult. Several options for dung collection are possible: (i) the entire dung sample made by one individual is collected and germinated (Blyth et al. 2013), (ii) dung samples from multiple individuals are pooled to obtain specific volume (Jaroszewicz et al. 2013b), (iii) all dung samples collected during one collection period are pooled (Kuiters & Huiskes 2010). These three approaches are impossible to compare unless all samples are dried after collection and weighed before germination.

The germination experiment is usually performed in greenhouse conditions but there are studies using common garden experiment as well and comparing the results. Significantly smaller number of species emerged in the common garden experiment compared to greenhouse conditions (Karimi et al. 2020). However, shrub species germinated better under natural conditions (Karimi et al. 2020). This experiment suggests a potential bias when only studying germination under controlled greenhouse conditions (potential endozoochorous dispersal) compared to germination under natural conditions (realised endozoochorous dispersal) (Karimi et al. 2020).

The primary information obtained from the germination experiment is (i) species composition, and (ii) the number of germinated seedlings per species. In the case when dung samples were collected per individual and not pooled, it is also possible to obtain the frequency of species, that is the number of dung samples in which the species occurred. Considering the fact that the majority of seedlings germinating in these experiments belong to one or a few species, the species frequency may provide additional information. The question remains whether or not to take into account species emerging with a very low number of individuals — such species may be dispersed only accidentally and in the field, such dispersal may not play any role.

0.5.2 Herbivore feeding preferences

The animals' feeding preferences are necessary for our understanding of endozoochorous dispersal because they provide one of the key filters. Expert knowledge can be provided by local managers or hunters about preferred and avoided species (Ceacero et al. 2015) but this information cannot be obtained for the entire local flora. We can thus assume the preferences based on information about plant palatability, or more precisely, using proxy variables describing the palatability: plant nutrient content. Animals can detect, and thus prefer or avoid, species with favourable nutrients (nitrogen, phosphorus) or toxic minerals (sulphur). However, there is a severe lack of cafeteria-type studies on wild species of herbivores and our knowledge is limited to a few species.

0.5.3 Herbivore digestive system

To successfully survive the passage through the digestive system is demanding because the seeds need to overcome several difficulties: (i) avoid the molar mill and not be chewed or ruminated (mechanical stress), and (ii) survive the passage through the acidic environment of the stomach or stomachs (chemical stress). It is possible to test the species' survival in two ways: either by performing laboratory simulations using a predefined protocol to simulate the mechanical and chemical conditions (Milotić & Hoffmann 2016b), or by performing a feeding experiment (Cosyns et al. 2005b).

Laboratory experiments are relatively easy to perform with basic laboratory equipment. However, also here there are different approaches and various methods used for the mechanical treatment: using sandpaper (Milotić & Hoffmann 2016b), real animal skulls (Milotić & Hoffmann 2016b), or an iron stick to simulate chewing (Kleyer et al. 2008). But different methods often give different results (Milotić & Hoffmann 2016b). Furthermore, it is impossible to simulate all substances influencing the seeds in the stomach or intestine and various enzymes are not included in the chemical treatments.

Feeding experiments are thus a more reliable approach to establishing the species' adaptation to the passage through guts. For the experiment, a predefined number of seeds is used and fed to the animals kept in enclosures. The animals are fed preferably sterile forage (silage, haylage) or forage without the species of interest (alfalfa hay) for several consecutive days prior to the experiment. The length of this period is based on the mean retention time of the animal of interest. The experimental seeds are fed to the animals either by hand when the animals are tamed (Mouissie et al. 2005a) or in a mixture of suitable forage. After the feeding, the dung samples are collected for a time longer than the retention time to ensure all seeds passed through the digestive tract. The collected dung is germinated in the greenhouse similarly to the process described above to ensure that the maximum number of seedlings emerge.

It is useful when individual animals are kept in separate pens or stalls because it allows the researchers to study the inter-individual differences. However, this is not usually possible in wild species which have strong herd needs.

0.5.4 Seedling establishment

The establishment of seedlings emerging from dung samples can be studied: (i) in a greenhouse (Milotić & Hoffmann 2016a), (ii) using a common garden experiment (Karimi et al. 2020), or (iii) directly in the field (Malo & Suárez 1995a). Only the last approach ensures all effects of the natural system including biotic and abiotic conditions.

In the field experiments, fresh dung samples are marked throughout the vegetation season to cover the seasonality of seed availability. The marked samples are then observed for up to several years until the dung completely disintegrates. According to the research question, the species composition growing on the dung samples or around them is recorded regularly. Individual seedlings can be marked as they emerge and their establishment and growth can be observed for several seasons.

0.6 Aims

The aim of this thesis was to take a closer and more detailed look into plant dispersal by free ranging herbivores (red deer and wild boar). I ask four main questions:

(i) what is the species composition dispersed in the dung of deer and boars? (Paper I)

(ii) what is the quantified effect of deer dung deposition on the vegetation of dry grassland? (Paper II)

(iii) how are seeds pre-adapted to dispersal? Do the dispersed species have specific traits? (Paper I, Paper III)

(iv) what is driving the species composition dispersed in dung? (Paper IV)

I performed three experiments to answer these questions and disentangle the individual filters depicted in Fig. 1. Paper I describes the dispersed species composition. For this paper, I collected dung samples in the study area and used a germination experiment to establish the composition of species in the samples. Furthermore, I added data on plant traits and characteristics from databases and searched for patterns in dispersed and non-dispersed plant species. Paper II demonstrates the effect of dung deposition on vegetation via a field experiment. I calculated community-weighted means of Ellenberg Indicator Values and tested the effect of dung deposition on species composition and succession. Paper III reports the results of a feeding experiment which I conducted with four species of herbivores and almost forty species of plants. I added data on seed nutrient content and seed traits to check whether these help the seeds to survive the passage through the digestive system of various animals. In Paper IV I took advantage of a comprehensive set of data from one study area. Combining detailed vegetation information together with data from the previous studies, I aimed to disentangle the individual drivers of endozoochorous seed dispersal: available vegetation (filter I), animals' feeding preferences (filter II), and species survival in the digestive system (filter III).

0.7 Study system

The research presented in this thesis was conducted in Military Training Area Hradiště in Western Bohemia as a part of a long-term project studying the effect of herbivores on vegetation in the abandoned landscape. The area is suitable for research on herbivore-vegetation interaction for several reasons: animals are in high abundance, they do not have access to agricultural fields, and they are not given extra forage during the vegetation season.

0.7.1 History

The Military area Hradiště is located in The Doupov Mountains (Duppauer Gebirge) close to the border with Germany. The mountains are of tertiary volcanic origin with a caldera in the centre and a chain of peaks surrounding it. Until the late forties, the area was heavily populated by mainly German inhabitants. In 1946, the German inhabitants were forcefully displaced and the majority of villages were abandoned. The remaining inhabitants were then relocated in 1953 when the Military Training Area was established. Currently, military training occurs only in the central part of the Training Area. The research presented in this thesis was conducted in the buffer zone not directly affected by military activities.

0.7.2 Vegetation

The vegetation of our study area consists of a mosaic of dry grasslands (class *Festuco-Brometea*) and shrublands with the dominance of *Crataegus* spp. (*Prunion spinosae*), see Fig. 2. This mosaic ensures the animals with sufficient cover in the shrublands and enough feeding ground in the open patches with dry grassland. This mosaic of grasslands and shrublands is where data were collected for Paper I and where the field experiment of Paper II was conducted. Furthermore, part of the area is under forestry management by *Vojenské lesy a statky* (~ Military forests and estates).

Specific vegetation grows in the areas of the former villages (Vojta 2007). These secondary forests are usually dominated by *Fraxinus excelsior* in the canopy layer and by nitrophilous species in the herb layer (*Urtica dioica*, *Geum urbanum*) (Kopecký & Vojta 2009). Some formerly cultivated species can be still found in the areas of the abandoned villages, e.g., *Hesperis matronalis* or *Syringa vulgaris* (Vojta 2007).

0.7.3 Animals

Game animals are present in the area in high numbers. The most abundant species are red deer (*Cervus elaphus*), introduced sika deer (*Cervus nippon*), and wild boar (*Sus scrofa*) similarly to what species are the most common in Central Europe (Linnell et al. 2020), see Fig. 3. The two species of deer belong to the family *Cervidae* and both are ruminants. Wild boar belongs to the family *Suidae* and has an omnivorous diet. Unless stated otherwise, the following review is taken from Anděra & Gaisler (2012).

Red deer are intermediate feeders and their diet lies between grazers and browsers (Hofmann 1989). They feed on grasses and shoots, and in winter, significant portion of their diet is bark peeled off of coniferous trees. Red deer inhabit a wide variety of habitats from the lowest altitudes to areas above the tree line but the most favoured environment is forested uplands and mountains (mean altitude 520 m. a. s. l.). Red deer are under a strong influence of human population: in the Czech Republic, red deer inhabited almost the entire area until the Late Middle Ages when the populations became patchy due to strong deforestation. At the beginning of the 19th century, the populations were heavily decimated because of high economic losses in forests and fields. The unfavourable effect of red deer on forests and fields remains a problem today when the animals cause significant economic losses.

The introduced sika deer (*Cervus nippon*) is originally from east and south-east Asia and they have been introduced in 1890 or 1891 as game animals. In the first half of the 20th century, some of the game parks were shut down and the animals spread out into the wildlife. Sika deer are similar in appearance to red deer but smaller, with smaller antlers, and a slightly different colour. Sika are not highly demanding when it comes to environmental conditions. They only need some type of closed vegetation as cover but this can be shrublands or even populations of invasive *Heracleum mantegazzianum*. The sika deer present a significant problem for nature conservation because of their frequent hybridization with the native red deer (Bartoš & Žirovnický 1981). Sika deer were not included as a separate species in the presented studies (with the exception of Paper III) because it was not possible to differentiate sika dung pellets from those of red deer due to the high frequency of hybrids. For this reason, I use the term 'deer' which refers to both red deer and sika deer.

The third most common herbivore is wild boar (*Sus scrofa*, *Suidae*). Contrary to deer, boars are not ruminants but are considered selective omnivores. However, during vegetation season majority of their diet consists of vegetative parts (Genov 1981). Boars inhabit almost any habitat from agricultural and anthropogenic areas to mountainous regions. It is not uncommon for boars to change habitats during the year. When inhabiting cultural landscape, boars are often observed in fields of agricultural crops: oilseed rape, sunflower, and most commonly maize. The wild boars are a native species in Central Europe but spread to almost the entire world and became invasive in many areas. The area of the wild boar population is one of the largest in all mammals.

Even though the animals — deer versus wild boar — differ in both habitat (Anděra & Gaisler 2012) and forage selection (Hofmann 1989), in general, we can summarize that they prefer closed habitats for rest (forests, shrublands) and open habitats for feeding (grasslands, fields).

Fig. 2 The abandoned landscape in the Doupov Mountains where the field experiments took place.

Fig. 3 The most common animal species from the area as captured by camera traps (© Eva Horčičková). Top left: deer male. Top right: roaring deer male. Bottom left: deer female with fawns. Bottom right: wild boar family with piglets.

0.8 Research summary

0.8.1 Paper I Endozoochorous seed dispersal by free-ranging herbivores in an abandoned landscape

The number of papers studying seed dispersal by free-ranging wild herbivorous animals has been increasing in the recent past. However, only a few of them compare between various animals and different areas. From these studies, we know there are significant differences both between animal dispersers and between study areas suggesting it is unwise to draw general conclusions about different habitats. Thus, I planned a basic experiment to study seed dispersal by free-ranging herbivores in our study area: collection and germination of dung samples from deer and wild boar. The dung samples were collected five times during the vegetation season capturing the seasonality in dispersed species composition. Furthermore, I added species composition of the neighbouring vegetation and plant characteristics and functional traits to search for patterns in the dispersed species.

The collected data allowed me to ask: (i) what is the plant species composition dispersed by different herbivorous dispersal vectors? (ii) Are the plant species dispersed primarily according to their availability to the herbivores? (iii) Have the dispersed plant species got a specific set of characteristics or functional traits?

Deer and wild boar dispersed 75 and 33 plant species, respectively, with a total of almost thirty thousand seedlings emerging from the dung samples and *Urtica dioica* as the most commonly dispersed species. The two herbivores differed significantly in the dispersed species composition of plants. Deer dispersed more species and seeds per sample but when the number of species was rarefied per five seedlings, there was no significant difference between the dispersing animals. Furthermore, I found contrasting trends between deer and boar in the dispersed numbers of species during the season: in deer, the highest number of species occurred in dung at the beginning of the season (June) and declined steadily, whereas in boar, the number of species remained constant throughout the season. The frequency of plant species in dung samples was significantly affected by different variables for deer and boar. In deer, we found only a positive effect of seed longevity index and the presence of mucilaginous appendages suggesting the consumed seeds have long-term persistence in the soil seed bank. Mucilage might act as protection during the passage through the digestive tract. On the other hand, dispersal by wild boar was significantly affected by more variables: positively by the frequency of plant species in the vegetation, the longevity index and nutritious appendages. Furthermore, it was negatively affected by the seed mass and elongated appendages. Our results suggest that wild boar is on one hand grazing on the species that are available to them but on the other hand, they may choose plants with nutritious seeds preferentially.

The results of my study showed that deer and wild boar are complementary dispersal vectors. They not only disperse different species composition but also species with different traits and other characteristics. Furthermore, the study showed there are large differences in species composition and number of dispersed seedlings per sample caused by seasonality.

0.8.2 Paper II Shit happens! Red deer dung increases species diversity but does not influence species composition of open grasslands

Following the results of Paper I, I asked: what is the direct effect of deer dung on the vegetation of dry grasslands? Much of the published literature on dispersal by herbivores is limited to dung collection and seedling germination (usually under greenhouse conditions). However, the greenhouse experiments give us only an idea of what species are dispersed by the studied animal because the herbivores may graze in various environments and disperse seeds which are then deposited in unfavourable habitats. Unfortunately, the dispersal potential from greenhouse experiments is often considered proof the germinated species is dispersed via the studied vector. Studies comparing greenhouse and common-garden experiments suggest there are significant differences even when seeds are germinated outside the greenhouse and yet still under very favourable conditions. There are even fewer studies looking into dispersal directly in the field, and fewer even studying dispersal by free-ranging wild herbivores.

To fill these gaps in knowledge, I planned an experiment in the study area where our previous research took place. Deer dung samples were deposited in plots with and without soil removal and vegetation development was studied for six years. The experiment was performed in a partially crossed design. I tested the following hypotheses: (i) dung addition increases the species diversity; (ii) dung addition increases the establishment of species dispersed in dung; (iii) dung addition increases the establishment of species with high nutrient demands. Furthermore, I hypothesized all the effects of dung addition to be stronger in plots with soil removal (disturbance).

I found partial support for our hypotheses. The dung deposition had significant positive effect on a number of variables: species richness, species diversity, and Ellenberg Indicator

Values for nutrients. However, the result remained significant only in the first year after the experiment was established and only in the disturbed plots. Contrary to my previous results (Paper I), there was no significant effect of the time of dung deposition which followed the same temporal pattern in both studies.

The results of my study suggested that red deer has a significant effect on the vegetation of dry grasslands but the effect is very weak and only temporary. Furthermore, it is necessary to take into account the total seed load available in one dung sample. The lack of significant results suggests that a portion of seeds might migrate to the soil seed bank. Considering the results of my previous study (Paper I) that seeds in dung samples have high longevity index, we can expect a cumulative effect of seed dispersal on the soil seed bank.

0.8.3 Paper III Herbivore digestion as environmental filter — which seed traits help species survive?

Plant functional traits and other characteristics are often used to characterize the dispersal syndrome — a set of these traits and characteristics typical for a specific type of dispersal or for specific species of disperser. The most common approach is searching for patterns in the dispersed composition of species and comparing it to patterns in the overall vegetation (Paper I). In this study, I aimed to look into the subprocess in the dispersal: how seeds survive the passage through the digestive tract and how they are adapted to it (level III in Fig. 1). I chose the method of feeding experiment when a predefined number of seeds is given to the animals with their usual forage. The dung samples are then collected for several consecutive days and using a greenhouse experiment, the germination rate of seeds from the dung samples as well as unfed control seeds is established. Using the knowledge of both vegetation and dispersal in the study area, I chose almost forty plant species of dry grassland. Some of these species were frequently found in dung samples, whereas others were a common part of the vegetation and did not germinate from dung in the previous experiments.

The feeding experiment was performed with four species of herbivores: red deer, sika deer, mouflon and wild boar. This allowed me to look into differences between closely related species and different ruminants. I tested these hypotheses: (i) plant species with the best survival in the guts have seeds with round shape, mucilaginous surface, and low amounts of nutrient reserves; (ii) survival success of plant species differs between herbivores: (a) body size positively affects germination rate, (b) length of the digestive system negatively affects germination rate, or (c) rumination negatively affects germination rate.

None of the tested plant species had a higher germination rate after feeding compared to the unfed control. The species with the highest germination rate all belonged to the *Fabaceae* family (e.g., *Vicia cracca*). The plant species survival and the germination rate were positively correlated to seed nitrogen content and negatively to seed elongation. However, when the analysis was run separately for plant species from various families (*Poaceae*, *Fabaceae*, and other families) different trends were found. For example, in *Fabaceae* and other species, I found a positive effect of seed phosphorus content, whereas in *Poaceae* the effect was negative. As a result, the overall effect on all species was non-significant. Furthermore, the effect of seed shape showed varying trends: positive effect of seed elongation in the group of other species and negative in both *Fabaceae* and *Poaceae*. Contrary to my previous results, I did not find a significant effect of mucilaginous surface on the survival of seeds in the digestive system. The germination rate was the highest after passage through red deer which supported my hypothesis (a) that the germination rate is positively affected by the body size of the disperser.

My results suggest that seed survival during passage through the digestive system is a very complex process and seed traits alone cannot predict seed survival. It is possible a key seed trait was missing in my analyses which could help us understand survival better. One of these key traits may be the seed coat thickness, however, it is not an easily measurable trait and is missing from the databases. I thus conclude that ingestion by free-ranging herbivores does not act as an environmental filter.

0.8.4 Paper IV Untangling drivers of endozoochorous seed dispersal by wild herbivores

In Paper IV, I aimed to connect the understanding of the endozoochorous dispersal within one study area. I took advantage of the long-term monitoring of vegetation in the area as well as the previous experiments which took place there (Paper I) or were planned based on the local vegetation (Paper III). I compiled a dataset which is unique in the current literature: species composition of dung samples, species composition of available vegetation, and species germination rates after passage through the digestive systems of herbivores occurring in the area — deer and wild boar. Furthermore, I added leaf traits from TRY and Leda databases as a proxy for plant palatability.

This comprehensive dataset allowed me to ask: (i) what is driving the species dispersal in dung? Is the main driver the species availability or their palatability measured by the leaf traits? Or is the main driver the species' ability to survive the passage in the digestive system? (ii) How does it differ between dispersal vectors? (iii) Are there species well adapted to herbivore digestion but not occurring in dung samples of studied animals?

My results differed based on what statistical analysis was used. Using redundancy analysis with a stepwise selection of variables I found only a significant effect on the standardized number of seedlings per 100 g of dry dung but not on the species frequency in dung. The standardized number of seedlings was strongly influenced by the species frequency in forest vegetation and by the leaf phosphorus content. However, this was driven mainly by one forest species: *Urtica dioica*. No significant variable affected the species frequency in dung either in the RDA or when tested for boar and deer separately. Using linear models on the standardized number of seedlings for each species of herbivore, I found contrasting results for deer and boar. The standardized number of seedlings in boar dung was significantly affected by species survival after passage through the digestive tract. I also found a weak effect of the interaction between frequency in the forest and the production of seeds but it was below the significance threshold. Surprisingly, none of the tested variables had any effect on the dispersal by deer.

Furthermore, I found discrepancies in species frequency in dung samples and their germination rates after feeding. I discovered some species with very high frequency in dung had surprisingly low germination rates suggesting these species have to be grazed preferentially to allow for such high numbers to be present in the dung samples. Similarly, a group of species with a very high germination rate not occurring in dung was observed. I hypothesize that such species are adapted to endozoochory but their disperser is missing in the system because the herbivores in the area do not graze them. My results also suggest that seed dispersal is partially a random process, and partially driven by the animals' feeding preferences. Furthermore, I hypothesize that traits promoting plant palatability may be in contradict with traits allowing the seeds to survive the passage through the digestive system which may be the reason for a non-significant effect of the tested leaf traits.

0.9 Author contribution statement

The presented thesis consists of general introduction, one published paper, two manuscripts under review and one unpublished manuscript. Author contributions to individual paper are as follows.

Paper I Endozoochorous seed dispersal by free-ranging herbivores in an abandoned landscape. **Lepková Barbora**, Horčičková Eva, & Vojta Jaroslav (2018). Plant Ecology, 219(9), 1127–1138. https://doi.org/10.1007/s11258-018-0864-9

BL, EH and JV planned the data collection based on initial idea of EH and JV. BL collected and analysed the data. BL led the writing of the manuscript. All authors contributed to subsequent editing of the manuscript.

