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ECOLOGY OF (HEMI)PARASITIC PLANTS

Habilitation Thesis

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Abstrakt

Tato habilitační práce se věnuje ekologii parazitických a poloparazitických rostlin. V její první části probírám evoluční ekologii rostlinného parazitismu. Druhů část je zaměřena na ekologii kořenových poloparazitů, které jsou spolu se jmelími největší funkční skupinou parazitických rostlin. Jejich ekologii představuji z mnoha pohledů, od ekofyziologických mechanismů interakce mezi hostitelem a poloparazitem, přes aspekty ekologie společenstev a ekosystémové ekologie, po využití poloparazitů v ekologických aplikacích. Součástí habilitační práce je 10 vědeckých článků, které přispěly k rozvoji poznání ekologie (polo)parazitických rostlin.

Abstract

This thesis deals with the ecology of parasitic and hemiparasitic plants. In its first part, I introduce the evolutionary ecology of plant parasitism. The second part focuses on ecology of root hemiparasites, together with mistletoes the largest functional groups of parasitic plants. I adopt a comprehensive view on their ecology ranging from the ecophysiological mechanisms of the hemiparasite-host interaction to community and ecosystem ecology, including also applications of root-hemiparasites in ecological restoration. The thesis is supplemented by 10 research papers which contributed to understanding (hemi)parasitic plant ecology and evolution.

Acknowledgement

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1. Story of losers or winners? Evolutionary ecology of parasitic plants

Most plants are photoautotrophic organisms. Their vital processes depend on carbon from CO_2, energy from light and also water and mineral nutrients. While availability of CO_2 in the air is rarely limiting and can be considered as a condition for plant life, the other three factors are resources available in limited supply. Therefore, they are subject of competition between co-occurring plants. Plants have evolved a number of strategies to facilitate acquisition of these resources. Some of them involve ecological interactions with other organisms. Of these, mutualistic mycorrhizal associations with fungi contributing mineral nutrients in exchange for organic carbon are most common in vascular plants. In fact, most plant species do establish mycorrhizal association, a symbiosis which is likely to have facilitated land colonization by plants (Smith & Read, 2008). Several evolutionary lineages of vascular plants (including e.g. Fabaceae, Cycadales, and a number of Fagales) developed mutualistic symbiosis with (cyano)bacteria, which have ability to fix nitrogen, one of the principal biogenic elements, from the atmosphere (Stevens, 2001; Li *et al.*, 2015). While the strategies mentioned above involve bidirectional exchange of resources, others involve exclusively flows of resources towards plant recipients, i.e. they are purely heterotrophic. Mycoheterotrophy refers to the cases where the balance of the originally mutualistic symbiosis shifted towards the benefit of the plant partner (Merckx, 2013). While most mycoheterotrophs retain photosynthetic ability (partial mycoheterotrophs), several evolutionary lineages are non-green and their carbon nutrition is completely dependent on fungal partner (Selosse & Roy, 2009). Another example includes carnivorous plants which trap and digest animal prey to acquire mineral nutrients, in particular nitrogen (Givnish, 2015). Plant parasitism defined as direct parasitic interaction between plant individuals represents one of the heterotrophic resource-acquisition strategies which contributes not only mineral nutrients but also water and variable amount of organic carbon.

The mechanism of plant parasitism is based on connection between vascular bundles of two plant species – the parasite and the host. This connection is established by a specialized organ called the haustorium which is developed as a modification of roots or stems of parasitic plants. Parasitic plants comprise *ca.* 4500 species, which accounts for 1% of angiosperms (Těšitel, 2016). Parasitism has evolved at least 12 times independently in the angiosperm evolution (Barkman *et al.*, 2007; Naumann *et al.*, 2013). Species diversity is distributed very unevenly among individual evolutionary lineages of parasitic angiosperms. Two largest groups, the Orobanchaceae family and the Santalales order comprise *ca.* 90% of parasitic species while multiple groups (Hydnoraceae, Mitrastemonaceae, Cynomoriaceae and Lennooidae) contain less than 10 species (Těšitel, 2016). Individual lineages markedly differ also in their estimated phylogenetic age. Santalales are the oldest lineage of extant parasitic plants, origin of which is estimated to early Cretaceous *ca.* 110 Mya (Naumann *et al.*, 2013). By contrast, the youngest transitions to the parasitic strategy were reconstructed in the family Orobanchaceae and the parasitic genus *Cuscuta* of Convolvulaceae. These groups are of late Paleogene age (32 and 35 Mya, respectively) which also indicates the upper boundary of the age of transition to parasitism (Naumann *et al.*, 2013). The uneven species richness of individual parasitic plant lineages was recently related with their phylogenetic age to show that there is little correlation between the two parameters and the non-parasitic sister clades are mostly more species rich than the parasitic lineages (Hardy & Cook, 2012; Naumann *et al.*, 2013). Such pattern would indicate a strongly limited evolutionary potential of parasitic plants, a hypothesis suggested by Hardy & Cook (2012) on the basis of sister-clade comparisons of parasitic and mycoheterotrophic plants. Notable exceptions to this are however the two largest parasitic lineages of Santalales and Orobanchaceae, species richness of which is higher than that of their respective non-parasitic sister clades by several orders of magnitude (Hardy & Cook, 2012). Naumann et al. (2013) postulated an alternative temporal specialization hypothesis (TSH) explaining the evolution of parasitic plants through an assumption that efficiency of parasitic pathways increases in time together with host specificity. This should produce multiple small, specialized and relict groups of highly specialized parasites together with few relatively young, non-specialized and diversified groups. This hypothesis generally seems to fit well with the empirical evidence on phylogenetic age, trophic specialization and species richness of the parasitic angiosperm lineages (Naumann *et al.*, 2013); however there is one important exception. Santalales are the oldest and simultaneously most species-rich group of extant parasitic plants. They display a range of trophic modes ranging from non-parasitic to full heterotrophy (Naumann *et al.*, 2013; Těšitel, 2016) with predominance of photosynthetic hemiparasites. This was also acknowledged by Naumann et al. (2013) who however tried to incorporate existence of Santalales into their hypothesis by pointing on existence of small specialized groups within the clade. In contrast to these previous studies investigating exclusively the evolutionary patterns, I am convinced that the complex evolutionary pattern of parasitic angiosperms may only be understood through examination of the extensive functional diversity relating to their physiology (dependence on host species, ability to withdraw resources), life forms, ecological interactions and also geographical distribution.

The physiological functioning of individual parasitic plant species can be described by a set of functional traits (Těšitel, 2016). Ability of photosynthesis indicates autonomy in carbon acquisition and varies from fully functional to completely missing photosynthesis. Closely associated with photosynthetic ability is the anatomical structure of the host-parasite contact in the haustoria. Some parasites access host xylem only while others take up resources from both xylem and phloem (Hibberd & Jeschke, 2001). Non-photosynthetic species mostly access both types of vascular bundles due to the need of abundant carbon nutrition provided by phloem (Irving & Cameron, 2009); however, this is not a strict rule due to existence of xylem-only feeding holoparasitic plants (Těšitel & Tesařová, 2013; Světlíková *et al.*, 2015; Těšitel, 2016). Other important traits relate to germination, which may be either induced by host presence or not, and establishment of the initial connection to the host by primary (terminal) haustoria or secondary (lateral) haustoria. Location of haustoria on roots or stems of the host determine life forms of parasitic plants, that is root or stem-parasites. The most advanced parasitic species however form extensive endophytic structures which may extend from root to shoot (Heide-Jørgensen, 2008). As a result, the distinction between root and stem parasites is not relevant in their case. Four functional groups of parasitic plants may thus be distinguished in the basis of the functional traits mentioned above – root-hemiparasites, root-holoparasites, stem parasites and endophytic parasites (Těšitel, 2016).

These functional groups make sense from morphological, physiological and ontogenetic points of view. However differences between some of them lack distinctness from the perspective of ecological interactions, resource acquisition and use. For instance, there is little difference in the ecological interactions between root-holoparasites and endophytic parasites since both are (typically) completely dependent on host for carbon and do not compete with the host for light. Therefore, I introduce here a scheme displaying major adaptive evolutionary steps which can be traced in the parasitic plants evolution (Fig. 1). Each of the steps involves an evolutionary innovation addressing a major ecological constraint limiting occurrence/abundance of its predecessors which did not display such innovation.

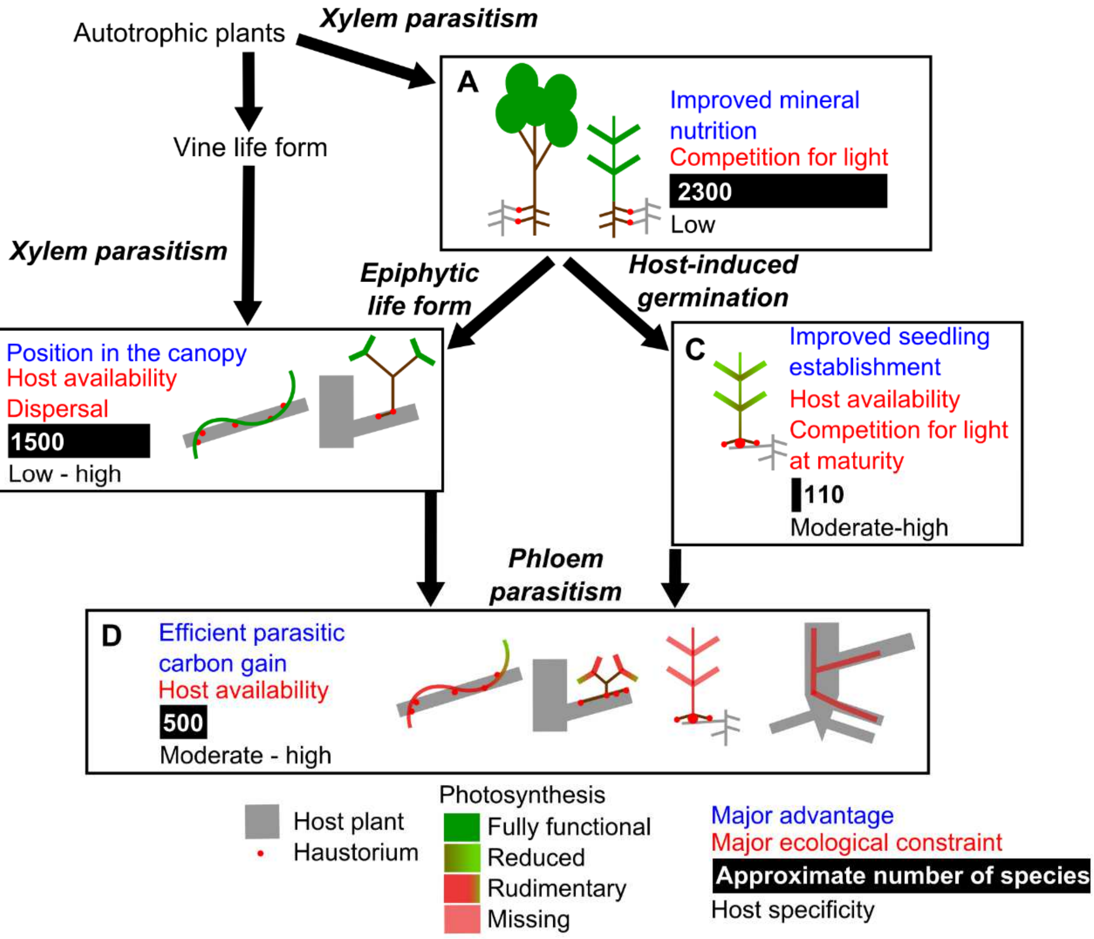

Fig. 1. Scheme of the evolution of parasitism in the angiosperms. Major evolutionary innovations facilitating parasitic resource acquisition are indicated by arrows. Rectangles encapsulate principal evolutionary steps which comprise plants differing in their physiological functioning, resource acquisition strategies and ecological interactions. Major ecological constraints overcome (i.e. advantages) and associated with respective evolutionary steps are provided together with the number of species and estimate of predominant host specificity. Illustration of photosynthetic ability refers to the state of a majority of species; some important exceptions, e.g. non-photosynthetic *Lathraea* in C are commented in the text. Note, that phloem continuity in the haustoria may be difficult to demonstrate in some less-well studied groups (Cynomoriaceae, Mirrastemonaceae, Hydnoraceae); as a result, their placement in C or D is not resolved to date.

Development of xylem connection to the host represents the first step in the evolution of plant parasitism (Fig. 1A). This provides access to the xylem-borne resources, namely mineral nutrients, water and also a limited amount of carbon (Hibberd & Jeschke, 2001; Irving & Cameron, 2009; Těšitel *et al.*, 2010a; Jiang *et al.*, 2010; Bell & Adams, 2011). This root-hemiparasitic strategy is characteristic of most Orbanchaceae, Krameriaceae and a substantial part of Santales. Access to host xylem largely facilitates mineral nutrition of the parasitic plants providing a great competitive advantage over non-parasitic species. This competitive advantage is however realized through own photosynthetic activity, rate of which is strongly increased by nutrients obtained heterotrophically (Těšitel *et al.*, 2015b). Therefore, the parasitic plants which display only this initial albeit crucial evolutionary innovation retain photosynthetic activity mostly in a fully functional state. As a result of this dependency on own photosynthesis, they are affected by competition for light from both the host species and other co-occurring species in the community. In fact, competition for light is the principal constraint limiting their performance (Fig. 1A; Matthies, 1995; Fibich *et al.* 2010; Těšitel *et al.*, 2011, 2013).

To address this constraint, some of these parasitic plants (hemiparasitic Santalales) evolved epiphytic mistletoe life form. Others (genus *Cassytha* of Lauraceae; also *Cuscuta* of Convolvulaceae, all extant species of which access host phloem) underwent the transition to parasitic xylem-feeding strategy from autotrophic epiphytic vine life forms. Either evolutionary pathway resulted in a photosynthetic xylem-feeding epiphyte (Fig. 1B), which addresses much of the constraint imposed by competition for light. Occurrence of such parasitic plants is however dependent on availability of suitable host species which are large and long-living enough to support the epiphytes. Dispersal is another important issue for mistletoes germinating on host branches (Mathiasen *et al.*, 2008). By contrast *Cassytha* germinates on the ground which however means that competition for light can still affect its establishment.

The effects of competition for light have most detrimental effects on the seedlings of the root-hemiparasitic plants (Fig. 1A; Těšitel *et al.* 2011) restricting their regeneration niche (Grubb, 1977). To address the seedling establishment constraint, multiple species evolved initiation of seed germination by presence of host root associated with a holoparasitic-seedling stage (Fig. 1C). Such strategy, intermediate between hemi- and holo- parasitism (Westwood *et al.*, 2010), is typical for a number of genera of Orobanchaceae, namely *Striga*, *Alectra*, *Tozzia* and perennial species of *Rhynchocorys*. The xylem-only connection to the host provides only a limited amount of carbon, which requires a transition to own photosynthesis either early in the subsequent ontogeny (*Striga*, *Alectra*) or a slow-growing, long-lasting underground non-green stage (*Tozzia, Rhynchocorys*). Most of these plants still produce green shoots which allow transformation of their mineral-nutrition benefits into offspring production by photosynthesis. The importance of the autotrophic pathway for generative-reproduction organs has indeed been demonstrated directly in *Striga* (Santos-Izquierdo *et al.*, 2008) or may be indirectly deduced from green shoots which are always fertile in *Tozzia* and *Rhynchocorys* (Těšitel *et al.*, 2010b). The evolutionary stability of their photosynthetic activity (albeit frequently lowered; Graves *et* *al.*, 1992) is thus secured by a reduction of fitness in eventual non-green mutants. Still, xylem only-feeding species of the genus *Lathraea* growing in closed-canopy forest lost photosynthesis probably as a result of minimal contribution of own photosythesis in the shady environment and lack of necessity to quickly complete its life cycle in a stable environment with plentiful resources contributed by long-living hosts. Moreover, the long-living xylem only-feeding holoparasite stage relies for carbon nutrition of xylem sap with low concentration of carbon. Sufficient carbon can be obtained only together with excessive amount of water, which cannot be discarded by transpiration. These species therefore feature specialized hydathode trichomes on scales of leaf origin covering their rhizomes, which actively excrete excess water (Ziegler, 1955; Renaudin & Garrigues, 1967; Weber, 1975; Těšitel & Tesařová, 2013; Světlíková *et al.*, 2015). This active process requires energy, which further decreases physiological efficiency of these parasitic plants.

The constraints related to carbon nutrition in xylem-only feeding hemiparasites, i.e. sensitivity to competition for light or the need for highly specialized and inefficient physiology, were addressed by the evolution of connection to host phloem in the haustoria (Fig. 1D). Phloem sap is rich in organic carbon providing sufficient nutrition to support both growth and reproduction of phloem feeding parasites which become practically independent of light conditions. None of the phloem-feeding parasitic plants displays functional photosynthesis which would contribute substantial proportion of its carbon budget. This indicates that photosynthetic ability is quickly lost after evolution of phloem connection due to genetic drift acting upon plastid genome devastating functionality of genes involved in synthesis of photosynthetic apparatus (Wicke *et al.*, 2013). While plastid genome itself is retained in most phloem feeding non-green species, recent research discovered that it may be eventually lost (Molina *et al.*, 2014). Most of the phloem-feeding holoparasitic lineages indeed lost the ability to photosynthesis in its entirety (Balanophoraceae, Cytinaceae, Cytinaceae, Lenooidae, most holoparasitic Orobanchaceae). By contrast, some species retain rudimentary photosynthetic ability. This is the case of the genus *Cuscuta*, where most species retain photosynthesis contributing to lipid synthesis which is crucial for offspring production as lipids constitute the seed reserves but their concentration in host phloem sap is low (McNeal *et al.*, 2007). The photosynthetic activity may also facilitate foraging for the host in the pre-attachment phase of *Cuscuta* seedlings (Švubová *et al.*, 2013). Similarly, in dwarf mistletoes (genus *Arceuthobium* of Viscaceae, Santalales), rudimentary photosynthesis may play role in seed production (Miller & Tocher, 1975) and establishment on host branches may be greatly facilitated by energy coming from photosynthesis of green endosperm present in all mistletoes (Heide-Jørgensen, 2008; Nickrent & García, 2009).

The efficient carbon nutrition allowed phloem-feeding parasites to colonize and thrive in habitats with conditions unfavourable for autotrophic plant life or largely exploitative, resource-wasting hemiparasitic strategy. These include e.g. dark forest understory or arid places, where holoparasitic phloem-feeding plants indeed frequently occur (Těšitel, 2016).. Given the efficiency of carbon nutrition by phloem feeding, an apparent question also arises: why did not all parasitic plants evolve this nutritional mode to avoid constraints imposed by competition for light? It seems that establishment of phloem contact is much more complicated compared to xylem contact – phloem is a living tissue and at least certain level of specific interaction with the host to facilitate transfer of the resources from host phloem to the parasite (Irving & Cameron, 2009). The higher specialization of phloem-feeding compared to xylem only-feeding haustoria implies also greater hosts specificity. This means that most of the phloem-feeding parasites are limited by host availability, which rarely limits distribution of xylem-feeding hemiparasites (in particular root-hemiparasites). Exemplified by the case of Orobanchaceae, horizontal gene transfer between host and parasite (which was documented in multiple phloem-feeding lineages; (Davis & Xi, 2015) has recently been suggested to play an important role in the evolution of phloem-feeding ability (Yang *et al.*, 2016). However, horizontal gene transfers are still rather rare events; obtaining the set of genes necessary for phloem-contact establishment may thus require substantial amount of time even on the evolutionary time scale. The evolution towards greater efficiency of heterotrophic carbon acquisition may moreover be hindered by the contrast between the hemiparasitic and holoparasitic strategies. Despite variable gain of host-derived carbon in the hemiparasites (Těšitel *et al.*, 2010a; Bell & Adams, 2011), they capitalize the benefits of their parasitism via own photosynthesis (Těšitel *et al.*, 2015b). Switching to holoparasitism means that the principal benefits of hemiparasitism, i.e. low-cost yet abundant mineral nutrition and water supply, are lost as holoparasites cannot benefit from their elevated availability.

Looking back to the evolutionary pattern of parasitic angiosperms, what can be concluded about the theories aiming at its explanation by the hypotheses on ecological limitation of parasitic plant diversification (Hardy & Cook, 2012) or temporal specialization (Naumann *et al.*, 2013)? Both of these hypotheses have a structure of universal laws of nature, which are however exercised on the evolutionary history. Such approach has been demonstrated inadequate from the philosophical perspective (Popper, 1957; Taleb, 2007). Moreover, both of these hypotheses are refuted by data available, most notably by the existence Santalales, a species-rich group containing mostly relatively non-specialized root- or stem-hemiparasitic plants (Těšitel, 2016). If the two proposed hypotheses are wrong and universal theory describing the evolution of plant parasitism is impossible, what can I offer instead? I propose an interpretation of the evolutionary pathways of individual parasitic plant lineages and their similarities.

Clearly, we can identify a number of small and functionally homogeneous lineages in the parasitic plants on the one hand and two highly successful species-rich and a functionally diverse groups on the other. This contrast will further increase when we consider their geographic distributions. The small holoparasitic lineages typically have a limited geographic distribution. The geographic ranges of Cytinaceae, Apodanthaceae and Mitrastemonaceae are also disjunct (Heide-Jørgensen, 2008), which indeed indicates their relict origin. By contrast, the two most successful lineages, Santalales and Orobanchaceae, have global distributions, which also applies for the genus *Cuscuta* (Heide-Jørgensen, 2008). These three groups represent an omnipresent component of principal terrestrial ecosystems worldwide. Root-hemiparasitic Santalales have been successful in both tropical woody and open temperate vegetation (Fig. 2A) , while Loranthaceae and Viscaceae, two independently evolved mistletoe lineages within the order (Nickrent *et al.*, 2010), colonized predominantly forest, savanna and other woody vegetation from the tropics to the temperate (Fig. 2B; Heide-Jørgensen, 2008). Root-parasitic Orobanchaceae have been highly successful in the open vegetation and diversified in both temperate and tropical grassland biomes (Fig. 2C,D; McNeal *et al.*, 2013). Hemiparasitic species of both Santalales and Orobanchaceae are moreover recognized as keystone species with profound effects on the communities and ecosystems, where they occur (Press, 1998; Watson, 2001, 2009, 2016; Phoenix & Press, 2004). Similarly, the genus *Cuscuta* has a global geographic distribution and its species were demonstrated to play a keystone role in structuring plant communities (Callaway & Pennings, 1996, 1998). Considering the other groups, species of the genus *Cassytha* are mostly restricted to Australia but *C. filiformis* has a remarkable pantropic distribution (Heide-Jørgensen, 2008). In their Australian homeland, the *Cassytha*

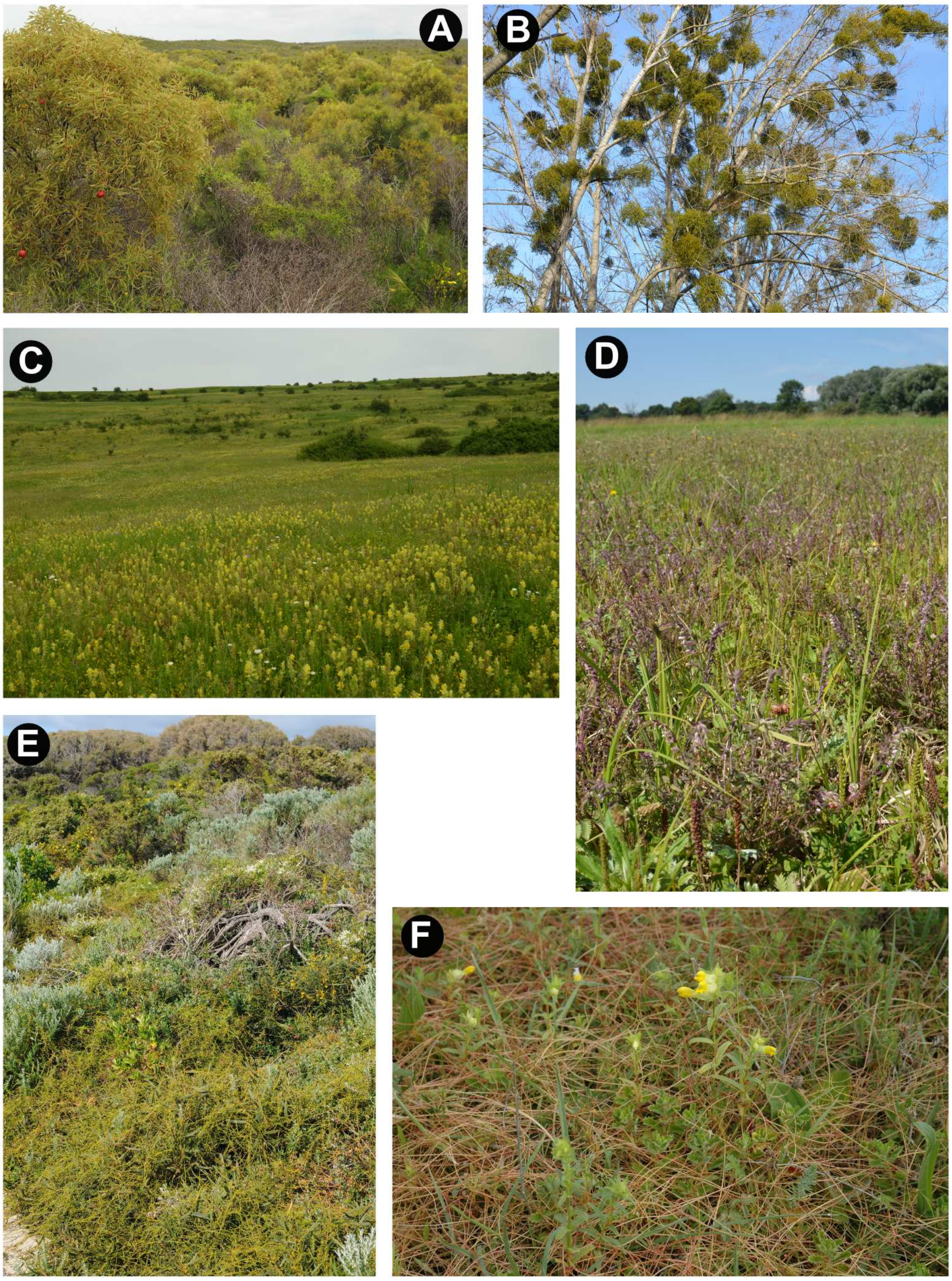

Fig. 2. Parasitic plants as vegetation dominants. A: *Santalum acuminatum* shrubs in Western Australian bush; B: *Viscum album* mistletoes parasitizing *Populus nigra* in floodplain forests, South Moravia; C: *Rhinanthus major* dominating a mesic meadow in Transsilvania; D: *Odontites vernus* dominance in a floodplain meadow in the regrowth stand after summer cut; E: *Cassytha* sp. in Western Australian bush; F: *Cuscuta* sp. (presumably *C. epithymum*) forming a dominance patch on a lowland meadow in Austrian part of Pannonia, note hyperparasitism on *Rhinanthus major*.

species may attain dominance in the communities (Fig. 2E) and recently have even been demonstrated to strongly suppress growth of leguminous invasive species (Prider *et al.*, 2009; Shen *et al.*, 2010). The parasitic-plant strategies which seem successful are thus rather diverse. Their success may be related to the history of global ecosystem and biota, i.e. the host environment. For instance, the evolution and radiation of Orobanchaceae coincides with the expansion of the grassland biome in the late Tertiary (Strömberg, 2011; Naumann *et al.*, 2013). The evolution and diversification of mistletoe life forms followed expansion of angiosperm-dominated forests in the late Cretaceous (Wang *et al.*, 2009). Loranthaceae mistletoes originated and radiated in the Oligocene following the expansion of savanna, their primary habitat at present (Vidal-Russell & Nickrent, 2008). The evolutionary success of *Cuscuta* may lay in the fact that most of the species of this genus are able to parasitize a wide range of hosts (Baráth & Csiky, 2012), which is otherwise rare among phloem-feeding parasites. This could possibly have been facilitated by the *Cuscuta* vine life form allowing interactions with many potential host species simultaneously (Fig. 2F) with possible repeated trials to attack also those hosts which were originally incompatible. Such situation may have triggered the evolution of a generalist phloem-feeding parasite. In summary, many different parasitic strategies underlie the success of individual parasitic plants. The lack of a universal hypothesis explaining the evolutionary pattern should not prevent us from acknowledging the evolutionary success and ecological roles of these successful groups of parasitic plants as well as extraordinary morphological and functional variability recognized in the whole functional group of parasitic plants.

2. Ecology of root-hemiparasitic plants

2.1. Introduction to root hemiparasites

Root-hemiparasitic plants are green photosynthetic plants which attack roots of other plants to withdraw resources of their xylem (Section 1; Irving & Cameron, 2009; Těšitel, 2016). To uptake the host resources they use a specialized organ, the haustorium which, in their case, is a modified root. Root hemiparasites comprise *ca* 2400 species (more than 50% of all parasitic plants, *ca* 0.5% of angiosperms) of three independent evolutionary lineages: Santalales, Orobanchaceae and Krameriaceae. Of these, the first two contain most of the diversity while Krameriaceae consist of a single genus with 18 species.