Paper II Shit happens! Red deer dung increases species diversity but does not influence species composition of open grasslands. **Lepková Barbora**, Horčičková Eva, & Herben Tomáš. Journal of Vegetation Science. (*under review*)

BL, EH and TH planned the data collection based on initial idea of BL and EH. BL collected the data with help from EH. BL analysed the data with help from TH. BL led the writing of the manuscript. All authors contributed to subsequent editing of the manuscript.

Paper III Herbivore digestion as environmental filter — which seed traits help species survive? **Lepková Barbora** & Mašková Tereza. Oecologia. (*under review*). https://doi.org/10.21203/rs.3.rs-1746037/v1

BL came with the initial idea for feeding experiment, planned and performed the data collection. TM came with the initial idea for seed content measurements, planned and performed the data collection. BL analysed the data based on initial idea from TM. BL led the writing of the manuscript. Both authors contributed to subsequent editing of the manuscript.

Paper IV Untangling drivers of endozoochorous seed dispersal by wild herbivores. **Lepková Barbora** & Herben Tomáš. (manuscript)

TH came with the initial idea. BL collected and analysed the data. BL led the writing of the manuscript. Both authors contributed to subsequent editing of the manuscript.

1 Paper I: Endozoochorous seed dispersal by free-ranging herbivores in an abandoned landscape

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1.1 Abstract

Seed dispersal is a crucial process for the dynamics and maintenance of plant populations. Free-ranging animals are effective dispersal vectors because they can move between similar habitats and transport seeds into favourable environments. Dung samples from two species of common free-ranging mammals — deer and wild boar — were used to study endozoochorous dispersal of seeds in a military training area in western Bohemia. The area was abandoned after WWII, and the military training area was established in 1953. The vegetation consists of shrublands and dry grasslands. Data on the local species pool of grassland herbs and forbs were collected to compare the characteristics of dispersed vs. non-dispersed plants. Deer and wild boar dispersed 84 plant species, however, species composition of seedlings emerging from dung samples showed significant differences between dispersal vectors and notable change across the growing season. 80 % of all seedlings extracted from the dung samples belonged to stinging nettle, *Urtica dioica*. From trait analyses, seeds of endozoochorous plants had a higher longevity index in the soil seed bank than non-endozoochorous plants and more often possessed a mucilaginous surface. Our results show that deer and boar are successful, though not substitutable dispersers.

Keywords: endozoochory, deer, wild boar, functional traits, *Urtica dioica*, species pool

1.2 Introduction

Endozoochory — the ability of seeds to be dispersed after animal ingestion — is common for many plant species across various habitats (Welch 1985; Malo & Suárez 1995c). A number of animal species can act as dispersal vectors, either domestic herbivores such as cattle, sheep, and horses (Cosyns et al. 2005a; Bartuszevige & Endress 2008; Kuiters & Huiskes 2010), or wild game such as deer (Oheimb et al. 2005; Jaroszewicz et al. 2009; Iravani et al. 2011 and others), wild boar (Dovrat et al. 2012), and even bears (Herrera 1989). The number of dispersed plant species often greatly differs with the species of disperser (e.g., 36 and 51 species from faeces of roe deer and wild boar, respectively, Schmidt et al. 2004; 47 species from red deer dung, Iravani et al. 2011). However, the majority of dispersed seeds often belong to one or a few species (e. g. *Urtica dioica* in the case of red deer, Oheimb et al. 2005; or *Juncus effusus* and *Chenopodium polyspermum* by wild boar, Schmidt et al. 2004). Most dispersed species produce a small number of individuals, often less than 1 % of all germinated seedlings (Jaroszewicz et al. 2013a). The limited richness of plants dispersed may reduce the importance of such dispersers in maintaining diversity.

The species composition of seeds dispersed in dung is highly area-specific (Oheimb et al. 2005) and primarily depends on the adjacent vegetation (Jaroszewicz et al. 2013a). This linkage suggests that a good understanding of endozoochory in an area also requires detailed vegetation data. Information on vegetation composition of potential food sources enables us to identify plant species common in the landscape but lacking in dung, or those species that are over-represented in dung relative to their local abundance. Thus, we can compare characteristics of plants found in the dung to those within the landscape to assess selectivity. Furthermore, as various animals in the landscape act as dispersal vectors, it is important to compare them to determine whether their role as dispersal vectors differs.

The number and composition of dispersed seedlings is often different between dispersal vectors because they differ in diet, digestive systems, and habitat preferences (Eycott et al. 2007). Furthermore, plant species may be preadapted to herbivore seed dispersal analogous to plants with fleshy fruits dispersed by frugivores (Janzen 1984) but using their foliage. The plant loses part of its biomass, but receives the benefit of dispersing its seeds (Quinn et al. 1994). Janzen (1984) suggested several characteristics should be taken into consideration when studying seed dispersal by ungulates. These include characteristics of maternal plants such as plant tissues being nutritious because they grow in nutrient-rich environments. Additionally, seeds should be protected mechanically or chemically as adaptations for passage through the digestive tract. Albert et al. (2015a) showed that seed dispersal by ungulates (both domestic and free-ranging) can act as an ecological filter supporting some of the traits suggested by Janzen. However, they did not distinguish between species or groups of herbivores despite the substantial evidence for differences among vector species (Eycott et al. 2007).

Research considering wild, free-ranging animals so far has focused on forest habitats, and our knowledge of endozoochory in non-forest habitats is limited. Therefore, the goal of this study was to assess endozoochorous seed dispersal within an unpopulated and mostly unmanaged landscape matrix of dry grasslands and shrublands. We hypothesised that firstly, obligate herbivores (deer) and facultative omnivores (wild boar) will act as different dispersal vectors — dispersing different species of plants driven by different plant characteristics. Secondly, frequency of plant species in dung samples will correspond to the frequency within the local vegetation and their availability to the vectors. Thirdly, endozoochorous species are consumed by herbivores because they are attractive as a reward (e.g. they are rich in nutrients). Lastly, endozoochorous species will possess seed characteristics that allow them to survive in the digestive tract.

To test these hypotheses, we collected dung samples from deer and wild boar during one vegetation season to analyse which plant species had the potential to be dispersed endozoochorously. These data were compared with inventories from previous surveys of the area including detailed local vegetation composition data. We compared the composition of dung samples to vegetation composition to determine which plant traits were more common for species in dung and in the vegetation, respectively.

1.3 Material and Methods

1.3.1 Study area and dispersers

The study was conducted in Military Training Area Hradiště in the Doupov Mountains in the western part of the Czech Republic. The area was largely inhabited by German inhabitants who were displaced in 1946 (Augustin 1994). Recolonization was stopped in 1953 when the Military Training Area was established and the remaining inhabitants were relocated. Before the abandonment and cessation of management, the region was used for agriculture and currently, only part of the area is forested and logged. The area is actively used by the military for training, so the research presented here took place in a buffer zone which is not affected by training activities. The abandonment resulted in a landscape generated by spontaneous succession creating a mosaic of shrublands dominated by *Crataegus* spp. with patches of dry grasslands (class *Festuco-Brometea*). High numbers of red deer (*Cervus elaphus*), sika deer (*Cervus nippon*) and wild boar (*Sus scrofa*) are present in the area. Furthermore, roe deer (*Capreolus capreolus*) are in the area, but their numbers are negligible (Horčičková, unpubl. data). Since the two species of deer hybridize (Bartoš and Žirovnický 1981), we merged the collected dung material into one deer category, *Cervus* spp. Dung material was collected from two areas (hills) which are approximately 3 km apart – Strážný vrch (50.3031464N, 13.0907986E) and Petrovský vrch (50.2963133N, 13.0470306E).

1.3.2 Data collection

Dung material was collected every four weeks from June to October from 15 transects during the growing season of 2012. The transects were set up on animal trails with a length of 200 to 300 m. All available dung from the selected herbivores was collected. Five collections were made with a total number of 277 dung samples. The samples were taken equally from red deer, sika deer, and wild boar but merging the two species of deer doubled the number of samples from deer compared to the number of samples from boar. Samples were also collected evenly during the growing season, with the exception of August when a smaller number of samples was found. This decrease was probably caused by hot and dry weather which caused the animals to be less active.

Samples were identified by herbivore species and photographed. After collection, the samples were stored at -14°C for a minimum of 48 hours to stop all invertebrate activity. Such a short exposure to cold should not be harmful to species of temperate region where winter temperatures can be substantially under 0°C. Furthermore, our subsequent experiments suggest that even longer exposure to temperature of -14°C (8 weeks) does not lower germination rates for species with transient seed bank (e.g. *Achillea millefolium*). Samples were concentrated under running water on fine sieves (2 mm and 200 μm) following the method of Ter Heerdt et al. (1996). Seeds larger than 2 mm were extracted manually with tweezers and added to the concentrated samples. Concentrated samples were air dried, weighed, stored and stratified for eight weeks at 4°C.

The content of viable seeds was checked by a greenhouse germination experiment over a period of twelve months. All 277 dung samples were sown on a commercial sterile substrate (potting soil supplied by Rašelina a.s.). At the beginning of the experiment the depth of sown material was up to 2 cm, but the material was very light and within several weeks, it had settled to a depth of several millimetres. Furthermore, the sown material was carefully mixed to allow germination of seeds in deeper layers but not enough to mix it with the substrate. Additional lighting was provided to produce 13 hours per day. Summer temperature in the greenhouse varied accordingly to weather conditions, while the temperature in the winter ranged between 15 and 20°C. Ventilators were installed in the summer to increase the movement of air to prevent the samples from becoming mouldy. The samples were watered as necessary with rain water. Additional pots with no samples were installed as controls and species that emerged from these trays were excluded from the analyses (three species). Whenever seed contamination was visible (new seeds in pots, usually large anemochorous seeds from trees), these were immediately removed.

Seedlings were identified and removed as soon as possible. When it was not possible to identify a species in the early stages, the seedling was transplanted to a separate pot and grown until identification was possible. Even after that, one individual remained unidentified. Nomenclature follows Kubát et al. (2002). In some cases, the individuals were not identified to the species level, but only to the genus level (e.g. *Fragaria* spp.).

Vegetation samples were taken in the same areas as the dung samples. During the summer growing seasons of 2008 and 2009, vegetation records were made on Strážný vrch, and in 2012 on Petrovský vrch. 133 and 40 plots were sampled on Strážný vrch and Petrovský vrch, respectively, with three subplots of 50*50 cm on every plot. For our analyses, we summed the three subplots into one sample and used only presence/absence data. Since the vegetation data included only herbaceous species, we excluded all woody species in the dung seed bank from all analyses. For the species list see Online resource 1.

1.3.3 Data analyses

A set of traits was used to explain plant species presence in the dung of herbivores including seed related traits (e.g. appendages on seed surface, seed dimensions, longevity index), Ellenberg indicator values, and plant traits, e.g. SLA (for full list see Tab. 1). Longevity index was calculated from the type of seed bank as recorded in the LEDA database (Kleyer et al. 2008). Only species with at least five records of seed bank type were used in the analyses. Longevity index (LI) was calculated following Bekker et al. (1998b):

$$LI = \frac{(SP + LP)}{(T + SP + LP)}$$

where T = no. of records of transient seed bank (surviving less than 1 year), SP = no. of short-term persistent seed bank records (1–4 years), and LP = no. of long-term persistent records (more than 4 years). The longevity index ranges from 0 (strictly transient species) to 1 (strictly persistent). For phylogenetic correction, we used the Daphne database (Durka & Michalski 2012). For simplification, all attributes are referred to as traits.

Table 1 Summary of traits and species characteristics analysed.

Source	Variables
Kubát et al. (2002)	Family
D3 database (Hintze et al. 2013)	Type of fruit, type of diaspore, exposure of diaspore, heterodiaspory, appendage rich with nutrients, ballochorous appendage, elongated appendage, appendage with hook, flat appendage, mucilage on the surface, none appendage, epizoochorous appendage, mass of diaspore, surface structure of diaspore, length/width/height of diaspore, shape of diaspore, form of diaspore, terminal velocity, rank for anemochory, rank for hydrochory, rank for epizoochory
Ellenberg et al. (2010)	Ellenberg Indicator Values (EIV) for light, temperature, continentality, moisture, pH and nutrients
LEDA traitbase (Kleyer et al. 2008)	Canopy height, SLA, Longevity index

We tested whether species occurrence found in the dung samples (in samples from deer and wild boar, respectively) can be explained by a set of explanatory characteristics using a bias-reduced logistic regression with Firth's bias correction method (logistf package in R, Heinze et al. 2013). All herbaceous species including grasses from the local species pool were analysed. In the trait analyses, presence/absence data of plant species in dung of individual herbivore were used as response variables. The predictors were first checked for collinearity, and for correlated variables (p-value < 0.05) we selected those with fewer missing values. Specifically, we removed terminal velocity, diaspore height, and diaspore width.

Phylogenetic correction was included in the analyses because the tested traits themselves may be phylogenetically conserved. To include phylogeny in the analyses, we used a method of phylogenetic eigenvectors (Diniz-Filho et al. 1998). First, a matrix of phylogenetic distances was calculated for all species within the local species pool. Then, the matrix was submitted to a principal coordinate analysis (PCoA) using the function dudi.pco from ade4 package in R (Dray & Dufour 2007) yielding 30 axes that represented 95 % of the total variation. These axes, referred to as phylovectors, were used as predictors and fitted in a bias-reduced logistic regression with plant species presence in dung of deer or wild boar as a response variable. The logistic regression was tested using backward selection based on penalized likelihood ratio test, generating three and one phylovector with significant effect ($P < 0.05$) for the analyses of deer and wild boar samples, respectively.

Individual traits were tested with and without phylogenetic correction (the significant phylovectors) using the logistf R package (Heinze et al. 2013). Response variables were the presence or absence of individual plant species in the dung of deer and wild boar, respectively. Traits were first tested as explanatory variables individually, then with phylovectors as covariates. Significant variables were then combined into one model, and by using a backward selection (based on penalized likelihood ratio test), we eliminated those variables which did not remain significant. Phylovectors were forced to stay in the final model. Logistic regressions were fitted following this formula:

logistf [presence of species in dung ~ trait 1 + trait 2 + trait 3 (+ phylovector 1 + phylovector 2 + phylovector 3)].

In addition to the trait analyses, the effect of environmental predictors on the number of seeds and on the rarefied number of species was tested using a linear mixed effect model (R packages lme4, Bates et al. 2015; and lmerTest, Kuznetsova et al. 2016). To reduce the effect of spatial autocorrelation, the place of collection (hill) was set as random effect in all linear mixed effect models. The number of seeds was expressed per gram of concentrated and dried dung. Environmental variables included the dispersing animal and the time of collection. Only samples with at least 5 seedlings were included in the analysis. The number of species were rarefied per five seedlings prior to analyses to generate a standardized measure for comparison. Rarefaction was calculated using the rarefy function from the vegan R package (Oksanen et al. 2016). Transformations were used to ensure Gaussian distribution, logarithmic transformation for the number of seedlings, and square root for the rarefied number of species. Trait analyses and all univariate analyses were carried out in R 3.3.1. beta (R Core Team 2016).

Influence of time and dispersing animal on species composition was tested using canonical correspondence analysis (CCA) in the Canoco 5 program (Braak & Šmilauer 2012) also using a logarithmic transformation. The place of collection was used as covariate in the analyses.

1.4 Results

A total of 29,663 seedlings emerged from the 277 dung samples. 25 samples, with the majority of them belonging to the wild boar (72 %), did not contain any viable seeds. The emerged seedlings were identified into 78 taxa of forbs and grasses (Tab. 2). Four woody species emerged but were not included in the analyses. None of the species were of agricultural origin. The neighbouring vegetation provided a species pool of 182 herbaceous plants, 61 of which (34 %) were present in the dung samples (for list of all species see Online Resource 1).

Table 2 Basic information about the material collected, and a summary of the average number of seedlings and species found in the samples of individual herbivore species. Number of species is rarefied per five seedlings.

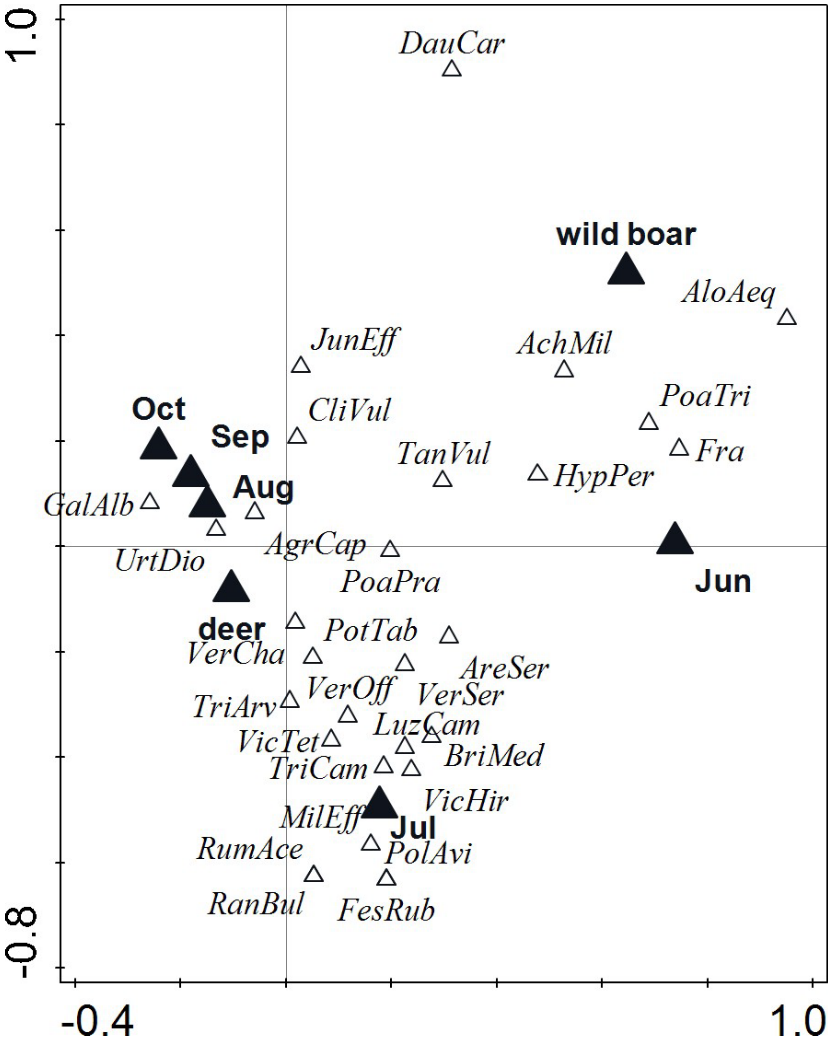
	Cervus spp.	*Sus scrofa*
Number of dung samples	190	87
Number of samples with viable seeds	183	69
Dry mass average	20.63 ± 1.09	15.43 ± 1.27
Number of seedlings		
Average (± SE)	137.78 ± 20.64	35.39 ± 11.04
Per 1 g dry mass (± SE)	7.14 ± 1.04	2.07 ± 0.54
Total	26940	2709
	29649	
Number of species		
Average per sample (± SE)	4.10 ± 0.20	2.02 ± 0.24
Average rarefied per 5 seedlings (± SE)	1.90 ± 0.07	1.94 ± 0.15
Total	75 (15 grasses)	33 (10 grasses)
	80 (17 grasses)	

The overwhelming majority of seedlings came from one species — *Urtica dioica* (appearing in 72 % of all samples and representing 81 % of all seedlings). Other frequent plant species included: *Poa pratensis*, *Veronica chamaedrys*, *Agrostis capillaris*, *Galium album*, *Dactylis glomerata* and *Trifolium repens*. Each of these species occurred in more than 10 % of all samples.

1.4.1 Differences between dispersal vectors

There were significant differences in species composition in the dung of individual herbivores (Fig. 1, partial CCA, P = 0.001). The most common species in deer dung were *Urtica dioica*, *Veronica chamaedrys*, *Poa pratensis*, *Agrostis capillaris*, *Galium album*, *Dactylis glomerata*, *Trifolium repens*, *Plantago media* and *Trifolium arvense* (in order of decreasing frequency emerging in at least 10 % of samples). Species composition in wild boar dung differed significantly with only 26 % of samples containing *Urtica dioica* (5 % of all seedlings in the boar samples). The most frequent taxon in the wild boar dung was *Poa pratensis* (in 40 % of samples, but only 6 % of seedlings). Other common species in boar were *Agrostis capillaris* and *Fragaria* spp. (appearing in more than 10 % of samples). However, *Fragaria* spp. was the most common species in the matter of absolute numbers (79 % of all seedlings, but only in 17 % of samples). Relative frequencies of plants in deer versus boar dung are depicted in Fig. 2 where three groups of species are recognizable. In the first group in the right top corner, there are species dispersed by both herbivores (*Veronica chamaedrys*, *Urtica dioica*, *Poa pratensis* and *Agrostis capillaris*). The second group (left top) are species primarily dispersed by deer (*Galium album*, *Trifolium arvense*, *Plantago media*). The third group in right bottom part of the figure is *Fragaria* which is almost entirely dispersed by wild boar.

Fig. 1 Partial CCA of species composition found in the dung samples. Names of months represent the time in which samples were collected. Deer and wild boar are the species of herbivores studied. The place of the collection (Strážný and Petrovský vrch) was also significant in the CCA with forward selection but is used as a covariate in the presented result. Only 30 species with the best fit are depicted. Species associated with an individual variable can be seen, e.g. *Fragaria* spp. was primarily found in samples from wild boar collected in June whereas *Urtica dioica* was mostly found in deer dung collected in autumn. Partial variation of the model is 15.24 and the explanatory variables account for 5 % of variability. Explained fitted variation of the first axis is 51 %. For full names of plant species, see Online Resource 1.



The two species of vectors also differed in the number of dispersed seedlings and species (Tab. 2). Wild boar dispersed approximately 2 seedlings per g of dry mass and 31 seedlings per sample whereas deer dispersed 7 and 142, respectively. Deer also dispersed more species per sample than wild boar, but when the number of species was standardized per five seedlings, they did not differ (see Tab. 2). Both the number of seedlings per g and the rarefied number of species showed a strong seasonal trend but these differed between dispersers. The number of seedlings in deer dung steadily increased throughout the season until September, after which it decreased. In contrast to this, wild boar dispersed more seeds at the beginning of the growing season (June), and the number of seedlings decreased after that (Fig. 3a). The number of dispersed seedlings per gram were significantly influenced by the species of disperser (linear mixed effect model; $F = 4.92$, $df = 4$, $P = 0.028$) and by the interaction of disperser and time ($F = 18.61$, $df = 4$, $P < 0.001$). The number of plant species found in the deer samples were the highest in June, and showed a steady decline for the rest of the season whereas in wild boar it did not show any peaks and maintained constant for the whole vegetation season (Fig. 3b). The rarefied number of species were significantly influenced by the interaction of disperser and time (linear mixed effect model; $F = 4.49$, $df = 4$, $P = 0.002$) while the direct effects remained non-significant.

Fig. 2 Frequencies of plant species in dung samples of wild boar vs. deer (proportion of deer/boar samples in which a species occurs). Species above the line are more common in deer samples, whereas species beneath the line are more frequent in the boar samples. The line is a model II regression line computed using major axis, MA (lmodel2 package in R, Legendre 2018). Data are shown in square root transformation. For clarification, only some species names are depicted. For full names of plant species, see Online Resource 1.

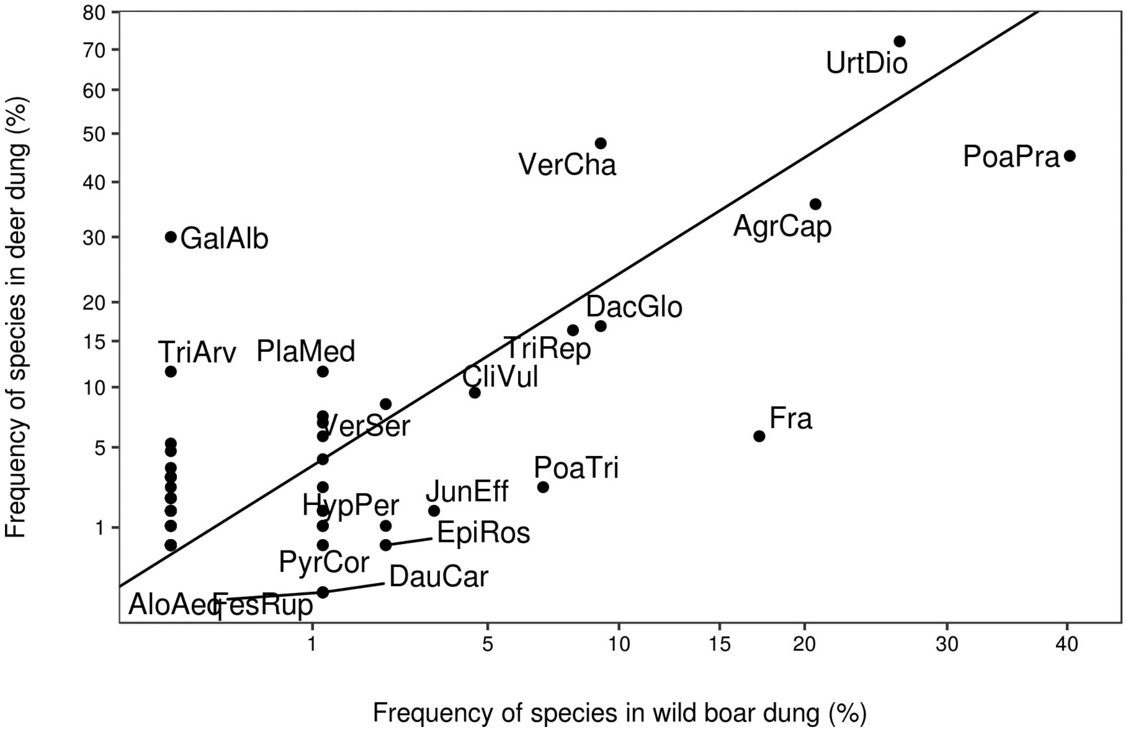
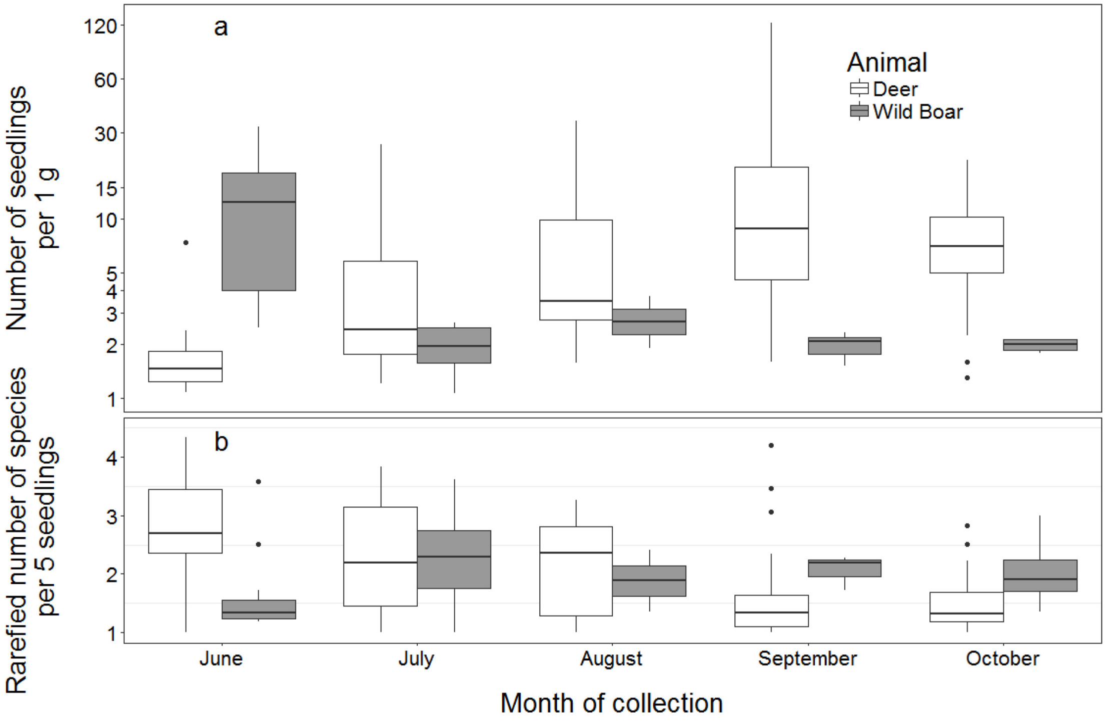


Fig. 3 Change in the number of seedling (a) and in the number of species (b, rarefied per 5 individuals) throughout the vegetation season between the individual herbivores. The numbers of seedlings were transformed using a common logarithm with a base of 10. Empty and grey boxplots represent deer and wild boar, respectively. The boxes range from the first to the third quartile, and the whiskers extend to the highest value 1.5 x inter-quantile range. Data outside this range are outliers, and plotted as points. Medians are shown as horizontal lines in the boxes. For full names of plant species, see Online Resource 1. The number of seedlings was significantly affected by animal species (P = 0.035) and by the interaction of animal and time (P < 0.001). The rarefied number of plant species was only influenced by the interaction of time and animal (P = 0.001).



1.4.2 The relationship between species composition found in dung samples and in the vegetation

Contrary to our expectations, many species showed significantly different frequencies in dung samples than in the vegetation (Fig. 4). Only one species, *Urtica dioica*, was more frequent in the dung than in the vegetation. The majority of species were much more common in vegetation than in the dung samples. Species common in dung samples more or less corresponded to their frequencies in the vegetation (e.g. *Agrostis capillaris*, *Poa pratensis*). Many species frequent in the vegetation did not appear in the dung or appeared in very low numbers. These absent species often belonged to the *Fabaceae* and *Poaceae* families (e. g. *Trifolium medium*, *Lathyrus pratensis*, *Brachypodium pinnatum* or *Arrhenatherum elatius*).

1.4.3 Trait analyses

All models tested included the same variables both with and without phylogenetic correction. Interestingly, phylogenetic correction did not alter the trait associations found (Tab. 3). The presence of species found in deer dung was positively influenced by the longevity index and the presence of a mucilaginous (sticky) surface (also a positive effect). The majority of plants appearing in the vegetation data that had a mucilaginous surface emerged from the dung samples (14 out of 18). Presence in wild boar faeces was influenced by different traits: negatively by seed mass and the presence of elongated appendages, and positively by presence of nutritious seeds and by species' frequency in vegetation. The only common plant characteristic for both models was the longevity index which had positive effect in both cases.

Table 3 The coefficients and effect directions of significant variables (p-value < 0.05) in the trait analyses models. Species occurrence in the dung of herbivores is driven by different plant characteristics with only longevity index in common. For precise p-values and model statistics see Online Resource 2.

	Deer	Boar	Deer + phylo	Boar + phylo
Freq. in vegetation		+ 0.404		+ 0.350
Seed mass		- 0.434		- 0.378
Longevity index	+ 2.891	+ 2.730	+ 2.709	+ 2.150
Mucilage	+ 1.688		+ 1.700	
Nutritious appendages		+ 1.160		+ 1.078
Elongated appendages		- 1.029		- 1.157

1.5 Discussion

Considerably larger numbers of seedlings emerged in our experiment than in previously published studies, regardless of the method of standardization (either per sample or per unit of dry mass). The species distribution was strongly skewed with the majority of dispersed seeds belonging to a small number of plant species (Eycott et al. 2007). The most common species was stinging nettle *Urtica dioica* (Schmidt et al. 2004; Oheimb et al. 2005; Jaroszewicz et al. 2013a; Steyaert et al. 2014) because it is a rather common species (Schmidt et al. 2004), it has high production of seeds (explaining the number of seedlings per sample, Jaroszewicz et al. 2013b), and moreover, the herbivores may graze it preferentially for its nutritional value or for its medicinal effects (Gülçin et al. 2004).

1.5.1 Differences between dispersal vectors

In support of our first hypothesis that herbivores and omnivores act as different dispersal vectors, the species composition of seeds dispersed in dung differed significantly between deer and wild boar, which has been previously shown in forest habitats (Eycott et al. 2007; Jaroszewicz et al. 2013b) but not for non-forest habitats. Even though the species are, based on the CCA (Fig. 1), clearly differentiated into boar-dispersed and deer-dispersed, some plant species are dispersed by both dispersers (Fig 2). This discrepancy is caused by the nature of the analyses, the CCA is based on common occurrence of multiple species in a sample (with the added information on the number of individuals), whereas the linear regression is based on frequencies of species in dung only. Difference in species composition is probably caused by differences in foraging behaviour and preferences. In contrast to purely herbivorous deer, wild boar is defined as an opportunistic omnivore (Schley & Roper 2003), with the majority of its diet consisting of plant tissues (Ballari & Barrios-García 2014). The most common plant species in boar faeces were *Poa* spp., *Urtica dioica* and *Agrostis capillaris* supporting that grasses and herbs are one of the most common components of boar diet (Genov 1981) together with fleshy fruits and agricultural crops (Schley & Roper 2003). Only one taxon with fleshy fruits was dispersed by boar — *Fragaria* spp. Even though it appeared in deer dung, it was predominantly dispersed by wild boar.

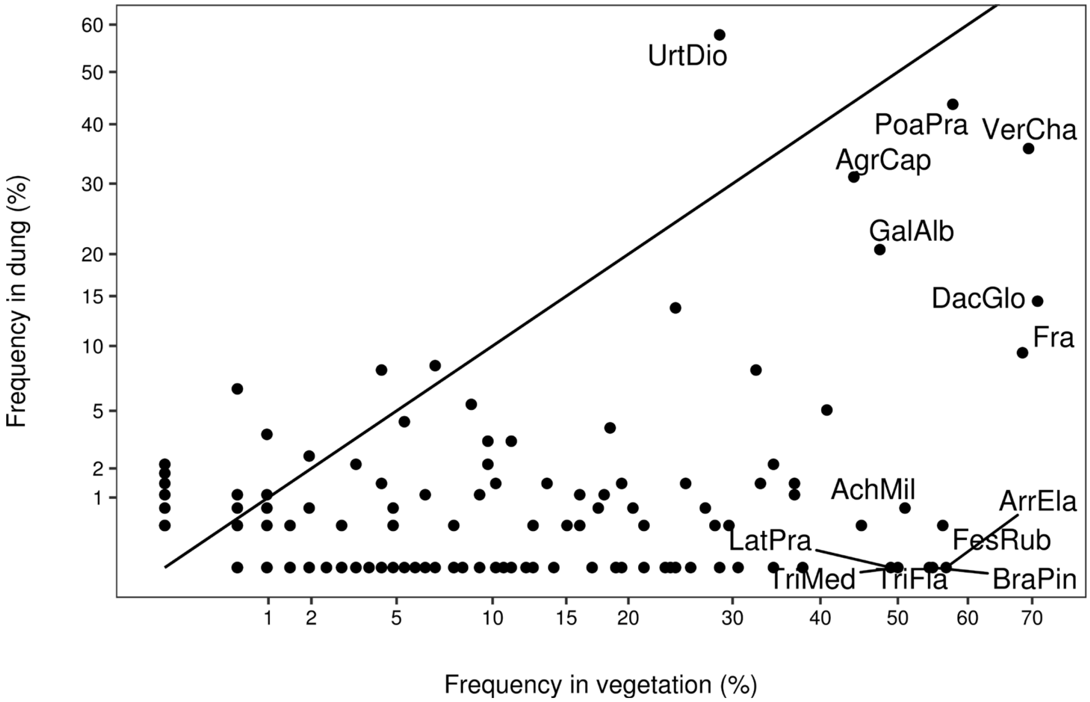
Dispersal vectors showed different temporal trends in the numbers of dispersed seeds and species (see also Oheimb et al. 2005). The dynamic of seedlings in deer dung was driven by the occurrence and phenology of *Urtica dioica*, and when this species was excluded, the peak of number of seedlings shifted. Temporal patterns in wild boar were dissimilar from those of deer due to phenology of dispersed species. Both the differences in species composition and in the numbers of dispersed species/seedlings support the following hypotheses: (i) deer and boar have different feeding preferences and/or (ii) due to the variation in their digestive systems, survival of different species is promoted. It is impossible to disentangle these processes without further study.

1.5.2 The influence of vegetation species pool

We found only a partial support for our second hypothesis that frequently dispersed plants should be also common in the surrounding vegetation. Species frequency in the landscape positively influenced presence in wild boar dung (see Schley & Roper 2003), but not in deer dung. This suggests that boar is foraging more as a generalist than deer, even though it disperses fewer species.

There are a number of species which did not appear in the dung but are fairly common in the landscape, e. g. *Arrhenatherum elatius* or *Trifolium medium*. Beside the fact that the plant species may not be grazed at all or may not be grazed when ripe, the species absence may be caused by other processes, firstly the seeds did not survive the passage through the digestive tract and were destroyed either by the molar mill (large seeds), or by the gastric fluids (thin seed coat). Secondly, the seed was consumed and survived the gut passage but did not germinate because the germination requirements were not met (which would be more likely in a field study), or the species has a very low production of seeds or short period of fruiting which makes it difficult to intercept. It is possible that certain species were not recorded in the dung samples because of the large time gaps (four weeks) between collections. However, we lack sufficient data on species' survival rate after ungulate gut passage, crucial information when establishing whether or not a species is endozoochorous.

Fig. 4 Difference in species frequency in dung samples and in the vegetation. Frequency = proportion of samples or plots in which a species occurs. The line shows an ideal relationship 1:1. Majority of species occurs more frequently in vegetation than in the dung samples. The most obvious exception is stinging nettle *Urtica dioica*¸ furthermore, *Plantago media*, *Trifolium arvense*, *Veronica serpylifolia* and *Cerastium holosteoides* are slightly more common in dung samples. On the other hand, some very common species did not emerge from the dung at all, e.g. *Trisetum flavescens*, *Arrhenaterum elatius* or *Trifolium medium*. Data are shown in square root transformation. For clarification, only some species names are depicted. For full names of plant species, see Online Resource 1.



1.5.3 Trait analyses

Analysing 39 plant traits, we identified which are important for endozoochorous dispersal by deer and boar in this landscape. In concordance with the hypothesis that seeds have traits protecting them from damage during the gut passage, we found positive effect of high longevity in seed bank and of presence of mucilaginous surface. The connection of seed longevity (potential to form a persistent seed bank) has been confirmed for other herbivores (Pakeman et al. 2002; Cosyns et al. 2005b; Albert et al. 2015a) but not for either deer or wild boar. Both traits may be connected to internal dispersal because the destructive processes in intestines and in the soil require similar adaptations (see Pakeman et al. 2002 for longevity index; and Hintze et al. 2013 for seed mucilage). However, species with mucilaginous surfaces may be eaten accidentally because they adhere to foliage when mature.

Our third hypothesis (that maternal plants are attractive rewards) was not fully supported by our data because we did not find significant effect of habitat characteristics as expressed by the Ellenberg Indicator Values (EIV) (see also Mouissie et al. 2005a). However, we did not have specific data on nutrient content of plants in our landscape to fully test this. On the other hand, we found a significant effect of nutrient enrichment of seeds on the occurrence of species in boar dung (but see Albert et al. 2015a). It is possible that boar selected species with nutrient rich seeds (not discriminating between types of nutritional value) and thus acted as a frugivore at times. In general, plant species dispersed by different dispersers had significantly different traits (supporting the hypothesis that herbivores and omnivores act differently as dispersal vectors). Trait analyses were performed with and without phylogenetic correction but both approaches yielded in the same results. This suggests there was no phylogenetic signal in the tested variables.

1.6 Conclusions and gaps in research

In this study, we illustrated the importance of distinguishing between dispersal vectors when studying endozoochorous seed dispersal. Deer and wild boar dispersal differed greatly in species composition and in the number and richness of dispersed seedlings. Furthermore, they differed in the plant traits which characterised the dispersed species. However, we must emphasize that results presented here are based on data from a greenhouse experiment and must be interpreted accordingly as potentially dispersed only. Furthermore, we lack knowledge about how the seeds survive passage through the digestive system. Exact data on species fate after ingestion are urgently needed to clarify whether species are missing in the dung material because they are not able to survive the passage. Experiments addressing effectivity of dispersal in the field, and the survival of species after ingestion would give us a better understanding of endozoochorous processes.

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Conflict of Interest The authors declare that they have no conflict of interest.

2 Paper II: Shit happens! Red deer dung increases species diversity but does not influence species composition of open grasslands

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2.1 Abstract

Questions: Free-ranging herbivores are often seen as important vehicles for plant propagules. In contrast to domestic animals, the dispersal by free-ranging animals and its effect on vegetation has rarely been studied. Considering other factors influencing seed dispersal, we ask: what is the effect of endozoochory and deposition of dung on open landscape vegetation? How does it influence vegetation development after soil removal? Does it change species richness or composition?

Location: The Doupov Mountains, Czech Republic

Methods: In 2015, the experiment consisting of 300 plots was set in five fenced open landscape sites. All plots were sampled at the beginning of the vegetation season in 2015 before the start of the experiment, for two consecutive years, and in 2021 (species composition and cover of all species). Treatments were implemented in partially crossed design: soil was removed (disturbance) and dung pellets were deposited, both was done every four weeks from June to October 2015.

Results: The dung deposition significantly increased the species richness, species diversity, and Ellenberg Indicator Values for nutrients but the effect was significant only in the first year after setup (2016) and in disturbed plots. We found similar effect on species composition. Disturbance had significant effect on all tested variables: species richness, diversity, composition, Ellenberg Indicator Values for nutrients, and the proportion of dung-dispersed species. The time of dung deposition did not have significant effect.

Conclusions: Our results indicate that the seed dispersal by red deer has significant effect on the vegetation of open landscape but it is extremely weak and only temporary. Considering the enormous seed load available in one dung dropping, it is possible the seeds from dung migrate to soil seed bank and wait for another opportunity to germinate.