Extensive morphological diversity exists in root-hemiparasites ranging from tiny annual *Euphrasia* species occurring in alpine grasslands to large tropical forest trees with *Okoubaka aubrevillei* of Santalales, their largest representative, reaching up to 40 m high (Veenendaal *et al.*, 1996). Root hemiparasites are a functional group distributed globally on all continents except the Antarctic and occurring in all major terrestrial habitats (Heide-Jørgensen, 2008). Most species however occur in open habitats such as temperate grasslands, alpine vegetation and (semi)arid vegetation (Těšitel, 2016).

Root hemiparasites have been demonstrated to substantially affect structure of plant communities and ecosystem processes (Press, 1998; Press & Phoenix, 2005; Watson, 2009) together with other parasitic plant groups, the mistletoes (Watson, 2001) and parasitic vines (Callaway & Pennings, 1996). Some species (e.g. *Rhinanthus* spp.) are recognized for their ecosystem engineering capacity which may increase community diversity (Pywell *et al.*, 2004; Těšitel *et al.*, 2017) while others (e.g. *Striga* spp.) are recognized as serious weeds threatening the production of agriculture (Pennisi, 2010; Parker, 2013).

In this text, I first aim to introduce the physiological and ecological mechanisms of the root-hemiparasitic interaction. Second, I explain how these fundamentals underlie community and ecosystem effects recognized in a series of root-hemiparasitic species. And third, I discuss ecological applications of root-hemparasitic plants, which are based on their effects on plant communities and ecosystems. I also outline possible future research directions and applications of root-hemiparasitic plants in ecological restorations and agriculture.

2.2. Ecophysiological mechanisms of the hemiparasite-host interaction

The life cycle of a hemiparasite starts by seed germination as in other plants. The germination may be spontaneous in favourable conditions or may require an external stimulus to break seed dormancy. In most species, the dormancy-breaking factor is environmental; germination is triggered by a period of low temperature (Curtis & Cantlon, 1968; ter Borg, 2005; Liebst & Schneller, 2008), which in temperate species secures that the seedlings appear in spring when the conditions are favorable for seedling survival. Following germination, the seedling grows independently of a host. It produces a root system to forage for host roots and uptake water and mineral nutrients from the soil. The formation of haustoria and establishment of host connection usually follows after a period lasting few days to few weeks and is facilitated by chemical clues (haustorial inducing factors) which are excreted from host roots (Yoshida *et al.*, 2016). The unattached seedlings of most hemiparasites display inefficient physiology with low rates of photosynthesis and frequently observed imbalance of mineral nutrient concentration. This results in in poor growth or even premature death if host attachment is not established (Mann & Musselman, 1981; Seel *et al.*, 1993; Fer *et al.*, 1994; Lechowski, 1996; Matthies, 1997; Jiang *et al.*, 2007). While, there are some exceptions of hemiparasites that are able to grow decently without a host, such as *Odontites vernus, Euphrasia minima* (Matthies, 1998) or *Santalum acuminatum* (Radomiljac *et al.*, 1999), there is no empirical evidence reporting unattached hemiparasites from natural conditions. Some root hemiparasites evolved an advanced strategy of host-contact establishment. These species require a chemical trigger from the host to initiate germination (Section 1, Fig. 1). The chemical signals used to trigger germination are called strigolactones (Cardoso *et al.*, 2011; Yoshida *et al.*, 2016) and were identified to play a crucial role in plant communication with arbuscular mycorhizal fungi (Akiyama *et al.*, 2005) and later became recognized as a previously unknown type of plant hormones (Gomez-Roldan *et al.*, 2008). Typically, these species also have a fully heterotrophic seedling. This means that they practically avoid the stage of unattached seedling which a critical phase of the life cycle when most of the mortality occurs caused either by adverse environmental conditions or competitive pressure from the host community (Ameloot *et al.*, 2006; Ducarme & Wesselingh, 2009; Těšitel *et al.*, 2011).

Root hemiparasites withdraw resources from xylem vessels in the host roots. This nature of the anatomical connection determines resources taken up from the host, i.e. water, mineral nutrients, but only a limited amount of organic carbon. The unidirectional flow of resources from the host to parasite is underlain by high concentration of sugar alcohols, which are osmotically active and lower the water potential of the hemiparasites (Hodgson, 1973; Press, 1995; Pageau *et al.*, 1998; Jiang *et al.*, 2005). Moreover, the resource flow may be further facilitated by some types of the xylem-xylem contact in the haustoria. In many hemiparasitic species (typically those of Santalales and Krameriaceae but also some Orobanchaceae) either cell wall or parenchyma cells are present on the host-parasite interface (Tennakoon *et al.*, 1997; Tennakoon & Cameron, 2006; Brokamp *et al.*, 2012). While water and some smaller molecules can pass through cell walls, the parenchyma cells may actively transport nutrients from host to the parasite facilitating uptake of greater molecules. A number of root-hemiparasitic species have however evolved open xylem continuity in the haustoria (Hibberd & Jeschke, 2001). Such continuity allows mass flow of xylem sap from the host to the parasite and is typical of species of Orobanchaceae genera *Striga* (Dörr, 1997) and *Rhinanthus* (Cameron *et al.*, 2006). These hemiparasites typically have highly elevated transpiration rates which greatly facilitates resource uptake from the hosts. Moreover, Orobanchaceae of the Rhinanthoid clade possess specialized hydathode-trichomes on their leaves which excrete water during night-time when transpiration does not proceed (Govier *et al.*, 1968; Světlíková *et al.*, 2015).

All water and mineral nutrition of root hemiparasites is typically of the host origin and may account for a substantial proportion of these resources taken up by the host (Ehleringer & Marshall, 1995; Jiang *et al.*, 2003, 2004). By contrast, carbon nutrition used to be hypothesized to be dependent solely or mostly on hemiparasites' photosynthesis. This was based on their morphology which in most cases resembles that of non-parasitic plants and in recent decades also on gas-exchange measurements indicating rates of photosynthesis comparable to those found in other plants (Fer *et al.*, 1994; Seel & Press, 1994; Lechowski, 1996; Těšitel *et al.*, 2011). Earlier reports on insufficiency of hemiparasites photosynthesis to secure carbon nutrition in a series of temperate hemiparasitic Orobanchaceae (Press *et al.*, 1988; Press, 1989) can nowadays be quite safely considered mistakes attributable to limitations of gas-exchange measurement instrumentation available at that time. Exceptions of this are however species of the genus *Striga*, photosynthesis of which was demonstrated to be generally low (Graves *et al.*, 1992; Cechin & Press, 1994). Similarly, species of genera *Tozzia* and *Rhynchocorys*, which are holoparasitic in the initial phase of their life (Section 1) may, be presumed to display reduced photosynthetic ability; however no empirical data are available in this respect.

Still, many root hemiparasites obtain organic carbon from their hosts despite the efficiency of photosynthesis and the exclusive connection to host xylem. This is because xylem sap of the hosts contains certain concentration of carbon (usually around 5-10 mmol org. C/l), mainly in the form of amino-acids or organic acids (Govier *et al.*, 1967; Seel & Jeschke, 1999; Alvarez *et al.*, 2008). The transfer of organic compounds from host to hemiparasite was first demonstrated by a ^{14}C-radioisotope labelling study of the model hemiparasitic associations of *Odontites vernus* with *Hordeum vulgare* and *Trifolium repens* hosts (Govier *et al.*, 1967). While this approach successfully identified numerous compounds translocated from the host to the parasite, it was largely qualitative and thus could not provide information on the quantitative contribution of host-derived carbon to the parasite carbon budget. The first quantitative assessment followed two decades later and was based on an analysis of carbon-stable isotopic composition of biomass of two *Striga* species attached to hosts with C_4 photosynthetic pathway (Press *et al.*, 1987). C_4 plants are known to differ in carbon stable-isotopic composition of assimilates from C_3 plants (Boecklen *et al.*, 2011). All hemiparasites use the C_3 pathway while a number of hosts are C_4 plants. Comparisons between the actual stable-isotopic composition of a hemiparasite attached to a C_4 host with that of hemiparasite attached to a C_3 host or a prediction based on gas-exchange measurement can thus be used to estimate proportion of host-derived carbon in hemiparasite biomass. The latter approach was used by Press et al. (1987), which resulted in an estimate of 28% and 35% of host-derived carbon in hemiparasite biomass in *S. hermonthica* and *S. asiatica*, respectively. Comparable proportions of heterotrophic carbon in hemiparasite biomass were identified also in a number other root-hemiparasitic species including temperate species of genera *Euphrasia* and *Rhinanthus* (Těšitel *et al.*, 2010a). Recent research has however identified extensive variability of heterotrophic carbon proportion present even within a single host-hemiprasite association. The host contribution to carbon budget was demonstrated to vary during ontogeny and also differ between below- and above- ground parts of the hemiparasite (Pageau *et al.*, 1998; Santos-Izquierdo *et al.*, 2008). Increased proportions of host-derived carbon were found in hemiparasites which were shaded, i.e. their photo-assimilation was limited by an external factor (Těšitel *et al.*, 2011). Most recently, a glasshouse experiment with *Rhinanthus alectorolophus* (Těšitel *et al.*, 2015b) has demonstrated that contributions of heterotrophic and autotrophic pathways to the carbon budget of hemiparasites largely depend on the availability of other resources and physiological balance between the host and parasite. Highest proportion of host-derived carbon was found in hemiparasites that were stressed by simultaneous low availability of water and mineral nutrients, which had a strong negative impact on their photosynthetic ability. Hemiparasite biomass also contained elevated proportions of host derived carbon when the balance of the hemiparasitic assotiation was shifted towards the hosts' side due to the simultaneous abundance of both abiotic resources,. This indicates that host-derived carbon is used as a back-up resource when hemiparasite's own photoassimilation is limited. It is also notable that the amount of carbon transferred from the host to the hemiparasite accounted for approximately 5% of total carbon in host above ground biomass (Těšitel *et al.*, 2015b) irrespective of experimental treatment. This indicates that carbon loss is not responsible for a major part of harm inflicted to the host by hemiparasitism.

2.3. Ecology of the hemiparasite-host interaction

The hemiparasitic interaction between a root hemiparasite and its host has an ambiguous nature. It is a clear case of parasitism below ground, where the parasite benefits from resources taken up from the host. Hemiparasites are mostly strong parasites which generally cover their needs for abiotic resources by parasitism. They also benefit from the fact that they do not need to invest into extensive root systems. Above-ground, however, a host and a hemiparasite are just two green plants that compete for light (Matthies, 1995; Fibich *et al.*, 2010), an interaction which is further strengthened by the spatial proximity of their shoots (Keith *et al.*, 2004). Root hemiparasites are mostly considered or assumed poor competitors (Hellström *et al.*, 2004; Cameron *et al.*, 2009; Fibich *et al.*, 2010; Borowicz & Armstrong, 2012). The effect of competition for light suppresses hemiparasite growth (Matthies, 1995; Těšitel *et al.*, 2011; Mardoian & Borowicz, 2016) but also increases seedling mortality (Těšitel *et al.*, 2011; Mardoian & Borowicz, 2016). The latter should have even stronger consequences for the population dynamics in particular considering the annual life history typical of many root-hemiparasitic species especially in Orobanchaceae (Tank & Olmstead, 2008; Těšitel *et al.*, 2010b). Elevated competitive pressure from the host (community) can largely be expected in ecosystems with favorable climate and with abundant below-ground resources (water and mineral nutrients), i.e. sites with high above-ground primary productivity (Hautier *et al.*, 2009). Hemiparasite populations have repeatedly been demonstrated to decrease in density at sites of elevated productivity; however the individuals having survived the critical seedling stage produced more biomass and/or more seeds (van Hulst *et al.*, 1987; Mudrák & Lepš, 2010; Těšitel *et al.*, 2013). It is likely that the surviving individuals were exceptionally well attached to their hosts (Keith *et al.*, 2004); their survival may hence be facilitated by heterotrophic carbon acquisition (Těšitel *et al.*, 2011). The elevated growth can then be attributed to elevated soil resources on which the hemiparasites response by an increase of photosynthesis similarly to non-parasitic plants (Simier *et al.*, 2006; Těšitel *et al.*, 2015b) and subsequent elevated individual fitness (Mudrák & Lepš, 2010; Hejcman *et al.* 2011; Těšitel *et al.* 2013). Still, the population may become exterminated since the elevated fecundity of the hemiparasites does not compensate for the decrease of population density under productivity level above certain threshold. For instance, *Rhinanthus minor* population persistence was largely dependent on import of seeds from the surrounding in a field fertilizer experimental setup if productivity of the meadow was higher than 5t dry mass per hectare (Hejcman *et al.*, 2011).

Both parasitic and competitive components of the root-hemiprasitic interaction were included in a model of hemiparasite population dynamics (Fibich *et al.*, 2010). This model identified equilibria of stable coexistence between a parasite and a host under low to moderate productivity. By contrast, parasite population was predicted to perish under very low and high productivity values, caused by insufficient host resources and competitive pressure from the host, respectively. Such model is largely in line with empirical evidence from field experiments described in the previous paragraph. However, there are two effects that may counter-act the effect of competition on root-hemiparasites. First, parasitism may contribute significant amount of heterotrophic carbon, which may support parasite vertical growth and facilitate escape from competition (Těšitel *et al.*, 2011). Some species with holoparasitic-seedling stage (*Tozzia alpina*, *Rhynchocorys elephas*, *Striga asiatica*) were even observed to be able to flower and reproduce under heavy light deficiency (Fig. 3A,B; Dörr, 1997). Second, infection by a root hemiparasite may inflict substantial harm to the host which may decrease its competitive ability. The suppression of host growth by hemiparasitism mostly impacts shoots more than roots (Těšitel *et al.*, 2015b; Matthies, 2017), which makes this effect a crucial determinant of above-ground competition. Hemiparasitism was recently suggested to have a disproportionally great impact on clonal hosts (Demey *et al.*, 2015; Mudrák *et al.*, 2016), which are typically strong competitors in temperate grasslands (Gough *et al.* 2012; Herben *et al.*, 2014). This strategy may strongly decrease the intensity of above-ground competition and create gaps for seed regeneration, which may facilitate persistence of hemiparasite populations (Lepš & Těšitel, 2015). An extreme case of hemiparasite causing harm to its hosts to decrease competitive pressure was reported for *Okoubaka aubrevillei*. This species of Santalales is a tree growing in tropical rainforests, a habitat characteristic by extreme intensity of competition for light (Grace & Tilman, 1990). To facilitate regeneration from seeds, *O. aubrevillei* saplings inflict extreme harm to its hosts, some of which may even be killed (Veenendaal *et al.*, 1996). Disproportionally greater negative effects are exerted upon fast-growing hosts, which has clear benefit for the parasite in terms of avoiding competition. The population dynamics model of Fibich *et al.* (2010) thus needs further generalization to account for these cases which apparently contradict its prediction.

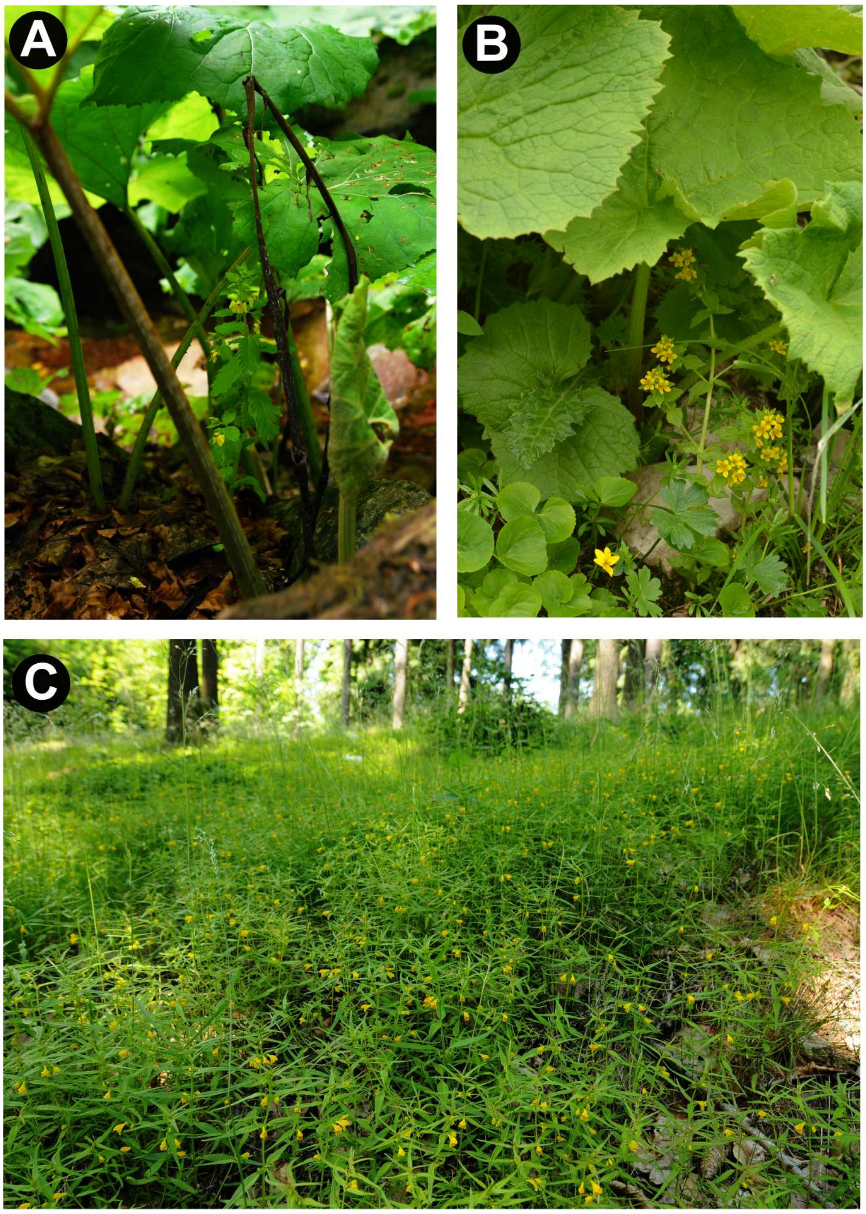

Fig. 3. Hemiparasitic strategy in environment of heavy light deficiency. A,B: *Rhynchocorys elephas* and *Tozzia alpina* growing under the canopy of *Petasistes* spp.; both species have long-lasting underground heterotrophic stage. Shoots are only produced for the purpose of generative reproduction. C: Dense stand of *Melampyrum sylvaticum* in a shady spruce-forest understory.

Root hemiparasites are mostly recognized as generalist parasites though the host range may be narrower in those species requiring host induction for germination, e.g. the *Striga* species (Parker, 2013). However, individual hosts markedly differ in their quality, i.e. how much they support hemiparasite growth. Some species may be even resistant to hemiparasitic infection. This may be based either on pre-attachment mechanism related to chemical signaling before the haustorial initiation or on post-attachment mechanisms when the host blocks resource transfer from its vascular bundles (Cameron *et al.*, 2006; Yoder & Scholes, 2010). The post-attachment resistance mechanism may be recognized by inspection of haustorial anatomy as was exemplified on the interaction between *Rhinanthus minor* and *Plantago lanceolata* resistant to *Rhinanthus* infection (Cameron *et al.*, 2006; Rümer *et al.*, 2007). Interestingly, *P. lanceolata* had earlier been demonstrated as a very good host of several hemiparasitic *Euphrasia* species (Yeo, 1964). This indicates that the root-hemiparasitic interaction depends on species identity of the host and the hemiparasite and there are no universal good or bad hosts. Nitrogen-fixing legumes and grasses have been repeatedly suggested as better hosts than 'forbs' at least for a majority of hemiparasitic Orobanchaceae, which was also supported by certain empirical evidence (Seel & Press, 1993, 1994; Svensson *et al.*, 2001; Cameron *et al.*, 2006). However, recent studies investigating multiple host associations of *Rhinanthus minor* (Rowntree *et al.*, 2014) and *Melampyrum arvense* (Schädler *et al.*, 2005; Matthies, 2017) did not support such hypothesis. Similarly, host quality of hemiparasitic Santalales is not determined by any simple difference between functional groups. Instead, there seems to be a continuous pattern high quality hosts to hosts of very poor quality (Radomiljac *et al.*, 1999; Calladine *et al.*, 2000; Guo & Luo, 2010), presence of which may even suppress hemiparasite growth compared to unattached individuals (Radomiljac *et al.*, 1999). Below the species level, an interplay between host and hemiparasite genotypes was identified to significantly affect the outcome of the interaction (Rowntree *et al.*, 2011). Similarly, genotypes resistant to hemiparasitic infection are known from many field crops attacked by *Striga* species (Yoder & Scholes, 2010), which highlights the importance of genetic variability for hemiparasitism.

Field trials generally identified a large number of species to which root-hemiparasitic plants attach. In addition, certain selectivity for some taxonomic groups (and avoidance of others) is also frequently observed. For instance, Suetsugu *et al.* (2008) reported *Thesium chinense* attached to 22 species of 11 families with grasses to be positively selected while the haustoria formed on legumes were of larger size. By contrast, Dostálek & Münzbergová (2010) reported frequency of *Thesium linophyllon* attachments to host species largely proportional their root abundance. Gibson & Watkinson (1989) reported a number of significantly avoided hosts of *Rhinanthus minor* while a few were selected positively. Interestingly, *Plantago lanceolata*, known to be a resistant host (Cameron *et al.*, 2006), was shown to be positively selected in this study (though the haustoria can be assumed to be non-functional). Most recently, (Holá *et al.*, 2017) used a DNA-barcoding approach to identify host associations of three root-hemiparasitic Orobanchaceae (*Rhinanthus minor, R. major* and *Melampyrum nemorosum*), which co-occurred at one site. The three species notably differed in selectivity for hosts at the family taxonomic level. Interestingly, many haustorial connections were identified between hemiparasitic species themselves both between and within species. Detailed field surveys are however rare due to methodological difficulties of associated with root-system inspection. In this respect, the DNA-barcoding approach may facilitate the workflow though there is still a lot of space for improvement and refinement of this method (Holá *et al.*, 2017). Deficiency of direct data on host identity is a critical issue of almost all studies aiming at community ecology of root hemiparasites. There are only few studies where above ground patterns of vegetation or results of manipulative experiments are compared with parasitic associations below-ground. An example of such a study is represented by a removal experiment with *Pedicularis kansuensis* in which positively selected hosts, grasses and legumes, significantly increased after weeding of the hemiparasite (Bao *et al.*, 2015).

The hosts' side of the interaction is characterized by harm inflicted by hemiparasitism, which is mostly measured as a reduction of its biomass production (or crop reduction in the case of hosts important for agriculture). The negative effects of hemiparasites on the hosts are very variable, ranging from negligible to lethal. Besides the extreme host damage inflicted by *Okoubaka aubrevillei* described above, hemiparasitic species with open xylem contacts in their haustoria, such as *Striga* or *Rhinanthus*, tend also to be highly host-damaging (Parker, 2013; Těšitel *et al.*, 2015b). Multiple physiological mechanisms are involved in the harmful effect of the hemiparasites on their hosts. Loss of abiotic resources is certainly the principal factor in most cases while loss of organic carbon seems of minor importance (Těšitel *et al.*, 2015b). Recent experiment with manipulated availability of water and mineral nutrients identified strong interactive effects of these two resources on harm inflicted to maize and wheat hosts of *Rhinanthus alectorolophus* (Těšitel *et al.*, 2015b). This indicates that loss of either resource due to parasitism may underlie the host damage. In this experiment, the largest harm was inflicted when either of the resources was provided in abundance while the other was deficient. Even damage to host photosynthesis (maximum quantum yield) was identified under dry and nutrient rich conditions. Simultaneous abundance of both resources largely alleviated the effect of parasitism on the host, while simultaneous deficiency was stressful for the parasites which consequently limited the harmful effect on the hosts (Těšitel *et al.*, 2015b). While individual hemiparasitic species differ in their intrinsic harmfulness the damage inflicted to the hosts depends also on host species. Obviously, resistant hosts are harmed less than susceptible ones (Cameron *et al.*, 2006). Several studies actually reported a tight correlation between host quality and damage inflicted by hemiparasitism (Matthies, 1996; Marvier, 1998). However, a recent extensive study with *Melampyrum arvense* (Matthies, 2017) testing 27 different hosts identified a group of hosts which supported vigorous growth of the hemiparasites but suffered relatively little harm. The growth of two legumes, *Trifolium pratense* and *Lotus corniculatus*, was actually negligibly reduced while supporting a decent growth of the parasite. Such pattern was also reported for some legume hosts also in studies with *Rhinanthus* spp. (Davies *et al.*, 1997; Joshi *et al.*, 2000; Cameron *et al.*, 2006). These results are mostly based on simple cultivation trials comparing host performance between infected and uninfected individuals. Largely ignored in such experimental setups, the litter input from hemiparasitic plants may however positively affect productivity of host community (see the next section for details). A recent study has found that high quality hosts of a hemiparasite may obtain disproportional benefit, which may partly compensate for the negative effect of parasitism (Fisher *et al.*, 2013). In real communities, competitive interactions between host and non-host plants and with other trophic levels are also involved together with the effect of the environment, all of which may have strong modifying effects on hemiparasitic interactions.

2.4. Community and ecosystem ecology of parasitic plants

Root-hemiaprasitic plants are a generally omnipresent component of terrestrial vegetation, in particular open (i.e. non-forest) plant communities (Heide-Jørgensen, 2008; Těšitel *et al.* 2015a). Due to their unique ecophysiology, trophic status and interactions with the hosts they have been recognized for their ability to influence structure of plant communities (Phoenix & Press, 2004). The community and ecosystem effects of some root-hemiparasitic species are so pronounced that they are even considered keystone species or ecosystem engineers (Phoenix & Press, 2004; Cameron *et al.*, 2005; Press & Phoenix, 2005; Watson, 2009; Decleer *et al.*, 2013; Těšitel *et al.*, 2017).

Root-hemiparasites may be expected to occur mostly in low-productiveand nutrient poor environments. Under such conditions, they would benefit most from the parasitism, providing low cost access to mineral nutrients (Phoenix & Press, 2004, 2005; Fibich *et al.*, 2010; Borowicz & Armstrong, 2012). In addition, corresponding plant communities tend to be of relatively low sward or stem density which implies good light availability. As a result, the effects of above-ground competition, a crucial factor limiting occurrence of root-hemiparasites (see section 2.3), are rather low. Most Krameriaceae and many of root-hemiparasitic Santalales largely conform to this scheme occurring in low-productive (semi-)arid habitats (Giannini *et al.*, 2011; Heide-Jørgensen, 2013). Still, a number of Santalales are tropical-rainforest trees detailed ecology of which has yet to be described. In temperate regions, a recent analysis of ecological niches of root-hemiparasites in the Czech Republic (Central Europe) identified a relatively wide range of habitats suitable for at least some root-hemiparasitic species (Těšitel *et al.*, 2015a). While some species were indeed found to occur at sites of low-productivity limited by nutrient availability, water deficiency, short vegetation season or a combination of these (e.g. *Euphrasia stricta*, *Thesium linophyllon*m, *Odontites luteus, Pedicularis sylvatica*, *Rhinanthus riphaeus*), some others grow at sites with relatively high productivity. *Euphrasia officinalis Melampyrum arvense, M. pratense, Rhinanthus alectorolophus, R. major, R. minor* can be found over most of the range of the soil fertility gradient but only if such sites are relatively dry as indicated by Ellenberg indicator values. Some of these species may also be found on moist sites but their niche is restricted to oligotrophic places at this end of moisture gradient. Such pattern corresponds well to the experimental study manipulating water and mineral nutrients, which demonstrated loss of advantage of parasitism and alleviation of its effect on the host when both resources were provided in abundance (Těšitel *et al.*, 2015b). Two root-hemiparasitic species, *Odontites vernus* and *Pedicularis palustris*, may however grow even in these conditions, which is underlain either by disturbance, which limits competition and creates regeneration gaps in the sward or by the selectivity of the hemiparasite for dominant competitors which are extremely harmed by parasitism (Decleer *et al.*, 2013), respectively. Three species of *Melampyrum* (*M. nemorosum*, *M. pratense*, *M. sylvaticum*) grow in understory of closed-canopy forests (Fig. 3C), where they are also unique by their annual life history (Těšitel *et al.*, 2015a). A recent model of *M. pratense* carbon budget based on gas-exchange data and light-condition monitoring throughout the growth season however suggests insufficiency of their autotrophic carbon assimilation pathway (Světlíková et al. *unpublished*). In particular, the predicted carbon balance was close to zero in summer, when energy- and carbon-demanding seed production is in progress. This points to possible importance of heterotrophic carbon acquisition in these species, which is however difficult to demonstrate directly.

Presence of root-hemiparasites usually results in possibly strong negative effects on host community productivity (Davies *et al.*, 1997; Joshi *et al.*, 2000; Ameloot *et al.*, 2005; Bardgett *et al.*, 2006; Stein *et al.*, 2009; Mudrák & Lepš, 2010; Borowicz & Armstrong, 2012; Demey *et al.*, 2013a; Bao *et al.*, 2015)., which is underlain by suppression of host growth (Phoenix & Press, 2004; Press & Phoenix, 2005). Moreover, in many cases, a decrease of total productivity (i.e. host + hemiparasite biomass) was observed (Davies *et al.*, 1997; Ameloot *et al.*, 2005;

Stein *et al.*, 2009; Mudrák & Lepš, 2010; Demey *et al.*, 2013a; Bao *et al.*, 2015), which means that parasite biomass did not compensate for the loss on the hosts' side. This effect is usually attributed to lower photosynthetic resource-use efficiency in hemiparasites (Seel & Press, 1994; Westbury, 2004). However, this effect on total community productivity is far from universal. In some cases, higher community productivity was observed, e.g. in case of species-poor assemblages in a biodiversity experiment (Joshi *et al.*, 2000). In an observation-based study, hemiparasitic *Castilleja occidentalis* was found to be associated with high-productivity patches in alpine tundra (Spasojevic & Suding, 2011). The positive influence of root-hemiparasites may largely be attributed to the effect of their litter on the community. Due to their ability to withdraw mineral nutrients from the hosts and relatively low photosynthetic resource-use efficiency (Seel & Press, 1994; Press & Phoenix, 2005), litter of hemiparasites displays high mineral nutrient concentration, often higher than that of co-occurring species (Quested *et al.*, 2002, 2003a,b, 2005). This implies higher rates of litter decomposition and consequent release of nutrients (Quested *et al.*, 2003b; Demey *et al.*, 2013b). The nutrients are also frequently released from litter during the growth season, when the demand for them is high (Quested *et al.*, 2005; Demey *et al.*, 2013b). These positive effects of the litter pathway on community productivity may at least partly compensate for the negative effect of parasitism. Recent experiments have moreover demonstrated that the largest benefits from the litter pathway are acquired by host plants species (Fisher *et al.*, 2013) or plants with fast-growth strategy (Demey *et al.*, 2013b), which may either compensate for the losses to parasitism or even further increase biomass production, respectively. In addition, hemiparasites have been demonstrated to increase average nutrient concentration in biomass (Ameloot *et al.*, 2008; Fisher *et al.*, 2013), which may have additional impacts on nutrient cycling. It is notable that many accounts on reduction of productivity by hemiparasites are based on peak biomass production in early to mid-summer. In annual hemiparasites, such as *Rhinanthus* spp., this captures predominantly the effects of parasitism, while the effect of nutrient release from litter should affect the community predominantly in the second half of the season (late summer, autumn), which is usually not monitored.

Root hemiparasites do not influence only the productivity but exert complex direct and indirect effects on the community structure. Suppression of host species may result in altered competitive hierarchy in the communities (Gibson & Watkinson, 1991; Marvier, 1998; Mudrák & Lepš, 2010; Borowicz & Armstrong, 2012; Bao *et al.*, 2015; Demey *et al.*, 2015). If host species are competitive dominants and a large reduction of their growth is inflicted, hemiparasites may open space for subordinate species and consequently increase community diversity (Davies *et al.*, 1997; Pywell *et al.*, 2004; Decleer *et al.*, 2013; Těšitel *et al.*, 2017). This is further supported by creation of gaps following annual hemiparasite die back in mid- to late summer, which may facilitate their and other species establishment (Joshi *et al.*, 2000; Lepš & Těšitel, 2015). However, if a subordinate species is parasitized or the suppressed dominant is replaced by another one, biodiversity may remain unchanged or may be even lower at plots with the parasites (Gibson & Watkinson, 1992; Mudrák & Lepš, 2010). Nonetheless, a recent analysis based on extensive vegetation plot database demonstrated that most root hemiparasites occur in significantly more species-rich vegetation than expected by chance and some of the hemiparasitic species are among the species most tightly associated with high community diversity (Fibich *et al.*, 2017). While this pattern is probably not caused solely by the positive effect of hemiparasites on diversity and other mechanisms are involved (namely the preference of hemiparasites to grow in low-competitive habitats which are frequently species rich), this results represent an upscale of the hemiparasite-diversity relationship from the local level of single site experiments. Taken together with their effect on productivity, it seems that hemiparasites may decrease productivity in rather productive environments via the parasitic pathway and harm inflicted to the fast-growing dominants, while in low productive environments, the positive effect of the litter pathway could prevail. Such hypothesis was introduced by Watson (2009) suggesting that root-hemiparasites create environment of intermediate productivity and open opportunities for generative reproduction, which is largely beneficial for them in terms of host resource availability and limited effect of above-ground competition. Eventually, communities of intermediate productivity are also those which display highest biodiversity worldwide (Fraser *et al.*, 2015).

The ecological interactions of root hemiparasites are not limited to the plant component of the ecosystems. Strong interactions with organisms of other trophic levels were observed and described in the literature. Root hemiparasites are mostly non-mycorrhizal. In recently described notable exceptions to this, two species of *Pedicularis* were demonstrated to have low-colonization level of arbuscular mycorrhiza in their roots, which may contribute up to few percent of their phosphorus nutrition while the rest is provided by the host (Li *et al.*, 2013). However, strong indirect interactions with arbuscular mycorrhiza were reported. In a glasshouse experiment, a strong benefit was observed for *Rhinanthus minor* if attached to a mycorrhizal host, which was also more suppressed by hemiparasitism (Davies & Graves, 1998). In another experiment, interactive effects of mycorrhizal fungi and parasitism on community structure were observed, with the effect of parasitism only apparent in mycorrhiza-infected communities (Stein *et al.*, 2009). Apart from mycorrhiza, presence of hemiparasite was demonstrated to influence fungal-to-bacterial ratio in soil, a major pattern of soil microbial communities (Bardgett *et al.*, 2006). A complicated four-member interaction was described between *Rhinanthus major*, its grass host (*Festuca pratensis*), alkaloid-producing endophyte present in grass roots and an aphid herbivore (Lehtonen *et al.*, 2005). The hemiparasite took up alkaloids of the host, which are produced by the symbiotic endophyte and protect the host against herbivory if it is not parasitized. If parasitized, the hemiparasite benefited from the herbivory protection while the grass host did not. Thus, the hemiparasite changed the originally mutualistic interaction between the grass and the endophyte to parasitic. Alkaloids, but this time of intrinsic host origin, also played an important role in another complex interaction. Two *Castilleja* species were found to benefit from lupin hosts (*Lupinus albus, L. argenteus*) and an uptake of alkaloids from them due to improved pollination and reduced herbivory (Adler, 2000, 2002). Hemiparasites may also mediate herbivory of hosts plants. In a glasshouse experiment, *Rhinanthus major* was demonstrated to decrease herbivory of acyanogenic *Trifolium repens* hosts while no such effect was observed in the case of cyanogenic plants (Puustinen & Mutikainen, 2001). In a multi-host experiment. Palatability of *Melampyrum arvense* biomass was found to strongly differ if attached to different host species but unrelated to host species palatability, which indicates a complicated underlying mechanism based on the interaction of hemiparasitism and host anti-herbivory defense (Schädler *et al.*, 2005). Concerning root-hemiparasitic Santalales, vegetation containing shrubby hemiparasite *Exocarpos strictus* was demonstrated to host more species of birds and a higher abundance of arthropods compared to vegetation without the hemiparasite (Watson *et al.*, 2011).

To sum up, a number of root-hemiparasitic species were demonstrated to affect the community structure, productivity and diversity via either parasitic or facilitative interaction. These effects may reach beyond the plant community to the scale of the whole ecosystem. Some of them may also offer opportunities for ecological applications. More on that in the next section.

2.5. Applications of root hemiparasites in ecological restoration

The idea on application of root-hemiparasitic plants in ecological restoration and nature conservation stems from the experimental work of Davies *et al.* (1997) who demonstrated that presence of *Rhinanthus* species is associated with lower grassland productivity. This study comprised also an experiment manipulating *Rhinanthus* density by weeding, which demonstrated suppression of grasses by the hemiparasites and reduction of sward density. Following this pioneering study, a number of further trials were done to test the applicability of *Rhinanthus* spp. (mostly *Rhinanthus minor*) in grassland restoration in Western Europe. The typical situations included either grassland sites which had been used for intensive agriculture and managed accordingly or former field used in agriculture. Either of these had been fertilized and the meadows also mown several times (>2) a year. Such high land-use intensity is known to greatly decrease diversity of the grassland ecosystem (Allan *et al.*, 2014, 2015; Lepš, 2014). Cease of fertilizer application combined with a decrease of mowing intensity may help restoring community diversity of meadows; however, this is largely impeded by high amount of residual soil nutrients and dominance of fast-growing clonal competitive grasses (Gough *et al.*, 2012; Lepš, 2014), which prevent establishment of other species. Similarly, in abandoned fields, competitive species may dominate after several years of succession even if the fields are seeded by dedicated seed mixtures of regional provenance (Prach *et al.*, 2014). Drastic measures such as topsoil removal or gramicide chemicals may be used to decrease dominant grass abundance and/or remove residual nutrients but these are costly and questionable from the environmental impact perspective. Therefore, the use of *Rhinanthus* sowing was tested as an alternative biological option. Application of *Rhinanthus minor* was demonstrated more beneficial to community diversity compared to the selective gramicide chemicals (Westbury & Dunnett, 2008) and supported establishment of significantly more target species sown at the beginning of the experiment. Another experiment identified a positive effect of supplementary soil surface scarification measure on establishment of *R. minor* on an abandoned field where a mixture of target grassland species was sown (Westbury *et al.*, 2006). In the same time, *Rhinanthus* itself was demonstrated to decrease grass dominance in the community and to increase community diversity. A similar experiment on a newly established meadow on ex-arable field demonstrated that *R. minor* can decrease grass dominance even at sites with high soil nitrogen concentration but only at high sowing density (1000 seeds $^{-2}$;Westbury & Dunnett, 2007). The most detailed experiment among these was conducted on a meadow of moderate productivity, which had been fertilized in the past and was dominated by a few grasses and forbs at the start of the experiment (Pywell *et al.*, 2007). The experimental treatments included sowing of *Rhinanthus minor* in different densities and sowing of a mixture of target species combined in a factorial design. *Rhinanthus* established at the plots where sown and later invaded also the unsown plots. A strong negative effect of *Rhinanthus* was found on sward height, which decreased by 50% at plots with a high hemiparasite density. By contrast, the effect on overall diversity as well as diversity of sown species was significantly positive. Analysis of species composition changes identified a positive effect of *Rhinanthus* on a number of forb species, such as *Leucanthemum vulgare*, *Leontodon autumnalis*, *Plantago lanceolata, Prunella vulgaris*. The utility of *Rhinanthus minor* on grassland restoration was also tested in a multi-site experiment scaling up the findings of the other studies to the landscape scale (Hellström *et al.*, 2011). The success of these experiments with *Rhinanthus minor* resulted in its regular application in grassland restoration in Western European countries, in particular the UK (Blakesley & Buckley, 2016).

In addition to high land use intensity, grassland biodiversity is also threatened by abandonment, i.e. cessation of mowing. This process leads to a similar community structure with a few competitive dominants as in the case of high land-use intensity. However, species identity is usually different. Abandonment and associated expansion of dominants is a particular problem in species-rich meadow grasslands occurring in Central and Eastern Europe, some of which represent the most species-rich grassland from the global perspective (Wilson *et al.*, 2012; Dengler *et al.*, 2014; Chytrý *et al.*, 2015). Particular issue there is the expansion of *Calamagrostis epigejos*, a dominant clonal grass, which spreads not only to abandoned sites but also to vegetation managed by low-intensity mowing, i.e. the management type recommended to maintain biodiversity (Dengler *et al.*, 2014). The competitive strategy of this clonal species involves spread by rhizomes and resource storage in the roots, which make it robust against targeted measures applied above ground. However, a recent multi-site study (Těšitel *et al.*, 2017) demonstrated that the grass may be infected by hemiparasites of the genus *Rhinanthus*, namely *R. alectorolophus. R. alectorolophus* exerts a drastic effect on the grass and may even exclude it from the community within two years, in particular if its application is coupled with a moderate increase of mowing intensity. In addition, species diversity may be restored in a rather short term. Such effect was only identified at one site out of three; however this represented a typical case of abandoned intermittently wet meadow, which expands the potential of this finding. Based on these results and also other similar experiments waiting for publication, nature conservation in the Czech Republic has started to use the hemiparasites as a restoration measure at sites infested by *Calamagrostis epigejos*. Another example of a root hemiparasite suppressing a particular competitive dominant is a report of *Pediculartis palustris* which drastically reduced tall sedges (mainly *Carex acuta*) and transformed a tall-sedge vegetation into a fen meadow, a highly valuable community from the nature conservation perspective (Decleer *et al.*, 2013).

All these studies indicate a great potential of the use of root-hemiparasitic plants in ecological restoration and biodiversity conservation, which is further facilitated by detailed application guidelines (Mudrák *et al.*, 2014; Těšitel, 2015; Blakesley & Buckley, 2016) However, there are always controversies connected with introduction of seeds of species on sites from which they were previously absent. Therefore, the hemiparasites should be used with caution. Preferably local seed sources should be used where possible. Ideal situation includes seeds transfers within a single site, which complies with the most strict requirements of nature conservation to preserve genetic resources and thus can be used even in nature reserves.

2.6. Future perspectives

The ecological interactions of root hemiparasites with their hosts are well understood particularly in grassland habitats and in the case of agricultural weeds. This knowledge is based on observations and experiments conducted *in situ*, which provides a good basis for understanding the ecology in the context of real communities and ecosystems as well as on glasshouse experiments which aim at identification of the nderlying physiological mechanisms. However, we still know very little about the ecology and ecophysiology of root hemiparasites growing in the forest vegetation. This includes e.g. *Melampyrum* species of Orobanchaceae and many woody species of Santalales. More information on their ecology, resource acquisition from the host and especially strategies of carbon acquisition in the shady environment may reveal new and possibly surprising ecological stories such as that of *Okoubaka aubrevillei* and its extreme effect on the hosts (see section 2.3).

The contemporary literature summarized here also contains a number of accounts on complicated ecological interactions where root hemiparasites play a central role. Most of these reports are however based on experiments in glasshouse conditions. The extent to which, these effects may be present in the context of real ecosystems remains questionable. Certainly some effects may be quite strong and frequent as demonstrated e.g. by field studies on stem-parasitic mistletoes which have a profound effects on nutrient cycling, productivity and community composition of several trophic levels (Watson, 2016). More studies aiming e.g. at direct and indirect interactions with mycorrhizal fungi, pollinators, invertebrate and vertebrate herbivores in the field conditions are thus needed to develop this topic.

One of the appealing question is the true nature of the interaction between root hemiparasites and Fabaceae. Both groups display specialized strategies of nutrient acquisition and Fabaceae frequently serve as hosts of the hemiparasites. While mostly high quality hosts, harm inflicted to them seems rather restricted at least in some species. A number of field experiments also did identify none or only moderately negative effect of hemiparasites on legume abundance in the community. Therefore, they may be rather tolerant hosts. In such case, they may actually establish an alliance with the hemiparasites to suppress competitively superior grasses. Such hypothesis may be supported by existence of vegetation patches, where these groups co-occur in abundance (Fig. 4A) and both look rather vigorous while grasses seem strongly suppressed (Fig. 4B). Although such patches may be transient, repeated informal observations of the same site suggest that they persist for at least several years. If such community could be established on a large scale, there may be a number of applications in agri- and horticulture, such as designing low-intervention high-benefit grassland communities for orchard understory (Fig. 4C).

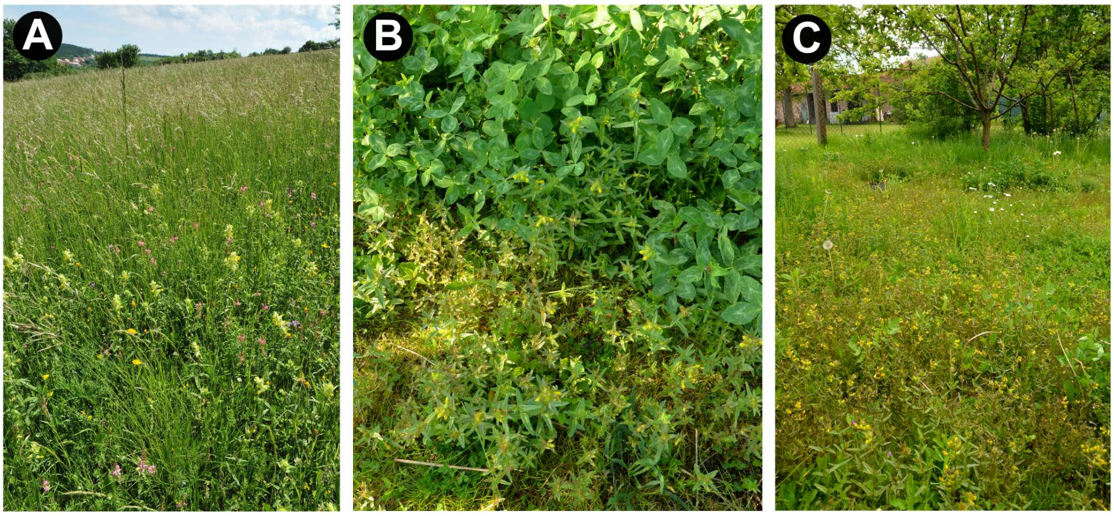

Fig. 4. Illustrations of possible alliances between root hemiparasites and legumes (A,B). A: Patch of *Rhinanthus alectorolophus* and *Onobrychys viciifolia* on a restored meadow near Suchov, Bílé Karpaty Mts.; B: Intraction of *Rhinanthus minor*, *Trifolium pratense* and the grass *Festuca rubra*. *Trifolium* seems to support vigorous growth of *Rhinanthus*, while *Festuca* looks strongly supressed. C: pilot application of the hemiparasites (*R. minor*, *R, minor*) in apricot orchard understory.

The role of hemiparasitic plants in ecological restoration and nature conservation is generally established due to recent strong development of the topic. However, practical experience indicates success at some sites, mixed effects at others and complete failure elsewhere. Scaling the research up to the large landscape scale is therefore needed to assess suitability of sites for hemiparasite application and identify underlying biotic and abiotic factors. This would greatly facilitate further use of hemiparasites by nature conservation and possibly also stakeholders.

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4. List of papers

1. **Těšitel J**. **2016**. Functional biology of parasitic plants: a review. *Plant Ecology and Evolution* **149**: 5–20.
2. **Těšitel J, Těšitelová T, Fisher JP, Lepš J, Cameron DD**. **2015**. Integrating ecology and physiology of root-hemiparasitic interaction: interactive effects of abiotic resources shape the interplay between parasitism and autotrophy. *New Phytologist* **205**: 350–360.
3. **Světlíková P, Hájek T, Těšitel J**. **2015**. Hydathode trichomes actively secreting water from leaves play a key role in the physiology and evolution of root-parasitic rhinanthoid Orobanchaceae. *Annals of Botany* **116**: 61–68.
4. **Těšitel J, Hejcman M, Lepš J, Cameron DD**. **2013**. How does elevated grassland productivity influence populations of root hemiparasites? Commentary on Borowicz and Armstrong (Oecologia 2012). *Oecologia* **172**: 933–936.
5. **Těšitel J, Fibich P, de Bello F, Chytrý M, Lepš J**. **2015**. Habitats and ecological niches of root-hemiparasitic plants: an assessment based on a large database of vegetation plots. *Preslia* **87**: 87–108.
6. **Fibich P, Lepš J, Chytrý M, Těšitel J**. **2017**. Root hemiparasitic plants are associated with high diversity in temperate grasslands. *Journal of Vegetation Science* **28**: 184–191.
7. **Lepš J, Těšitel J**. **2015**. Root hemiparasites in productive communities should attack competitive host, and harm them to make regeneration gaps. *Journal of Vegetation Science* **26**: 407–408.
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REVIEW

Functional biology of parasitic plants: a review

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Background – Parasitic plants are functionally specialized to acquire at least some essential resources from other plants via specialized organs called haustoria. Parasitism evolved 12 times independently in the evolution of angiosperms of which approximately 1% (4500 species) are parasitic. Not only are parasitic plants diverse in terms of evolutionary origins but also in terms of their physiological functioning and ecological behaviour.

Methods – Here, I review the importance of principal functional traits which underlie the physiology and ecology of individual parasitic plants. These include the ability to perform photosynthesis, anatomical details of the vascular connection to the host determining the quality of resources acquired from the host, location of the haustoria on the host, which is closely connected with the parasite life form, and the mode of germination (either triggered by environmental condition or induced by presence of host roots).

Results and conclusions – Based on the distribution of all these traits in parasitic plants, I introduce their functional classification into root hemiparasites, root holoparasites, stem parasites and endophytic parasites. In addition to the classification, I also present an evolutionary hypothesis explaining the evolution of advanced parasitic plant forms from root hemiparasites. This hypothesis is based on ecological constraints from which the parasites are released with increasing ability to acquire resources from the host. This evolutionary process also implies increasing host specificity which imposes new constraints on the ability to establish host connection. This explains the evolutionary stability of photosynthetic hemiparasites and their species richness which is one order of magnitude higher than that of holoparasites.

Key words – Ecology, evolution, haustorium, hemiparasite, germination, mistletoe, Orobanchaceae, parasitic plant, Santalales.

INTRODUCTION

Parasitic plants are a specialized plant functional group defined by parasitic acquisition of at least some essential resources from other plants. The parasitic resource acquisition proceeds via haustoria, specialized organs which penetrate host vascular bundles. Parasitic plants comprise approximately 4500 species which accounts for c. 1% of angiosperms (Heide-Jørgensen 2008, Nickrent 2012). Parasitism evolved several times independently during angiosperm evolution (Barkman et al. 2007, Naumann et al. 2013). Therefore, parasitic plants do not form a monophyletic group but are defined functionally by their physiology and ecological interactions, which include parasitic uptake of resources and interaction with other plant species. However, individual species differ widely in mechanisms of parasitism and other details of their biology.

A distinct functional and evolutionary difference exists between hemiparasites, which retain photosynthetic activity, and non-green holoparasites, which fully depend on their hosts for all essential resources. Parasitic plants nevertheless display many other functional traits that are largely variable among species and underlie biological differences among them. The location of the attachment to the host defines root and stem parasites and substantial variation in growth forms exists even within these groups. Either induced by presence of the host or relying on environmental or internal germination signals, seed germination mechanisms determine the strategy of establishment on the host. Anatomical details of the connection to the host vascular bundles underpin the quality and quantity of resources acquired from the host.

Parasitism evolved twelve times during angiosperm evolution (fig. 1; Barkman et al. 2007, Nickrent et al. 2005). Individual independent lineages largely differ in size (number of species), phylogenetic age and the degree of trophic specialization (Barkman et al. 2007, Nickrent 2012, Naumann et al. 2013). Of these, Orobanchaceae and Santalales are the largest monophyletic groups of parasitic plants, both of which also contain both hemi- and holoparasites. By contrast all the other lineages are small in terms of number of species and genera and uniform in terms of the trophic strategy of their species (table 1, fig. 1). Many of these small groups are holoparasitic and display a highly specialized morphology including extreme modifications of flowers.

In this review, I introduce the key functional traits of parasitic plants and explain their significance. On the basis of functional trait distribution among individual phylogenetic lineages or functionally defined groups within them, I propose a new functional classification of parasitic plants. This is aimed to fill a gap in current literature with the last comprehensive review on functional biology of parasitic plants published more than a decade ago (Nickrent 2002). Since then, there has been a great advancement in reconstruction of phylogenetic relations (in terms of both phylogenetic placement of parasitic plants: Nickrent et al. 2005, Barkman et al. 2007, Naumann et al. 2013, and phylogenetic relations within particular lineages, e.g. Nickrent et al. 2010, García et al. 2014, McNeal et al. 2013) and other aspects of parasitic plants, e.g. ecophysiology (Irving & Cameron 2009, Bell & Adams 2011, Těšitel et al. 2015) or reproductive biology (Bellot & Renner 2013). In addition, a book describing biological features of many parasitic plant species of all phylogenetic lineages has been published (Heide-Jørgensen 2008). A review on key functional aspects of parasitic plants as a whole and considering the evolutionary perspective is, however, still missing.

KEY FUNCTIONAL TRAITS OF PARASITIC PLANTS

Photosynthesis and carbon nutrition

Photosynthesis is generally viewed as a principal characteristic of land plants. It uses light as the energy source for the chemical process in which carbon in CO_2 is reduced to organic substances. This makes light the principal resource for plants and competition for light the dominant interaction occurring between plant individuals. Deficiency of this principal resource also prevents photosynthetic plants from inhabiting dark habitats. Holoparasitic species that lack the

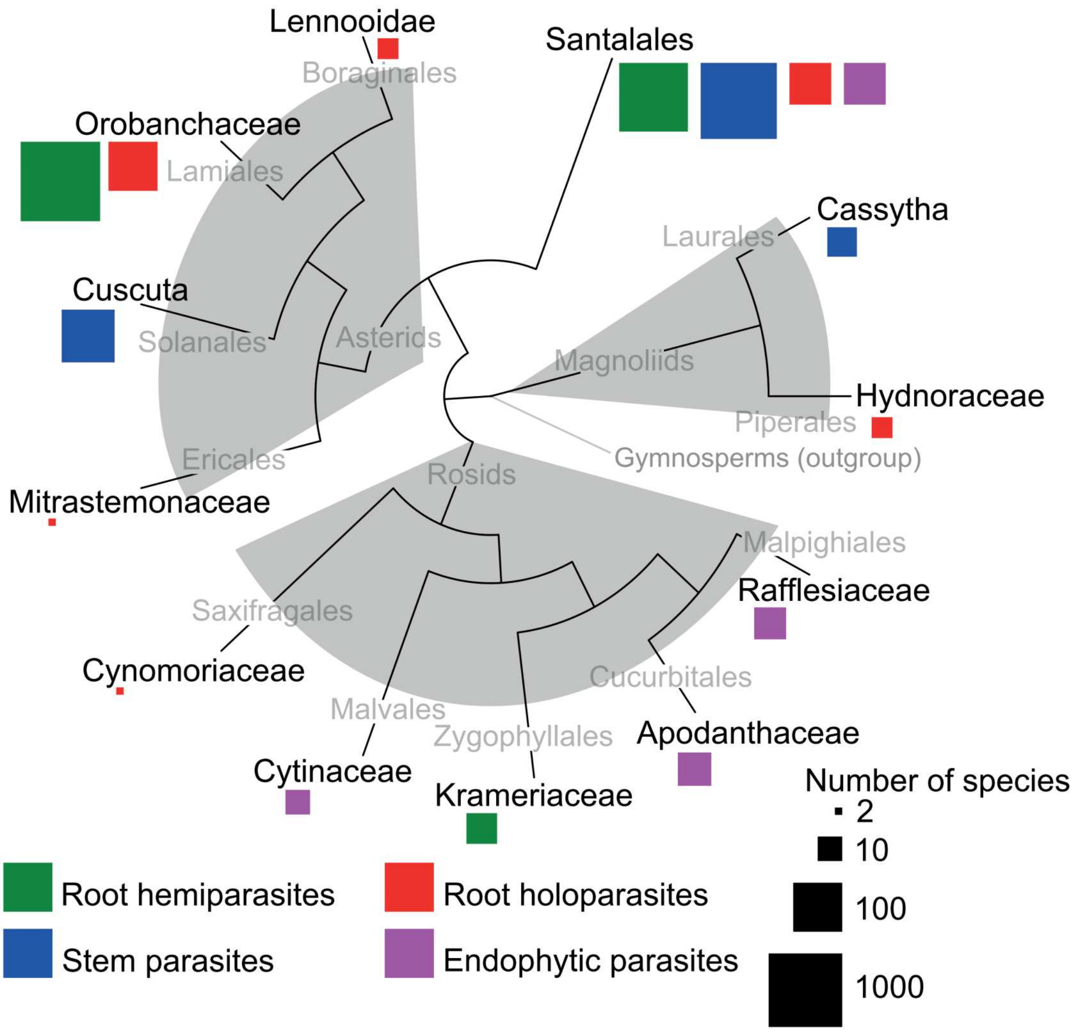

Figure 1 – Phylogenetic origins of parasitic angiosperms. The relationships among monophyletic parasitic plant lineages are based on APG III (2009). Functional classification of species of each monophyletic group (see fig. 2 for details) together with corresponding estimates of number of species (table 1) is illustrated by colour squares.

Table 1 – **Summary of the current knowledge of the functional biology of parasitic plant groups defined on the basis of phylogenetic relatedness and functional similarity.** Note that the groups are not always monophyletic. - absent, + present, +/- present in some species or reduced (value 0.5 used in PCA; fig. 2); (+) present in rudimentary form/only a small fraction of species or of minor importance (value 0.25 used in PCA), +/(-) present in most species; in some species present but not fully developed (value 0.75 in PCA), ? not known (mean substitution used in PCA); * Based on Heide-Jørgensen (2008), ** typical representatives: *Arceuthobium* (most species), *Phacellaria*, *Tristerix aphyllus*, *Viscum minimum*, *Phoradendron perredactum*, *** *Pedicularieae* = Clade IV in McNeal et al. (2013); † Biology of many species/genera is not known in sufficient detail. Unequivocal classification between Orobanchaceae 1 and 2 is therefore impossible in these cases making the estimates of genus and species numbers uncertain. ‡ listed taxa indicate typical examples/largest groups, not a complete list.