Key words: endozoochory, deer, seed dispersal, dry grassland, disturbance, seedling germination

2.2 Introduction

The population of red deer in Europe has increased almost double in the past forty years (Burbaite & Csányi 2010). All herbivorous mammals influence vegetation in a number of ways including direct biomass reduction via grazing, creation of vegetation gaps through trampling and rooting, and relocation of nutrients in dung (Gill & Beardall 2001; Eichberg & Donath 2018). Even though overabundant wild herbivores affect growth and survival of plants, reduce diversity and change nutrient cycling (Côté et al. 2004), they are also capable of dispersal of plant propagules — seeds and fruits — which pass undamaged through the digestive system (Janzen 1984). When the dung with seeds is deposited in a favorable environment, e.g. freshly disturbed soil, seeds have higher probability of germinating and establishing (Cosyns et al. 2006).

Large quantities of seeds were found in the dung of various herbivorous animals (cattle and horse, Auffret & Cousins 2013; deer, Eycott et al. 2007) suggesting internal passage — endozoochory — can provide dispersal for many plant species in high quantities. The passage through the digestive system can be beneficial to the dispersed plants in several ways: destroying seed parasites (Miller & Coe 1993), providing scarification necessary for germination (Russi et al. 1992), and protecting seeds from predators (Malo & Suárez 1995a). A large number of species are dispersed endozoochorously (44 % of all available species, Albert et al. 2015a) and the frequency of species in dung is closely connected to the species frequency in the landscape (Lepková et al. 2018). However, in some species, the frequency in the dung is much higher than in the vegetation (Lepková et al. 2018) suggesting preferential grazing which can cause increase of specific species after increase of dung deposition. Welch (1985) observed introduction of grasses into a moorland which he attributed to heavy deposition of dung by cattle. However, not all species were recorded as germinating from dung suggesting some were already present in the seedbank and germinated in the conditions after dung deposition.

Beside the deposition of seeds, the dung contains significant amounts of nutrients (Mohr et al. 2005; Aarons et al. 2009). The effect on vegetation has been measured indirectly by Ellenberg Indicator Values (Mouissie et al. 2005b), but also directly on the performance of individual plants: even though the germination inside the dung pat takes longer, once the individuals germinate, they grow faster and flower more (Milotić & Hoffmann 2017). After germination, the risk of grazing is decreased because the animals tend to avoid vegetation in the vicinity of dung piles (Castle & MacDaid 1972; Malo & Suárez 1995b; Gillet et al. 2010). But, due to the lower grazing pressure and higher input of nutrients (Milotić & Hoffmann 2016a), vegetation can overgrow the seedlings limiting their access to light (Bakker & Olff 2003). The effects of dung on germination and establishment differs between herbivorous species due to different foraging behavior or chemical and mechanical features of the dung itself, e.g. cattle is a generalist grazer and creates large dung piles which suffocate underlying vegetation (MacDiarmid & Watkin 1971; Welch 1985), in contrast to red deer: an intermediate feeder (Hofmann 1989) creating small droppings.

However, the animals' presence at a locality shall not be reduced to only one type of effect, e.g., grazing or dung deposition. As a result of various types of behaviour, the vegetation is disturbed and overturned, and plants are destroyed. Small-scale disturbances are created by biotic factors: e.g., wild boar rooting or burrowing by small mammals. Abiotic factors might cause disturbances on larger scale: tree uprooting, erosion. These disturbances create gaps with bare soil. It has been shown for seeds dispersed in the cattle dung: if they are deposited in vegetation gaps, they can easily germinate and establish (Oesterheld & Sala 1990; Cosyns et al. 2006). However, most of the current research has been done on domestic animals both in the field (e.g., Cosyns et al. 2006) and in the laboratory environment (Milotić & Hoffmann 2016a, 2017). Wild herbivores, increasingly important due to their numbers (Burbaite & Csányi 2010), are often overlooked.

In general, dung piles increase alpha diversity and decrease beta diversity of pastures (Malo & Suárez 1995b). Cattle and horse dung deposition leads to increase in small-scale species diversity and the establishment of seeds is more successful when they are deposited into gaps of disturbed vegetation (Cosyns et al. 2006). However, results from cattle pastures are not valid for other vegetation types inhabited by wild herbivores. Furthermore, the effect of endozoochorous dispersal by wild herbivores is usually assumed on the basis of data from greenhouse experiments without field testing (Milotić & Hoffmann 2016c). Long-term field experiments are even more needed since the presence of dung has various effects on different stages of plant life as well as different plant species (Milotić & Hoffmann 2016a). We aim to address these knowledge gaps: the lack of field studies and the lack of studies on wild, free-ranging herbivores.

To disentangle the effect of seed addition, nutrient addition, and disturbance creation by wild herbivores, we performed a field experiment with red deer dung. The main objective was to test these hypotheses: (i) Dung addition increases the species diversity through seed dispersal. The effect is stronger when dung is deposited into a vegetation gap (disturbance). (ii) Dung addition increases the establishment of species previously known to be dispersed in dung. Dung-dispersed species establish better in disturbance. (iii) Dung addition leads to increased establishment of species with high nutrient demands (as measured by Ellenberg indicator values, EIV, for nutrients). To test our hypotheses, we started a sowing experiment in partially crossed design with deposition of red deer dung and disturbance treatment. To investigate the effect of nutrient deposition without the deposition of seeds, we used red deer dung collected from farm animals with no access to fresh forage. Furthermore, we tested the effect of seasonality which strongly influences the species composition of dung seed bank. We recorded vegetation for two consecutive years and again six years after the setup.

2.3 Material and methods

In a military area in western Bohemia we established plots with dung addition and disturbance treatment by soil removal. Samples of dung from deer in the area and from a deer farm (expected to be seed-free) were sown in the plots leaving one third as a control. Vegetation records were taken before the start of the experiment in 2015, for two consecutive years, and six years after the establishment. The change in species composition, species diversity, and abiotic conditions of plots according to treatment was tested.

2.3.1 Study area

The study took place in the Doupov Mountains in the Western Bohemia. The area is part of the active Military Area Hradiště which was established in 1953 after the displacement of German inhabitants in 1946 (Augustin 1994). Before abandonment, the area had mainly agricultural use, and currently part of the area is under forestry management. Wild, free-ranging animals have high population densities and provide the only grazing pressure. The research took place in a buffer zone outside the active military training area. The vegetation consists of shrublands (*Prunion spinosae*) and open vegetation sites with dry grasslands (*Festuco-Brometea* class). Game animals are in high abundances in the area, primarily red deer (*Cervus elaphus*), sika deer (*Cervus nippon*) and wild boar (*Sus scrofa*, not included in this study). In our previous research, we studied the effect of wild boar rooting (Horčičková et al. 2019), seed dispersal by deer and wild boars (Lepková et al. 2018) and the general effect of grazing by free-ranging animals (Horčičková, unpublished data). For more detailed description of the area, see Kopecký & Vojta (2009).

2.3.2 Data collection

The experiment was set in May 2015 on five open landscape sites. Sites where chosen non-randomly and following these criteria: sufficient open area and flat surface to avoid nutrient leaching (only five enclaves following these criteria were found and the experiment was set on all of them). On each site, a rectangle (app. 8 m * 3.5 m) was fenced to deter animals (height of fence 1.5 m). In each rectangle, 60 plots of 50 cm * 50 cm were established in a grid six times ten plots. In each 50 cm * 50 cm plot, a smaller subplot was marked with nails (20 cm * 20 cm) which was further used for vegetation sampling. A total of 300 plots of 20 cm * 20 cm were set.

The effect of dung deposition was studied on disturbed plots (top 20-30 cm of soil layer was completely removed with a spade imitating wild boar disturbance, Horčičková et al. 2019) and in undisturbed vegetation (top soil layer left untouched). For the plot design, see Fig. 1. Plots were arranged in ten rows of six plots with an alley after every two rows. Each double-row presented a treatment block of dung deposition, disturbance and time, i.e. half of plots from rows one and two were disturbed in June and dung samples were deposited in these rows at the same time. The next month, rows three and four where treated in the same way, etc.

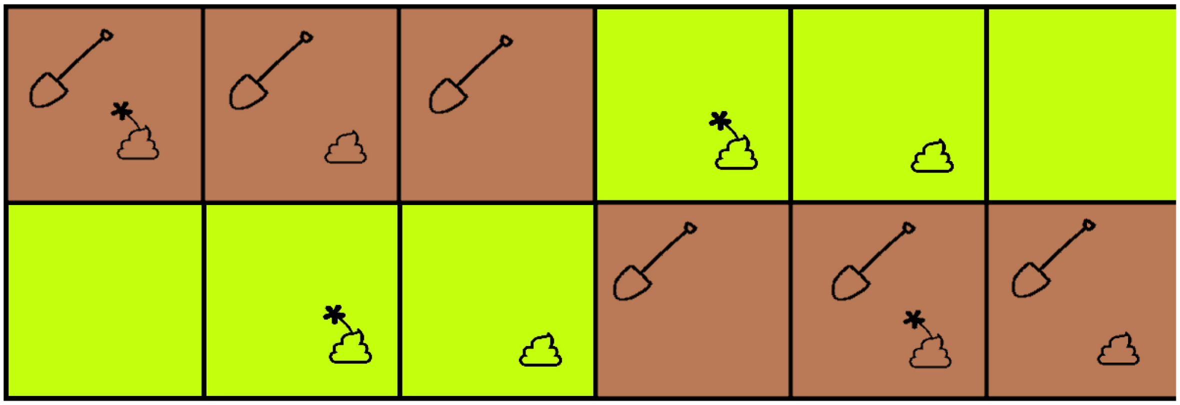
Dung was deposited five times during vegetation season, and the disturbances were created at the same time. The dung was deposited every four weeks starting at the end of June, the deposition dates are therefore referred to as June, July, August, September and October. We chose two sources of dung: experimental farm Podlesko and local population of deer. The dung material from local animals was collected at the same time as the disturbances were made at the end of each month (Tab. 1). Fresh looking samples were collected. Due to frequent hybridization of the two species of deer (Macháček et al. 2014), we did not differentiate between red and sika deer and for the purposes of this study we refer to the animals as to deer. The dung from the experimental farm was used as a control to the dung from the field. From each collection date, several dung samples were tested in the greenhouse for germination and species composition (Appendix S1). The farm dung had a significantly lower numbers of seeds compared to the samples from the field. The dung material from farm animals was collected directly before establishing a disturbance in the field, with the exception of the last sampling for which we used dung material collected in September (due to security reasons — deer males are aggressive during the rut). In the meantime, the samples were stored in freezer (-18°C) which should not influence the germinability or dung properties (Lepková 2014). The herd on experimental farm Podlesko consisted primarily of red deer with one individual of sika deer. Dung samples were collected from both males and females without recognition.

All studied treatments — disturbance versus vegetation, dung deposition and month of deposition — were applied in partially crossed design (see Fig. 1). Every month, two rows of plots (six disturbances and six with undisturbed vegetation) in each site were treated — disturbances were created and dung was deposited. The dung was deposited without any treatment, fresh dung sample was collected and then deposited in the plot. One third of all plots (both disturbed and undisturbed) were left as controls and nothing was deposited there. Vegetation records of all plots were taken during June and early July (visual estimates of percentages of species cover) before the start of the experiment in 2015, and then in 2016, 2017 and 2021. All vascular plants were recorded. Three species remained unidentified and some species were identified only to a genus level (seedlings, non-flowering adult plants, species with difficult taxonomy, e.g. *Alchemilla*). Total cover, litter, moss layer cover and percentage of bare soil were recorded as well and in the later years also percentage covered by the dung pellet. In the last recording in 2021, total cover of herb and shrub layer were recorded separately.

Table 1 Time overview of the experiment setup

time	action
2015: May, June	vegetation sampling
2015: June	dung collection, disturbance creation, dung deposition
2015: July	dung collection, disturbance creation, dung deposition
2015: August	dung collection, disturbance creation, dung deposition
2015: September	dung collection, disturbance creation, dung deposition
2015: October	dung collection, disturbance creation, dung deposition
2016: June, July	vegetation sampling
2017: June, July	vegetation sampling
2021: June, July	vegetation sampling

Fig. 1 Overview of the experiment setup. Each site consisted of five replicates of these twelve plots where half of plots were disturbed with spade (top soil removed, in brown) and half were left undisturbed (green). Dung samples were deposited into plots at the same time as the disturbance was created: dung collected in the field (depicted with flower) and dung collected at a farm which contained much lower numbers of seeds (depicted without flower). One third of plots was left as control. This treatment was done on all five sites five times per vegetation season (see Tab. 1).



2.3.3 Data analyses

Before building models, we checked for spatial variation in our data. On the control dataset from 2015 before the experiment was established, we checked for between-site (site ID) and within-site (row and column ID) variability and its effect on all tested variables. We performed linear mixed effect models (for number of species, Shannon diversity index, Ellenberg Indicator Values, and the percentage of dung-dispersed species) and stepwise selection for the species composition. We revealed both between- and within- site variation and for this reason, site ID and row ID were used in all models as random factors and covariates.

To test the effect of treatment (dung addition and disturbance) we used these response variables: total number of species, Shannon diversity index, Ellenberg Indicator Values (EIV) for nutrients (average values per plot weighted by species abundance, using function functcomp from the *FD* package, Laliberté & Legendre 2010; Laliberté et al. 2014), and proportion of dung-dispersed species from the total number of species. A species was considered as dung-dispersed if it had occurred in the deer dung samples as published in Lepková et al. (2018). The effect of dung deposition and disturbance was tested using generalized linear mixed-effect models. Data from 2015 were excluded from the analyses.

The effect of treatment on the species composition was tested using Redundancy analysis RDA. Some plots were damaged, destroyed, or impossible to localize precisely in the last recording in 2021 which resulted in a slightly unbalanced design as multivariate analyses cannot handle missing values and such samples were left out of these analyses. Data from 2015 were excluded from the analysis and were used only to test for differences between plots before the experiment was established.

All analyses were done in R Core (R Core Team 2022). Linear mixed effect models were performed using package *lme4* (Bates et al. 2015) and *lmerTest* (Kuznetsova et al. 2016). The effect of treatment on the species composition was tested using RDA in package *vegan* (Oksanen et al. 2022). Prior to the analyses, the species cover data were square root transformed. Nomenclature of plant species follows Kubát et al. (2002).

2.4 Results

In total, we found 125 species over the six years. The most common species were generally grasses (*Alopecurus pratensis*, *Agrostis capillaris*, *Poa pratensis*, *Brachypodium pinnatum*) together with some very common herbs (e.g. *Galium album*, *Tanacetum vulgare* and *Clinopodium vulgare*). The most common shrub species was *Rubus* sp. All response variables showed change in time, i.e. the year of the sampling had significant effect in all cases (Tab. 2). Furthermore, we found a significant effect of the interaction between the year of the sampling and the disturbance in all tested variables (see Tab. 2).

2.4.1 Species diversity

The number of species showed significant increase after dung addition compared to plots without dung (Tab. 2). Post hoc testing showed significant differences between the presence of dung from farm and the absence of dung but the difference between plots with field dung and without dung were on the verge of significance (Fig. 2). Testing each year separately revealed that the effect of dung addition was significant only in the first year, i.e., in 2016 (Fig. 2). Furthermore, we found significant effect of the interaction between dung addition and disturbance (Tab. 2) even though the effect of disturbance itself did not have a significant influence. Contrary to the number of species, Shannon diversity index did not change significantly with the dung addition (Tab. 2). Similarly to the previous tests, the effect of dung addition on the diversity index was only significant in the first year and in disturbed plots (Fig. 2).

The species composition was not significantly affected by treatment with dung addition. It was significantly affected by the disturbance, the year of sampling, and by both interactions with disturbance and dung deposition (Tab. 3). The dung addition had significant effect on species composition only when disturbed plots were analyzed separately and no significant effect was observed in non-disturbed plots.

Table 2 Results of all linear mixed effect models. The response variable of individual models is in bold. In all tests, the site ID were used as random effect and data from 2015 were omitted. For the EIV for nutrients we used community weighted means (calculated using package FD, Laliberté et al. 2014) Pseudo R2 per model was calculated using the package MuMIn (Bartoń 2020). Significant results are emphasized in bold.

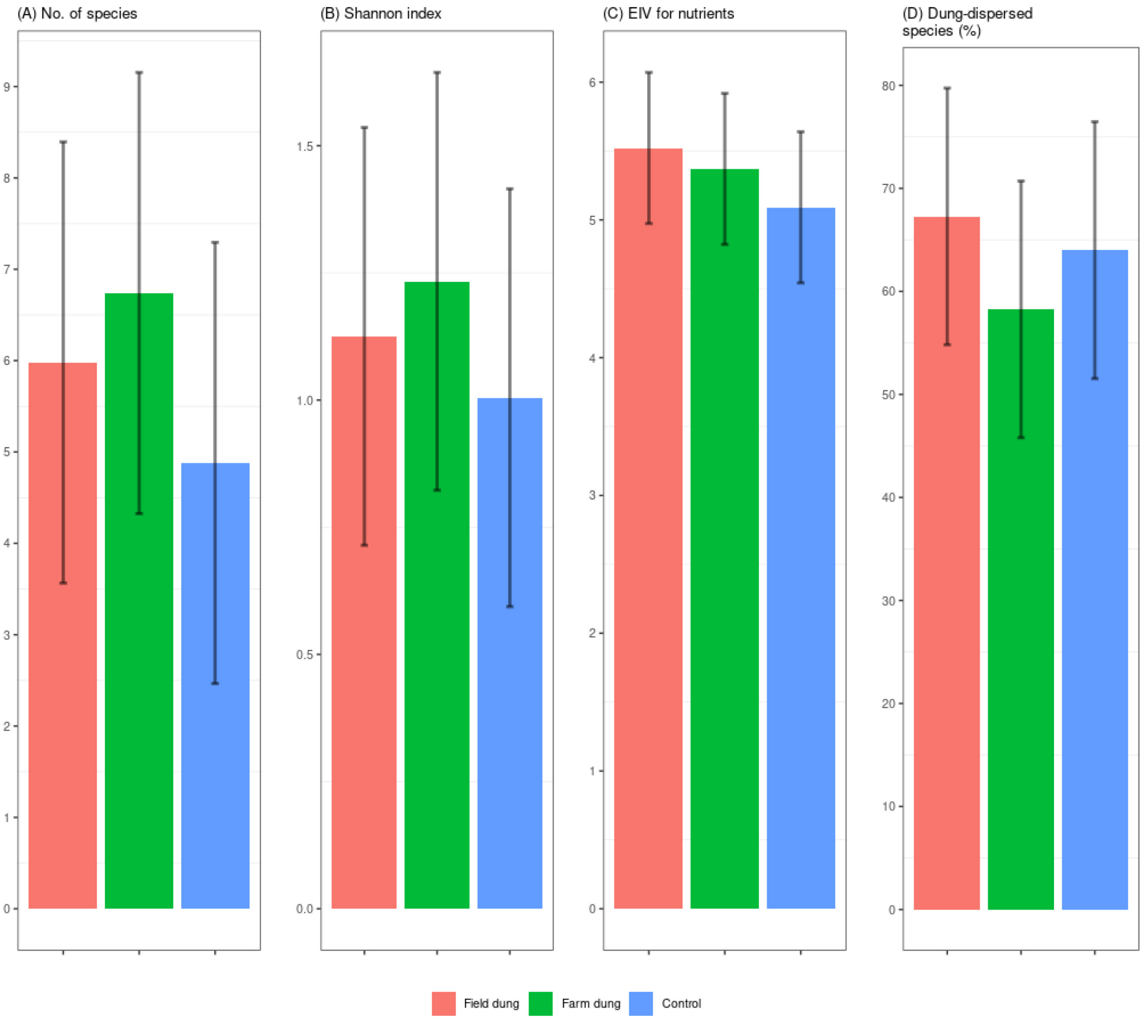
	No. of species			Shannon index		
	F	Df	p-value	F	Df	p-value
dung addition	4.500	2	**0.011**	2.697	2	0.068
disturbance	0.718	1	0.397	4.110	1	**0.043**
year	9.396	2	**< .001**	17.742	2	**< .001**
dung:disturbance	7.254	2	**0.001**	1.108	2	0.331
dung:year	1.892	4	0.11	1.620	4	0.167
disturbance:year	63.816	2	**< .001**	32.161	2	**< .001**
pseudo R2	0.347			0.312		

	EIV for nutrients			Proportion of dung-dispersed species		
	F	Df	p-value	F	Df	p-value
dung addition	3.271	2	**0.038**	0.266	2	0.766
disturbance	35.705	1	**< .001**	52.790	1	**< .001**
year	11.023	2	**< .001**	13.894	2	**< .001**
dung:disturbance	2.125	2	0.12	0.032	2	0.968
dung:year	0.887	4	0.471	2.442	4	0.045
disturbance:year	45.714	2	**< .001**	10.784	2	**< .001**
pseudo R2	0.414			0.277		

Table 3 Anova table of the redundancy analysis: effect of explanatory variables on the species composition. Site ID and row ID were included as covariates. Three variables were used as explanatory: dung deposition (three-level factor: dung from field, dung from farm, control without dung), disturbance (two-level factor: top soil removed or left intact) and year of sampling (four-level factor: years 2016, 2017, 2018, and 2021). Variables explained 11.4 % of all variance. Model significance was 0.001.

	F	Df	Variance	p-value
dung addition	1.232	2	0.109	0.139
disturbance	73.908	1	3.272	**0.001**
year	14.279	2	1.264	**0.001**
dung:disturbance	1.857	2	0.164	**0.002**
dung:year	0.522	4	0.092	1.000
disturbance:year	13.924	2	1.233	**0.001**
Residual		873	38.644	

Fig. 2 The effect of dung addition treatment on (A) species numbers per plot, (B) Shannon Diversity Index, (C) the average Ellenberg Indicator Values for nutrients, and (D) proportion of dung-dispersed species from the total number of species. For simplicity, only data from disturbed plots and from 2016 are depicted. The pink boxplots show results for plots with dung collected in the field, the green boxplots show results for plots with dung collected on the farm, and the blue boxplots show results for plots with no dung addition (controls).



2.4.2 Dung-dispersed species

The proportion of dung-dispersed species from the total number of species was not influenced by the dung addition but we found significant effect of disturbance, and the proportion of dung-dispersed species changed in time (Tab. 2). Furthermore, when we tested the disturbed plots only, the proportion of dung-dispersed species was significantly affected by the year of the sampling and its interaction with dung addition, but not with dung addition itself.

2.4.3 Nutrient addition

We found only a very weak relationship between Ellenberg Indicator Values (EIV) for nutrients and dung addition (table 2). There has been a slight increase of EIV in plots with addition of farm dung compared to the dung-free plots (Fig. 2). However, this has been true only in the first year after experiment establishment and in the following years the effect disappeared (Fig. 2). We also found significant decrease of EIV for nutrients in the disturbed plots (Fig. 2) but contrary to the previous variables, there was no significant effect of dung addition in the disturbed plots (Tab. 2).

2.5 Discussion

In our six-year experiment we tested the effect of endozoochory by red deer on the vegetation dynamics of dry grassland in connection to a common phenomenon of vegetation disturbance. Interestingly, we found only partial support for our hypotheses. The dung deposition had significant effect on most of the tested variables but the effect was significant only in the first year after the setup of the experiment. Furthermore, the effect of dung addition showed a strong interaction with disturbance, i.e. creation of vegetation gaps, which is in concordance with previous research (Bakker & Olff 2003; Eichberg et al. 2007; Blyth et al. 2013). The strongest driver of change in both the species richness and the species composition was the disturbance itself which supports findings of earlier studies (Bakker & Olff 2003; Cosyns et al. 2006).

2.5.1 Species diversity

The deposition of dung significantly increased the number of species per plot similarly to previous studies (Cosyns et al. 2006), but there was no effect on the Shannon Diversity index suggesting there was no difference in community evenness between the dung-addition treatments. The increase was significant only in the first year after experiment setup suggesting at least some seeds germinated within 12 months after the deposition. However, there was almost no germination in the same vegetation season when the experiment started (Williams & Haynes 1995; but see Cosyns et al. 2006). Seedling counts were planned to be performed four weeks after dung deposition but the germination was so low that the seedling counts were discarded. This was due to very high temperatures and low precipitation in the growing season 2015 (86 % of the long-term average precipitation) which could influence the seedling recruitment. Also, it slowed the dung decomposition because dung pellets developed a hard coat which might (i) be difficult for seeds to penetrate (Ishikawa 2011); and (ii) cause anoxic and toxic conditions killing the seeds inside (Traveset et al. 2007; but see Jaroszewicz & Pirożnikow 2011).

The effect of dung deposition on the number of species did not remain significant in the later years (two and six years after experiment setup) so we presume the seedlings either died or were outcompeted (Gower 2008). This is also supported by the fact that species composition was not significantly affected by dung deposition. Experiments with cattle and horse dung showed that germination probability decreased significantly when seeds were deposited in the dung (Milotić & Hoffmann 2016c). If the seed germinates shortly after deposition, the nutrient rich environment of the dung pat gives it advantage over surrounding vegetation and such individual grows faster and produces more flowers (Milotić & Hoffmann 2017) which can boost it for the following year. However, no effect was observed in the later years suggesting some new species appeared in the first year after deposition but disappeared within the next 12 months. Cumulative effects are, thus, not probable but it is possible seeds migrate to the soil seed bank (Jaroszewicz 2013) because seeds dispersed in herbivore dung exhibit high values of longevity index suggesting they are pre-adapted to long-term survival in the soil seed bank (Albert et al. 2015a). Such seeds then await germination for longer period than six years of our monitoring. However, after six years, the vegetation is closing again and the seeds might need another disturbance to open it suggesting the importance of vegetation gaps.

2.5.2 Dung-dispersed species

Interestingly, we did not find significantly more dung-dispersed species in plots with dung addition. Furthermore, this was not affected by the disturbance status. Two explanations are possible: (i) dung-dispersed species are in general common and already present in the surrounding vegetation and thus, their recolonization from dung pats does not play any significant role. This is probable for species e.g. from *Poaceae* family or common herbs like *Veronica chamaedrys* and *Galium album* which are frequent both in dung samples and vegetation. (ii) Such species have very different environmental requirements and thus, when they are dispersed in dung and manage to germinate, they are not able to successfully establish. This is probable for the most commonly dispersed species in deer dung, *Urtica dioica*.

2.5.3 Nutrient addition

We found differences between the effect of field-collected dung and dung from the farm. Post-hoc testing revealed significant difference only between plots with farm dung and plots without dung treatment, the plots with field-collected dung did not differ from any of these controls. The farm dung was used to control for the effect of nutrient addition as these samples contained several orders of magnitude fewer seeds compared to the field-collected dung (greenhouse experiment, Appendix S1). In addition to that, we must expect that the farm dung also contained larger amounts of nutrients as a result of the intensive supplementary feeding in the paddocks (Jaroszewicz et al. 2017). This suggests the increase in species number in plots with dung is caused by nutrient addition rather than dispersal (Jaroszewicz et al. 2017). However, the effect of nutrients, indirectly measured by Ellenberg Indicator Values for nutrients, was very weak and only short-term (but see Mouissie et al. 2005b).

Our results suggest that seed dispersal by red deer does not effectively influence established vegetation of dry grasslands even though it has significant effect on vegetation development on freshly disturbed plots. Animals in the study area choose their forage largely based on plant frequency in the landscape (Lepková et al. 2018), i.e., abundant species have larger probability of being dispersed. However, the study area is under the influence of herbivorous ungulates for decades (Dvořák et al. 2014) suggesting an interplay between herbivory, animal abundance, and vegetation development. Different results can be obtained from a similar experiment in area where animals are newly introduced or excluded.

2.6 Conclusion

In our six-year experiment we tested for the effect of deposition of red deer dung on the vegetation of dry grassland. We conclude that, contrary to predictions based on experiments germinating dung content, the tested effect on vegetation dynamics is extremely weak and does not effectively influence vegetation. However, we expect a portion of seeds migrates to the soil seed bank where it awaits for another opportunity to germinate. Furthermore, the species which survive the passage through digestive system are well adapted to the long-term survival in the seed bank as well and thus, we expect there can be a long-term cumulative effect of seed dispersal through the soil seed bank which could not be detected with our experiment.

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Author contributions BL and EH conceived the original idea of the experiment and collected the data. BL and TH performed statistical analyses. BL wrote the manuscript with the contribution from EH and TH. All authors discussed the results and commented on the manuscript.

Data availability statement Data produced from this study are provided in this manuscript as Supporting Information (Appendix S2).

3 Paper III: Herbivore digestion as environmental filter — which seed traits help species survive?

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3.1 Abstract

1. Herbivorous animals are one of the vectors for seed dispersal of open-landscape plant species. The plant species are adapted to this type of dispersal and show specific seed traits. However, most literature focuses on domestic animals or laboratory simulations.
2. We conducted a comprehensive feeding experiment with almost forty species of plants and four species of wild herbivores. We fed specified numbers of seeds to the animals, collected the dung and germinated the dung content. We explored whether seed morphological traits and seed nutrient contents are good predictors of seed survival after passage through the herbivore digestive system. We also tested how the seed survival differed after the passage through different herbivore species.
3. We found that species survival and germination success was positively correlated to seed nitrogen content and negatively to seed elongation. However, when we tested species from main families separately, i.e., legumes, grasses, and all other species, these trends changed directions. Furthermore, plant species had much higher success after passage through red deer compared to other herbivores, which we attribute to larger body size of the animal.

3.2 Introduction

Effective dispersal of plants is crucial for the connection between different habitat patches, survival of plant populations and the metapopulation dynamics, and their genetic diversity (Lozada-Gobilard et al. 2021). Large animals, e.g., mammals, are potential drivers for both short- and long-distance dispersal (Cain et al. 1998). A simplified model of endozoochorous dispersal can be expressed by this equation:

$$N_{dispersed} = N_{shoots} * N_{seeds/shoot} * p_{eaten} * p_{survival}$$

The effectivity of dispersal as measured by the number of dispersed and germinated seeds ($N_{dispersed}$) is dependent on the characteristics of both parties: the dispersed plant species, here the number of shoots and the number of seeds per shoot (N_{shoots} and $N_{seeds/shoot}$), and the dispersal vector (animal), which influences the probability the seed is eaten in the first place (p_{eaten}). Both plants and animal vector characteristics affect the probability with which the seed survives the passage through the digestive tract ($p_{survival}$).

From characteristics influencing survival in the digestive tract, seed traits are the main driver. Contrary to epizoochory, where the morphology of dispersed propagule clearly affects its ability to attach and remain in fur (Tackenberg et al. 2006; Albert et al. 2015a), in endozoochory the traits supporting successful passage through the digestive tract are less clear. The propagule must be able to survive rough mechanical and chemical conditions, e.g., molar mill and acidic environment in the stomach, taking into account it is actually eaten in the first place. Based on previous research, successful endozoochorously dispersed seeds are small and without appendages (to escape the molar mill), and exhibit long survival in the soil bank which is similarly destructive as the chemical conditions in the stomach, Pakeman et al. (2002); Albert et al. (2015a)]. The effect of seed shape received contradictory support with better survival of round seeds (Mouissie et al. 2005a) as well as elongated seeds (Cosyns et al. 2005b) and no effect of seed shape whatsoever (D'hondt & Hoffmann 2011).

However, what is rarely taken into account are the constraints of the herbivore species, e.g., how morphological constraints of the animal body influence dispersal (Illius & Gordon 1992) — small animals are more likely to disperse only small seeds, whereas large-bodied animals are able to ingest both small and large seeds (Chen & Moles 2015). The body size influences another aspect of the digestive system. That is the speed by which particles travel through the tract: large animals have alonger digestive system, and it takes longer for the particles to pass through it (Clauss et al. 2006). Hand in hand with feeding preferences go the feeding strategies and nutrient demands of herbivore species. Unlike hind-gut fermenters, ruminants are able to digest phytic acid from the seeds thus disintegrating the seeds in the process. Furthermore, Cervids (e.g., deer) have higher phosphorus demands because they store it in their antlers, whereas other ruminants (e.g., Bovids) excrete the excess phosphorus in the dung (Sitters et al. 2014). All of these — speed of passage, digestive type, and nutrient demands — directly affect the probability with which seeds survive the travel through the digestive tract.

Furthermore, the effect of seed traits on survival in the digestive tract is usually tested on the species composition found in dung samples in comparison with available vegetation (Albert et al. 2015a). This approach is used to suggest which plant traits help the species to be eaten and successfully survive the passage. But the probability of being eaten (p_{eaten}) is a function of several factors including plant frequency in the landscape (Lepková et al. 2018), seed production (Bruun & Poschlod 2006), and feeding preferences of the herbivore (Hofmann 1989). The feeding preferences are further influenced by a number of plant traits, e.g., the nutritional value of foliage (Hejcmanová et al. 2016). When species found in dung are compared with the available vegetation, all these filters are included and direct deduction of traits enabling endozoochory is thus questionable. Different type of experiments is available to study the traits influencing seed survival per se — comparing germination rates of seeds fed directly to animals in known quantities.

In this paper, we aim to address the question of which seed traits drive the survival and germination success of species after passage through the digestive tract of four species of wild animals: red deer, sika deer, mouflon, and wild boar. Specifically, we asked two main questions: (i) which seed morphological traits and nutrient contents drive the species' ability to survive the passage through the digestive tract? We hypothesized that the best surviving species have a round shape, mucilaginous surface, and low amount of nutrients. (ii) Does the survival success differ between herbivore species? We tested which of our hypotheses is valid for our set of herbivores: (a) body size has a positive effect on germination rate (red deer > sika deer > wild boar > mouflon). (b) Length of digestive tract has a negative effect on germination rate (mouflon > wild boar > sika deer > red deer). (c) Rumination has a negative effect on germination rate (wild boar > others). We chose thirty-eight species of plants from the landscape of dry grassland, both previously found in dung and from the same area but absent from dung (Lepková et al. 2018). A predefined number of seeds was fed to four different herbivores and their germination rate was established with a greenhouse experiment.

3.3 Material and Methods

We selected the plant species from the vegetation of dry grasslands in the Doupov Mountains where endozoochorous dispersal by deer and boars has been studied before (Lepková et al. 2018). Species were picked based on two criteria: frequency of species in dung samples (> 5%) and vegetation (> 20%). Not all species were available on the market resulting in a list of 39 species from which 23 species were previously known as dispersed in dung. One species — *Knautia arvensis* — did not germinate from control pots or dung samples. It was included in the feeding experiment, but it was excluded from all analyses. The analysed number of species was, therefore, thirty-eight.

All seeds were obtained from the commercial source Planta naturalis (plantanaturalis.com, Markvartice, CZ) from the harvest of 2015. Random amounts of seeds were weighed and counted to find out an exact number of seeds per gram. The obtained seed numbers per gram were then used to weigh the amount of seeds to be fed. Plant species were divided into four groups according to their weight: extra heavy (more than 10 mg), heavy (1–10 mg), medium (0,1–1 mg) and light (less than 0,1 mg) (data from D3 and LEDA databases; Hintze et al. (2013) and Kleyer et al. (2008); respectively). Total numbers of seeds in fed mixtures reflected their absolute weight so that animals were not fed too large quantities of large and heavy seeds (for numbers of seeds fed to one individual, see Tab. 1). Not enough seeds were available for *Vicia tetrasperma*, and we used 400 and 200 seeds for deer and mouflon/wild boar, respectively. Weight data from the databases were used because this influenced the amount of seeds ordered from the commercial supplier.

The experiment was conducted in forest ZOO Malá Chuchle in Prague, Czech Republic. Four species of ungulates were used for the experiment: three individuals of red deer (*Cervus elaphus*), three individuals of sika deer (*Cervus nippon*), seven individuals of mouflon (*Ovis musimon*), and one young individual of wild boar (*Sus scrofa*). Even though three of the chosen herbivore species are ruminants with a similar digestive system, both species of deer belong to the group of intermediate feeders, whereas mouflon is a more selective browser (Hofmann 1989). Compared to the ruminants, wild boar is an omnivore who chooses plant-based forage primarily during the vegetation season (Schley & Roper 2003). Different digestive systems and different feeding behaviors lead to differences in the processing of forage in the digestive system (Baker & Hobbs 1987).

For the feeding experiment, we divided the plant species into two groups to make the identification of seedlings easier and not to feed the animals such large quantities of seeds. The groups of plant species were fed separately in two different phases: on December 4, 2016 and on February 20, 2017. All individuals of one animal species were held in an enclosure together (pen in the case of boar in the first phase). The seeds were fed in a mixture of oat, barley, carrot and apples with lukewarm water and all animals were fed all species of plants. The animals were fed alfalfa hay and their usual forage (fruit, dry bread, and cereals) for three days before the experiment, during the experiment, and until the end of the dung collection. All dung was removed from the paddocks before the experiment and it was used as a control sample to check for germination from dung before the feeding started. After the feeding, the dung was collected every day for four days, with few exceptions (see Tab. 2) so as not to disturb the untamed animals. After collections, the dung samples were stratified in a freezer for two months and then crumbled and air-dried.

Seed survival was checked by a greenhouse emergence experiment over a period of two and half years. The germination pots were filled with perlite, covered by non-woven fabric and the air-dried crumbled dung samples were put on top. This allowed the samples to be mixed without mixing the substrate as well and burying the seeds at the bottom of the pot. The emergence took place under natural light conditions from May and June 2017 for the first and second phases, respectively. Pots were watered as necessary with rain-water and shifted around the greenhouse at random. In the last months of the experiment, when germination decreased, samples were left to dry out several times and mixed to let seeds from lower layers to germinate. Three different controls were set: (i) pots with dung samples collected from each enclosure before the feeding started, (ii) pots with no seeds and no samples to control for seed rain (small amount of potting soil was used on top of the non-woven fabric as substrate), and (iii) pots with 500 seeds per species to establish germination rate without passing through the guts (mixed with small amount of potting soil). In all cases, pots were filled with perlite and covered with non-woven fabric. All controls were stratified for the same period as the samples.

Table 1 The number of seeds fed to each animal. The numbers of individuals per animal species are in brackets. Thus, the number of seeds of a light plant species fed to all individuals of red deer was 30 000.

Herbivore	Extra heavy	Heavy	Medium	Light	*V. tetrasperma*
Red deer (3)/Sika deer (3)	500	1000	5000	10000	400
Mouflon (7)/Wild boar (1)	250	500	2000	5000	200

Table 2 Results of the generalized linear mixed-effect model testing the effect of herbivore species on the germination success of ingested seeds of 38 plant species. Post-hoc pairwise comparison was performed using package emmeans and revealed that survival of seeds after passage through red deer differed from all other animal species. The remaining animal species did not differ from each other.

contrast	estimate	SE	df	t.ratio	p.value
Mouflon - Red Deer	-1.884	0.213	146	-8.837	**< .001**
Mouflon - Sika Deer	-0.531	0.231	146	-2.298	0.103
Mouflon - Wild Boar	-0.361	0.263	146	-1.372	0.519
Red Deer - Sika Deer	1.353	0.181	146	7.463	**< .001**
Red Deer - Wild Boar	1.523	0.214	146	7.103	**< .001**
Sika Deer - Wild Boar	0.170	0.241	146	0.704	0.896

3.3.1 Seed traits

Seed nutrient reserves were measured as the content of nitrogen (N), phosphorus (P), carbohydrates, and oils. Carbohydrates content was calculated as the sum of fructans and starch. N content was measured by flow injection analysis after Kjeldahl mineralization. P content was measured by flow injection analysis after perchloric acid mineralization. Starch and fructans were measured with the enzymatic procedure Megazyme (McCleary et al. 1994). Oil content was measured by Soxhlet extractor (International Organization for Standartization 2016). See Mašková & Herben (2021) for more details. To calculate the index of shape (Thompson et al. 1993) we used seed dimension from LEDA database (Kleyer et al. 2008). The index is the variance in dimensions and it ranges from 0 (perfectly round seeds) to 1 (elongated seeds). The following equation was used to calculate the index:

$$\frac{\sum (x_i - \bar{x})^2}{n}$$

To make the understanding of the index simpler, it is further referred to as seed elongation. We used the information about the presence of mucilaginous surface from D3 database (Hintze et al. 2013).

3.3.2 Data analyses

No plant species germinated better after passing through the digestive system than the unpassed controls. We thus used the germination rate of controls as the maximum possible germination potential for seeds fed to animals and corrected the number of fed seeds for this germination potential.

We used a generalized linear mixed-effect model (GLMM) to test the effect of seed traits on species germination rate. Two models were built: a model not accounting for phylogeny using package glmmTMB (version 1.1.2.9000, Brooks et al. 2017) and a model with phylogenetic correction using package phyloglmm (developer version from GitHub, DOI: 10.5281/zenodo.2639887, Li & Bolker 2021). We used beta-binomial distribution to account for overdispersion and animal species as a random factor. To test for the differences between animal species, we used GLMM from the same package glmmTMB with betabinomial distribution (Brooks et al. 2017). For the tests with betabinomial distribution, we made a matrix of successes (germinated seeds) and failures (germinated seeds subtracted from the fed seeds). Furthermore, we separated species into three major groups and tested these separately: *Fabaceae* (9 species), *Poaceae* (14 species), and all other species (15). All models were made in the same manner (GLMM with betabinomial distribution and animal species as random factor).

We used the RLQ method (Kleyer et al. 2012) from the package ade4 (Thioulouse & Dray 2007) to test the correlation between seed traits, seed germination rate, and animal species. These three types of information were all stored separately in three matrices. The RLQ analysis consists of several steps: first, individual matrices were analysed using principal component analysis (seed trait matrix and matrix of "environmental" variables, here the species of animal) and correspondence analysis (species germination rate matrix). The final RLQ model was then tested using fourth-corner statistics (function forthcorner.rlq, package ade4). All analyses were performed in R (version 4.1.1., R Core Team 2021). Nomenclature of plant species follows Kubát et al. (2002).