Group	**Number of genera / species**	**Photo-synthesis**	**Pholem connection**	**Growth form**	**Location of haustoria on host**	**Endophytic at maturity**	**Germination**	**Primary haustorium**	**Predominant habitat ***	**Key references**
Hydnoraceae	2/7	-	?	root-parasite	root	-	host-induced	?	desert	Bolin et al. 2009, 2010, Tennakoon et al. 2007
Cassytha	1/16	+	-	parasitic vine	stem	-	autonomous	-	tropical and subtropic shrublands	Cartellieri 1928, de la Harpe et al. 1980, Li et al. 2012, Prider et al. 2009, Shen et al. 2005
Santalales	151/ c. 2000									Nickrent et al. 2010, Su et al. 2015
- root hemiparasites	60/ c. 600	+	-	root-parasite	root	-	autonomous	-	subtropic shrubland, temperate grasslands, tropical forests	Radomiljac et al. 1998, 1999a, 1999b, Suetsugu et al. 2008, Tennakoon & Cameron 2006, Tennakoon & Pate 1996, Tennakoon et al. 1997, Veenendaal et al. 1996, Watson et al. 2011
- mistletoes	84/ c. 1500	+	-	mistletoe	stem	(+)	autonomous	+	tropical, subtropic and temperate forests and shrublands	Calvin & Wilson 2006, Ehleringer et al. 1986, Glatzel & Geils 2009, Mathiasen et al. 2008, Shaw et al. 2004, Strong et al. 2000, Watson & Herring 2012
- endophytic mistletoes **	5/ c. 50	(+)	+	mistletoe endophytic	stem	+/(-)	autonomous	+	temperate forests, deserts	Hawksworth & Wiens 1996, Hull & Leonard 1964a, 1964b, Kraus et al. 1995, Kuijt 2011, Mauseth 1990, Mauseth et al. 1984
- Balanophoraceae and Mystropetalaceae	17/50	-	+	root-parasite	root	-	host-induced	+	tropical forests, subtropic shrubland	Eberwein et al. 2009, Ecroyd 1996, Gedalovich-Shedletzky & Kuijt 1990, Holzapfel 2001, Kuijt & Dong 1990, Shivamurthy et al. 1981, Su et al. 2015
Cytinaceae	3/11	-	+	endophytic	root	+	host-induced	?	subtropic shrublands, tropical forests	Alvarado-Cárdenas 2009, Fernándes-Alonso & Cuardos-Villalobos 2012, de Vega et al. 2007, 2010
Krameriaceae	1/18	+	-	root-parasite	root	-	autonomous	-	deserts, grasslands, (forests)	Brokamp et al. 2012, Giannini et al. 2011

Table 1 (continued) – **Summary of the current knowledge of the functional biology of parasitic plant groups defined on the basis of phylogenetic relatedness and functional similarity.**

Group	Number of genera / species	Photo-synthesis	Pholem connection	Growth form	Location of haustoria on host	Endophytic at maturity	Germination	Primary haustorium	Predominant habitat *	Key references
Cynomoriaceae	1/2	-	?	root-parasite	root	-	?	?	salty grasslands	Heide-Jørgensen 2008, Zhang et al. 2009, Nickrent et al. 2005
Rafflesiaceae	3/20	-	+	endophytic	root	+	?	?	tropical forests	Bellot & Renner 2013, Davis et al. 2007, Fernandes et al. 1998, Filipowicz & Renner 2010, Hidayati et al. 2000, Nickrent et al. 2004, Nikolov et al. 2014, Patiño et al. 2002
Apodanthaceae	2/23	-	?	endophytic	stem	+	?	?	tropical forests, deserts	
Cuscuta	1/145	(+)	+	parasitic vine	stem	-	autonomous	-	open vegetation from tropics to temperate	Birschwilks et al. 2006, Clayson et al. 2014, García et al. 2014, Hibberd.et al. 1998a, Jeschke et al. 1997, Koch et al. 2004, Lee & Jernstedt 2013, McNeal et al. 2007, Meulebrouck et al. 2009, Švubová et al. 2013
Lennoiodae	3/7	-	+	root-parasite	root	-	+	?	desert, dry forests	Kuijt 1966, Kuijt 1967, Yatskievych 1982
Mitrastemonaceae	1/2	-		root-parasite	root	-	?	?	tropical and subtropic forests	Heide-Jørgensen 2008
Orobanchaceae	96/ c. 2000									Bennett & Mathews 2006, Joel et al. 2013, McNeal et al. 2013, Westwood et al. 2010
- 1: *Rhinanthus*, *Melampyrum*, *Euphrasia*, *Odontites*, *Bartsia*, *Pedicularieae****, *Cymbariae* ‡	c. 70†/ c. 1650†	+	-	root-parasite	root	-	autonomous	-	mostly grasslands, forest understory	Cameron et al. 2005, Cameron & Seel 2007, Fisher et al. 2013, Govier et al. 1967, 1968, Jiang et al. 2010, Klaren & Janssen 1978, Li et al. 2013, Mann & Musselmann 1981, Matthies 1995, 1997, Mudrák & Lepš 2010, Světlíková et al. 2015, Těšitel et al. 2015, Těšitel & Tesařová 2013, Weber 1981
- 2: *Striga*, *Alectra*, *Cycnium*, *Tozzia*, perennial *Rhynchocorys* ‡	c. 15†/ c. 100†	+/(-)	-	root-parasite	root	-	host-induced	+/-	mostly grasslands, stream edges	Cardoso et al. 2011, Dörr 1997, Gomez-Roldan et al. 2008, Graves et al. 1992, de la Harpe et al. 1980, Jamil et al. 2012, Kubat & Weber 1987, Simier et al. 2006, Weber 1973

Table 1 (continued) – Summary of the current knowledge of the functional biology of parasitic plant groups defined on the basis of phylogenetic relatedness and functional similarity.

Group	Number of genera / species	Photo-synthesis	Pholem connection	Growth form	Location of haustoria on host	Endophytic at maturity	Germination	Primary haustorium	Predominant habitat *	Key references
- 3: *Orobanche, Cistanche, Harveya, Hyobanche, Epifagus* ‡	15/ c. 250	-	+	root-parasite	root	-	host-induced	+	grasslands, deserts, forest understory	Hibberd et al. 1998b, 1999, Schneeweiss 2007, Thorogood & Hiscock 2010
- 4: *Lathraea, Boschniakia*	2/11	-	-	root-parasite	root	-	host-induced	-	temperate forest understory	Fay 2010, Heinricher 1894, Kuijt & Toth 1985, Renaudin & Larher 1981, Světlíková et al. 2015, Těšitel & Tesařová 2013, Ziegler 1955

photosynthetic ability and essentially acquire all organic carbon from the host represent an exception of this rule (together with fully mycoheterotrophic plants; Selosse & Roy 2009).

Most parasitic species are hemiparasites with functional photosynthesis (table 1, fig. 1). The efficiency of their photosynthetic activity can vary largely, not only between species but also within species depending on host quality and environmental conditions (Cechin & Press 1993, Seel & Press 1994, Radomiljac et al. 1999a, Strong et al. 2000, Těšitel et al. 2015). In general, hemiparasite photosynthesis can vary from levels hardly exceeding the compensation point to levels comparable to those of non-parasitic plants. Despite possibly efficient photosynthesis, hemiparasites uptake organic carbon from the host in the form of xylem-mobile organic elements (Těšitel et al. 2010a, Bell & Adams 2011). This host-derived carbon can substantially contribute to hemiparasite biomass. Nevertheless, the importance of host-derived carbon as an energy resource seems highest when the hemiparasite's own photosynthesis is limited, either because of competition for light or inefficient photochemistry caused by stress such as mineral nutrient deficiency (Těšitel et al. 2015). The host-derived carbon can thus be viewed as a backup resource for hemiparasites.

Some hemiparasitic species are non-green and thus completely dependent on their host during the initial period of their life. Such a strategy evolved at least three times independently in the Orobanchaceae. It is typical of closely related *Striga* and *Alectra* species, which evolved dust seeds with minimal reserves, thus requiring immediate contact with host roots after germination (Dörr 1997, Irving & Cameron 2009, Westwood et al. 2010). By contrast, the life cycles of *Tozzia* and perennial species of *Rhynchocorys* include a long-term underground holoparasitic stage (which evolved independently in these two genera) and they produce green photosynthetic shoots only for the purpose of sexual reproduction (Těšitel et al. 2010b). In all of these cases, the holoparasite stage occurs early in the ontogeny and this ability to acquire all necessary resources is likely to substantially increase establishment success in communities with intense competition for light. Seedling establishment of related hemiparasitic species not displaying such holoparasite stages tends to be the most significant factor limiting their occurrence in competitive environments (Těšitel et al. 2011, 2013).

Several intermediate strategies between hemi- and holoparasitism can be distinguished in parasitic plants. Species of the genus *Cuscuta* are functionally holoparasitic and acquire all saccharides from the host via phloem connections, but most of them display rudimentary photosynthetic activity (Hibberd et al. 1998a, Clayson et al. 2014) and their plastid genome evolution is functionally constrained (McNeal et al. 2007). Despite its low intensity and efficiency (Hibberd et al. 1998a), photosynthetic activity plays an important role in the biosynthesis of lipids which serve as energy reserves. These are stored in *Cuscuta* seeds and used by seedlings actively searching for suitable host stems in their surroundings (McNeal et al. 2007, Švubová et al. 2013). Similarly, in mistletoe species of the genus *Arceuthobium* (Viscaceae), the limited photosynthesis produces assimilates supplementing host-derived carbon in nutrition of exophytic shoots bearing flowers and fruits (Miller & Tocher 1975), while extensive endophytic structures are completely dependent on host carbon (Hull & Leonard 1964a, 1964b). In summary, a parasite's own photosynthetic activity plays an important role in providing resources for sexual reproduction in all species on the edge between hemi- and holoparasitism. That is likely to be the cause why maintaining even a rudimentary and inefficient photosynthetic activity is evolutionarily stable despite efficient carbon uptake from the host, which generally meets the requirements for vegetative growth of the adult parasite.

Interestingly, all hemiparasitic mistletoes (e.g. *Viscum album*) have evolved photosynthesis in the endosperm (Heide-Jørgensen 2008, Nickrent & García 2009), which is highly unusual within the angiosperms. This adaptation helps the seedling to penetrate through possibly thick host bark, which requires a large amount of energy. This unusual location of photosynthesis thus facilitates the establishment of a parasite with a free pre-attachment stage, which is quite similar to the situation in *Cuscuta*.

Anatomy of haustorial connections and resource uptake from the host

The details of the anatomical connection to host vascular bundles hidden in the haustorium are as important to parasitic plant biology as the ability to photosynthesize. All parasitic plant species have access to host xylem, but only some of them have the ability to also withdraw nutrients from the phloem (table 1; Hibberd & Jeschke 2001, Irving & Cameron 2009). The anatomy of the vascular connection to the host underlies not only the quality and quantity of resources acquired, but is also associated with host specificity. The importance of this trait led to a suggestion of a functional classification of parasitic plants into xylem- and phloem-feeders instead of hemi- and holoparasites (Irving & Cameron 2009).

Haustorial anatomy is indeed largely correlated with the ability to photosynthesize or with photosynthetic efficiency. Typical hemiparasites with efficient photosynthesis (hemiparasitic Santalales, Orobanchaceae, Krameriaceae) access host xylem only (table 1). This provides parasitic uptake of mineral nutrients and water but only a limited amount of organic carbon available as xylem-mobile organic elements (Bell & Adams 2011, Těšitel et al. 2010a). Thus, photosynthesis appears to be a requirement for an efficient xylem-feeding strategy. This is however not true for the holoparasites of the genera *Lathraea* and *Boschniakia*, which display a holoparasitic xylem-only feeding strategy (Kuijt & Toth 1985, Ziegler 1955). In *Lathraea*, this is underpinned by acquisition of xylem-mobile organic elements and the ability to actively secrete excess water using hydathode trichomes located on underground leaf scales (Renaudin & Garrigues 1967, Světlíková et al. 2015, Těšitel & Tesařová 2013, Weber 1975). Although providing only a limited spectrum of resources, the xylem parasitism has a largely mechanical nature; i.e. penetrating a host vessel can be viewed as a simple penetration of a dead tube in which resources are transported. Establishment of a xylem connection usually imposes little constraint on the host ranges of xylem-feeding parasites (Gibson & Watkinson 1989, Radomiljac et al. 1999b, Suetsugu et al. 2008), although host defence reactions based on root tissue lignification were identified as a cause of distinct host preferences in some hemiparasitic species (Cameron et al. 2006).

In holoparasites, parasitic uptake of phloem sap rich in assimilates can cover the demand for carbohydrates not provided by autonomous photosynthesis. Such independence from photosynthesis presents the main advantage of phloem parasitism, releasing the parasites from competition for light. In contrast to xylem, phloem is a living tissue. Its parasitism thus requires biochemical compatibility between host and parasite (Thorogood & Hiscock 2010). Therefore, phloem-feeders tend to be more host-specific (Heide-Jørgensen 2008, Thorogood et al. 2009). Their occurrence is consequently limited by their ability to find a compatible host. Although phloem connections are closely associated with holoparasitism, their presence in haustoria of some holoparasitic lineages (Apodanthaceae, Hydnoraceae, Cynomoriaceae, Mitrastemonaceae) remains to be confirmed (table 1). The existence of the holoparasitic xylem-feeding *Lathraea* and *Boschniakia* species (see above) prevents extrapolating phloem parasitism to other lineages based just on the lack of photosynthesis.

Growth habit and location of the haustoria on the host

Parasitic plants display a variety of growth habits (table 1). Root-parasites attach to the host below ground and seem to grow independently of the host from the above-ground perspective. Stem parasites that attach to the host above ground can be further divided into mistletoes and parasitic vines. Mistletoes are parasitic epiphytic shrubs, which attach to the host stem immediately after germination. Parasitic vines are herbs which germinate on the ground and their seedling attaches to the host stems after a certain period of independent growth, which can last from a few days up to several months (Heide-Jørgensen 2008). The most extreme modification is represented by parasitic plants which are completely endophytic at maturity except for their reproductive organs (endophytic parasites; table 1). These parasites produce haustoria only after germination when entering the host.

The location of the haustoria on the host is an important trait which has been used to classify parasitic plants into the functional groups of root and stem parasites (Nickrent 2002). It is really important for photosynthetic hemiparasites, which, if established from an epiphytic seedling, acquire not only the resources from xylem but also a position in the canopy with much more favourable light conditions compared to the understory. In contrast, the position of haustoria makes little difference to non-photosynthetic parasites that do not use light as a resource. Moreover, the endophytic parasites might enter host roots or stems during their establishment and then spread throughout the host body (e.g. in *Rafflesia*; Heide-Jørgensen 2008). Considering the whole diversity of growth habits of parasitic plants might be more ecologically meaningful than just using the location of the haustoria. Nevertheless, the location of haustoria is a simple binary trait more suitable when multiple traits are analysed, which is why I retain it in a multitrait analysis (fig. 2).

The evolution of parasitic plant growth form started from perennial hemiparasitic woody plants (shrubs or trees) in

Santalales (Nickrent et al. 2010) and Krameriaceae (Carlquist 2005). The Orobanchaceae contain few woody taxa (shrubs: *Brandisia*, *Asepalum*, *Cyclocheilon*, *Pterygiella suffruticosa*; woody herbs or subshrubs: *Hedbergia*, *Nothobartsia*, *Sopubia*, *Graderia*; Morawetz et al. 2010, Těšitel et al. 2010b, Dong et al. 2013, McNeal et al. 2013). These are mostly phylogenetically unrelated and many of them form either isolated lineages within the family (*Brandisia*; McNeal et al. 2013) or sister groups to the major clades within the family (*Cyclocheilon*, *Asepalum*, *Sopubia*, *Graderia*; Morawetz et al. 2010; in part also *Pterygiella*; Dong et al. 2013). Such an evolutionary pattern together with the woody habit of the Paulowniaceae, the sister family to Orobanchaceae (APG III 2009), may suggest a possibility of a hemiparasitic woody ancestor also in Orobanchaceae. Given the current knowledge, it difficult to conclude whether the first plant which evolved hemiparasitism in this family was a shrub or an herbaceous plant. By contrast, it is certain that the parasitic vines of the genus *Cuscuta* evolved from non-woody Convolvulaceae vines (García et al. 2014). Other groups of parasitic plants are too distant from their non-parasitic relatives and their vegetative morphology is strongly modified, which prevents drawing conclusions on their growth form evolution.

Germination and establishment

Seed germination and establishment of the connection to the host represent critical points of the parasitic plant life cycle. Individual parasitic plant species have adopted one of two distinct germination strategies. Germination can be either autonomous or induced by chemical signals released by the host.

The autonomous germination might be started just by conditions favourable for seedling survival (e.g. sufficient humidity) or might require specific environmental germination clues to break seed dormancy. This is well-documented for some temperate hemiparasitic Orobanchaceae (e.g. *Rhinanthus*, *Melampyrum*, *Odontites*, *Euphrasia*, *Cordylanthus*, *Orthocarpus*, some species of *Castilleja* or *Agalinis*) which require variable periods of low temperature to initiate germination (Royal Botanical Gardens Kew 2015). This ensures

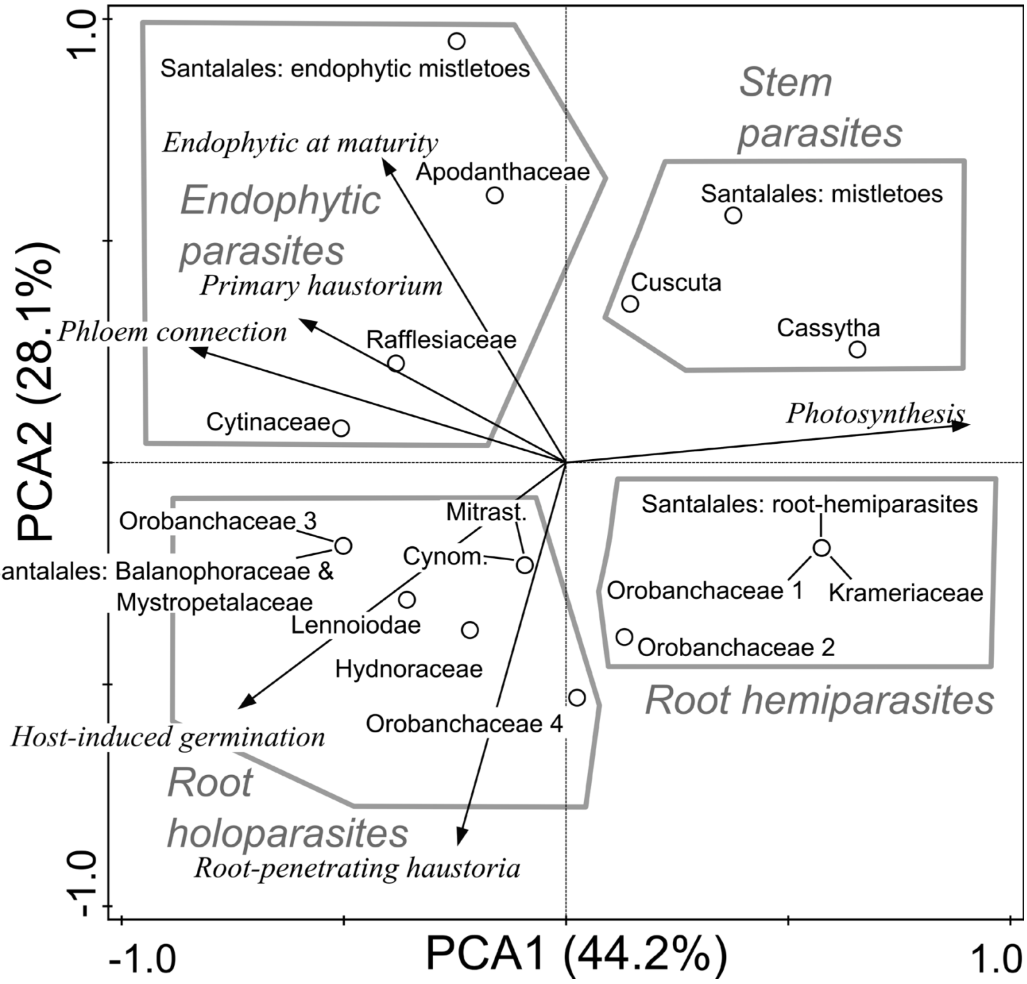

Figure 2 – Principal component analysis plot displaying the functional similarities among individual parasitic angiosperm lineages. The analysis is based on the functional trait values summarized in table 1, which are also displayed in the ordination space. Four principal functional groups of parasitic plants are defined on the plot by grey envelopes. The PCA was computed in Canoco 5 (ter Braak & Šmilauer 2012). Mean substitution was applied in case of missing data. See electronic appendix for exact data table which served as the basis for the PCA.

that germination occurs in periods when most plant species are dormant and thus the seedlings at least in part avoid above-ground competitive pressure from the surrounding vegetation (Těšitel et al. 2011). Germination dynamics in response to temperature can be largely variable among closely related species and to a lesser extent also among populations of a single species, as documented for *Rhinanthus* by ter Borg (2005). Attachment to the host occurs after an independent seedling stage which can last up to many weeks. This germination strategy is typical of many root hemiparasites and stem parasites, seedlings of which can support their growth by own photosynthesis and/or abundant seed reserves. Despite limited development of the root-hemiparasitic seedling root system, it still allows foraging for a suitable host in a sizeable volume of soil. Combined with low host specificity, this strategy provides a good chance to find a suitable host. In mistletoes, the chance of establishing a host connection is increased by specialized dispersal mechanisms (mostly endozoochory by birds, but also explosive seed dispersal in *Arceuthobium*) and sticky seeds. Seedlings of parasitic vines use all their energy to forage for a host above-ground across a distance of tens of centimetres. Active foraging based on volatiles produced by the host has been demonstrated in *Cuscuta* (Koch et al. 2004) together with selection of hosts with higher nutritional status (Kelly 1992).

Host induction of germination combined with long-term seed dormancy can be expected in all host-specific parasitic plants as a trait reducing wasteful seed germination in the absence of a suitable host. Still, it has only been documented in a few species. The best-known examples include dust-seeded Orobanchaceae such as *Striga*, *Alectra*, *Orobanche* and *Phelipanche*. Their germination is induced by strigolactones, plant hormones responsible for signalling with arbuscular mycorhizal fungi (Akiyama et al. 2005, Cardoso et al. 2011), but also affecting plant architecture (Gomez-Roldan et al. 2008, Cardoso et al. 2011). In the Orobanchaceae, host-induced germination was also reported in *Lathraea* and *Epifagus* (Heinricher 1894, Williams & Zuck 1986 reviewed in Bolin et al. 2009). Bolin et al. (2009) experimentally demonstrated host-induced germination in *Hydnora* (Hydnoraceae) and reviewed this phenomenon in *Bdallophytum* (Cytinaceae), *Dactylanthus* (Mystropetalaceae; formerly Balanophoraceae; Su et al. 2015) and *Pholisma* (Boraginaceae - Lenooideae). Despite the great importance for understanding biology of parasitic plants, data on germination of many holoparasites and in particular endophytic holoparasites are still largely missing (table 1).

Another important establishment trait is the ability to form a primary (terminal) haustorium. Haustoria of this type are produced by seedlings of mistletoes and some Orobachaceae (*Striga*, *Orobanche*) to establish the first contact with the host. By contrast, most parasitic plant species produce only secondary (lateral) haustoria, which is typical of species with self-sustained seedling including root-hemiparasitic Santalales, parasitic vines and most Orobanchaceae (table 1). It has been suggested that the ability to form a primary haustorium is closely related to host-induced germination, as is the case in some Orobanchaceae such as *Striga* and *Orobanche* (Westwood et al. 2010). However, this is not true in mistletoes, which produce a primary haustorium but their seedlings germinate autonomously (table 1), nor in root-parasitic *Lathraea*, which requires host germination cues but does not produce a primary haustorium (Ziegler 1955). Functional roles and evolutionary pathways of these establishment traits can thus be diverse, making such generalizations difficult. Unfortunately, the difficulty in observing germination and initial life stages of many parasitic plants results in a large data deficiency for this trait (table 1).

FUNCTIONAL CLASSIFICATION OF PARASITIC PLANTS

A principal component analysis (PCA) was performed to summarize functional trait distribution across parasitic plant lineages and establish a new functional classification of parasitic plants (fig. 2). On the basis of the PCA results, it is possible to define four functional groups, located in the quadrants of the first two principal components: root hemiparasites, root holoparasites, stem parasites and endophytic parasites. The major advantage of this classification is that it is based on variability of all key functional traits across all parasitic angiosperms. Every parasitic plant species can thus be assigned to one of the functional groups based on current knowledge of their biology. The functional groups are not homogeneous (i.e. a certain variability in functional traits is present within the group) but that is always the case in such categories comprising species of different evolutionary lineages.

Probably the largest heterogeneity is present in stem parasites including both mistletoes and parasitic vines. There are however substantial differences even between functionally similar *Cuscuta* and *Cassytha* (presence/absence of phloem connections in haustoria, differential degree of photosynthetic ability) which justifies the concept of a single heterogenous functional group of stem parasites.

Endophytic parasites are newly distinguished here as a functional group of parasitic plants. They are defined by the dominance of an endophytic stage in their life cycle. Typically, these species form a haustorium immediately after germination to penetrate the host. They then form extensive endophytic structures and produce exophytes only for flowering and seed production. The exophytes may be just flowers or inflorescences (Rafflesiaceae, Apodanthaceae, Cytinaceae), or larger shoots (most endophytic mistletoes). There is an entire gradient of parasitic strategies and host dependence in mistletoes. A majority of the species attach to their host by haustoria, each of which forms a single connection to host vascular bundles (species with epicortical roots, clasping unions, and wood roses; Calvin & Wilson 2006, Mathiasen et al. 2008). These can be clearly classified as stem parasites. Other species produce endophytic bark strands with multiple connections to the host vasculature. The extent of the endophytic system is rather limited and smaller in size compared to the exophytic shoots in many species (e.g. *Viscum album*; Zuber 2004). These species are considered stem-parasitic mistletoes here. By contrast, other species may cause systemic infections and the endophyte of some of them is even isophasic, i.e. it proliferates into the apical buds of the hosts and displays growth synchrony with the host (Calvin & Wilson 1996, Kuijt 2011, Lye 2006). Photosynthesis tends to be reduced in these species and they acquire most of the organic carbon from the host (Hull & Leonard 1964a, 1964b). Such species are considered as endophytic mistletoes here. Typical representatives include most *Arceuthobium* species (e.g. *A. americanum*, *A. pusillum*, *A. douglasi*; Lye 2006), *Phoradendron perredactum* (Kuijt 2011), *Viscum minimum* of Viscaceae (Engler & Krause 1908), *Phacellaria* (Amphorogynaceae; Nickrent et al. 2010) and *Tristerix aphyllus* (Loranthaceae; Mauseth et al. 1984, Kraus et al. 1995, Mauseth 1990). Most *Arceuthobium* species that do not show isophasic growth still have a large endophyte and a low photosynthetic capacity and can be assigned to this group. *Arceuthobium oxycedri*, which is seemingly the most photosynthetic species of the genus acquiring c. 50% of its carbon by its own photosynthesis (Hawksworth & Wiens 1996, Rey et al. 1991), is difficult to classify and should be probably considered a transitional case between stem and endophytic parasites.

Root hemiparasites are considered a single group here without further classification to facultative and obligate as suggested by Nickrent (2002). Facultative parasitism, which, in a strict ecological sense, means the ability to keep per-capita population growth rate $r > 0$ in the absence of a host, is very rare in parasitic plants and difficult to demonstrate. It probably exists e.g. in *Triphysaria* (Westwood et al. 2010) and *Odontites vernus* (Weber 1981, Geppert 2012). Most of the species suggested as facultative hemiparasites (Nickrent 2002) are nevertheless unable to survive, produce flowers or their growth is largely reduced and flower production is minute in host-free cultivation (Mann & Musselmann 1981, Matthies 1997, Weber 1981) unless high doses of mineral nutrients are applied (Mann & Musselmann 1981). In addition, no hemiparasite has been reported to grow without a host under natural conditions (Heide-Jørgensen 2013). By contrast, even *Striga asiatica*, member of a genus comprising typical “obligate root-hemiparasites” (Westwood et al. 2010) can grow and flower without host in an axenic culture if provided with nutrients and germination stimulants (Yoshida & Shirasu 2012). The cultivation studies hence demonstrated that both “facultative” and “obligate” root-hemiparasites can grow and reproduce under artificial conditions, although the latter require a higher level of condition control. In summary, there is apparently a large variability in host-dependence among hemiparasitic species. *Triphysaria* and *Odontites* discussed above represent one extreme root-hemiparasitism while species with holoparasitic seedlings, like *Striga*, *Alectra*, *Tozzia* and the perennial species of *Rhynchocorys* represent the other. Most of the other root-hemiparasitic species lie between these extremes and it is difficult to make a clear border line between “facultative” and “obligate” root hemiparasites. Therefore, I suggest viewing root hemiparasites as a single, yet variable functional group, members of which are dependent on their hosts to various extents.

COMMUNITY ECOLOGY AND HABITAT PREFERENCES OF PARASITIC PLANTS

Parasitic plants occur in all terrestrial ecosystems ranging from tropical rainforests and hot deserts to temperate grasslands and arctic tundra (Heide-Jørgensen 2008). Numerous species are known to act as keystone species in the ecosystems they inhabit. This is based on their specialized nutritional or reproductive strategies interacting with organisms in many different ways (Press & Phoenix 2005). The most important mechanisms of the ecosystem effects include: (1) harm inflicted to the host species by parasitism, which can modify competitive relations in plant communities (Cameron et al. 2005, Li et al. 2012, Prider et al. 2009, Shen et al. 2005); (2) effects on nutrient cycling via modifications of soil microbial community structure (Bardgett et al. 2006, Quested et al. 2003, Spasojevic & Suding 2011) and (3) provision of important resources for animals, such as birds or insects (Watson 2001, Watson et al. 2011, Watson & Herring 2012).