3.4 Results

None of the studied species showed increased germination after passage through the digestive tract, and eight species did not germinate from dung samples at all. The germination rate ranged from 0 % to 39 % (percentage of germinated seedlings from the total number of fed seeds corrected for germination rate of seeds in the control lot). The most successful species belonged to the *Fabaceae* family: *Vicia cracca*, *Trifolium repens*, *Securigera varia*. However, even in the species with the highest germination rate, it varied between animals: the best surviving *Vicia cracca* showed a germination rate of almost 40 % after passage through red deer but less than 6 % after passage through mouflon. Plant species survived the passage through red deer better than through other herbivores (Fig. 3).

3.4.1 Seed traits and nutrient contents

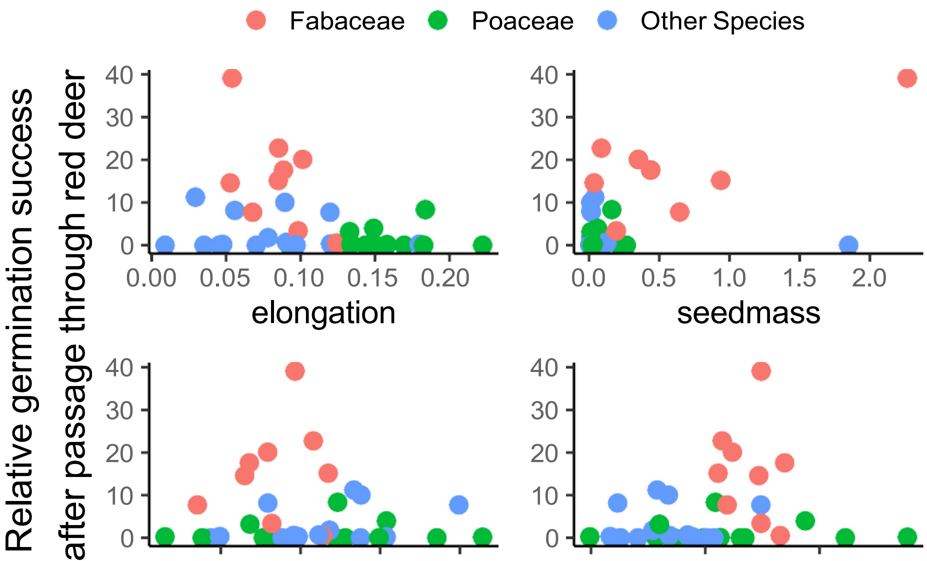
The effect of seed traits on germination rate was tested both with and without phylogeny. Accounting for phylogeny resulted in a non-significant effect of any of the tested variables. Without accounting for phylogeny, the germination rate was positively affected by the amount of nitrogen and negatively by seed elongation, i.e., round seeds had better survival than elongated seeds (see Tab. 3, first column). In all variables, the plant species with zero or extremely low germination rates were distributed along the entire measured gradient. However, no model with zero inflation argument improved the fit.

The trait analyses have also been done separately for the families with the highest number of species: *Fabaceae* and *Poaceae*, and for the remaining species. Different results were found for individual groups of species. In some cases, we found a positive effect of a trait or nutrient in one group and a negative in another (see Tab. 3). The effect of seed phosphorus content which was non-significant in the test for all species, was significant for all groups separately: a positive effect was found in *Fabaceae* and other species, and negative in *Poaceae*. The seed nitrogen content had a significantly positive effect when tested for all species but negative in both *Fabaceae* and the group of other species, and non-significant for *Poaceae*. Last but not least, the effect of seed elongation was negative (better survival of round seeds) in all but the group of other species where it had a positive effect (better survival of elongated seeds). Contrary to our predictions, none of the tests revealed any relationship between seed germination rate and the presence of the mucilaginous surface. Results of all tests for all species as well as individual groups of species are summarized in Tab. 3.

Table 3 Results of the GLMM of all species, only *Fabaceae*, only *Poaceae*, and all other species. P-values for the significant variables are shown with arrows indicating the direction of effect (↑ for positive effect and ↓ for negative effect). A different trend is visible for the most important driver of species germination success - the seed elongation with a negative effect in both *Fabaceae* and *Poaceae*, and a positive effect in the remaining species.

	All Species		Fabaceae		Poaceae		Other species	
Seedmass	ns.	-	0.001	↑	ns.	-	0.01	↑
P	ns.	-	0.01	↑	0.05	↓	< 0.001	↑
N	0.01	↑	< 0.001	↓	ns.	-	< 0.001	↓
Starch	ns.	-	ns.	-	0.02	↑	ns.	-
Oil	0.06	-	ns.	-	ns.	-	ns.	-
Mucilage	ns.	-	–	–	ns.	-	ns.	-
Elongation	0.01	↓	< 0.001	↓	0.08	-	< 0.001	↑

Fig. 1 The relationship between significant seed traits and nutrient values, and the relative germination success for passage through red deer. The plant species were divided into three groups based on their family: *Fabaceae* species in pink, *Poaceae* species in green, and all species from other families in blue (Other Species).



3.4.2 Differences between herbivores

There was no significant relationship between seed traits and the species survival after passage through different species of animals as tested by RLQ and fourth corner analysis. However, there were significant differences in the survival of seeds after passage through different species of herbivores (GLMM, $p < 0.001$). Post hoc testing revealed that after passage through red deer, germination was significantly higher than after passage through any other tested herbivore (Tab. 2, Fig. 3).

Interestingly, red deer dung samples collected after four days from the feeding trials still contained seeds of some species suggesting the retention time was underestimated. This was the case for *Alopecurus pratensis*, *Trifolium arvense*, and *Clinopodium vulgare*, and at a lower rate for *Hypericum perforatum*, *Plantago media*, *Potentilla argentea*, and *Securigera varia* (Fig. 3). However, this has not been the case for other animal species, even though in sika deer, the collection was omitted on day 3 in phase 2.

Fig. 2 The germination rate of seeds after passage through four tested species of herbivore: red deer, sika deer, mouflon, and wild boar. The letters above boxplots show the significant differences between animals as tested by a mixed-effect model with a beta-binomial family where the plant species was set as a random factor. The only significant difference was in red deer, which showed higher germination success than all other animals.

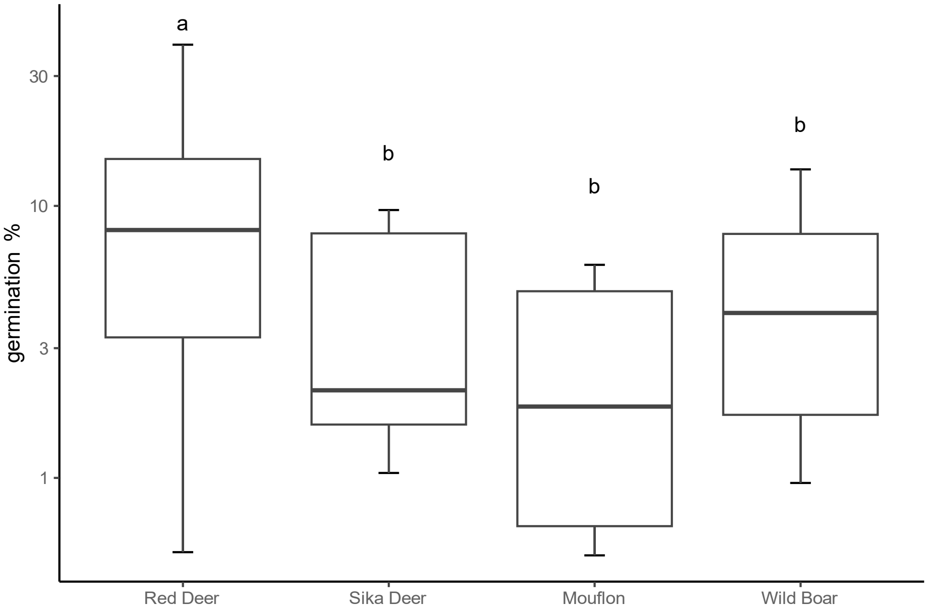
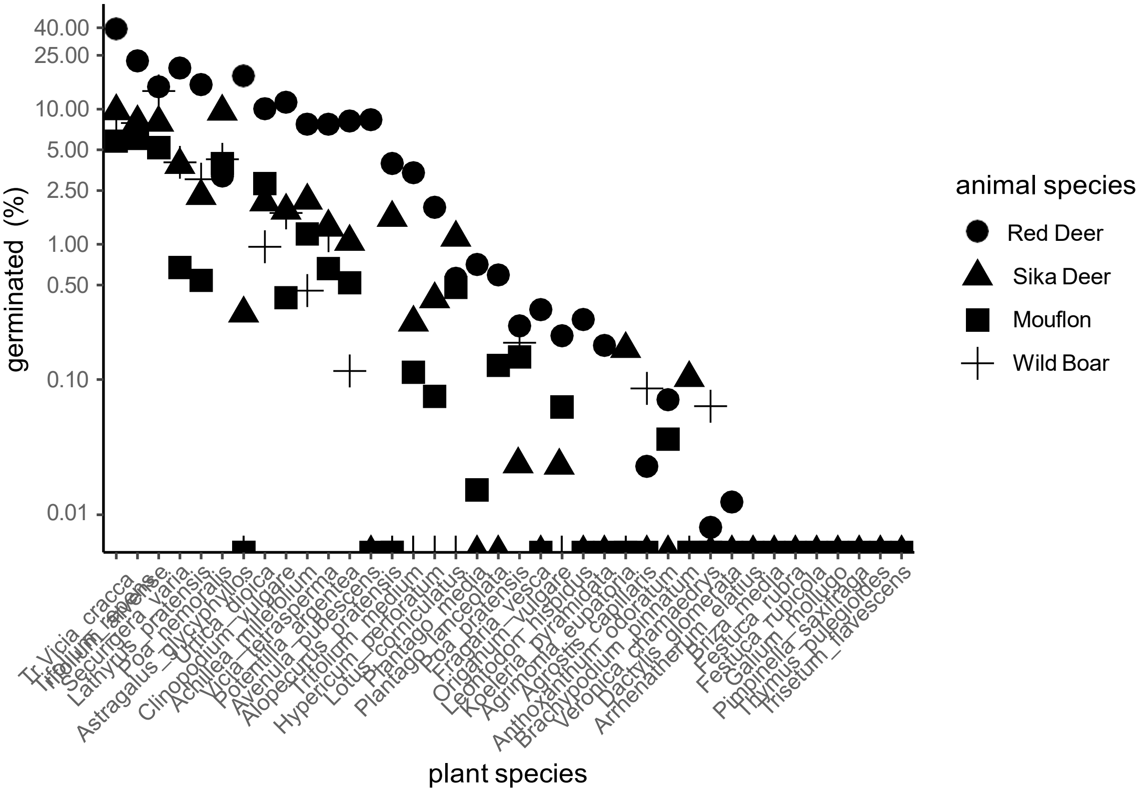


Fig. 3 The seed germination rate after passage through herbivore guts and how the survival differs between herbivore species: red deer (full circle), sika deer (full triangle), mouflon (full square), and wild boar (plus sign, +). *Vicia cracca* was the best surviving plant species, with germination after passage through red deer nearing 40 %. Species with zero germination after passage through all four herbivore species were not plotted. The survival rate is depicted as a percentage of the number of seeds ingested corrected for the non-ingested seeds. For full names of plants, see Supp. Mat.



3.5 Discussion

None of the species showed increased germination after passing through the digestive tract. In agreement with our predictions, we found that round seeds were more likely to survive the passage through the digestive tract. This held true when tested for all species together as well as for *Fabaceae* separately. However, when *Fabaceae* and *Poaceae* were excluded, the trend was opposite. The seed germination rate was higher after passage through red deer compared to any other herbivore supporting hypothesis (a) "body size has a positive effect on germination rate". Contrary to our prediction, the seed nutrient content had different effect based on the plant species group and the nutrient in question. Contrary to our previous results, we did not find support for the hypothesis that seeds with the mucilaginous surface will survive the passage through the digestive tract better.

Only one tested trait showed a sufficiently significant effect on the germination success of all species: the seed elongation (index of shape). We found that, in general, round seeds survived better than elongated seeds. When tested separately for *Fabaceae*, *Poaceae*, and all other species, this held true for all but the last where survival and subsequent germination success were better for the species with elongated rather than round seeds. This could also be an explanation for contrasting support in literature: better survival of round seeds was reported by Mouissie et al. (2005a), better survival of elongated seeds in Cosyns et al. (2005b), and no significant relationship between seed survival and shape in D'hondt & Hoffmann (2011). The cited studies used a range of plant species but only in the last case did the authors take phylogenetic relationships into account. Even though seed traits are often phylogenetically conserved (Moles et al. 2005), analyses with phylogeny might not receive significant effects (Bello et al. 2015), as was also our case. This suggests that both the tested traits and the environmental filter (here, the passage through herbivore guts) are phylogenetically conserved (Bello et al. 2015). Furthermore, in the cited studies (Cosyns et al. 2005b; Mouissie et al. 2005a; D'hondt & Hoffmann 2011), the seed shape measurements used were made on seeds with no appendages, whereas in the presented study we used data from the LEDA database where seeds were measured with appendages. When grazing, herbivores feed on vegetation and the seeds they consume are not cleaned of glumes or pericarps. However, such structures can provide extra protection for seeds in the digestive tract, and therefore, they should be included in the measurements.

Our results are partially supporting our hypothesis that seed survival and subsequent germination success will be directly influenced by seed roundness and the presence of mucilaginous surface. The effect of shape has been addressed above, but we have found no effect of mucilaginous surface on seed survival and germination rate, which is in contrast to some previous findings (Lepková et al. 2018). The mucilaginous surface is a rarely occurring trait which is believed to have a connection to seed survival in the digestive tract (Hintze et al. 2013). Some species exhibiting mucilage were very common in field-collected dung, e.g., *Poa pratensis* and *Veronica chamaedrys*, but here, all tested species with mucilage showed very low germination rates (less than 1 %). However, the low germination rate of frequently dispersed species is common throughout the studied species set. Two reasons are plausible: (i) seeds from the commercial supplier were of insufficient quality. This is not probable because the species showed successful germination in the control pots. (ii) The animals in the field ingest numbers of seeds several orders of magnitude higher than what we fed them in the experiment. This suggests preferential grazing which has nothing to do with seed traits or their nutritional content but can be driven by other traits of mother plants which influence palatability.

Previous research showed that even a very similar setting of feeding experiment does not guarantee similar outputs. For example, in a multi-species study with a number of plant species fed to cattle, Cosyns et al. (2005b) and D'hondt & Hoffmann (2011) found vastly different results in the species of plants included in both studies, e.g., the relative germination rate of *Agrostis capillaris* was 17 and 54 %, respectively. In the presented study, the measured germination rate for the same plant species was effectively zero, no matter the herbivore species. However, this species is one of the most common grasses dispersed by wild herbivores (present in 35 % and 20 % of deer and wild boar dung samples, respectively, Lepková et al. 2018). This example only emphasizes the high discrepancy between different types of experiments and also between similar experiments but under the influence of naturally behaving animals.

The most successful species were members of the *Fabaceae* family (similarly to Gardener et al. 1993a), which is in contrast to often used laboratory experiments (Milotić & Hoffmann 2016b) and shows the importance of testing with real animals. The success of the *Fabaceae* family is often explained by their mechanical characteristics (e.g., thick seed coat, Gardener et al. 1993b). In the case of the presented study, the best surviving *Fabaceae* had round and large seeds. The effect of seed mass is counterintuitive and unexpected as endozoochorous seeds are usually small (Bruun & Poschlod 2006; Albert et al. 2015a). This result has been clearly driven by one species: *Vicia cracca* with the highest germination success, and when the species was excluded, the seed mass became non-significant.

Our experiment revealed a complex relationship between seed survival in the guts and the content of available nutrients. In both cases of nitrogen and phosphorus, we found diverging results when groups of plant species were tested separately (Tab. 3). Both legumes and grasses are known for specific amounts of seed nutrients (Mašková & Herben 2021), and we expected these nutrient contents to affect the seed survival, or more precisely, the effectivity with which animals extract the nutrients and thus kill the seeds. However, we revealed relationships contrary to our predictions. In *Fabaceae* and the group of other species (legumes and grasses excluded), we found a significant positive effect of phosphorus content on seed survival even though ruminant herbivores are preadapted to digest the phytic acid in which phosphorus is stored (Klopfenstein et al. 2002). Only the effect of phosphorus on the survival of grasses was significantly negative and, as a result, the overall test of all species did not show a statistically significant effect. This suggests the herbivore species included in this study are more adapted to the digestion of graminoid seeds, which are more common in their diet in Central Europe (Spitzer et al. 2020).

3.5.1 Differences between herbivore species

Four herbivores exhibiting different feeding and digestive behavior were used for the experiment, and differences based on body size, length of the digestive system, and/or feeding style were expected. Since at the time of the feeding experiment, only one individual of omnivorous wild boar was available, comparing herbivores and omnivores is outside the scope of the presented study. Our results support our hypothesis (a), which states that the driving force is the size of the animal. Dung samples from red deer, as the largest animal (Anděra & Horáček 2005), showed the highest germination rates of seedlings. However, the effect of body size is contradictory to other literature using feeding experiments (Simao Neto et al. 1987; Cosyns et al. 2005b).

Experiments with multiple animal species are rare, and the majority of published data are on domestic animals (Bonn 2004; Cosyns et al. 2005b). Therefore, it is difficult to further address the effect of body size on seed survival. Chen & Moles (2015) performed a meta-analysis on the relationship between seed size, seed dispersal, and disperser size. They found out that in large ungulates in particular the relationship with seed size is negative, i.e., the large animals primarily ingest small-seeded species. The relationship is even more complicated because ruminants spit large seeds which are not digested at all (Castañeda et al. 2018). This complexity can also be the reason for the non-significant effect of seed size in our dataset.

3.5.2 Speed of passage

Since some species were still germinating in significant numbers from samples from the fourth day of collection, we must assume the retention time was longer than 96 hours (but see Cosyns et al. 2005b). Our results show that *Fabaceae* had a slower passage through the guts (Gardener et al. 1993a). Combined with the fact that *Fabaceae* also had the highest measured survival success, we can assume the survival was still underestimated. This is in contrast to our prediction that long passage through the digestive tract shall be more destructive for the seeds. However, this result also means that seeds, which stay in the digestive tract for this long, can be dispersed further away from the mother plant which can even compensate for losses during the passage (Janzen 1984).

3.6 Conclusions

Our experiment with almost forty species of plants and four herbivores did not reveal a single seed trait that predicts seeds survival when passing through the herbivore digestive system. The most important trait was the seed shape, but we found contrasting effect on species from different families. In general the best survival was found in round seeds but when legumes and grasses were excluded, we found the survival to be better in elongated seeds. Furthermore, an important piece of knowledge may be missing to understand the survival of seeds better, e.g., seed coat thickness. However, it is vital not to focus too much on plants and take into account the constraints of the animal body and digestive system.

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Authors contributions BL planned and performed the feeding and germination experiments. TM collected the data on seed nutrient content. BL analysed the data and wrote the manuscript, TM provided feedback for data analyses and revised the manuscript. Both authors approved the final version.

Conflict of Interest The authors declare no conflict of interest.

4 Paper IV: Untangling drivers of endozoochorous seed dispersal by wild herbivores

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4.1 Abstract

1. Large herbivores are effective dispersers for a number of species via their digestive system. However, the seeds of plants dispersed this way need to be pre-adapted to the environment in the guts to survive and successfully germinate, that is if they are eaten in the first place. These conditions together with species availability present filters seeds need to get through to be successfully dispersed. Here we aimed to disentangle the filters and we ask: what is driving the species dispersal in dung? How does it differ between herbivore species? Are some species dispersed yet not adapted?

2. We built a unique dataset by combining data on the species composition of dung samples, the composition of available vegetation, and relative germination rates after passage through the digestive system of deer and boar, all coming from one study area. Further, we added plant traits from databases (TRY and LEDA) as proxies for plant palatability.

3. We found a significant effect of the availability of forest species on dispersal by both herbivores, however, this result was largely driven by a few species not occurring in grassland habitats. When tested separately, we found contrasting results for boar and deer. Dispersal by boar was significantly affected by the germination rate after passage through digestion, whereas none of the variables had a significant effect in deer.

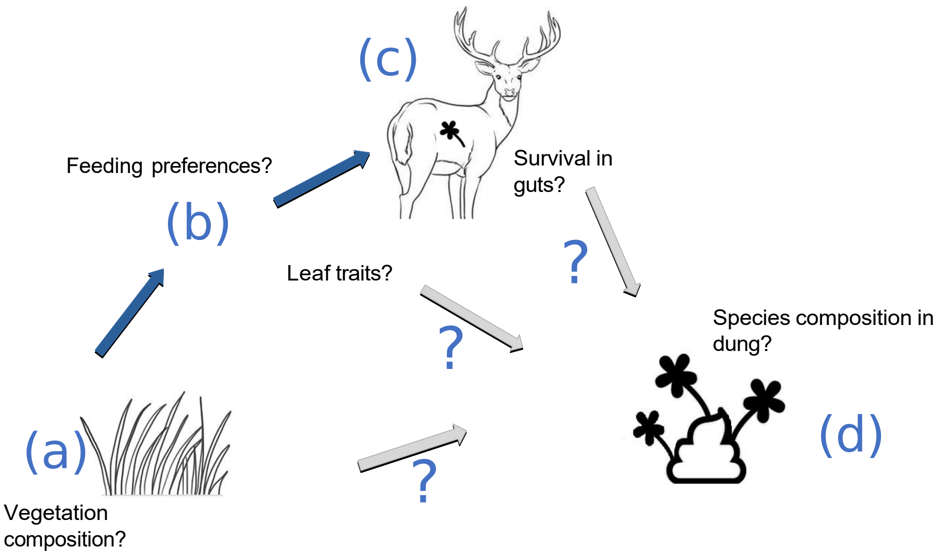
4. Synthesis. Our results suggest that drivers of seed dispersal by deer and boar in our study area differ between plant species. One species in particular — the stinging nettle — was driving our results. This plant is grazed preferentially: often and in high numbers even though it is not adapted to survive the digestive tract very well. We also found a number of species (mainly legumes) with high adaptation to the passage through the digestive tract but they are not dispersed by our studied animals. Disperser of these species may be missing from our system.

4.2 Introduction

Large herbivores are an inseparable component of grassland ecology throughout the evolution. Due to their herbivorous diet, they also are a good vector for seed dispersal via their digestive tract — endozoochory — which happens anywhere the herbivores forage on vegetation bearing mature seeds. If the plants are consumed when their seeds are mature, they can take advantage of it and use the herbivore as a vector for seed dispersal (Janzen 1984). This is supported by evidence from both domestic and wild herbivores which have been described as effective endozoochorous vectors (e.g., Eichberg et al. 2007; Eycott et al. 2007, respectively) although wild herbivores are studied less compared to domestic animals. Furthermore, the majority of previous research focused on studying the content of dung samples (e.g., Jaroszewicz et al. 2013b). This represents a black box view which is useful when asking what species and how often are dispersed in dung. However, it does not help us untangle the individual factors influencing seed dispersal.

The endozoochorous dispersal is driven by several factors: (i) available vegetation in the home range (common vs. rare species), (ii) plant adaptations to grazing and dispersal (plant nutrient content, the timing of seed maturation, etc.) including how frequent the species is in the landscape, (iii) feeding preferences of the herbivore (grazer vs. browser), and (iv) the probability with which seeds survive passage through digestive tract (Janzen 1984) (see Fig. 1). Untangling the importance of each of these factors is a way how to look inside the endozoochorous black box because the factors act as filters and on each level, only a subset of plant species passes through. However, the question remains: which of these filters is the driver for plant dispersal in herbivore dung?

Fig. 1 Diagram showing factors influencing the species composition in dung and how they relate to each other.



The first filter is the pool of available plant species at the locality (Fig 1a) because the animals cannot feed on plant species not present in their home range. Wild herbivores can choose between habitats in their home range based on their behavioural needs. In the landscape of Central Europe, the most abundant wild herbivores are deer (red deer, roe deer) and wild boar (Linnell et al. 2020). During the vegetation season, both deer and boar show feeding preference for vegetation of open landscape (Krojerová-Prokešová et al. 2010; and Genov 1981 respectively) even though they have different digestive systems and feeding preferences (Hofmann 1989). Furthermore, within the preferred habitat, plant species with high abundance have a higher probability of encountering the herbivore (Bailey et al. 1996).

The second filter is the probability with which the plant is grazed. This includes plant adaptation to grazing and the animal's feeding preferences. The most frequently grazed species are from two families: *Poaceae* and *Fabaceae* (Krojerová-Prokešová et al. 2010; Spitzer et al. 2020; Potter et al. 2022). However, the animals have preferences towards particular species. How picky the herbivores are in their diet and how much they graze, and thus disperse, on whatever is available, largely remains a question. One proxy helping us to understand herbivore selection of forage is the foliar nutrient content. Deer prefer forage with high levels of nitrogen (Forsyth et al. 2002) and avoid species with high levels of sulphur (Ceacero et al. 2015). We also know that deer, compared to boar, are in dire need of nutrients like calcium and phosphorus in order to grow their antlers (French et al. 1956; Landete-Castillejos et al. 2007). In addition, herbivores with different feeding preferences can distinguish their forage based on other plant functional traits like leaf thickness (Potter et al. 2022). Since we cannot infer the herbivores' preferences in detail, we assume that leaf traits act as a proxy for plant palatability (Fig. 1b) which in turn is a proxy for animal preference.

The last filter is the seeds' adaptation to survival in the harsh environment of the digestive tract (Fig. 1c). The seeds need to be protected mechanically (from mastication and rumination) and chemically (from the acidic environment) (Janzen 1984). Various seed traits have been found as having a significant effect on survival in the digestive system (Albert et al. 2015a) (for example seed shape or size). Seed production is one of the significant traits — species with high production of seeds are more frequently dispersed (Bruun & Poschlod 2006). Such species have smaller seeds which give them a higher probability of surviving in the guts (Pakeman et al. 2002), but also because the animals may consume them accidentally when grazing on neighbouring vegetation. However, the best measure of species success in the digestive system is an actual survival and consequent seed germination rate after the passage which can be obtained by a feeding experiment (e.g., Mouissie et al. 2005a).

As mentioned before, endozoochorous dispersal is often studied by the means of germinating dung samples without looking deeper into this black box. However, due to the multidisciplinary nature of this topic, particularly in wild herbivores, information on its individual components is often scattered and incomplete. Here we present a unique dataset which comes from one geographical area and contains data about individual filters, thus permitting us to look inside the black box and to disentangle individual processes outlined in Fig. 1. We combine data on vegetation composition (Fig. 1a), plant functional traits (Fig. 1b), and seed ability to survive in the digestive tract (Fig. 1c) with the dung bank composition (Fig. 1d). Using this dataset we aim to look inside the black box and ask: (i) what are the drivers of plant dispersal in dung? (ii) How do the drivers differ between herbivore dispersers? (iii) Are there plant species well adapted to dispersal but not present in dung samples? We used linear and generalized linear models, and redundancy analysis with a stepwise selection of variables to explore the relationship between species occurrence in dung and all the listed variables.

Terms used in this paper and their definitions

palatability = a set of plant traits suggesting the species have an increased probability of being grazed (as opposed to species which are avoided)

preference = a set of animal characteristics which affects the animal's choice of forage

adapted species = plant species showing germination success after passage through the digestive tract higher than average. Plant species may be adapted to the digestive tract of a specific herbivore.

dispersed species = plant species frequently (more than average) occurring in dung samples of studied animals. Plant species may be dispersed by one or several dispersing animals.

4.3 Material and Methods

4.3.1 Data assembly

For this study, we combined several existing datasets from the Doupov Mountains in Western Bohemia. The mountains are of tertiary volcanic origin and until the late forties, the entire area has been heavily populated by mainly German inhabitants who were forcefully displaced after WWII (Augustin 1994). Since the establishment of the military area in 1953, the vegetation has been developing mostly freely in the buffer zone of the active military area where all the datasets come from. The vegetation there consists of a mosaic of open dry grasslands (*Festuco-Brometea*) and shrublands (*Prunion spinosae*). In areas of former villages, the vegetation consists of secondary forests with the dominance of *Fraxinus excelsior* (Vojta 2007). The mean annual precipitation is approximately 670 mm and the mean annual temperature is about 6°C (Vesecký, A., Briedoň, V., Karský, V., Petrovič 1961). The most common herbivores in this area are red deer, sika deer, and wild boar (Horčičková, unpubl. data).

We combined previously published datasets from the area and added data on plant traits from databases (see Tab. 1 for a summary of all variables). All these datasets were collected as a part of a long-term project on plant-animal interactions and vegetation change after abandonment. The species composition of dung samples was taken from Lepková et al. (2018) and was used to calculate species frequency in dung and the standardized number of seedlings. In this study, the dung samples were collected throughout the vegetation season 2012 on 15 transects. The samples were collected every four weeks from June to October with a total of five collections. The dung samples were collected for deer and boar separately (no. of samples 190 and 87, respectively). The species composition was diagnosed via a greenhouse germination experiment.

Using these data we defined two response variables used in our analyses: (i) frequency of plant species in dung samples and (ii) number of seedlings per plant species standardized per 100 g of dry dung mass (Lepková et al. 2018). For the purposes of our analyses two types of species frequencies were used: (i) the number of dung samples where the species was found from the total number of samples (this allowed us to create a matrix of successes and failures for binomial model); and (ii) relative frequencies of all species (in percentages, this allowed us to perform multivariate analyses).

The explanatory variables were species frequency in the landscape, leaf traits, seed production and germination rate after passage through the herbivore's digestive tract. The species composition of grasslands was taken from Lepková et al. (2018) and was used to calculate species frequency in the grassland habitat. The species composition of secondary forests was taken from Vojta (2007) and was used to calculate species frequency in the forest habitat. Different sampling method was used for respective habitats, and thus, the data were analysed separately. We used a number of samples with species present from the total number of samples and relative frequencies of all species. Species not found in vegetation records were coded as zeros.

Leaf traits were used as a proxy of plant palatability. Leaf nitrogen content, leaf phosphorus content and leaf mass were downloaded from the TRY database (Kattge et al. 2020). Multiple measurements per trait per species were averaged. Strawberry (*Fragaria*) was not identified into species level in the dung samples, and thus, we used average trait values for two most common species in the area: *Fragaria vesca* and *Fragaria viridis* and the species is referred to as *Fragaria vesca*. Data on specific leaf area (SLA) were taken from the LEDA Traitbase (Kleyer et al. 2008). Data on seed production were taken from Šerá & Šerý (2004). When data was not available for the species, it was coded as NA.

The last explanatory variable was the species germination rate after passage through the digestive tract of different herbivores which was taken from Lepková & Mašková (2022). The original study included wild boar, red deer, sika deer, and mouflon. For the present study, we used data on wild boar and summed the data on red and sika deer. Thirty-eight plant species were included in the feeding experiment, twenty-three of them were previously known to be dispersed in dung in the area (Lepková et al. 2018). The species were selected from the grassland vegetation of the area to cover a whole range of frequency of occurrence in dung pats in the region (Lepková et al. 2018). See Lepková & Mašková (2022) for a more detailed description of species selection. For the data analyses, relative germination rates were used.

The combined dataset of species occurrence in dung and in vegetation consisted of 202 plant species. However, the statistical analyses were limited by the smallest dataset: the germination rates after passage through the digestive system. Thus, the actual number of species for analyses was thirty-eight for which the data on plant traits were almost complete.

4.3.2 Data analysis

Correlation matrix was calculated to explore the direct relationship between all variables considering plant frequency. These included: species frequency in grassland, species frequency in forest, species frequency in dung samples from both deer and boar, seedling concentration in dung samples from both deer and boar, and species germination rate after passage through deer and boar digestive systems. Prior to calculating the correlation matrix, the variables were log-transformed.

We used two response variables: species frequency in dung samples and seedling concentration expressed by the number of seedlings per 100 g of dry mass (see Tab. 1). The number of seedlings was first standardized per g of dry mass per sample, and then the mean across all samples was calculated. Separate values per deer and boar were used for the analyses. As explanatory variables, we used species frequency in the vegetation (grassland and forest), plant traits (leaf nutrient content, leaf mass, and seed production) and species germination rate after passage through the digestive tract (separate values for deer and boar) (see Tab. 1).

The response variables were analysed separately using different methods. The species frequency in the dung was tested using generalized linear models with the package glmmTMB (version 1.1.5, Brooks et al. 2017). Since the relative frequencies of species did not have a normal distribution, binomial distribution was used. A matrix of "successes" and "failures" was built: success equalled the number of samples with species present, failure was calculated as the number of samples where the species was missing. All explanatory variables excluding leaf nutrient contents were log-transformed. Due to overdispersion and convergence problems, betabinomial distribution was used in the GLM analyses. To test for overdispersion, we used the package DHARMa (Hartig 2022). The seedling concentration in dung samples was tested using a linear model. Separate models were built for deer and boar, respectively.

Furthermore, we combined both response variables into one multivariate regression and ran redundancy analysis with stepwise selection of explanatory variables (one model for species frequency with both deer and boar present, second model for seedling concentration in samples of both animals). Missing values for the tested traits were imputed using the function imputePCA (package missMDA, Josse & Husson 2016) with the regularised iterative PCA algorithm. The maximum number of imputed values was for the leaf content of phosphorus where seven species had missing values. The response data were square-root transformed.

Last, we classified plant species based on their frequency in dung and their germination rate after passage through the digestion of studied animals. Species were classified based on values smaller or larger than the mean of the given variable (see Tab. 2). Three groups were established. Group (i) included species which were well adapted to dispersal (high germination rate from the feeding experiment) and at the same time were often dispersed by the studied animals (high frequency in dung). Group (ii) included species well adapted to dispersal but not frequently dispersed (presumably because the animals do not graze on them). Finally, group (iii) included species which were often dispersed but showed low adaptation to dispersal as measured by the germination rate after feeding. This group contained species which were presumably grazed preferentially.

Table 1 Summary of variables used in the analyses. In the column *reference*, see the original studies where the data were published.

type of variable	variable name	reference
response variables	species frequency in dung	Lepková et al. 2018
	number of seedling per 100 g of dry dung	Lepková et al. 2018
explanatory variables	species frequency in grassland	Lepková et al. 2018
	species frequency in forest	Vojta 2007
	leaf traits: leaf nutrient content, leaf mass	Kattge et al. 2022
	leaf traits: SLA	Kleyer et al. 2008
	seed production	Kleyer et al. 2008
	species germination rate after passage through digestive tract	Lepková & Mašková 2022

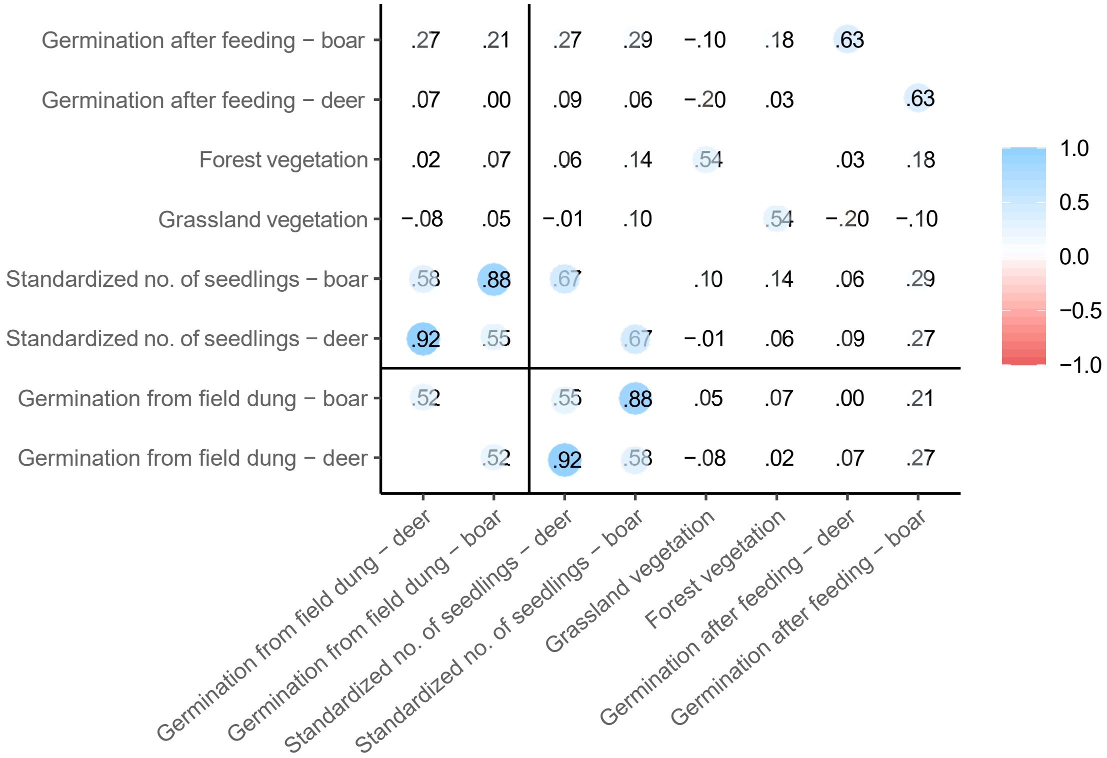
Table 2 Plant species were sorted into functional groups according to how well they were dispersed and adapted to dispersal: group (i) = well adapted and often dispersed; group (ii) = well adapted but not dispersed; group (iii) = not adapted but dispersed, thus preferred. Species were sorted based on values smaller or larger than the mean of the variable: adaptation to dispersal (germination rate after feeding) and frequency of dispersal by the studied animals (frequency in dung). The group (iv), which summarizes species not dispersed in dung and not adapted to endozoochory, consists of the majority of species from the vegetation and is not discussed.

	frequency in dung > mean	frequency in dung < mean
deer		
	group (i):	group (ii):
germination rate > mean	*Clinopodium vulgare, Trifolium arvense, Trifolium repens, Urtica dioica*	*Achillea millefolium, Astragalus glycyphyllos, Avenula pubescens, Lathyrus pratensis, Poa nemoralis, Potentilla argentea, Securigera varia, Vicia cracca, Vicia tetrasperma*
	group (iii):	group (iv):
germination rate < mean	*Agrostis capillaris, Dactylis glomerata, Galium mollugo, Plantago media, Poa pratensis, Veronica chamaedrys*	
boar		
	group (i):	group (ii):
germination rate > mean	*Clinopodium vulgare, Trifolium arvense*	*Lathyrus pratensis, Poa nemoralis, Securigera varia, Trifolium arvense, Vicia cracca*
	group (iii):	group (iv):
germination rate < mean	*Agrostis capillaris, Dactylis glomerata, Fragaria vesca, Poa pratensis, Urtica dioica, Veronica chamaedrys*	

4.4 Results

Using a correlation matrix we found that species frequencies from different sources (landscape, dung composition, and germination rate after feeding) do not correlate with each other but we found that the frequency of species in deer dung correlates with species frequency in boar dung (Fig. 2). Similarly, the species germination rate after passage through digestive tract correlates to rates after passage through different animals (Fig. 2). Furthermore, the species frequency in field dung correlated to seedling concentration.

Fig. 2 Correlation matrix of species frequencies and seedling concentration in dung samples from the field, in different types of available vegetation, and species germination rates after passage through digestive system of deer and boar. The analysed number of species was 38 (as per the feeding experiment).



4.4.1 Plant frequency in dung samples

The species frequency in wild boar dung was significantly and positively affected by species germination rate after passage (p-value = 0.045, Fig. 3, Tab. 3). Furthermore, we found a weak non-significant effect of the interaction between plant frequency in forest vegetation with the production of seeds (p = 0.065, Tab. 3). On the other hand, we did not find any relationship between tested variables and species frequency in deer dung. Furthermore, we did not find a significant effect of any of the tested variables on seedling concentration per 100 g of dry dung mass for either wild boar or for deer (Tab. 4).

Using redundancy analysis with stepwise selection of explanatory variables we found results different from the univariate models (Fig. 4). None of the tested variables had a significant effect on the species frequency in dung samples but we found a significant effect of frequency in forest vegetation on the seedlings concentration (model p-value = 0.004, variable p-value = 0.02, Fig. 4)

Table 3 Results of the generalized linear model for species frequency in dung of deer and wild boar. Significant values are in bold. Values between 0.1 and 0.05 are in italics. n = 38

Animal	Predictor	Chisq	Df	p-value
Deer	Grassland vegetation	1.737	1	0.188
	Forest vegetation	1.949	1	0.163
	Survival after feeding	0.073	1	0.786
	SLA	0.900	1	0.343
	Leaf nitrogen	2.615	1	0.106
	Leaf phophorus	0.499	1	0.480
	Leaf mass	0.880	1	0.348
	Grassland vegetation : Seed production	0.620	1	0.431
	Forest vegetation : Seed production	2.422	1	0.120
Wild Boar	Grassland vegetation	0.001	1	0.971
	Forest vegetation	0.400	1	0.527
	Survival after feeding	4.025	1	**0.045**
	SLA	0.749	1	0.387
	Leaf nitrogen	0.215	1	0.643
	Leaf phophorus	0.300	1	0.584
	Leaf mass	0.021	1	0.884
	Grassland vegetation : Seed production	1.315	1	0.252
	Forest vegetation : Seed production	3.408	1	*0.065*

Table 4 Results of the linear model for seedling concentration in deer and wild boar dung. None of the predictors had significant effect. n = 38

Animal	Predictor	Sum Sq	Df	F value	p-value
Deer	Grassland vegetation	8.571	1	0.530	0.476
	Forest vegetation	45.628	1	2.821	0.109
	Survival after feeding	0.331	1	0.020	0.888
	SLA	1.118	1	0.069	0.795
	Leaf nitrogen	17.798	1	1.101	0.307
	Leaf phophorus	32.013	1	1.979	0.176
	Leaf mass	20.177	1	1.248	0.278
	Grassland vegetation : Seed production	19.776	1	1.223	0.283
	Forest vegetation : Seed production	48.017	1	2.969	0.101
	Residuals	307.282	19		
Wild Boar	Grassland vegetation	1.386	1	0.105	0.749
	Forest vegetation	24.592	1	1.868	0.188
	Survival after feeding	14.220	1	1.080	0.312
	SLA	8.122	1	0.617	0.442
	Leaf nitrogen	0.314	1	0.024	0.879
	Leaf phophorus	32.037	1	2.433	0.135
	Leaf mass	3.783	1	0.287	0.598
	Grassland vegetation : Seed production	6.044	1	0.459	0.506
	Forest vegetation : Seed production	27.996	1	2.126	0.161
	Residuals	250.165	19		

Fig. 3 The relationship between species frequency in wild boar dung (%) and species germination rate after passage through the wild boar digestive system (%). The germination after digestion had a significant positive effect on the species frequency in boar dung. The plot shows two groups of species which are differently adapted to endozoochory by boar: in the top left corner there are species with high germination from field dung but lower germination after direct feeding. These species are either grazed preferentially (*U. dioica*) or accidentally due to their high frequency in vegetation (*V. chamaedrys*). The bottom right corner show species better adapted to endozoochory (*Fabaceae*) but less frequently dispersed (because their ideal disperser may be missing from the system). The plot is in a log-log scale. Only eight species germinated from the dung samples from the field as well as from dung after passage through the digestive tract.