Despite the general omnipresence of parasitic plants in terrestrial habitats, individual species, parasitic plant lineages and functional groups often show contrasting habitat preferences (table 1). The low number of independent evolutionary origins of parasitic plants does not allow a formal testing of these differences and relating them to functional traits or groups. Still, some patterns are clear and can be interpreted using the knowledge of ecology and physiology of individual parasitic plant groups.

The greatest benefit of parasitism for root hemiparasites lies in the uptake of mineral nutrients, although they also acquire water and organic carbon from the host (Těšitel et al. 2015). Hemiparasites require light to transform this benefit into fitness by photoassimilation. This implies that root hemiparasitism should be most advantageous in habitats where mineral nutrients are limiting and light is available in abundance (Matthies 1995, Těšitel et al. 2011). It is not so straightforward, since the hemiparasites’ growth can be increased by abundant mineral nutrients to an extent similar to non-parasitic plants and light deficiency may be in part compensated by heterotrophic carbon acquisition (Těšitel et al. 2015). Still, root-hemiparasitism provides only limited advantages in habitats such as closed canopy forest where competition for light is the major ecological constraint restricting recruitment ability (e.g. Whitmore 1990). That is why open habitats host the greatest diversity of root hemiparasites.

This association of root hemiparasites with open habitats is clear in Orobanchaceae root-hemiparasitic species of which occur mostly in grasslands and only a tiny fraction them (such as some species of *Melampyrum*) grow in closed-canopy forests. Similarly, most *Krameria* species are restricted to open habitats (though e.g. *K. lappacea* and *K. lanceolata* occur also in forests; Giannini et al. 2011). In Santalales, root-hemiparasitism has probably evolved in tropical trees (Nickrent et al. 2010). Apart from the major clades (see below), extant root-hemiparasitic Santalales comprise multiple mostly small phylogenetic lineages whose species grow in tropical forests. Of these, the family Aptandraceae is probably the largest group containing 34 predominantly forest species; Nickrent et al. 2010). Another example includes the genus *Okoubaka* (Cervantesiaceae) occurring in tropical forests of Africa. *Okoubaka aubrevillei*, the largest hemiparasitic tree, is known to reduce the competitive pressure from the surrounding vegetation by strong reduction of growth or even killing the trees it parasitizes (Veenendaal et al. 1996). Nevertheless, the major radiation events in root-hemiparasitic Santalales are associated with lineages of open habitats

(*Thesium* with c. 350 species is the largest genus of Santalales, *Olax* with forty species; Nickrent et al. 2010). Such evolutionary pattern implies that the fact that the root-hemiparasitic strategy *per se* provides the largest benefits in habitats where light is available in abundance may apply also to Santalales. Why Santalean root hemiparasitism evolved in tree species growing in tropical forests, i.e. a habitat where light deficiency is a limiting factor (Whitmore 1990), remains a question. This might be answered by a detailed analysis of habitat preferences and ecological niches of the forest species of the small basal clades of Santalales.

The limitation by competition for light is largely overcome in stem parasites which grow epiphytically. This is clear in mistletoes which start their life as seedlings attached to host branches and many species of which indeed grow in forest canopy. By contrast, parasitic vines have a ground-based, at least partially photosynthetic seedling, which probably underlies their occurrence in open habitats.

Root holoparasites and endophytic parasites occur mostly in forest understory and arid habitats. Only a few of them (e.g. some *Orobanche* species) grow in grasslands and other open habitats inhabited by root hemiparasites. The non-photosynthetic parasites can thus be viewed as ecological vicariants of root-hemiparasites at least in non-arid habitats and the evolution of holoparasitism as a strategy to colonize habitats not accessible to hemiparasites.

EVOLUTIONARY TRENDS IN FUNCTIONAL TRAITS

The evolution of parasitism in the angiosperms must have started from a non-parasitic ancestor in all parasitic plant lineages. It is now largely accepted that except in parasitic vines the first parasitic stage was a root-hemiparasitic species (Westwood et al. 2010, Naumann et al. 2013). This is also supported by the evolutionary trends in Orobanchaceae and Santalales, the only two extant monophyletic parasitic plant lineages that comprise species of multiple functional groups (table 1, fig. 2). It is likely that further evolution towards more specialized forms was triggered by their ability to colonize habitats unsuitable for their root-hemiparasitic ancestors. This resulted in the repeated evolution of epiphytic mistletoes in Santalales (Nickrent et al. 2010) and of root-holoparasitism in both Santalales (Su et al. 2015) and Orobanchaceae (Bennett & Mathews 2006, McNeal et al. 2013).

Root hemiparasitism and stem parasitism are very successful strategies measured by both the number of species and their profound impact on plant communities and ecosystems. Ecosystem effects are based on the primary consequences of parasitism, but also on secondary effects such as enrichment of ecosystems by nutrient-rich litter and consequent enhancement of nutrient cycling (Phoenix & Press 2005, Cameron et al. 2005, Press & Phoenix 2005, Quested et al. 2005, Prider et al. 2009, Watson 2009, Shen et al. 2010, Li et al. 2012, Watson & Herring 2012, Demey et al. 2013, Fisher et al. 2013). This contrasts with generally low species richness recorded in lineages of root holoparasites and endophytic parasites. Profound ecosystem effects of species of these functional groups are also rather exceptional (well documented only in the case of *Arceuthobium* dwarf mistletoes; Shaw et al. 2004).

Such evolutionary patterns suggest that the greatest benefits causing evolutionary radiations were associated with the evolution of haustoria and connection to host xylem. These evolutionary innovations, defining the initial phase of the evolution of parasitism in land plants, released the hemiparasitic plants from nutrient limitation, which is likely to be the major cause of their radiation and spread across the Earth. Further constraints based on competition for light and seedling establishment were addressed at least to some extent by the evolution of stem parasitism and especially epiphytic seedlings. This caused the great evolutionary success of mistletoes as indicated by multiple origins of this growth form and intense radiation in some mistletoe lineages. The evolution of host-induced germination, phloem connection, loss of photosynthesis and tendency to grow endophytically is likely also a reaction to establishment-related constraints. Although these advanced evolutionary innovations allowed parasitic plants to colonize habitats inaccessible to root hemiparasites, they produced highly specialized forms (often host-specific) with a limited evolutionary potential. Such multistep evolution associated with changes in ecology is also likely to trigger the evolution of extreme morphological modifications typical of many extant root-holoparasitic and endophytic lineages.

CONCLUSION

This paper summarizes current knowledge on functional biology of parasitic plants. Identification of key functional traits and an analysis of their distribution in parasitic plant lineages underpin a new functional classification of parasitic plants into four principal groups: root hemiparasites, stem parasites, root holoparasites and endophytic parasites. These categories have been used in literature on parasitic plants, but have never been combined in a comprehensive functional classification. Despite being heterogeneous, these functional groups allow each parasitic plant species to be classified into one of them, which is the key advantage over previous concepts based on model species biology. This functional classification is particularly useful in the global view on biology of parasitic plants. Focussing on just one of the key traits underlying biological differences within individual lineages or genera might, however, be a more pragmatic approach on a finer taxonomic scale.

I also attempted to relate the functional biology and classification of parasitic plants with their habitat preferences and community ecology. In addition, an evolutionary scenario is presented to explain diversity and functional trait patterns observed in parasitic plants. Still, the ecological and evolutionary hypotheses presented in this paper are only of an informal nature. Although some formal modelling approaches might seem available, the low number of independent origins of parasitism in the angiosperms largely disqualifies their use at the global perspective adopted in this review. Still, they might be perfectly useful for detailed analyses within a particular parasitic plant lineage.

Parasitic plants are often considered a fascinating group of organisms. That is, however, based on many fascinating stories on individual parasitic plant lineages displaying unique biological features or evolutionary patterns rather than on any general trends typical of the biology of parasitic plants. This is why studying a particular parasitic plant species is always important and might reveal unexpected natural processes.

SUPPLEMENTARY DATA

Supplementary data are available at *Plant Ecology and Evolution*, Supplementary Data Site (http://www.ingentaconnect.com/content/botbel/plecevo/supp-data), and consist of the data table that served as the basis for the principal component analysis (Excel spreadsheet).

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Integrating ecology and physiology of root-hemiparasitic interaction: interactive effects of abiotic resources shape the interplay between parasitism and autotrophy

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Summary

- Root hemiparasites are green photosynthetic plants, which parasitically acquire resources from host xylem. Mineral nutrients and water, two principal below-ground abiotic resources, were assumed to affect the interaction between hemiparasites and their hosts. The shape of these effects and the underlying physiological mechanisms have, however, remained unclear.
- We conducted a glasshouse experiment with root-hemiparasitic *Rhinanthus alectorolophus*, in which we manipulated the availability of mineral nutrients and water. Biomass production and Chl fluorescence of the hemiparasites and hosts were recorded, together with proportion of host-derived carbon in hemiparasite biomass.
- The abiotic resources had profound interactive effects on the performance of both the hemiparasite and the hosts, as well as the balance of above-ground biomass between them. These effects were mainly based on an increase of growth and photosynthetic efficiency under high nutrient concentrations, on the hemiparasite's ability to induce strong water stress on the hosts if water is limiting, and on release of the host from parasitism by simultaneous abundance of both resources.
- Hemiparasitism is a highly variable interaction, in which environmental conditions affect both the parasitic and autotrophic (and thus competitive) components. A hemiparasite's own photosynthesis plays a crucial role in the assimilation of parasitized mineral resources and their transformation into growth and fitness.

Introduction

Metabolism based on photoautotrophy and acquisition of energy and inorganic compounds from the environment are considered the principal defining features of plants. Several flowering plant lineages, however, have evolved strategies to acquire some or all of their fundamental resources (water, mineral nutrients, organic carbon (C)) by parasitizing other organisms. Approximately 1% of all plant species directly parasitize the vasculature of other plants via specialized transfer organs called haustoria (haustorial parasites). A further 10% of angiosperms have evolved to parasitize mycorrhizal fungi at some point in their lifecycle (mycoheterotrophs; Leake & Cameron, 2010). In both groups, an evolutionary continuum exists where the evolution of fully heterotrophic species is preceded by intermediate mixotrophic steps, hemiparasitic or partially mycoheterotrophic plants which obtain C from their host but also retain photosynthetic ability (Irving & Cameron, 2009; Selosse & Roy, 2009; Westwood *et al.*, 2010).

The mechanism of parasitic resource acquisition in partial mycoheterotrophs is unlikely to differ substantially from that in full mycoheterotrophs and the same fungal lineage can provide resources to both partially and fully mycoheterotrophic plants (Dearnaley *et al.*, 2013). By contrast, the physiology of C acquisition by hemiparasitic and holoparasitic plants is remarkably different, specifically in terms of the structure of their respective haustoria. Typical hemiparasites withdraw resources exclusively from host xylem, while holoparasites have access to host phloem as well as the xylem (Irving & Cameron, 2009), although exceptions and intermediate forms exist. These include the xylem-only feeding holoparasitic genus *Lathraea* (Ziegler, 1955; Těšitel & Tesařová, 2013) and the largely heterotrophic phloem-feeding genus *Cuscuta*, which retains limited photosynthetic capacity (Hibberd *et al.*, 1998; Švubová *et al.*, 2013). Hemiparasites constitute *c.* 90% of the estimated 4500 species of haustorial parasitic plants (Heide-Jørgensen, 2008). This asymmetry in species richness highlights the ecological success and evolutionary stability of the hemiparasites and indicates that they are not simply an intermediate evolutionary step towards the full heterotrophy (Selosse & Roy, 2009; Westwood *et al.*, 2010). Consequently, a detailed understanding of the uses of individual resources by hemiparasites in the context of their ecological interaction with their hosts is crucial to understanding their evolutionary success and stability, as well as the evolution of parasitism in land plants in general.

Central to this understanding is the recognition of two distinctive functional groups within the hemiparasites defined by the location of their attachment to the host – root and stem hemiparasites. Growing as epiphytes, stem hemiparasites attack host stems, and the host also provides them with a favourable position with access to light required for photosynthesis. Root hemiparasites, by contrast, attack host roots below ground, and their shoot morphology is usually very similar to that of co-occurring nonparasitic plants. As a result, root hemiparasites are subjected to intense shade and are therefore simultaneously competing with their hosts for light and stealing water and nutrients.

The traditional view that hemiparasites simply acquire water and mineral nutrients from the hosts while producing all assimilates through their own photosynthesis has been challenged by recent evidence demonstrating C flow from hosts to hemiparasites in the form of xylem-mobile organic elements (for reviews, see Těšitel *et al.*, 2010; Bell & Adams, 2011). This reveals the possibility for substantial heterotrophic C acquisition by hemiparasites, but the ecological relevance of this is unclear. Regardless of the heterotrophic C acquisition, the consensus is that root hemiparasitism should be most beneficial in nutrient-poor environments where low-cost, yet efficient, acquisition of mineral nutrients represents a key advantage over co-occurring nonparasitic plants. Abundance of mineral nutrients should diminish this advantage and, moreover, increase the above-ground competition for light (Fibich *et al.*, 2010), the dominant (negative) ecological interaction occurring among shoots of green plants (Schwinning & Weiner, 1998) also affecting root hemiparasites (Matthies, 1995; Keith *et al.*, 2004; Těšitel *et al.*, 2011). This resource-competition hypothesis has been supported by observations of a lower population density of root hemiparasites on sites of elevated productivity (van Hulst *et al.*, 1987; Fibich *et al.*, 2010; Těšitel *et al.*, 2013) and by their decrease in communities following fertilizer application (Mudrák & Lepš, 2010). Although the heterotrophic acquisition of organic C can relieve the adverse effect of competition for light and potentially increases the seedling survival rate, the host–hemiparasite C flow is not sufficient to completely counteract the effect of competition (Těšitel *et al.*, 2011).

The effect of mineral nutrient availability and/or productivity on hemiparasite populations is not as straightforward as this, however, as many root-hemiparasitic species occur in mesotrophic rather than genuinely nutrient-poor habitats (e.g. Westbury, 2004; Mudrák *et al.*, 2014). Moreover, a range of studies also report an increase in growth or fecundity of hemiparasite individuals under elevated nutrient availability/productivity (van Hulst *et al.*, 1987; Simier *et al.*, 2006; Mudrák & Lepš, 2010; Borowicz & Armstrong, 2012; Těšitel *et al.*, 2013). There are two possible explanations for this, neither of which is mutually exclusive: faster-growing hosts of higher nutrient status provide more/higher-quality organic resources to hemiparasites (Hautier *et al.*, 2010); or hemiparasites benefit from elevated nutrient status in a similar way to nonparasitic plants, that is, through an enhancement of photosynthesis (Lambers *et al.*, 2008; Taiz & Zeiger, 2010). In order to understand how much these processes contribute to the resource budget of hemiparasites and how this contribution varies with availability of the resources, it is vital that we integrate the physiology of root hemiparasites with the ecology of their interaction with their hosts.

We investigated the response of the resource budget of the root hemiparasite *Rhinanthus alectorolophus* (Orobanchaceae) to limitation in nutrients and water in order to determine the impact of resource variability on the outcome of host–parasite interactions. We conducted a glasshouse experiment with root-hemiparasitic *R. alectorolophus* in which we aimed to separate the effects of availability of mineral nutrients, water and host–hemiparasite C flows. These three components are the principal resources the hemiparasite acquires from the host, and all of them were shown to affect the performance of hemiparasites (Boukar *et al.*, 1996; Simier *et al.*, 2006; Ducarme & Wesselingh, 2009; Mudrák & Lepš, 2010; Těšitel *et al.*, 2011, 2013; Borowicz & Armstrong, 2012). However, their effects have never been studied simultaneously in a single manipulative experiment focusing on the performance of both host and hemiparasite together with underlying physiological principles. Biomass production of the hemiparasites and the infected and uninfected hosts under all experimental treatments was monitored, and the resulting patterns could be interpreted as root-hemiparasitic interaction components (parasitic resource acquisition, harm inflicted to the host and above-ground competition). Maize (*Zea mays*, C_4 grass) and wheat (*Triticum aestivum*, C_3 grass) were used as hosts, which allowed us to monitor organic C flows from the host to the hemiparasite using C stable isotope analysis.

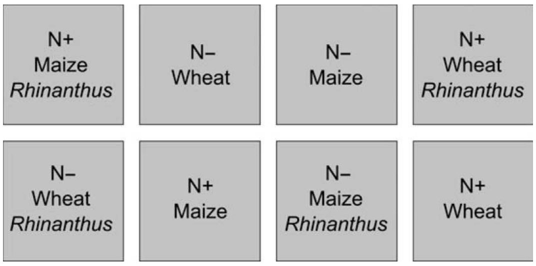
Materials and Methods

Seeds of *Rhinanthus alectorolophus* (Scop.) Pollich used in this study were collected from fruiting plants of a natural population in Zechovice, Czech Republic (49°09′28″N, 13°52′13″E, 510 m above sea level). Seeds of maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) used as the host species were obtained from the school farm of the Faculty of Agriculture, University of South Bohemia.

The substrate for cultivation consisted of a mixture of perlite and Levington John Innes no. 2 compost (82/18, v/v; Scotts Miracle-Gro Co., Marysville, OH, USA). The total volume of substrate was divided into two halves, the first of which corresponded to the low-nutrient treatment with no additional nutrients, while 3.08 g (l^{-1} substrate) of Osmocote Exact Standard (5–6 months) fertilizer (Scotts Miracle-Gro Company) was added to the second half-volume of the substrate (high-nutrient treatment). The mean macronutrient compositions calculated on the basis of the substrate and fertilizer composition provided by the manufacturer were (l^{-1} substrate): 0.078 g N, 0.034 g soluble P, 0.019 g insoluble P, and 0.13 g K in the low-nutrient treatment, and 0.570 g N, 0.127 g soluble P, 0.042 g insoluble P, and 0.437 g K in the high-nutrient treatment. The amount of added nutrients in the N+ treatments were approximately comparable to those applied annually in the fertilized plots of the long-term Rengen grassland experiment (Hejcman *et al.*, 2010). Pots (0.8 l) were filled with either high- or low-nutrient substrate and used for cultivation. Pots were placed in an air-conditioned glasshouse at the University of Sheffield (UK). The pots received natural sunlight which was supplemented with sodium bulbs providing an additional homogeneous irradiation of 250 μmol photons m^{-2} s^{-1}. This additional light source was necessary, because the experiment was conducted in the winter period (January–March) when the natural sunlight intensity is substantially lower than during the natural growth period of the hemiparasites and their hosts. Light : dark diurnal cycles of 12 : 12 h, 18 : 15°C were set during the course of the experiment.

Seeds of host plants were germinated on moist filter paper at room temperature for 3 d and then sown into the pots (a single seed per pot). A single seedling of *R. alectorolophus* (germinated on wet filter paper at +4°C for 8 wk) was added to half the pots to create the infected treatment. Following infection, 15 blocks consisting of eight pots were formed to include all combinations of infection by the hemiparasite (infected/noninfected), host species (maize/wheat, M/W) and nutrient concentration (low/high, N−/N+) arranged in a factorial design (Fig. 1). Pots in all blocks were well irrigated every day for 10 d following the introduction of *Rhinanthus*. Several additional pots of all infected treatments were set up, which were used to replace pots in blocks where preattachment mortality occurred. Thus, we ensured *Rhinanthus* attachment in all infected pots, which corresponds to high success of establishment under natural conditions if not limited by adverse abiotic conditions or competition (up to 200 hemiparasite plants established from 300 seeds sown m^{-2} in a recent field experiment; Mudrák *et al.*, 2014) Irrigation treatments commenced after this initial 10 d period, as all the hemiparasites showed clear signs of attachment (rapidly expanding leaves and vigorous growth). All pots in eight of the 15 blocks received 200 ml of water, corresponding to the full saturation of the substrate (determined based on a mean of five measurements) three times a week (high irrigation; W+), while all pots in the seven remaining blocks received 70 ml of water at the same time (low-irrigation treatment; W−) until harvest. Blocks of the two irrigation treatments were arranged at random in the glasshouse. The experiment was hence arranged in a split-plot design where the irrigation treatment was tested at the between-block level (i.e. whole plots) and the other factors and all the interactions at the within-block level (i.e. split plots). The protocol based on constant water supply in each of the irrigation treatments irrespective of nutrient concentration simulates conditions occurring at natural sites with different nutrient availability and receiving similar amounts of precipitation. However, a complete separation of the pure physiological effects of water and nutrient availability is not possible because of intrinsic confounding effects of water supply on nutrient release from substrate and bulk flow of nutrients into the root-depletion zone (Taiz & Zeiger, 2010) and higher water use of faster-growing fertilized plants apparent from the pattern of pot substrate water content measurement (Supporting Information, Fig. S1). This limitation, unavoidable in analogous glasshouse or field experiments, is taken into account in the interpretation of the data, which considers the resources in terms of their supply to the system rather than their exact physiological effects.

In general, our experimental protocol created conditions located in contrasting positions on the water and mineral nutrient availability gradients. This is directly documented for water availability by relative soil water content measurements (Fig. S1). The range of the soil moisture values recorded before a scheduled irrigation event corresponds to a gradient from very dry (as was also apparent from observed loss of turgor of plants in the N+W− pots) to moist conditions (i.e. elevated moisture content but well below waterlogged conditions). For mineral nutrient availability, a direct chemical analysis of the substrate would not be informative because of the use of the slow-releasing fertilizer. The nutrient status can, however, be approximated form the biomass production of the uninfected hosts. The mean biomass production of uninfected N− wheat and maize was close to 1 g DW, which is similar to values of a series of hosts cultivated under 42% summer daylight (comparable to our light conditions) by Hautier *et al.* (2010) in a nutrient-poor mixture of peat and sand after 60 d of cultivation. This similarity in uninfected host performance indicates oligotrophic conditions in our N− pots, which were close to the lower limit of nutrient availability gradient in grasslands. The size of the difference between the N+ and N− conditions is also indicated by a large difference in biomass production of the uninfected hosts, which produced approximately five times more biomass in the N+ pots than in the N− pots (if compared within the same irrigation treatment; Fig. 2a).



N+ Maize *Rhinanthus*	N− Wheat	N− Maize	N+ Wheat *Rhinanthus*
N− Wheat *Rhinanthus*	N+ Maize	N− Maize *Rhinanthus*	N+ Wheat

Fig. 1 Scheme of pot setup within a single block of the experiment. The pots were arranged at random within blocks; hence the positions of individual treatments differed between blocks. The irrigation treatment was applied at the between-block level.

Survival of hemiparasites after their attachment was monitored over the course of the experiment to record the rate of postattachment mortality. Pots containing dead hemiparasites were omitted from all analyses except for the analysis of hemiparasite mortality. Chl fluorescence measurements were taken on both hemiparasite and host plants using a MINI-PAM photosynthesis yield analyser (Heinz Walz GmbH, Effeltrich, Germany) 50 d after the start of the irrigation treatments and just before a scheduled irrigation event. The maximal quantum yield of photosystem II (F_v/F_m) was measured on mature dark-acclimated (10 min) leaves. The fluorescence measurements could not include all experimental plants because of a temporal constraint: the readings needed to be taken in a short time period of a maximum of a few h to avoid the bias caused by a gradual loss of water by the experimental plants and hence an increase in drought stress. Instead, a subset of blocks ($n = 10$) was used. After completing the Chl fluorescence measurements, the relative water content of the substrate (v/v ratio) was measured in each pot using an HH2 moisture

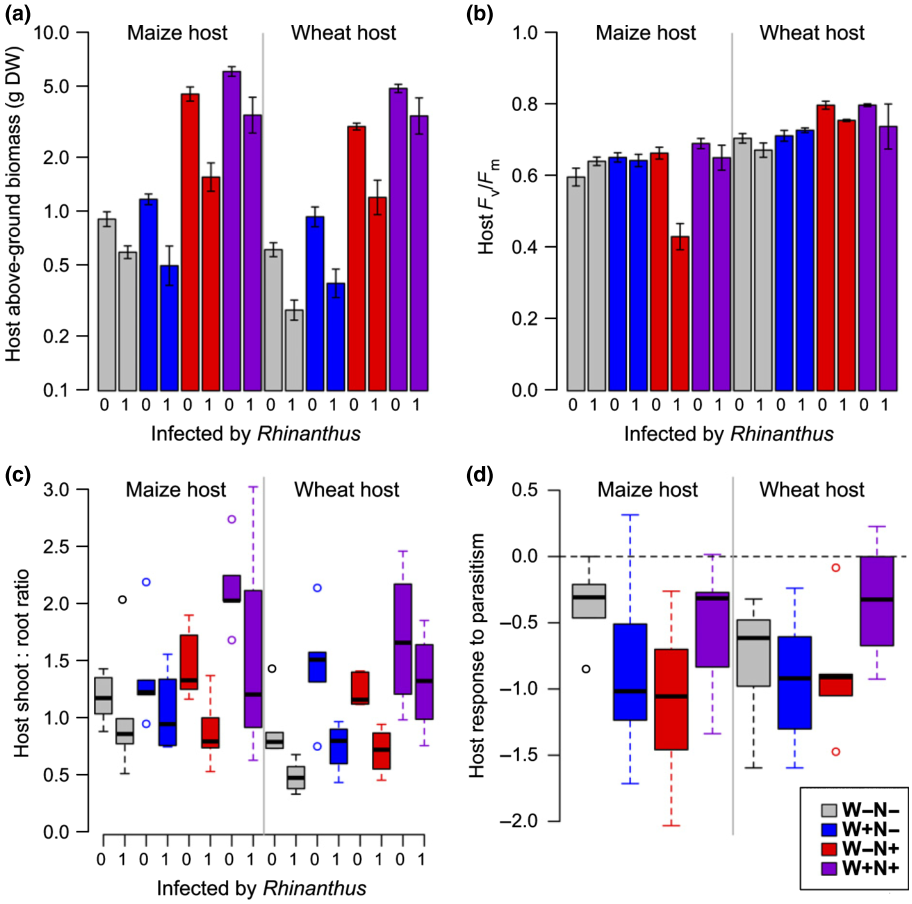


Fig. 2 Host performance parameters as affected by host species identity, infection by *Rhinanthus*, irrigation and nutrient levels. (a) Host dry above-ground biomass; (b) maximum quantum yield of photosystem II (F_v/F_m); (c) host shoot : root ratio; and (d) host response to parasitism expressed as the ratio of above-ground biomass production between infected and control hosts of the same treatment combination within the same block. '−', indicates low and '+' high amounts of irrigation (W) and nutrient availability (N). See Table 1 for summaries of mixed-effect model analyses. Bar plots with linear (b) and logarithmic (a) *y*-axes represent arithmetic and geometric means, respectively, with associated standard errors. Boxplots (c, d) represent median, quartiles and nonoutlier ranges, with outliers displayed as points outside the nonoutlier ranges.

meter with an SM200 sensor (Delta-T Devices Ltd, Cambridge, UK). Three readings per pot were taken and their mean value was used in subsequent analyses.

All experimental plants were harvested on the day following the Chl fluorescence measurements. Above-ground biomass of the host and the hemiparasite (where applicable) was sampled in each of the pots, oven-dried at 80°C for 120 h, and weighed. The host response to parasitism (i.e. reduction of growth as a result of parasitism) was measured as the ratio of above-ground biomass production between infected and control hosts of the same treatment combination within the same block. This ratio was transformed by natural logarithm, providing a measure that is independent of the actual host size and has distribution properties that comply with the requirements of statistical analyses (normal distribution, homogeneity of variances). Below-ground host biomass was also sampled and processed in the same way across a subset of blocks ($n = 11$), because of a temporal constraint. Roots were initially manually separated from dry substrate (mostly coarse perlite particles) and were then rinsed with water to remove smaller particles.

Leaves of hosts and whole shoots of hemiparasites were processed for C stable isotope analysis. Analysis of host leaves provides a good estimate of C-isotopic composition of the assimilates (Bowling *et al.*, 2008) that might be taken up by the hemiparasite. Analysis of the bulk above-ground biomass of the hemiparasite provides data on mean isotopic composition of the whole shoots, which is crucial for between-plant comparisons. Although certain differences in C-isotopic composition can be expected between individual parts of the shoot (e.g. leaves vs stems), these would be rather small (see Těšitel *et al.*, 2011 for details) and an analysis of separate parts would not allow a simultaneous determination of the mean isotopic composition of the shoot. Host leaves and whole shoots of hemiparasites were processed for C stable isotope analysis. Each sample was homogenized and a 5 mg subsample of each constituent part was analysed for ^{13}C content by continuous-flow mass spectrometry (PDZ Europa 2020 Isotope Ratio Mass Spectrometer coupled to a PDZ ANCA GSL preparation unit; SerCon Ltd, Cheshire, UK). Data were collected as atom % ^{13}C and re-expressed as delta values relative to the Pee Dee Belemnite standard (δ) using:

$$\delta^{13}C = R_{\mathrm{Sample}}/R_{\mathrm{Standard}} - 1 \qquad \text{Eqn 1}$$

where $R_{\mathrm{Sample}} = {}^{13}C/{}^{12}C$ ratio in the sample and $R_{\mathrm{Standard}} = {}^{13}C/{}^{12}C$ ratio in the Pee Dee Belemnite standard.

Rhinanthus alectorolophus and wheat display the C_3 photosynthetic pathway, while maize performs C_4 photosynthesis. As a result of their different photochemical processes, C_4 plants are usually significantly more enriched in ^{13}C than C_3 plants. This difference allows the proportion of host-derived C in hemiparasite biomass to be inferred from measurements of the difference in the $\delta^{13}C$ value of biomass of the C_3 parasites attached to C_3 and C_4 hosts. The calculation is based on an isotope mixing model relating the excess of ^{13}C in hemiparasites attached to the C_4 host compared with those attached to the C_3 host to the difference in isotope composition between the C_3 and C_4 hosts themselves (see Těšitel *et al.*, 2011 for more details).

$$\%H = \left(\frac{\delta_{P(C_4)} - \delta_{P(C_3)}}{\delta_{H(C_4)} - \delta_{H(C_3)}}\right) \times 100\% \qquad \text{Eqn 2}$$

where $\%H$ = the percentage of C in parasite biomass that is derived from the host, $\delta_{P(C_4)} = \delta^{13}C$ of the parasite attached to the C_4 maize, $\delta_{H(C_4)} = \delta^{13}C$ of the infected maize host, $\delta_{P(C_3)}$ = mean $\delta^{13}C$ of the parasites growing on the C_3 wheat hosts growing at the same nutrient concentration and irrigation treatment, and $\delta_{H(C_3)}$ = mean $\delta^{13}C$ of the infected wheat hosts growing at the same nutrient concentration and irrigation treatment. The absolute amount of biomass accumulated in the shoot of the hemiparasites from the host-derived C was calculated as the product of the proportion of host-derived C in biomass and the above-ground biomass (DW) of the respective hemiparasite.

Linear mixed-effect models were used to analyse relative water content, biomass production, host response to parasitism, Chl fluorescence, biomass C stable isotope composition, and the proportion of host-derived C in hemiparasite biomass. All models contained block as a random effect term to account for the structure of the data. Thus the model formulas followed the general pattern: response ~ host species × irrigation × nutrients × infected + (1|block). Some of the fixed predictor terms were not applicable to some cases, however (e.g. the 'infected' predictor in analyses focusing on the hemiparasites), which is reflected in summaries of the models (Tables 1, S1). Biomass data were transformed by natural logarithm before the analyses in order to improve normality and homoscedasticity of residuals. Distribution of residuals was screened visually after fitting each of the models to verify that the assumptions of normality and homoscedasticity were not violated. Hemiparasite mortality between attachment and harvest of the experiment was analysed using a generalized mixed-effect model with binomial distribution and included the same model structure as the models described earlier. Selection of the best (i.e. minimal adequate) models followed the backward selection procedure. R, version 3.02 (R Core Team, 2013), was used for all statistical analyses. Linear and generalized linear mixed-effect models were fitted using R packages nlme (version 3.11; Pinheiro *et al.*, 2013) and lme4 (version 0.99; Bates *et al.*, 2013), respectively. Summaries of the models with tests of regression coefficients of individual terms are reported in the Results section and the Supporting Information. Further details of the models and individual steps of model selection are provided in Methods S1 (main results analyses) and Methods S2 (supplementary analyses).

Results

Host performance

Growth of uninfected hosts was much greater at both higher nutrient concentrations and higher irrigation (Fig. 2a; Table 1). The effects of the abiotic conditions were additive on the log-biomass scale (which implies multiplicativity on the original biomass

Table 1 Summary of the mixed-effect models describing the effects of host species identity, abitotic resources and infection by the hemiparasite on parameters of host and hemiparasite performance

	Hosts					*Rhinanthus*			
	Biomass production	Biomass production (uninfected hosts)	F_v/F_m	Shoot : root ratio	Response to parasitism	Survival	Biomass production	F_v/F_m	Proportion of host-derived C in biomass
Host species	*** M	*** M	*** W	** M			ns	** W	▨
Irrigation	* ↑	*** ↑	ns	** ↑	ns		ns		ns
Nutrients	*** ↑	*** ↑	* ↑	*** ↑	ns	* ↓	*** ↑	*** ↑	** ↓
Infected	*** ↓	▨	ns	*** ↓	▨	▨	▨	▨	▨
Host × irrigation			ns				ns		▨
Host × nutrients			ns				ns	**	▨
Irrigation × nutrients	ns		ns		**		*		**
Host × infected		▨	ns		▨	▨	▨	▨	▨
Irrigation × infected	ns	▨	ns		▨	▨	▨	▨	▨
Nutrients × infected	ns	▨	***		▨	▨	▨	▨	▨
Host × irrigation × nutrients			ns						▨
Host × irrigation × infected		▨	ns		▨	▨	▨	▨	▨
Host × nutrients × infected		▨	***		▨	▨	▨	▨	▨
Irrigation × nutrients × infected	**	▨	***		▨	▨	▨	▨	▨
Host × irrigation × nutrients × infected		▨	***		▨	▨	▨	▨	▨

Empty and grey fields, terms omitted from the model during model selection and terms which are not available for a particular model, respectively. Arrows, the direction of change in the response variable for statistically significant main effects (an up or down arrow indicates that a response variable increases or decreases with that factor, respectively); M or W, the host species (maize or wheat) associated with the higher values of a response variable.
***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, terms with nonsignificant regression slopes retained in the model due to significant higher-order interactions.

scale based on the mathematical rule log a + log b = log ab) as indicated by the nonsignificant irrigation × nutrients interaction ($F_{1,40} = 0.18$, $P = 0.67$; unparazitised hosts only); that is, autotrophic plant growth was limited by both, either or none of the abiotic resources depending on the actual treatment. The observed additivity is important for interpretation of the effect of the irrigation × nutrients interaction on other parameters of the hemiparasitic association, as the main effects of nutrient and water availability on plants cannot be unequivocally separated, because of confounding intrinsic processes (see the Materials and Methods section for more details).

Host growth was significantly depressed in pots containing the hemiparasite (Fig. 2a; Table 1). This suppression of growth affected the host shoots more than the roots (Fig. S2), which resulted in a lower host shoot : root ratio in all infected pots compared with uninfected plants (Fig. 2c). Hemiparasite infection also added complexity to the pattern of host biomass production, as indicated by the significant irrigation × nutrients × parasite interaction (Table 1). This complexity is most visible in the pattern of the host response to parasitism (Fig. 2d) where it corresponds to the irrigation × nutrients interaction. The largest negative host response in host biomass occurred under conditions when supply of one of the abiotic resources was restricted while the other was abundant. The effect of hemiparasitism on the host was generally lower if either both or none of the resources were abundant. The pattern of host growth did not coincide with the pattern of maximum quantum yield of photosystem II (F_v/F_m ratio; Fig. 2b), which was dominated by a prominent decrease of the F_v/F_m value of maize under the W−N+ conditions. Not only did infection by the hemiparasite decrease biomass production of the host, but also the total biomass production in individual pots (Fig. S3), because hemiparasite biomass did not compensate for the biomass loss inflicted on the host through infection.

Hemiparasite performance

Attached *R. alectorolophus* plants suffered an overall 19.3% mortality rate between attachment and the harvest of the experiment. The incidence of premature death was significantly higher in hemiparasites growing at high nutrient concentrations (Fig. 3a; Table 1). Divergent amounts of nutrients and irrigation had significant and mostly interactive effects on the growth of the surviving hemiparasites and their photosynthetic ability (Table 1). Under low-nutrient conditions, the hemiparasites produced a small amount of above-ground biomass, but their growth was increased by high irrigation (Fig. 3b). The high nutrient concentration strongly increased biomass production of the hemiparasites but the effect of irrigation was reversed compared with the low-nutrient conditions (Fig. 3b; significant nutrients × irrigation term in Table 1). As a result, the highest hemiparasite biomass production was recorded under the W−N+ conditions. In contrast to the hosts, the pattern of biomass production of the hemiparasites coincided with the pattern of maximum quantum yield of photosystem II (F_v/F_m ratio; Fig. 3c) and there was a

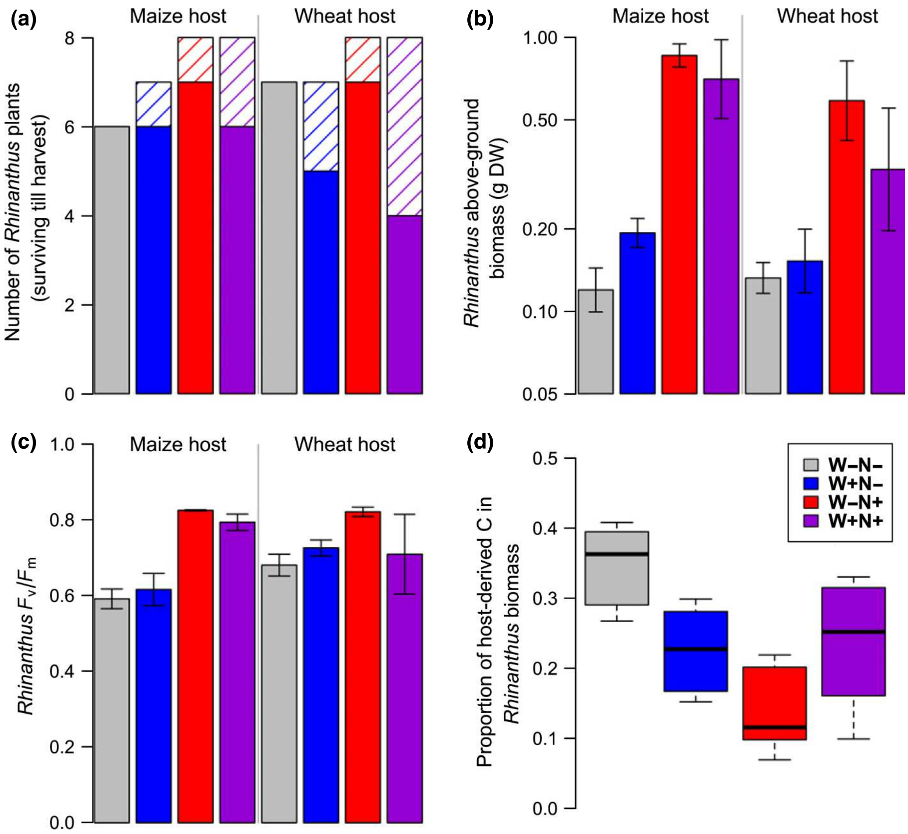


Fig. 3 *Rhinanthus alectorolophus* performance parameters as affected by host species identity, and amount of irrigation and nutrient content. (a) Survival of *Rhinanthus* plants between their attachment to the host and harvest of the experiment. Raw counts of hemiparasite individuals are displayed; closed and hatched parts of bars indicate surviving and dead hemiparasites, respectively. (b) *Rhinanthus* dry above-ground biomass. (c) Maximum quantum yield of photosystem II (F_v/F_m). (d) Proportion of host-derived carbon (C) in *Rhinanthus* above-ground biomass. '−' indicates low and '+' high amounts of irrigation (W) and nutrient availability (N). See Table 1 for summaries of mixed-effect model analyses. Bar plots with linear (c) and logarithmic (b) *y*-axes represent arithmetic and geometric means, respectively, with associated standard errors. Boxplot (d) represents median, quartiles, and ranges.

significant positive correlation between the F_v/F_m ratio and log-transformed biomass production ($r = 0.713$, $t_{26} = 5.18$, $P < 0.001$).

Carbon flows in the host–hemiparasite association

Both hosts and hemiparasites displayed notable patterns in C stable isotopic composition of their biomass (Figs S4, S5; Table S1). The differences in isotopic composition of *Rhinanthus* biomass when attached to maize allowed the determination of the proportion of host-derived C under different treatment combinations. The proportion of host-derived C ranged from *c.* 5 to 40% and was significantly affected by nutrient concentration and its interaction with irrigation (Fig. 3d). The highest proportions of host-derived C were detected in hemiparasites growing under low amounts of both abiotic resources (W−N−), while the lowest proportions were recorded in hemiparasites growing under the W−N+ conditions. The proportion of host-derived C was negatively correlated with biomass production of the hemiparasites ($r = -0.712$, $t_{22} = -4.76$, $P < 0.001$).

Despite a substantial proportion of host-derived C in hemiparasite biomass, its absolute amount accounted for only *c.* 4–7% of biomass produced by the hosts (Fig. 4). The total above-ground biomass of the whole association was dominated by host biomass. However, this dominance was reduced under the W−N+ and W+N−, where hemiparasite biomass (both autotrophic and heterotrophic) accounted in average for 35 and 24.5% of the total biomass, respectively (Fig. 4).

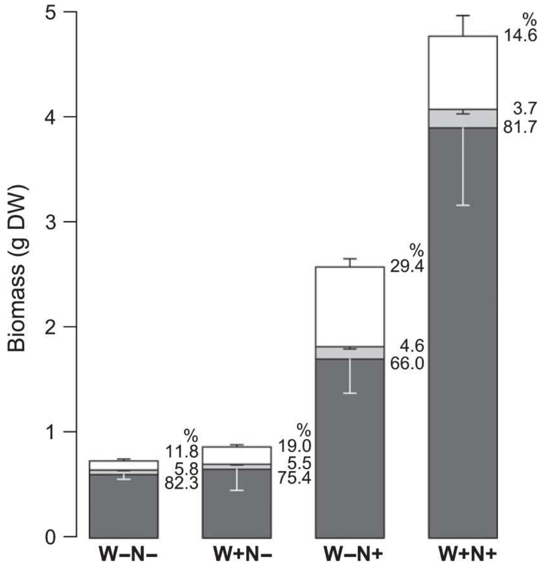


Fig. 4 Partitioning of above-ground biomass of the *Rhinanthus alectorolophus*–maize root-hemiparasitic association as affected by amount of irrigation and nutrient concentration. Dark grey bars, host biomass; light grey bars, heterotrophic hemiparasite biomass; white bars, autotrophic hemiparasite biomass. The biomass partitions are based on a combination of the proportion of host-derived carbon in hemiparasite biomass (Fig. 2d) and biomass production of hemiparasites and their hosts (Figs 1a, 2b). Mean above-ground biomass values are shown together with associated standard errors. In addition, percentages of biomass are displayed for each of the experimental treatments. '−' indicates low and '+' high amounts of irrigation (W) and nutrient availability (N).

Discussion

Influence of abiotic resources on hemiparasitic interaction

Survival of attached hemiparasites was the only key variable for which a significant main effect of nutrients was detected without additional significant interactive effects, suggesting its general validity irrespective of hydric conditions. The survival was significantly lower in the N+ treatments as observed in field experiments (van Hulst *et al.*, 1987; Mudrák & Lepš, 2010; Těšitel *et al.*, 2013), which can be attributed to the effect of above-ground competition from the hosts (Fibich *et al.*, 2010). Similarly, the decrease of hemiparasite performance in the W+N+ treatment compared with N+W− indicates competitive pressure of host shoot on the hemiparasite. Thus, despite the fact that we used a simplified system consisting of a single pair of host and hemiparasite, above-ground competition was present in our experimental setup, albeit at an intensity lower than that recorded in the field.

For the first time, we provide evidence that mineral nutrient and water availability have an interactive effect on the key parameters of root-hemiparasitic association (host response to parasitism, hemiparasite photosynthetic efficiency and biomass production, and proportion of host-derived C in hemiparasite biomass; Figs 2d, 3b,c), while the same treatments had only an additive effect on the growth of nonparasitic plants. Although both of these abiotic resources have been demonstrated to affect the hemiparasitic interaction (Cechin & Press, 1994; Simier *et al.*, 2006; Cameron *et al.*, 2009; Ducarme & Wesselingh, 2009; Mudrák & Lepš, 2010), their significant interaction means that the explanatory power of experiments manipulating one of them is limited.

The significant interaction between water and mineral nutrient availability indicates that the direction of changes in the interaction between hemiparasite and its host induced by elevated nutrient concentration is affected by the availability of water. Although both growth of the host (Fig. 2a) and hemiparasite (Fig. 3b), as well as the hemiparasite's photosynthetic efficiency (Fig. 3c), were increased by high nutrient concentrations under both irrigation treatments, the balance (i.e. above-ground biomass ratio) between the host and hemiparasite was different. Under the high-irrigation treatments, the association showed trends that were expected based on the resource-competition hypothesis; that is, the balance between host and hemiparasite biomass was shifted towards the host under the W+N+ conditions compared with W+N−. Simultaneously abundant resources (W+N+) supported vigorous growth of the host and apparently relieved the hosts from the effect of parasitism. Nevertheless, such hosts also provided abundant organic resources to the hemiparasites, which partly counteracted the host competitive pressure on them. Under moist conditions, the increase of growth of hemiparasites induced by abundance of mineral nutrients is thus caused by both an increase in their photosynthetic efficiency (Fig. 3c) and acquisition of more organic resources from the host (Fig. 4).

The low-irrigation treatments showed a substantially different trend. The balance between host and hemiparasite biomass was largely shifted in favour of the hemiparasite under the W−N+ conditions, where the host suffered from a severe water stress. The hemiparasite benefited from an increase of its photosynthesis induced by abundance of mineral nutrients (Fig. 3c), while the competitive pressure from water-stressed hosts was lower compared with the W+N+ treatment (Fig. 3b). Although the absolute amount of C translocated from the host to the hemiparasite in W−N+ was higher than in the W−N− conditions (Fig. 4), its concentration in hemiparasite biomass (Fig. 3d) was the lowest among all treatments because of a high efficiency of parasite photoassimilation induced by abundant mineral nutrients (Fig. 3c).

This complexity and variability in the hemiparasitic interaction can be illustrated by a two-dimensional scheme depicting importance of the interaction components on the environmental gradients of moisture and mineral nutrient availability (Fig. 5). The extensive qualitative dynamics of the interaction outcome caused by environmental conditions updates the rather static traditional view of the hemiparasitism.

Physiological principles

The contrasting outcomes of the hemiparasitic interaction described earlier are based simply on quantitative adjustments of physiological processes that are well known to occur in root hemiparasites. Despite infecting its hosts below ground, parasitic resource acquisition in *Rhinanthus* is mostly driven by the shoot, which contains osmotically active sugar alcohols (Jiang *et al.*, 2005), displays a high transpiration rate (Klaren & van de Dijk, 1976; Jiang *et al.*, 2003) and can even actively secrete water by hydathode trichomes (Govier *et al.*, 1968; Těšitel & Tesařová, 2013), together generating a water potential gradient between host and *Rhinanthus* and so facilitating mass flow of nutrients to the parasite. The hemiparasite maintains this water potential difference, directing resource flow from the host through open vascular connections in the haustoria (Jiang *et al.*, 2003; Cameron *et al.*, 2006). Although not captured by our physiological measurements, it is likely that the water potential difference between the host and hemiparasite was strongly increased by contrasting behaviour of their stomata under water-stressed conditions (Ehleringer & Marshall, 1995). This probably increased the hemiparasite's scavenging of host xylem-borne resources, further supporting its growth under the W−N+ conditions in our experiment. Such positive feedback can explain both the vigorous growth of the hemiparasites and harm inflicted to the hosts under W−N+ conditions. By contrast, limited parasite shoot growth as a result of nutrient deficiency (N− conditions) decreased the absolute amount of resources acquired from the host, because of lower total leaf area for transpiration (Fig. 4). Host-derived C, however, constituted a relatively high proportion of the hemiparasite's biomass under such limited growth conditions as a result of the low rate of parasite photosynthesis (Fig. 3c,d) caused by low water and photosynthetic nutrient-use efficiency when nutrients are deficient (Seel & Press, 1994). A similar increase of host-derived C proportion in hemiparasite biomass occurs if its own photosynthesis is limited by competition for light (Fig. 3d: W+N+ conditions; see also Těšitel *et al.*, 2011). In all, this demonstrates that host-derived C can make a substantial contribution to hemiparasite C budget; however, this resource is of the highest importance in stress conditions when the hemiparasite's own photosynthetic activity is limited. Thus, it can be considered as a backup resource. By contrast, the hemiparasite's own photosynthesis plays a crucial role in the exploitative hemiparasitic strategy of *Rhinanthus* by vastly increasing hemiparasite fitness if mineral nutrients and light are abundant. This conflicts with an earlier view from Press (1989), who reported low amounts of photosynthesis, hardly exceeding dark respiration, and pointed out its insufficiency for sustaining growth of several hemiparasitic species of Orobanchaceae. Our experimental data suggest that such physiological behaviour might be recorded only in hemiparasites growing under nutrient-deficient conditions.

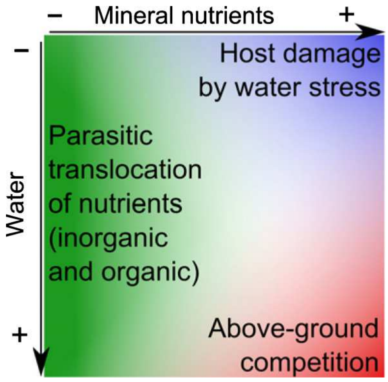


Fig. 5 Proposed general scheme of the ecological interaction between a root hemiparasite and its host on the gradients of nutrient availability and soil moisture. The colours and their intensities indicate the relative importance of individual components of the interaction in relation to abundance of the below-ground abiotic resources; that is, how much the individual components contribute to hemiparasite growth and hemiparasitism-induced reduction of host growth relative to the other components. Note that the green colour intensity does not indicate the rate of nutrient translocation from host to hemiparasite. (See the Discussion section for further details).

Variation in root hemiparasite physiology and ecology

The exploitative hemiparasitic mechanism of *Rhinanthus* based on an open vascular connection appears most efficient under conditions with contrasting availability of water and mineral nutrients or when these resources are available at moderate levels. This strategy appears highly successful as indicated by the large number of species, global distribution and the variety of habitats that are colonized by the hemiparasitic Orobanchaceae (Heide-Jørgensen, 2008), which share their core hemiparasitism-related physiological traits, such as elevated transpiration rate lowering the hemiparasite water potential (Press *et al.*, 1988).

Simultaneous abundance of both water and mineral nutrients diminishes the advantage provided by hemiparasitism and results in the exclusion of root hemiparasites from the host community by above-ground competition. Root-hemiparasitic plants are therefore generally missing from humid and eutrophic habitats, except for those affected by disturbance where the intensity of competition is reduced (e.g. hemiparasitic *Odontites vulgaris* growing at various ruderal sites across Europe; Koutecký *et al.*, 2012). The hemiparasitic tree *Okoubaka aubrevillei* (Santalaceae) presents another exception to this trend. Growing in tropical rainforest, where competition for light is extremely limiting (Whitmore, 1990), this species evolved high virulence (of unknown mechanism), which increases the mortality of co-occurring fast-growing pioneer species (Veenendaal *et al.*, 1996).

By contrast, if both water and mineral nutrients are deficient, for example in some (semi)desert habitats, autotrophic plant growth becomes highly compromised. Although appropriate stress tolerance strategies evolved in the angiosperms to cope with such conditions (e.g. water-conserving succulents), these are largely incompatible with the exploitative root-hemiparasitic strategy of *Rhinanthus*. Root hemiparasitism still exists in nutrient-poor arid habitats represented by a number of species of two independently evolved hemiparasitic groups – Santalales and Krameriaceae. However, their haustoria do not feature an open vascular connection with the host xylem, and the host–hemiparasite water potential difference is maintained exclusively by an increased concentration of osmotically active compounds, while the rate of hemiparasite transpiration is similar to or even lower than that of the host (Tennakoon *et al.*, 1997; Tennakoon & Cameron, 2006; Brokamp *et al.*, 2012).

Another type of hemiparasitic interaction is present in perennial root hemiparasites growing in nutrient-poor environments, which are also limited by a cold climate with a short growing season. These species (exemplified by *Bartsia alpina* and *Castilleja occidentalis*; Quested *et al.*, 2003 and Spasojevic & Suding, 2011, respectively) have the potential to increase nutrient cycling and thus productivity of their plant communities by producing nutrient-rich litter, which not only fertilizes other species (including the hosts; Demey *et al.*, 2013; Fisher *et al.*, 2013) but also enhances the decomposition of other species' litter (Quested *et al.*, 2003; Spasojevic & Suding, 2011). Owing to the low intensity of competition for light at such sites, these hemiparasites can afford to increase the productivity of their community via the litter pathway, which also increases the abundance and/or quality of host resources.

Parallels and contrasts with partial mycopheterotrophs

Both root hemiparasites and partial mycoheterotrophs are green plants that acquire most mineral nutrients and a variable amount of organic C from their partners in hemiparasitic or mycoheterotrophic association, respectively. As such, they represent an unparalleled trophic level on the edge between autotrophy and heterotrophy. The proportions of autotrophic and heterotrophic assimilates in their C budget are affected by light availability in a similar way (Těšitel *et al.*, 2011; Gonneau *et al.*, 2014). Own assimilation represents an important resource contribution for partial mycoheterotrophs without which their fitness becomes depressed (Roy *et al.*, 2013). However, for root hemiparasites, it is a crucial process which transforms the resources acquired by xylem parasitism into growth and fitness, which is probably the basis of its evolutionary stability. In our study, we have demonstrated that autotrophy allows hemiparasites to utilize abundant mineral nutrients, which is a substantial update of the traditional view of hemiparasitism as a strategy that is most advantageous in oligotrophic environments. In addition, the hemiparasites' ability to act as ecosystem engineers by manipulating abiotic resources and suppressing host growth might in fact improve the conditions for further persistence (or even expansion) of their populations. This makes the apparent functional parallel between root hemiparasites and partial mycoheterotrophs much less straightforward than suggested by Selosse & Roy (2009).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Relative water content (in volumetric percent) in the substrate of the experimental pots measured before the final irrigation of the experiment.

Fig. S2 Root biomass of the two host species cultivated under individual experimental treatments.

Fig. S3 Pattern of the total above-ground biomass production in individual pots (i.e. sum of host + hemiparasite).

Fig. S4 Carbon stable isotope composition ($\delta^{13}C$) of bulk above-ground biomass of the two host species cultivated under individual experimental treatments.

Fig. S5 Carbon stable isotope composition ($\delta^{13}C$) of bulk above-ground biomass of *Rhinanthus alectorolophus* attached to either of the host species and cultivated under individual experimental treatments.

Table S1 Summary of mixed-effect models describing the effects of host species identity, abiotic resources and infection by the hemiparasite on the supplementary parameters of the experimental hemiparasitic associations

Methods S1 R code of model selection and summaries for the results reported in the main text.

Methods S2 R code of model selection and summaries for the results reported in the Supporting Information.

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Hydathode trichomes actively secreting water from leaves play a key role in the physiology and evolution of root-parasitic rhinanthoid Orobanchaceae

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- **Background and Aims** Root hemiparasites from the rhinanthoid clade of Orobanchaceae possess metabolically active glandular trichomes that have been suggested to function as hydathode trichomes actively secreting water, a process that may facilitate resource acquisition from the host plant's root xylem. However, no direct evidence relating the trichomes to water secretion exists, and carbon budgets associated with this energy-demanding process have not been determined.
- **Methods** Macro- and microscopic observations of the leaves of hemiparasitic *Rhinanthus alectorolophus* were conducted and night-time gas exchange was measured. Correlations were examined among the intensity of guttation, respiration and transpiration, and analysis of these correlations allowed the carbon budget of the trichome activity to be quantified. We examined the intensity of guttation, respiration and transpiration, correlations among which indicate active water secretion.
- **Key Results** Guttation was observed on the leaves of 50 % of the young, non-flowering plants that were examined, and microscopic observations revealed water secretion from the glandular trichomes present on the abaxial leaf side. Night-time rates of respiration and transpiration and the presence of guttation drops were positively correlated, which is a clear indicator of hydathode trichome activity. Subsequent physiological measurements on older, flowering plants indicated neither intense guttation nor the presence of correlations, which suggests that the peak activity of hydathodes is in the juvenile stage.
- **Conclusions** This study provides the first unequivocal evidence for the physiological role of the hydathode trichomes in active water secretion in the rhinanthoid Orobanchaceae. Depending on the concentration of organic elements calculated to be in the host xylem sap, the direct effect of water secretion on carbon balance ranges from close to neutral to positive. However, it is likely to be positive in the xylem-only feeding holoparasites of the genus *Lathraea*, which is closely related to *Rhinanthus*. Thus, water secretion by the hydathodes might be viewed as a physiological pre-adaptation in the evolution of holoparasitism in the rhinanthoid lineage of Orobanchaceae.

Key words: Ecophysiology, holoparasite, hydathode trichome, *Lathraea*, parasitic plant, respiration, *Rhinanthus alectorolophus*, rhinanthoid Orobanchaceae, orobanche, root hemiparasite, transpiration, *Triticum aestivum*, water regime, water secretion, xylem.

INTRODUCTION

About 1 % of flowering plants corresponding to 4500 species parasitize other plants by specialized organs called haustoria to acquire essential resources (Heide-Jørgensen, 2008). The majority of parasitic plant species are hemiparasites, green photosynthetic plants acquiring water, mineral nutrients and a certain amount of heterotrophic carbon from the host xylem (Press, 1989; Irving and Cameron, 2009; Těšitel *et al.*, 2010*a*; Heide-Jørgensen, 2013). In contrast, holoparasites completely lack photosynthetic ability and thus acquire all essential resources heterotrophically from the host (Hibberd and Jeschke, 2001; Irving and Cameron, 2009).