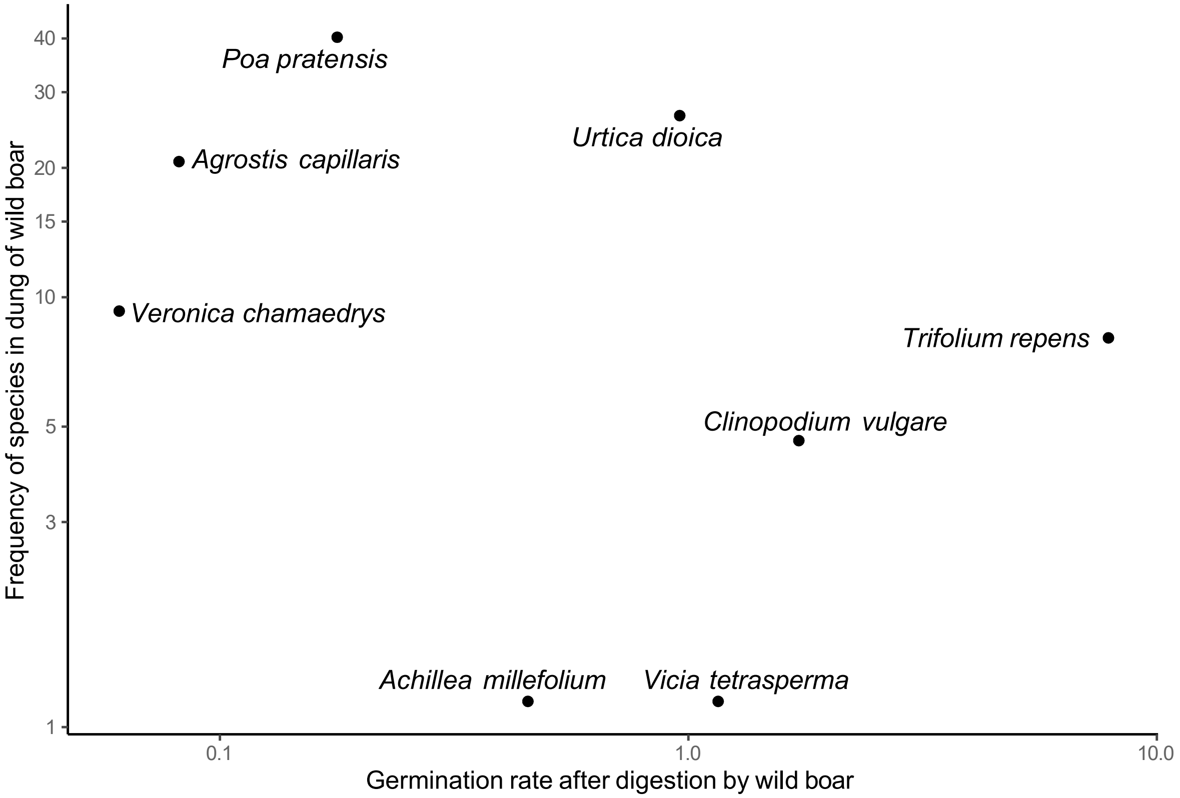
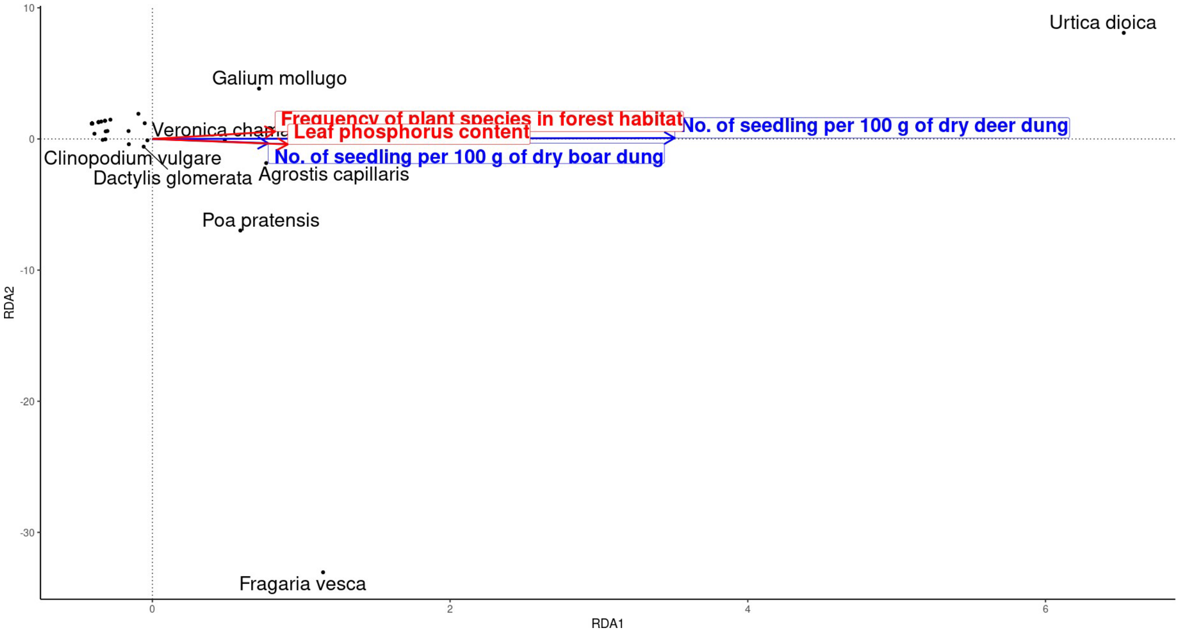


Fig. 4 Results of stepwise RDA. The model significance was 0.007. The only significant variable influencing the species frequency in dung of deer and wild boar was the frequency of plant species in forest habitats explaining 46.7 % of the variance.



4.4.2 Plant traits

Contrary to our predictions, we did not find any significant effect of the tested plant traits (leaf traits and seed production) on the response variables when tested separately for deer and boar. The seed production, which was tested in interaction with plant frequency, had a weak effect in interaction with forest vegetation but it was below the significance threshold. None of the variables had an effect on the seedling concentration. Similarly to our previous result, we found a significant effect of leaf traits when tested using RDA: the seedling concentration was significantly affected by leaf phosphorus content (model p-value = 0.004, variable p-value = 0.003, Fig. 4).

4.4.3 Plant adaptation to dispersal

Species were sorted into individual groups based on values smaller or larger than the variable mean (Tab. 2). There was a large overlap of species in individual groups dispersed by deer and boar (Tab. 2). Species well adapted to passage through deer and at the same time often dispersed were *Trifolium arvense* and *T. repens*. The group of adapted but not dispersed species included a mixture of plants from *Poaceae* and *Fabaceae* family (e.g., *Poa nemoralis* and *Securigera varia*). The third group of dispersed species lacking adaptation to passage through guts were typically very common in the vegetation of dry grasslands of the studied area: e.g., *Dactylis glomerata*, *Veronica chamaedrys*, or *Galium mollugo*.

4.5 Discussion

Interestingly, we found inconsistent results for our hypothesis that seed dispersal by free ranging herbivores is driven by a combination of plant availability, palatability, and survival in the guts. The results differed not only between the statistical methods (redundancy analysis and linear model) but also between the measures of dispersal — frequency of species in dung samples versus the standardized number of seedlings per 100 g of dry dung mass as a measure of seed concentration in dung. The frequency of species in dung was not strongly affected by any of the tested variables. We only found a weak effect of germination rate after passage through the digestive tract on seedling concentration in boar dung. When the standardized number of seedlings was tested using multivariate methods, we found a significant effect of plant species frequency in forest vegetation and a significant effect of foliar phosphorus content.

Our results suggest that there are effects overriding possible drivers of the analysed species frequencies and plant traits. The most important seems to be the animals' feeding preferences. We used leaf traits to predict the plant palatability which we used as a proxy for feeding preference. However, the lack of a strong driver among the leaf traits suggests that either there are different, more important traits driving the preferences, or the feeding preferences are so complex that we cannot predict them using simple plant traits at all.

4.5.1 Frequency in the landscape

Using linear models, we did not find a strong driver of seed dispersal either measured by the plant frequency in dung or measured by the standardized number of seedlings. We found only a very weak effect of germination after passage on the plant frequency in wild boar dung samples and it was on the verge of significance. In contrast, when using RDA with stepwise selection, we found a significant effect of frequency in forest vegetation and foliar phosphorus in the standardized number of seedlings (Fig. 4). These variables explained over 45 % of the variance. The first unconstrained axis represented 35 % of the variance suggesting these variables are of high importance to seedling concentration in dung samples.

In both cases — linear models and RDA — the most important species driving the results was the stinging nettle *Urtica dioica*. It is the most dominant and common species found in the dung samples (Jaroszewicz 2013) and also one of the species found primarily in forest vegetation compared to the remaining species composition which occurs mainly in the grassland vegetation (Chytrý et al. 2010). *U. dioica* acted as an outlier in both analyses: in RDA it was driving the significance of forest vegetation, whereas in the linear models, the significance of germination after passage through wild boar guts increased when the nettle was excluded. These results suggest the animals come to forage on nettle on purpose, probably due to its high nutritional value (Humphries & Reynolds 2014) or medicinal effects (Gülçin et al. 2004). This is further supported by the combination of results from the feeding experiment and from the field dung samples: in the feeding experiment, the nettle showed a surprisingly low germination rate after gut passage. However, the dung samples from the field contained up to thousands of seeds in one pellet. This further supports our hypothesis that the stinging nettle is grazed by both deer and boar preferentially.

The weak effect of species frequency in the surrounding vegetation is in contradiction to other literature, e.g., Iravani et al. (2011), Picard et al. (2016), where the relationship between seed dispersal and neighbouring vegetation was repeatedly confirmed. Even though the studies differ in the sampling resolution of the vegetation or statistical analyses, there seems to be a discrepancy between different regions which may not be connected to vegetation composition. It may be more connected to animal behaviour and feeding preferences which are difficult to measure.

4.5.2 Leaf traits and grazing preferences

Similarly to the effect of surrounding vegetation, we did not find a strong driver of endozoochorous dispersal between the tested leaf traits. The only exception was leaf phosphorus content when the dispersal measured by seedling concentration was analysed using RDA (Fig. 4). Similarly to the other variable significantly affecting dispersal — frequency in the forest vegetation — this was also driven by the outlying *Urtica dioica*. This result agrees with our hypothesis that leaf nutrient content is a predictor of palatability.

However, several things must be taken into account: first, various plant species may be sensitive to individual filters differently. For example, some species appearing in dung samples very often may get there accidentally. *Veronica chamaedrys*, a very common species in the grassland vegetation of the study area, is also the second most common species in deer dung (Lepková et al. 2018). Due to its procumbent growth form with upstanding flowering stalks, it is more likely that the stalks with seeds are grazed accidentally with other vegetation. In such case, the feeding preferences for this particular species do not play any role. On the other hand, species may be grazed preferentially for their foliar phosphorus (Forsyth et al. 2002) but at time when their seeds are not mature or the seeds are not able to survive the passage through the digestive system.

4.5.3 Germination after digestion

The germination rate after digestion had a significant effect only on species frequency in wild boar dung (Fig. 3). This confirms our hypothesis that survival in the digestive tract is one of the conditions of successful dispersal (Janzen 1984). However, no effect was found for species in deer dung suggesting that dispersal by deer is either (i) driven by different traits than species adaptation to passage (e.g. feeding preferences), or (ii) a completely random process. Since the feeding experiment was performed with a high number of seed replicates, it is unlikely the source of the insignificant result. Considering the enormous numbers of seeds of some species per dung sample and the big differences between samples (Lepková et al. 2018), individual preferences are more likely the cause. Some individuals preferentially feed on certain species (e.g. *Urtica dioica*) consuming tens or hundreds of thousands of seeds overriding the differences between species' survival in the guts.

4.5.4 Plant adaptation to dispersal

Based on species frequency in the dung and their survival after passage, we classified species into three groups (Tab. 2):

(i) well adapted to dispersal and often dispersed by studied animals;

(ii) well adapted but not dispersed by studied animals;

(iii) not adapted but often dispersed.

Logically, a group (iv) exists — species not adapted and not dispersed. The majority of species in the available vegetation belong to this group and are not discussed here.

There is a large overlap of species in individual groups dispersed by deer and boar suggesting similar filtering processes. However, there are species in the landscape which are pre-adapted to endozoochorous dispersal but their animal disperser is missing (group (ii)), for example, *Securigera varia* or *Poa nemoralis*. Almost all species from the group (ii) are from families *Fabaceae* or *Poaceae*. This agrees with our previous findings (Lepková & Mašková 2022). Legumes and grasses are the most commonly grazed plant families (Krojerová-Prokešová et al. 2010) and our findings suggest each of these families chose a different evolutionary path to cope with the grazing pressure. Furthermore, the tested herbivores show strong preferences to consume certain plant species (group (iii)) but this preference could not be related to leaf nutrient values or specific leaf area. This group included *Urtica dioica* or *Veronica chamaedrys*.

4.6 Conclusions

In contrast to our predictions, none of the tested variables was a strong predictor of seed dispersal by deer and boar. Our results suggest that leaf traits are not useful proxies of herbivore feeding preferences which are either driven by different plant traits or the effect of traits is neutralized by selectivity on the level of the animal individual. Although filters driving the endozoochorous seed dispersal act sequentially on all species (adaptation to the digestive tract does not guarantee dispersal if the plant is not grazed), they are likely to affect the individual plant species differently. Without more detailed data on animal feeding preferences, the process of seed dispersal is too stochastic to be predicted.

Furthermore, specific plant species showed a surprising level of adaptation which was different from what we expected and measured. Mainly the species from the *Fabaceae* family showed a high level of adaptation to the passage through the digestive tract even though these species were not found in the dung. This group of plants is therefore pre-adapted to endozoochorous dispersal but possibly to a different disperser which may have disappeared from the landscape and in the future, these species may become dispersal-limited. Also, they may be adapted to other ecological processes which are correlated with endozoochory (granivory or survival in the soil seed bank). We also found a number of species which were dispersed frequently and in high numbers, but this could be explained by neither plant traits, nor by species survival in the guts. This suggests that such species are grazed preferentially by the animals for their nutritional value or medicinal effects (*Urtica dioica*), or dispersed accidentally (*Veronica chamaedrys*).

The results of our study show that endozoochorous dispersal is, similarly to other ecological processes, highly species-specific both on the side of the dispersed plant and on the side of the dispersing animal. The studied filters appear to be of different importance to various plant species which may be a result of different evolutionary paths the plants took to cope with herbivorous pressure or a side effect of other ecological processes (persistence in soil seed bank). To deepen our understanding of endozoochorous dispersal, more information on herbivore feeding preferences are needed in relation to plant functional traits. Furthermore, studies on individual filters from different habitats and with various species of herbivores will help us understand the differences between vegetation types, regions, and herbivore species.

5 General conclusions

The presented thesis provides insight into endozoochorous seed dispersal in the abandoned landscape in Western Bohemia. A set of various experimental approaches was used to study the endozoochorous dispersal providing information about the individual components of the process. The results are summarized in four papers investigating various aspects of the dispersal process — individual filters as depicted in Fig. 1.

In Paper I, I found there are extreme numbers of seeds dispersed in dung samples - up to thousands of seeds were found in one dung pile. This was particularly true for samples from deer which also dispersed larger number of species compared to boar. The results suggested seed dispersal by wild herbivores and particularly by deer is very strong in the study area and has a potentially significant impact on vegetation composition.

To test the hypothesis that seed dispersal strongly affects species composition in the area, I performed a field experiment reported in Paper II. My results indicate the realized effect of dung deposition and endozoochorous seed dispersal is much lower than I anticipated based on published literature and the results of Paper I. I hypothesized that some seeds may be transferred to the soil seed bank where they wait for more favourable conditions but more research in this direction is needed.

To complement our understanding of the dispersal process, I performed a feeding experiment which is reported in Paper III. I found contrasting trends in species from different families, particularly between *Fabaceae* and *Poaceae*. Since the legumes and grasses are the most frequently grazed plants, these results suggest that different evolutionary adaptations have evolved in respective families in order for the plants to benefit from the grazing pressure by dispersing seeds. However, some seed traits were not included because they are not easily measured or available in the databases, for example the seed coat thickness. Such traits may explain the survival of seeds in the digestive tract. However, it is necessary to bear in mind the evolutionary differences between the most commonly foraged plant groups — grasses and legumes.

Ultimately, my aim was to disentangle the Filters I-III in Fig. 1. I took advantage of the published information about the vegetation in the area and my previous findings about the dispersal process. The results summarized in Paper IV brought me to conclude: (i) some species in the vegetation are dispersed because they are grazed preferentially and in high numbers, (ii) some species in the vegetation are adapted to dispersal but not grazed by the studied animals, i.e., their disperser is missing or their adaptation to the digestive tract is correlated to another trait, (iii) the dispersal process is either random or is driven by traits not included in this study, (iv) leaf traits as a proxy for palatability are not useful proxy for animal feeding preferences or their effect is overriden by different traits (e.g., survival in the digestive tract).

To summarize my results, I found out that despite the enormous numbers of seeds contained in one dung sample, the realized effect on vegetation is weak and some seeds may be transferred to the soil seed bank where they wait for favourable conditions. However, this was confirmed for dispersal by deer and the result cannot be interpreted similarly for the case of wild boar which differs in the dispersed species composition. The difference between dispersal vectors is further supported by the fact that the species composition and plant frequency in deer and wild boar dung is driven by different factors, e.g., the frequency of plants in forest vegetation influenced their occurrence in wild boar dung but no tested variables affected dispersal by deer. I found contrasting effects of seed traits and seed nutrient content on the survival of plant species from various families. Particularly the difference between *Fabaceae* and *Poaceae* was striking suggesting different evolutionary paths these groups of species took to deal with the grazing pressure.

Based on the results of my research, I present several ideas where the future research might embark. First of all, it is necessary to abandon the idea that high numbers of seeds found in dung via a greenhouse experiment logically mean high level of dispersal in the study area. Even though information on what species from the local species pool have the dispersal potential for endozoochory is important, it is not sufficient to estimate the realised dispersal or effect on vegetation communities. Thus, second, field experiments with various animal species are needed, especially to study dispersal by free-ranging wild herbivores. Third, field experiment should not focus on the dispersal phase only but take into account the subsequent phases of seed germination and establishment, the possibility of seed migration to the soil seed bank, and also the associated effects (deposition of nutrients) and their influence on vegetation and seedling recruitment. Fourth, more information on functional ecology of seeds is essential to estimate and predict the endozoochorous potential. Last, information on animal behaviour and especially feeding preferences are necessary to uncover the drivers of grazing pressure. Such information is often available only for domestic herbivores. Information on wild animals is often collected only in relation to crop damage, which is not very helpful for understanding processes taking place in natural habitats.

To conclude, it is important to remember that dung pile is a black box. To look at the black box from afar is important and may give us important information (on the species composition with dispersal potential) but it is not sufficient. New approaches to look inside the black box are needed. Genetic methods are gaining more attention in the recent years, e.g., DNA-barcoding. These methods may uncover not only the species dispersed in the dung but also the full spectrum of forage. Differences between the two sets of species will answer the question which traits help the species survive. Furthermore, certain components of the dispersal by herbivores has been omitted in the past even though it is frequently studied in frugivorous endozoochory, e.g., the phase of foraging. Last, subsequent phases after dung and seed deposition shall be studied in depth. This does not mean only seedling establishment and vegetation change several years after dung deposition but also other processes not mentioned in this thesis — effect of dung beetles and other organisms involved in dung decomposition and the subsequent seed transport.

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