Holoparasites are generally thought to have evolved repeatedly from hemiparasites (Westwood *et al.*, 2010; McNeal *et al.*, 2013; Naumann *et al.*, 2013), but such an evolutionary transition can rarely be documented or studied due to the extinction of assumed hemiparasitic ancestors (Nickrent and Duff, 1996; Nickrent *et al.*, 1998; Naumann *et al.*, 2013). However, the family Orobanchaceae provides an opportunity to study the macroevolutionary transition between the trophic strategies of parasitic plants as it encompasses closely related non-parasitic, hemiparasitic and holoparasitic species (Bennett and Mathews, 2006; Heide-Jørgensen, 2008; Westwood *et al.*, 2010; McNeal *et al.*, 2013; Naumann *et al.*, 2013). This is the case of the sister genera *Rhinanthus* and *Lathraea*, and closely related *Rhynchocorys* which form a separate sub-clade within the Rhinanthoid clade of Orobanchaceae (Těšitel *et al.*, 2010*c*). Moreover, *Tozzia alpina*, another related Rhinanthoid species, displays a parallel evolutionary tendency towards holoparasitism (Těšitel *et al.*, 2010*c*).

Rhinanthus species are hemiparasitic annuals possessing a highly efficient resource acquisition strategy based on an open vascular connection with the host xylem (Cameron *et al.*, 2006) and a high transpiration rate directing the xylem stream from the host (Klaren and Janssen, 1978; Stewart and Press, 1990; Jiang *et al*., 2010). Despite the acquisition of substantial amount of carbon from the host in the form of xylem-mobile organic elements (Těšitel *et al*., 2010*a*, 2011), the hemiparasite's own photosynthesis plays a crucial role in realization of its fitness (Těšitel *et al*., 2015). Most of the species of the Rhinanthoid clade are in principal physiologically similar to *Rhinanthus*, i.e. they are photosynthetic root hemiparasites acquiring resources from the host root xylem (Těšitel *et al*., 2010*a*; McNeal *et al*., 2013).

In contrast, *Lathraea*, *T. alpina* and the perennial species *Rhynchocorys* are holoparasitic, at least in early ontogenic stages of underground individuals, but unlike most other holoparasitic species (Irving and Cameron, 2009) they do not feature a connection to the host phloem in their haustoria. *Lathraea* species are characterized by extensive perennial underground rhizomes covered by fleshy scales of leaf origin (Ziegler, 1955; Renaudin, 1966). Shoots are short lived and their only function is flowering and seed production. The third genus of the sub-clade, *Rhynchocorys*, contains both species which are morphologically similar to *Lathraea* (rhizomes with scales, e.g. *R. elephas*), but retain photosynthetic activity in their green above-ground shoots (Kubat and Weber, 1987), and annual species which are closely similar to *Rhinanthus* (e.g. *R. orientalis*) (Těšitel *et al*., 2010*c*). The plant architecture and physiological functioning of the more distantly related *T. alpina* are closely similar to those of perennial *Rhynchocorys* species and the species is also known to have only a xylem connection in its haustoria (Weber, 1973). As a result of the underground growth habit, these species cannot transpire to discharge excess water taken up from the host xylem, which requires an alternative mechanism of water secretion for their physiological functioning.

Hemiparasites of the Rhinanthoid clade of Orobanchaceae were shown to have glandular trichomes on the abaxial side of their leaves (Fedorowicz, 1915; Kaplan and Inceoglu, 2003; Těšitel and Tesařová, 2013), frequently located close to leaf veins (Govier *et al*., 1968). Anatomically identical trichomes were also revealed on the scales of the below-ground rhizomes of *Lathraea* and *Rhynchocorys* (Groom, 1897; Ziegler, 1955; Renaudin, 1966; Kubat and Weber, 1987). The ultrastructure of these trichomes revealed numerous mitochondria, labyrinthine cell walls and plasmodesmata, structures suggesting their high metabolic activity (Schnepf, 1964; Renaudin and Garrigues, 1967; Těšitel and Tesařová, 2013). Govier *et al*. (1968) suggested a function of the trichomes as hydathode trichomes actively secreting water based on their observation of guttation from the leaves of hemiparasitic *Odontites vernus* Dumort. and a radioisotope tracing experiment. Moreover, extensive water secretion was also observed from the underground scale-like leaves of *Lathraea*. First reported by Darwin (1880), the secretion was later suggested to be associated with the glandular trichomes (Renaudin and Garrigues, 1967). To sum up, there is convincing evidence of the presence of metabolically active glandular trichomes in the Rhinanthoid Orobanchaceae and of an intense water secretion from the leaves of these parasitic plants. However, direct evidence relating the trichomes to water secretion and the carbon budget of the assumed, energy-demanding water secretion is yet to be revealed.

In this study, we aim to present conclusive direct evidence on the physiological role of the assumed hydathode trichomes and integrate their function into the physiology of hemiparasites. Macroscopic and microscopic observations were combined with gas exchange measurements to capture the physiological activity of the trichomes on the leaves of hemiparasitic *Rhinanthus alectorolophus*. Using the gas exchange measurements, we were able to estimate the carbon budget of the hydathode trichome activity. Moreover, our experimental set-up allowed testing of the effects of the hemiparasite developmental stage and availability of below-ground abiotic resources on the hydathode trichome activity.

MATERIALS AND METHODS

Plant material

Seeds of *Rhinanthus alectorolophus* (Scop.) Pollich were collected from the natural population near Zechovice, Czech Republic (49 °09′28″N, 13 °52′13″E; 510 m a.s.l.). Seeds of wheat (*Triticum aestivum* L.) used as a host species were obtained from the school farm of the Faculty of Agriculture, University of South Bohemia.

Experimental design and conditions

The experiment was carried out in a growth chamber at the Faculty of Science, University of South Bohemia from December 2013 to March 2014. Three-day-old seedlings of wheat germinated on a Petri dish with moist filter paper were sown to 0·8 L pots (one seedling per pot) filled with a mixture of sand and peat (1:1, v/v ratio). Half of the pots received 1 g of Osmocote Exact Standard 5–6 M fertilizer (Scotts Miracle-Gro Company, UK) per litre of substrate (high nutrient treatment, N+). According to the manufacturer's specifications, the fertilizer contains 150 mg N g^{-1}, 90 mg P g^{-1} and 120 mg K g^{-1}. The other half of the pots did not receive any additional nutrients (low nutrient treatment, N–). All pots ($n = 98$) were well watered and maintained in the growth chamber with a 12 h light/12 h dark cycle and temperature regime of 20–22 °C (light):17–18 °C (dark). The photosynthetically active radiation (PAR) intensity during the day period was from 400 to 500 µmol m^{-2} s^{-1}. The pots were randomized once a week to filter out possible heterogeneity in non-treatment cultivation conditions (mainly PAR intensity). Seedlings of *R. alectorolophus*, pre-germinated on moist filter paper at 4 °C after approx. 8 weeks, were added to the pots (two seedlings per pot) 1 d after wheat sowing. The hemiparasite seedlings were thinned to one per pot, and two contrasting water regimes were established 27 d after *Rhinanthus* sowing (DAS). High irrigation pots (W+) and low irrigation pots (W–) received 150 and 100 mL of tap water every fourth day, respectively. The nutrient and watering treatments were established in a full factorial design. The purpose of the nutrient and water treatments was to create certain environmental variability since hemiparasite physiology is known to be profoundly affected by the availability of these abiotic resources (Těšitel *et al*., 2015). However, the length of the simulated environmental gradients was much shorter than in the study of Těšitel *et al*. (2015) and was not of primary interest in our study.

Two sets consisting of 20 plants (i.e. five individual plants per each treatment combination, Supplementary Data Table S1) were selected for observations and physiological measurements conducted before and during the peak flowering period (55 and 73 DAS, Supplementary Data Fig. S1A, B). The plants were watered (following the watering protocol) several hours before the measurements. Repeated measurements on individual plants usually could not be performed due to frequent mortality of plants that had been subjected to the first measurement. Elevated plant mortality was probably caused by accidental mechanic damage.

Macroscopic and microscopic observations

The leaf surface of plants to be measured by gas exchange (see 'Gas exchange measurements') was examined for the density and size of guttation drops immediately before the measurements. Drops were classified on an ordinal scale (0, no drops; 0·5, small drops, i.e. $<$25 % leaf area covered by guttation drops; and 1, large drops, i. e. $>$25 % leaf area covered by guttation drops; Fig. 1). Leaves of *R. alectorolophus* were detached from some of the young non-flowering plants cultivated under each treatment combination and cut with a razor blade into thin sections. These sections were placed in either water or mineral oil as mounting media and subsequently subjected to light microscopy using an Olympus CX41 Microscope (Olympus Imaging America Inc., Center Valley, PA, USA) and INFINITY1-3 C 3.1 MP CMOS Color Camera (Lumenera Corp., Ottawa, Canada).

Gas exchange measurements

Night-time rates of respiration (μmol CO_2 m^{-2} s^{-1}) and transpiration (mmol H_2O m^{-2} s^{-1}) were measured on intact leaves with a Li-6400 Portable Photosynthetic System (Li-Cor, Lincoln, NE, USA) coupled to a 2 cm^2 circular leaf chamber. Each measurement was done between 0200 and 0900 h at ambient temperature and an air relative humidity of 65–70 % in the dark. Air relative humidity inside the measurement chamber and ambient CO_2 concentration were controlled at 60–75 % and 400 μmol mol^{-1}, respectively. The surface of the leaves subjected to measurements had been dried by filter paper prior to the gas exchange measurements. Dark respiration and transpiration rates were recorded in 5 s intervals for approx. 3 min after a steady-state gas exchange rate was achieved. The surface of the measured leaves was dry before and after the gas exchange measurements. Mean values of these measurement series were then used in the data analysis as respiration and transpiration rates of the corresponding plants.

In addition, the relative water content (RWC) of substrate was measured in the pots used in the gas exchange measurements with an HH2 Moisture Meter with an SM200 sensor (Delta-T Devices Ltd, Cambridge, UK).

Carbon budget calculations

Gas exchange measurements allowed us to estimate the concentration of organic carbon in the xylem sap of the hemiparasite necessary to compensate the carbon loss through respiration. Since no studies on the efficiency of carbon filtering from the xylem sap of hemiparasites were available, we assumed only the concentration of organic carbon in the xylem sap (i.e. filtering efficiency of 100 %) in the calculation of the carbon budget of the hydathode trichome activity (Supplementary Data Methods). Therefore, our carbon budget calculation indicates the maximal possible carbon acquisition from the xylem sap. In reality this might be lower, which is reflected in the discussion.

Data analysis

Linear (LM) and generalized linear models (GLM) were used to analyse the effect of developmental stage and water and nutrient treatments on the physiological parameters of *Rhinanthus* plants. Respiration and transpiration rates were analysed by LMs, while binomial GLM was used to analyse the presence and size of guttation drops, which was allowed by the quasi-binomial coding. The correlation between night-time transpiration and respiration rates was analysed as a linear regression (respiration–transpiration), which produces numerical results identical to Pearson correlation. All analyses were conducted in R, version 3.0.1 (R Core Team, 2013). The relationships among all treatments and parameters monitored were summarized by principal component analyses (one analysis for each of the two developmental stages) included as Supplementary Data Fig. S3. These analyses were based on the

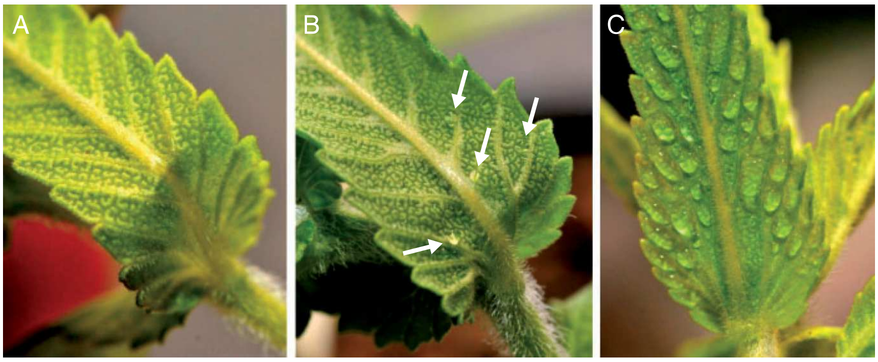

FIG. 1. The density and size of drops on the leaves of *Rhinanthus alectorolophus* (55 d after sowing) classified on an ordinal scale: (A) no drops (0), (B) small drops (0·5), (C) large drops (1). The plant was cultivated under (A) low irrigation and nutrient treatment, (B) low irrigation and high nutrient treatment, and (C) high irrigation and nutrient treatment. Images were taken immediately before the physiological measurement.

TABLE 1. *Summary of (generalized) linear models testing the effects of developmental stage, water and nutrient treatment on the presence and size of guttation drops, respiration and transpiration rates in* R. alectoroplophus

		Drops		Respiration			Transpiration		
Effect	d.f.	Deviance	*P*	Sum Sq.	*F*	*P*	Sum Sq.	*F*	*P*
Nutrients	1	4·39	**0·0362**	3·17	10·23	**0·0031**	0·36	0·51	0·48
Water	1	0·04	0·84	0·03	0·10	0·76	0·60	0·84	0·37
Stage	1	7·08	**0·0078**	0·0002	0·0005	0·98	9·28	13·02	**0·0010**
Nutrients × Water	1	0·65	0·42	0·16	0·52	0·48	0·76	1·07	0·31
Nutrients × Stage	1	0·50	0·48	0·29	0·95	0·34	0·01	0·01	0·93
Water × Stage	1	0·13	0·72	1·02	3·30	0·08	1·99	2·79	0·10
Nutrients × Water × Stage	1	0·00	1·00	0·56	1·79	0·19	0·50	0·70	0·41
Residuals	32	21·37		9·92			22·80		

Statistically significant results ($P < 0{\cdot}05$) are highlighted in bold.
Non-significant terms ($P > 0{\cdot}05$) were omitted from the final models.

variables centred by mean subtraction and standardized by dividing by the standard deviation, and were performed in Canoco for Windows, version 5 (ter Braak and Šmilauer, 2012).

RESULTS

Macroscopic and microscopic observations

Guttation drops were observed on the abaxial leaf surface of 50 % of non-flowering plants (55 DAS) and 15 % of flowering plants (73 DAS). The presence and size of drops were significantly ($P < 0{\cdot}05$) affected by the developmental stage of a plant and nutrient treatment (Table 1). The presence of large drops was significantly higher under the N+ treatment ($z = 2{\cdot}076$, $P = 0{\cdot}038$) and lower in flowering plants ($z = -2{\cdot}311$, $P = 0{\cdot}021$). No large drops were found on flowering plants (Supplementary Data Table S1). Both stalked and sessile hydathode trichomes were observed on the abaxial leaf surface of examined plants of all treatments. They were omnipresent on the abaxial surface, but sporadically occurred also on the adaxial surface. Microscopic observation in mineral oil revealed drops of liquid secreted from both trichome types (Figs 2A–D and 3A–F). No drops of liquid were observed in water as the mounting medium (Supplementary Data Fig. S2).

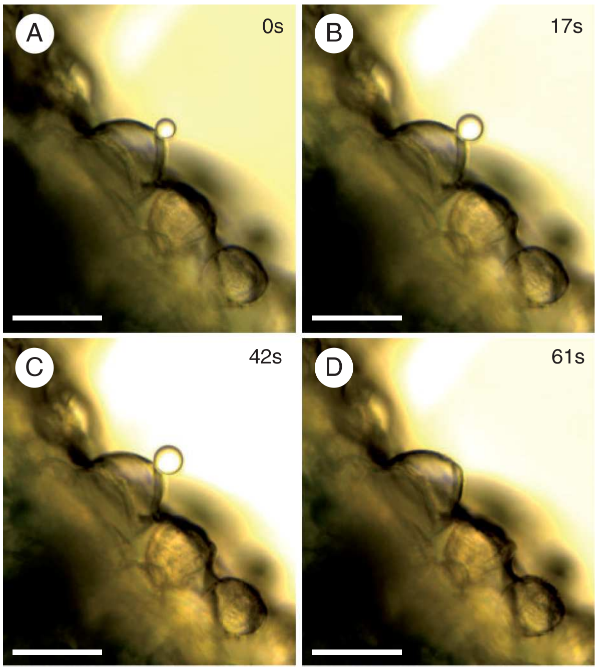

FIG. 2. Micrographs showing secretion from sessile hydathode trichomes on the abaxial leaf surface of *Rhinanthus alectorolophus*. The secretion was observed in oil shortly after immersion of the sample (0s, A) and in the time series as indicated (B–D). The drop of liquid finally detached from the trichome and moved out of view (D). The scale bars indicate 50 µm.

Gas exchange measurements

Dark respiration and transpiration rates were affected by the nutrient treatment and developmental stage, respectively (Table 1). Flowering *Rhinanthus* plants had lower transpiration rates than those measured before flowering ($t_{38} = -3{\cdot}613$, $P < 0{\cdot}001$). *Rhinanthus* cultivated under the N+ treatment displayed a higher dark respiration rate ($t_{38} = 3{\cdot}172$, $P = 0{\cdot}003$). Regardless of the significant effect of the water treatment on the RWC in pots (Welch two sample t-test: $t_{32.3} = 3{\cdot}005$, $P = 0{\cdot}005$), it did not have any significant effect on the gas exchange parameters (Table 1).

The gas exchange measurements revealed a strong positive relationship between night-time respiration and transpiration rates in non-flowering *R. alectorolophus* (Fig. 4A). The regression slope estimate was 0·55, which corresponds to 0·55 µmol respired carbon for the release of 1 mmol water in the form of guttation drops and stomatal transpiration. Moreover, both processes were also positively associated with the presence and size of guttation drops (Figs 4A and 5). The positive correlation among transpiration, respiration and size of the guttation drops is also demonstrated by the principal component analysis (Supplementary Data Fig. S3). In contrast, flowering hemiparasites exhibited no such relationship between the gas exchange physiological processes (Fig. 4B; Table S1; Fig. S3).

DISCUSSION

The combination of macroscopic and microscopic observations with the gas exchange measurements of *Rhinanthus* leaves

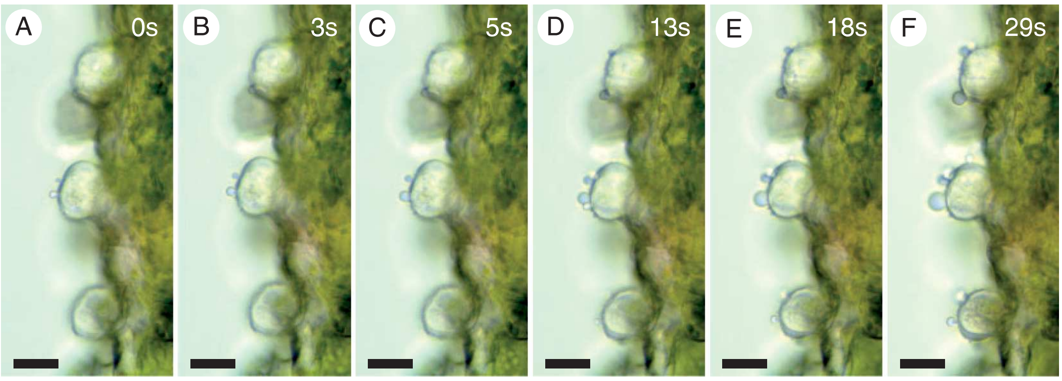

FIG. 3. Micrographs showing secretion from stalked hydathode trichomes on the abaxial leaf surface of *Rhinanthus alectorolophus*. The secretion was observed in oil shortly after immersion of the sample (0s, A) and in the time series as indicated (B–F). The scale bars indicate 25 µm.

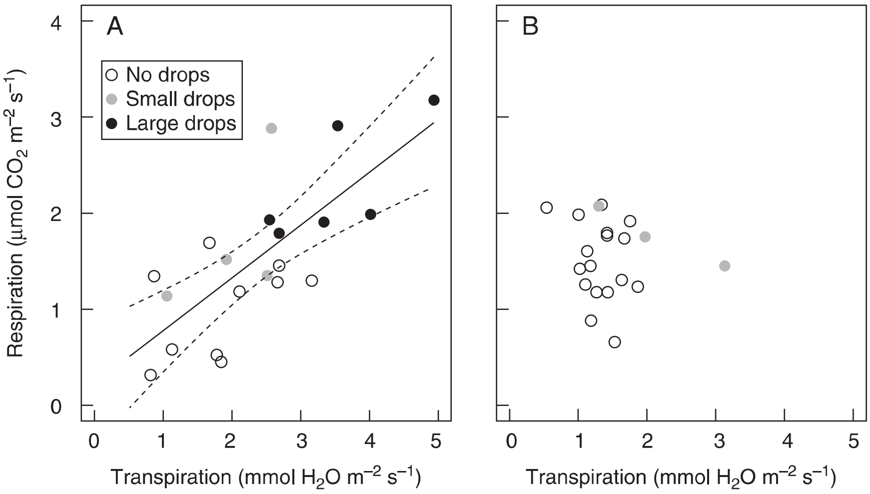

FIG. 4. The relationship between the night-time rates of respiration and transpiration in (A) non-flowering and (B) flowering *Rhinanthus alectorolophus*. Each circle relates to one individual plant. The size of drops observed on the leaves of examined plants immediately before the physiological measurement is indicated in the key. Linear regression ($r^2 = 0{\cdot}55$, $F_{1,18} = 22{\cdot}31$, $P < 0{\cdot}001$) and the 95 % confidence interval are presented by solid and dashed lines, respectively. No large drops were observed on the leaves of flowering plants.

provided the first unequivocal direct evidence on the physiological role of hydathode trichomes in water secretion in the Rhinanthoid Orobanchaceae. Their role is further supported by their ultrastructure (Schnepf, 1964; Renaudin and Garrigues, 1967; Těšitel and Tesařová, 2013) and explains earlier field measurements documenting an elevated night-time respiration and its correlation with night-time transpiration in multiple young hemiparasitic species (Press et al., 1988; Press, 1989). A similar relationship was found here in young leaves of *R. alectorolophus* and it was correlated with the presence and size of guttation drops secreted from hydathode trichomes.

The observed effects of developmental stage (young vs. flowering plants) and nutrient availability on the hydathode trichome activity provide a partial explanation of the high variability in the respiration rate and net photosynthesis reported in the Rhinanthoid hemiparasites (Press *et al.*, 1988; Press, 1989; Seel and Press, 1993; Lechowski, 1996; Těšitel *et al.*, 2011). The other part of the explanation lies in well-known effects of host species and nutrient availability on the photosynthetic efficiency and growth of hemiparasites (van Hulst *et al.*, 1987; Seel *et al.*, 1993; Cameron and Seel, 2007; Mudrák and Lepš, 2010; Těšitel *et al.*, 2013, 2015). Thus, the physiological functioning of attached hemiparasites is highly plastic, depending not only on the host quality and environmental conditions, but also on the developmental stage. This should be considered in all ecophysiological studies focusing on the Rhinanthoid hemiparasites as it is unlikely to capture the activity of hydathode trichomes during standard photosynthetic measurements (e.g. light response curves) of flowering specimens.

Resource acquisition from the host is driven by the water potential difference between the host and parasites in xylem-feeding parasitic plants (Ehleringer and Marshall, 1995; Seel and Jeschke, 1999; Hibberd and Jeschke, 2001). A strongly negative water potential is maintained by the high content of osmotically active compounds (such as sugar alcohols) and the elevated transpiration rate, physiological traits shared by many Rhinanthoid Orobanchaceae (Hodgson, 1973; Press *et al.*, 1988; Ehleringer and Marshall, 1995; Jiang *et al.*, 2003;

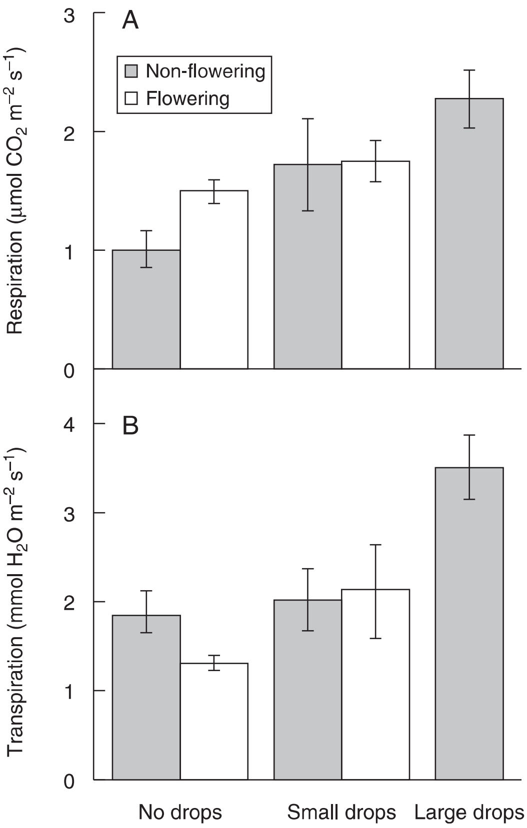

FIG. 5. Rates of respiration (A) and transpiration (B) measured on the leaves of *Rhinanthus alectorolophus* with various sizes of water drops at the two developmental stages of the plants. Means and standard errors are presented. Non-flowering plants (55 d after sowing) and flowering plants (73 d after sowing) are indicated in the key. No flowering plants with large drops were recorded.

Phoenix and Press, 2004). Stomata of some hemiparasitic species including *Rhinanthus* spp. are insensitive to abscisic acid and remain open even at night or under water stress (Smith and Stewart, 1990; Jiang *et al.*, 2003). Still, the hemiparasite's night transpiration rate is very low due to high ambient relative air humidity. Driving the xylem stream during night-time independently of air humidity, the active water secretion by hydathode trichomes can play a crucial role of an additional mechanism decreasing the water potential. The hemiparasite does not compete with the host shoot for the host xylem stream under these conditions, which results in an exclusive flow of the xylem sap to the hemiparasite strongly facilitating resource acquisition. Such a role for hydathode trichomes in plant mineral nutrition and water balance is not unique to the (hemi)parasitic plants discussed here. These structures were suggested to play a similar role in young leaves of some non-parasitic plants, in particular under the conditions when transpiration is low (Frey-Wyssling, 1941; Höhn 1950; Klepper and Kaufmann, 1966; Heide-Jørgensen, 1980). The mechanism of active water secretion from hydathode trichomes, when water is transported through the cell wall against its osmotic potential, is not known yet. Nevertheless, recent studies suggest that water secretion may be driven by a co-transport of water and ions through specialized protein co-transporters (Zeuthen and MacAulay, 2012; Wegner, 2014).

Despite requiring energy, the water secretion from the hydathode trichomes is highly efficient according to our gas exchange measurements (1 mmol water release per the loss of 0·55 µmol C) (Fig. 4A). The effect of water secretion on the carbon balance of hemiparasites depends on the concentration of carbon in the xylem sap (Těšitel *et al.*, 2010*b*, 2011; Bell and Adams, 2011) and the efficiency of its filtering from the sap on its way to the guttation fluid (Govier *et al.*, 1968). The organic carbon is contained in the xylem sap mostly in the form of organic acids, amino acids and sugars (Canny and McCully, 1988). The concentration of organic carbon (in terms of organic C atoms) in the xylem sap necessary to compensate the carbon loss through respiration is 31 mM (Supplementary Data Methods). Taking this concentration into account and considering the filtering efficiency of <100 %, we expect that the direct effect of water secretion on carbon balance would be close to neutral (Govier *et al.*, 1967; Seel and Jeschke, 1999; Alvarez *et al.*, 2008) to positive (Canny and McCully, 1988) in hemiparasites growing on grass species. Although the amount of organic carbon in the xylem sap of trees varied significantly between seasons, the effect of water secretion on carbon balance in holoparasitic *Lathraea* growing on tree species would be positive [Schill *et al.*, 1996; Heizmann *et al.*, 2001; Escher *et al.*, 2004; but not in all cases, see Furukawa *et al.* (2011); Supplementary Data Methods]. The positive carbon balance of the active water secretion by hydathode trichomes might be crucial for the evolution of the xylem-only feeding holoparasitic strategy of *Lathraea* (Ziegler, 1955) and early developmental stages of *Rhynchocorys* and *Tozzia* species (Weber, 1973; Kubat and Weber, 1987), which would not be able to compensate the negative carbon balance of the active water secretion by their own photosynthesis.

The increased activity of the hemiparasite hydathode trichomes under the N+ conditions probably reflects a generally better physiological performance of hemiparasitic plants. However, the host may also perform better under the N+ conditions and its competitive ability (in terms of competition for light) may increase. This can reduce the fitness of hemiparasites which are in general poor competitors (Matthies, 1995; Lepš, 1999; Mudrák and Lepš, 2010; Fibich *et al.*, 2010; Těšitel *et al.*, 2013) and decrease the effect of parasitism (Těšitel *et al.*, 2015). The increased activity of the hydathode trichomes might thus partially compensate this negative effect by facilitating host-derived carbon acquisition and also inflicting more harm to the host. Both of these effects would decrease the competitive ability of the host and shift the hemiparasite–host fitness balance in favour of the hemiparasite.

Conclusion

Hydathode trichomes might be seen as an evolutionary innovation facilitating the resource acquisition of hemiparasitic Rhinanthoid Orobanchaceae and decreasing the adverse effects of the competitive pressure from the host community. Given their ubiquity among the Rhinanthoid Orobanchaceae (Fedorowicz, 1915; Kaplan and Inceoglu, 2003), they might also be considered a physiological pre-adaptation allowing the evolution of the xylem-only feeding holoparasitic strategy. This xylem-only feeding holoparasitic strategy evolved two or three times independently within the Rhinanthoid clade, and the incomplete and complete transitions from hemiparasitism to holoparasitism in the Rhinanthoid clade represent relatively recent evolutionary events (Těšitel *et al*., 2010*c*; Scheunert *et al*., 2012; McNeal *et al*., 2013). The knowledge of the evolutionary mechanism of these transitions together with well-resolved phylogenetic relationships thus make the Rhinanthoid clade an ideal model group for studying the macroevolution of trophic strategies in parasitic plants.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournaljournal.org and consist of the following. Figure S1: images of hemiparasitic *Rhinanthus alectorolophus* before and during the peak flowering period. Figure S2: image of stalked and sessile hydathode trichomes on the abaxial leaf surface of *R. alectorolophus* in water as mounting medium. Figure S3: ordination diagrams correlating response data and environmental variables in non-flowering and flowering plants. Table S1: guttation, respiration, transpiration and relative water content data recorded in the study. Methods: carbon budget calculations regarding the activity of hydathode trichomes.

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VIEWS AND COMMENTS

How does elevated grassland productivity influence populations of root hemiparasites? Commentary on Borowicz and Armstrong (Oecologia 2012)

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Abstract In their recent study, Borowicz and Armstrong (Oecologia 169:783–792, 2012) investigated effects of nutrient availability and competition for light on a perennial root hemiparasite *Pedicularis canadensis*. Their study showed a reduction of community productivity as a result of hemiparasite infection independently of a clear positive effect of increased nutrients. In contrast, there was a minimal effect of increased competition for light on growth of the parasite. Here, we summarize the available data on the influence of nutrient availability (closely related to productivity) on temperate grassland root hemiparasites thus expanding the discussion presented by Borowicz and Armstrong (Oecologia 169:783–792, 2012). Most studies show that root hemiparasites are highly sensitive to elevated competition for light in productive environments, which is manifested as an increase in mortality coupled to a decrease in population density. Such responses reflect increased mortality of hemiparasite seedlings that are physiologically inefficient in terms of photosynthesis and nutrient acquisition owing to a limited root network and consequently, are highly sensitive to competition for light. However, the susceptibility of hemiparasites to competition for light tends to decrease for individuals that survive the critical seedling stage. Moreover, survivors benefit from elevated nutrient availability, resulting in increased growth and fecundity. Elevated productivity can thus have opposing effects on the survival and growth of hemiparasites depending on life stage. We conclude that the findings by Borowicz and Armstrong (Oecologia 169:783–792, 2012) are not in conflict with this general view that root hemiparasite population ecology is strongly influenced by competition for light in highly productive environments.

Keywords Competition · Eutrophication · Mineral nutrients · *Rhinanthus* · Seedling

In their recent paper, Borowicz and Armstrong (2012) investigate the effect of increased nutrient availability and competition for light (simulated by shading) on the growth and survival of *Pedicularis canadensis*, a perennial root hemiparasite growing in grassland and open woodland communities in eastern North America. Their study concluded that the growth of the parasite was positively affected by fertilizer addition, and in contrast, the parasite's effects on the community, namely suppression of host species and decrease of total biomass production, were independent of fertilizer application. The negative effect of shading on the parasite was only detected in one of the experiments. These results are in conflict with the authors'

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expectations that the hemiparasite should perform best, and have the highest impact on the host community under high light and low nutrient availability. According to these expectations, the fertilizer application and the imposition of shading should act to reduce the growth of the hemiparasite, resulting in a decrease of its effect on the plant community. The findings by Borowicz and Armstrong (2012) make a significant contribution to the literature on root hemiparasite ecology. However, we do not agree with the authors' conclusion that their results are in conflict with the current view on the role of nutrient limitation in the interactions between root hemiparasites and their hosts.

Elevated nutrient availability has been repeatedly shown to decrease hemiparasite abundance in plant communities (van Hulst et al. 1987; Cameron et al. 2009; Fibich et al. 2010—analysis of data collected by Hadač 1969; Hejcman et al. 2011a, b; Fig. 1a). This effect has been attributed to increased competition for light on eutrophic sites (Hautier et al. 2009) and a low competitive ability of hemiparasites relative to the surrounding plant community (Matthies 1995; Keith et al. 2004). While competitive ability is context specific, hemiparasite sensitivity to competition for light certainly varies with the developmental stage of the hemiparasite (Těšitel et al. 2011). Seedlings of hemiparasites that germinate without host induction and which live independently of a host in the initial period of their life are particularly sensitive to competition. This is due to the lack of abstraction of host-derived resources as well as inefficient photochemistry and nutrient acquisition (Seel et al. 1993; Lechowski 1996; Davies and Graves 1998). Moreover, Těšitel et al. (2011) demonstrated a substantial suppression of the growth of shaded young seedlings of *Rhinanthus alectorolophus* even after their attachment to the host, and in spite of their ability to acquire and metabolise a substantial amount of host-derived organic carbon (Těšitel et al. 2010a, 2011). However, shading of the same intensity typically had a lesser effect on older hemiparasitic plants, suggesting that the seedling stage (both prior to the attachment and those plants that had recently attached) represents a significant demographic bottleneck for populations of hemiparasites (similar to many non-parasitic grassland species; Grubb 1977). Increased competitive pressure on hemiparasites caused by elevated productivity therefore tends to manifest itself through increased seedling mortality (van Hulst et al. 1987; Mudrák and Lepš 2010; Hejcman et al. 2011a, b), resulting in a decrease of population density or even localized extinction of the hemiparasite. By contrast, studies investigating the effect of competition for light on survival and growth of hemiparasites at a later stage of development in the greenhouse (Hwangbo and Seel 2002) and in the field (Borowicz and Armstrong 2012) did not find any effect of decreased irradiation, which would be comparable to the detrimental effect of shading on seedlings. Nevertheless, in their greenhouse experiment, Borowicz and Armstrong (2012) detected a negative effect of light competition on *P. canadensis* seedlings, which indicates that the competition also affects the demography of this perennial species. In contrast to populations of annual *Rhinanthus* spp., the *Pedicularis* population does not need to establish itself each year, resulting in a long-term response of the population to fertilizer application.

The dynamics of hemiparasite populations along a productivity gradient are nonetheless more complex than simply a competitive interaction acting through increased

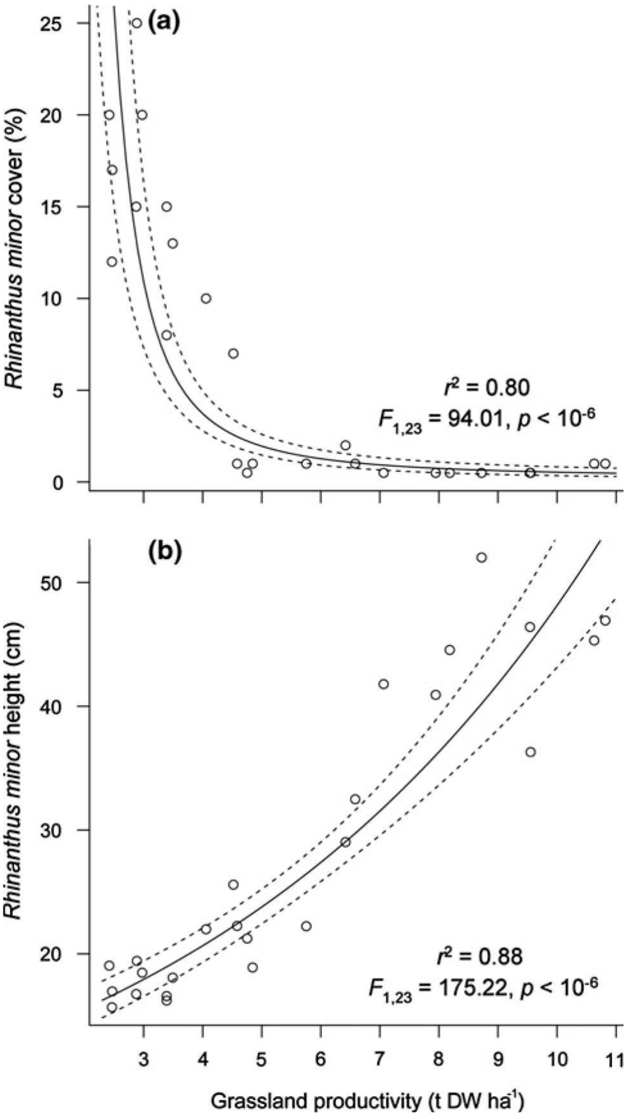

Fig. 1 Effect of grassland productivity on **a** percent cover and **b** mean height of spontaneously occurring *Rhinanthus minor* in the Rengen Grassland Experiment [see Hejcman et al. (2010) for further details on the experimental design]. For both, the response variable was log-transformed prior to the analysis and fitted values were back-transformed for the plotting purpose, $n = 25$. The model estimate (*solid line*) and 95 % confidence intervals are shown. *DW* Dry weight

seedling mortality. Hemiparasites are green, photosynthetic plants, acquiring most of their organic carbon via autotrophy (although a substantial fraction is also acquired heterotrophically from the host via the mass flow of organic and amino acids in the xylem stream; see Těšitel et al. 2010b for a review). Therefore, as with non-parasitic plants, hemiparasites require mineral nutrients such as nitrogen and phosphorus to synthesize constituents of their photosynthetic apparatus. Increasing the availability of these mineral nutrients can hence result in elevated rates of photosynthesis and growth in both hemiparasites and their hosts. Furthermore, high host growth rates can have an additional positive effect on hemiparasite performance (Hautier et al. 2010). The results from numerous experimental studies provide a rather ambiguous picture of hemiparasite response to elevated nutrient availability since both increased (van Hulst et al. 1987; Matthies and Egli 1999; Mudrák and Lepš 2010; Borowicz and Armstrong 2012; Hejcman et al. 2011b; Fig. 1b) and suppressed (Davies and Graves 2000; Jiang et al. 2010) growth or fecundity have been reported. While the first group of studies mostly represent field experiments or observations, the latter two studies are based exclusively on mesocosm experiments, in which the hosts were initially cultivated without hemiparasites and infected after a few weeks of development. This clearly contrasts to the establishment process under the natural conditions given that hemiparasites tend to germinate very early (at the end of the winter season) when their hosts are still dormant, which provides an important competitive advantage over co-existing species including the hosts (ter Borg 2005; Těšitel et al. 2011). As a result, these data, derived from field experiments, provide stronger evidence for a positive effect of productivity on performance of hemiparasite individuals compared to the mesocosm experiments suggesting the opposite trend. Moreover, hemiparasites in all the field studies reviewed, grew larger under increased nutrient availability (van Hulst et al. 1987; Mudrák and Lepš 2010; Hejcman et al. 2011b; Fig. 1b) despite facing increased competitive pressure from the surrounding vegetation, an interaction that was manifested via increased seedling mortality [except for Borowicz and Armstrong (2012), where this was not tested].

Elevated nutrient availability and increased community productivity have an inconsistent effect on root hemiparasites; on the one hand this can result in increased mortality of seedlings as a result of more intense competitive pressure, which can have even fatal consequences for the population. On the other hand, elevated nutrient availability supports the growth and fecundity of the survivors. In our opinion, the results presented by Borowicz and Armstrong (2012) fit well with this view of the ecology of grassland root hemiparasites. This is all the more remarkable given *Pedicularis canadensis*, the model species used in the study, displays a perennial life history as opposed to the annual life history of the hemiparasite species on which this concept is based.

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Habitats and ecological niches of root-hemiparasitic plants: an assessment based on a large database of vegetation plots

Biotopy a ekologické niky kořenových poloparazitů: zhodnocení na základě velké fytocenologické databáze

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Root hemiparasites are a specialized group of green photosynthetic plants that obtain resources from the roots of other plants. Some root hemiparasites are considered to be important keystone species in temperate grasslands while others are listed as endangered. In this study, we used vegetation-plot data from the Czech National Phytosociological Database to construct habitat suitability models for root hemiparasites occurring in the Czech Republic. These models were based on a formalized vegetation classification, species co-occurrence patterns in vegetation units and actual presence of hemiparasitic species in the database. The resulting habitat models defined as sets of suitable plots for each species were further described by a climatic gradient, community Ellenberg indicator values and the leaf-height-seed (LHS) plant ecology strategy scheme values characterizing the associated vegetation. Using the properties of each vegetation unit, descriptors of the habitat suitability models and information from experimental studies, we interpreted the habitat suitability models as axes and shapes of ecological niches of individual root-hemiparasitic species. The individual hemiparasites differed in their favoured type of vegetation but almost all types of vegetation in the Czech Republic could host some of them. Semi-natural and natural grasslands with moderate availability of mineral macronutrients and water were identified as types of vegetation with a high incidence of hemiparasites and the highest number of species of hemiparasites. High incidence but low species richness of hemiparasites was recorded in forests and scrub. In contrast, most species of root hemiparasites did not occur in extreme habitats with a high level of stress or disturbance and at nutrient-rich and moist sites dominated by fast-growing species, i.e. at sites with intense above-ground competition. This reflects the ecophysiological fundamentals of the hemiparasitic strategy, which provides efficient yet low-cost access to below-ground abiotic resources. On the one hand, this advantage diminishes at sites where primary macronutrients and soil moisture are abundant but on the other hand, exploitation of this advantage, however, requires non-extreme environmental conditions. Apart from this common pattern, individual species of hemiparasites differ in their ecological requirements, which frequently underlie their possible use as ecosystem engineers in grassland restoration or their conservation status.

Keywords: *Bartsia*, Beals index, *Euphrasia*, habitat suitability model, hemiparasite, *Melampyrum*, *Odontites*, *Pedicularis*, phytosociology, *Rhinanthus*, *Thesium*

Introduction

Identification and description of the habitats of individual species is one of the important goals of ecology. A model of habitat suitability can serve as the first step in identifying the ecological niche of a species (Kearney 2006). In addition, it is an invaluable tool in conservation management as it can identify habitat requirements of endangered species and suitable sites for its reintroduction (Hirzel & Le Lay 2008). The habitat of a plant species is defined in terms of the abiotic and biotic conditions of sites where a species grows (Kearney 2006). A number of stochastic factors such as fecundity, dispersal limitation and demographic stochasticity (Hirzel & Le Lay 2008, Chase & Myers 2011) cause that species may not occur at all sites with favourable conditions (Ozinga et al. 2005). Therefore, analysing the habitats of the species involves considering not only its observed but also potential distribution. This idea is summarized by the concepts of species pool (Eriksson 1993) and dark diversity (Pärtel et al. 2011), respectively, referring to the pool of species that can potentially grow at a given site and the set of species that are missing but have ecological requirements compatible with site conditions. The habitat definition and analysis should take this into account and consider differences in conditions between sites, which are suitable for species occurrence and sites where species cannot occur.

Exploring large sets of vegetation plots is one approach to habitat analysis, which can cover also the local and community aspects. Such vegetation plot data are increasingly available as extensive databases (Schaminée et al. 2009, Dengler et al. 2011) that are representative of vegetation across a defined territory. The data available for each plot usually consist of species composition and cover-abundance, location of the site and a few additional observations or measurements. Vegetation recorded in the plots can be classified and individual plots assigned to one of the vegetation units based on the species composition. Thus, co-occurrence of a given species with others and its incidence in vegetation units can be explored. Species co-occurrence patterns are crucial for definitions of species pools (Ewald 2002) and dark diversity (Pärtel et al. 2011). However, they can also be used to define a set of suitable but unoccupied sites based on species composition of plots where the species actually occurs (Münzbergová & Herben 2004). Three classes of vegetation plots can thus be defined for each of the species included in a database: occupied, suitable but unoccupied (hereafter referred to as suitable), and unsuitable and unoccupied (hereafter referred to as unsuitable; Fig. 1). The habitat of a species is then defined in terms of the set of plots comprised in the first two groups. The contrast of occupied and suitable vs unsuitable is crucial for exploring abiotic and biotic conditions defining the limits of a species habitat, since it filters out the stochastic effects of dispersal limitation and sampling (each vegetation plot is a spatial subsample of a stand and cannot contain all species present in a habitat). As a result, it should provide a more realistic picture of species habitats in comparison to the contrast between occupied vs unoccupied (= suitable + unsuitable) plots.

The vegetation databases can be used to model habitats in terms of a set of plots suitable for a given species. A habitat model defined in this way is, however, of limited informative value and predictive power. Its properties cannot be described in a straightforward manner and more importantly, habitats of individual species can hardly be compared or located on environmental gradients. Comparison of the habitats of the same species based on two different databases is also very complicated. Therefore, habitats need to be

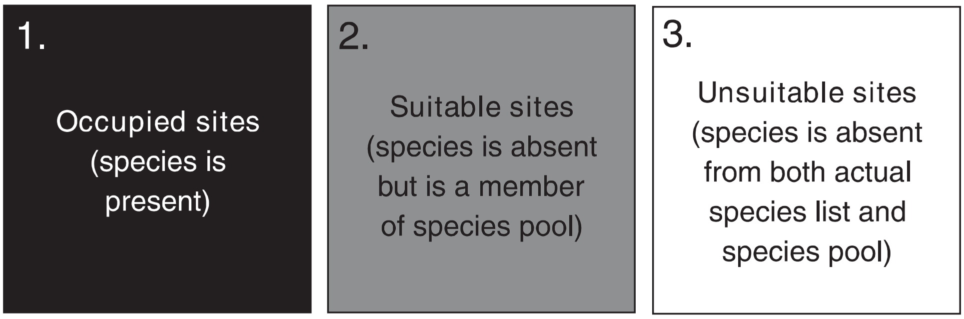

Fig. 1. – Three classes of sites: occupied, suitable (but unoccupied) and unsuitable based on the occurrence of individual species and their patterns of co-occurrence with other species in the vegetation-plot database.

described in terms of a few descriptors, which are biologically meaningful in relation to the environmental gradients at a given site. Description of climatic conditions is generally possible based on plot location (together with plot aspect and slope, a characteristic generally available in the vegetation-plot databases; Chytrý & Rafajová 2003). Expert-based systems of species environmental preferences (such as Ellenberg indicator values, EIVs; Ellenberg et al. 1991) can be used to estimate environmental conditions of plots on the basis of species composition. Functional traits of species (available from trait databases) can be used to identify ecological strategies of species occurring in individual plots. Such a two-step approach of model construction and consequent description is required by the fact that the EIVs cannot be used as predictors in models where the response contains information derived from species composition. This is because of an intrinsic interdependence of the predictors and the response in such a model resulting in biased outcomes as demonstrated by Zelený & Schaffers (2012). Due to the similar way of computation, the same issue applies for community weighted mean of functional trait values. Both EIVs and traits can, however, be used in descriptions to indicate the positions of suitable plots on environmental gradients and availability of resources. These descriptions consequently allow mechanistic (yet informal) interpretations of habitat models in relation to individual axes of species ecological niches (Fig. 2). Thus the correlative nature of the habitat models can be connected with mechanistic principles underlying the shape of a species' ecological niche, a concept proposed by Kearney (2006)

In this paper, we explore the habitats of species of root hemiparasites in the Czech Republic. Root hemiparasites form a distinct functional group of plants. They are green, photosynthetic species, which, however, use specialized root organs called haustoria to attach to the roots of other plants and withdraw resources from the host's xylem (Irving & Cameron 2009). Mineral nutrients, water and a limited amount of organic assimilates are thus acquired from their hosts (Irving & Cameron 2009, Těšitel et al. 2010a). Nevertheless, root hemiparasites tend to be dependent on their own photosynthesis for most organic carbon and are thus affected by above-ground competition (Matthies 1995, Mudrák & Lepš 2010, Těšitel et al. 2013, 2015). Several species of root hemiparasites are keystone species in some ecosystems due to their ability to suppress their hosts (Press & Phoenix 2005), thus affecting competitive relations in communities (Cameron et al. 2005) and altering nutrient cycling (Quested et al. 2003, Spasojevic & Suding 2011, Demey et al. 2014). Thus, it is suggested they play the role of ecosystem engineers in semi-natural grassland communities where they can reduce asymmetric competition,

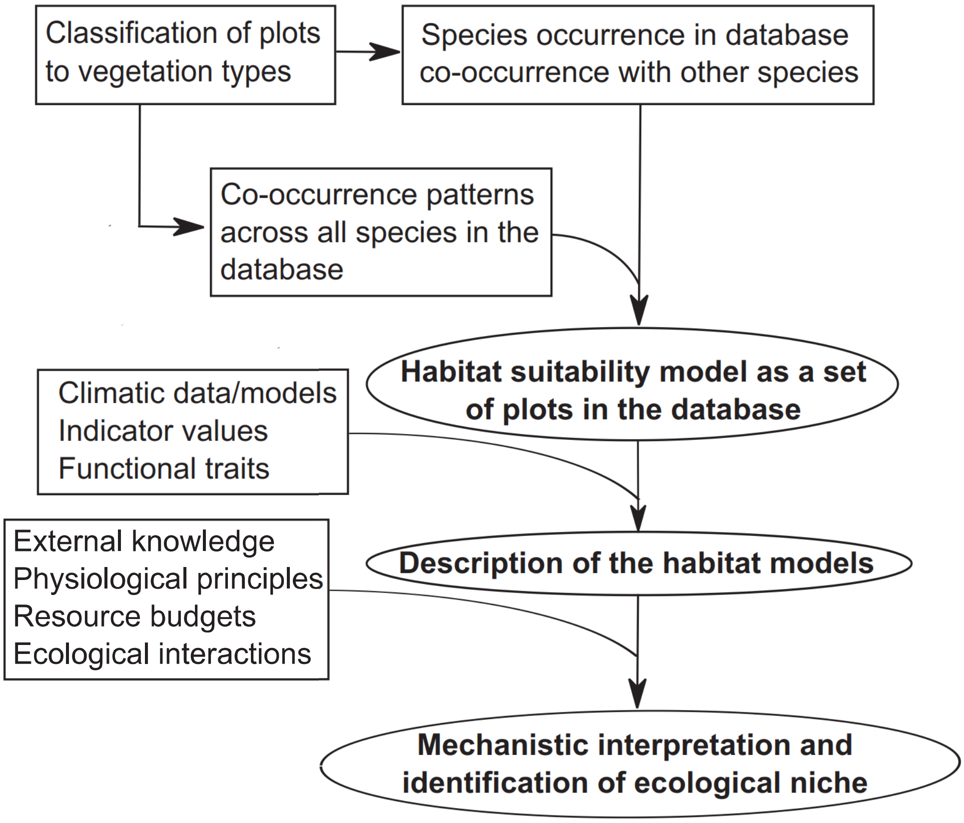

Fig. 2. – Conceptual scheme of the steps and data sources needed to construct a habitat suitability model, and describe and interpret it in terms of niche axes and shape.

facilitating species coexistence and increasing diversity (Westbury et al. 2006). The hemiparasites, in particular those of the genus *Rhinanthus*, are therefore currently used in grassland restoration (Westbury et al. 2006, Pywell et al. 2007, Westbury & Dunnett 2007, Hellström et al. 2011, Mudrák et al. 2014). In contrast, many other hemiparasitic species are considered threatened from the nature conservation perspective (Svensson & Carlsson 2005, Ramsay & Fotherby 2007, Schmalholz & Kiviniemi 2007, Grulich 2012). Knowledge of the favourable habitats and factors shaping the ecological niches of species of root hemiparasites is thus crucial for the development of both appropriate restoration strategies (identification of potentially suitable sites for introduction) and conservation.

In total, 42 species and subspecies of root hemiparasites belonging to the families *Orobanchaceae* and *Santalaceae* have been reported from the Czech Republic (Electronic Appendix 1; Danihelka et al. 2012). Their habitats and ecology are indicated in regional floras but this is based on various observations and has never been studied in a formal way based on the features of occupied or suitable sites. Using the data available in the Czech National Phytosociological Database (for 18 species of root hemiparasites, the others being rare or extinct), a climatic model (Tolasz et al. 2007), Ellenberg indicator values (EIVs; Ellenberg et al. 1991) and a set of functional traits (leaf-height-seed traits; Westoby 1998) we aim to (i) identify the types of vegetation in which individual species of root hemiparasites occur, (ii) construct habitat suitability models for each species of hemiparasites and (iii) interpret the habitat suitability models using knowledge of the biology of root hemiparasites revealed by experimental studies.

Methods

Data sources

Czech National Phytosociological Database containing records of vegetation plots (relevés) in the Czech Republic (Chytrý & Rafajová 2003) is the principal source of data for this study. For each plot there is a list of species of vascular plants with their cover-abundances and basic information on geographic location, habitat and vegetation structure. We used a stratified subsample of the database following the resampling criteria used by Chytrý et al. (2005; see also Knollová et al. 2005) in order to reduce local oversampling of some areas or habitats. This resulted in a set of 31,512 plots covering all the different types of vegetation in the country, which was used in this analysis. Ellenberg indicator values (EIVs) for each plot were calculated as unweighted means of the indicator values (from Ellenberg et al. 1991) for species present in the plots using the JUICE 6.5 program (Tichý 2002). Phytosociological class was determined for each relevé using an automated classification and an expert system based on the Cocktail method (Bruelheide 2000, Kočí et al. 2003) developed for the Czech national vegetation classification (Chytrý 2007–2013). The expert system can be downloaded and the database obtained upon request following instructions at www.sci.muni.cz/botany/vegsci. Climatic data were obtained from the national climatic atlas (Tolasz 2007), which includes spatial models of individual climatic variables based on interpolated values for climate stations.

We used the leaf-height-seed (LHS) plant ecology strategy scheme (Westoby 1998) to characterize the ecological strategies of species occurring in vegetation plots using functional traits. In addition, we considered the life spans of plants (proportion of annuals in the community). Most of the root hemiparasites studied are annuals and, remarkably, they are often the only annuals present in an otherwise perennial community (Strykstra et al. 2002). Here we want to explore the extent to which this applies to multiple species systems at a broad spatial scale. Values of specific leaf area (SLA) and shoot canopy height (Height) were acquired from the LEDA database (Kleyer et al. 2008). Data on seed weight and life span were obtained from the BiolFlor database (Klotz et al. 2002). Community weighted means (CWM) of traits were computed for each vegetation plot on the basis of species abundances and their trait values. Only herb-layer species were considered in computations of CWMs for all vegetation plots including forest plots since all root hemiparasites in the Czech Republic are herbaceous plants, and consequently, they potentially compete with other species in the herb layer, but not those in the shrub and tree layers.

We included in our study all the root hemiparasites occurring in the Czech Republic (see Electronic Appendix 1 for a list of taxa, their Red-List status and habitat descriptions in the Flora of the Czech Republic). Only species with more than 10 occurrences in the database were analysed (n = 18). Of these, all species with 10–30 occurrences (n = 4) were considered rare and the informative power of their habitat analyses should be interpreted with caution. Occurrences in phytosociological classes were also listed for species with at least one occurrence in the database (n = 8; Appendix 2). Eleven species were not recorded in the database. The nomenclature of plant taxa and syntaxa follows Danihelka et al. (2012) and Chytrý (2007–2013), respectively.

Taxonomy and nomenclature of the root hemiparasites studied

Most species studied are well defined taxonomically. Hybrids between them occur with rather low frequency (e.g. between *Rhinanthus major* and *R. minor*; Ducarme & Wesselingh 2005). Many species display ecotypic seasonal variation typical of annual hemiparasitic *Orobanchaceae* (Wettstein 1895), which could not be included in our analyses since it was not recorded in vegetation-plot records. In most cases, however, this variation is more or less continuous and there are no distinct ecotypes (e.g. in *Melampyrum pratense* and *M. sylvaticum*; Štech 1998), or the ecotypes share similar habitats (e.g. *Rhinanthus major*; Skála & Štech 2000). An exception to this is *M. nemorosum*, a species with very distinct ecotypes one of which grows in open habitats and the other at the edges and in forests (Štech 2000). The most complicated taxon studied is the *Odontites vernus* group, which consists of two cytotypes, diploids and tetraploids, and the cytotypic variation furthermore interacts with seasonal variation (Koutecký et al. 2012). However, the novel taxonomic concept based on the recognition of these patterns could not be used in our study because we used older data. Therefore, we only report results for the *Odontites vernus* group as an aggregate taxon. *Melampyrum sylvaticum* might be another taxonomically complicated species. *Melampyrum herbichii*, its closely related congener, was, however, rejected from a taxonomic perspective and all Czech populations previously referred to this taxon were assigned to *M. sylvaticum* (Těšitel et al. 2009)

Habitat modelling

Habitats of individual species consist of occupied and suitable sites. While the former group is directly available, suitable sites have to be identified using a probabilistic approach based on species co-occurrence patterns in the database. We adopted approach used by Münzbergová & Herben (2004), based on Beals' index of sociological favourability (Beals 1984, see also Ewald 2002), which measures the threshold for the suitability of unoccupied sites. The threshold is defined as a minimum of Beals' index values of occupied sites. Unoccupied sites with Beals' index higher than the threshold are considered to be suitable. This method computes thresholds of habitat suitability for individual species because the threshold depends on the frequency of occurrence (rare species should have lower thresholds than common species).

We made two modifications to the method of Münzbergová & Herben (2004): (i) the threshold for suitable sites was defined as the 10th percentile of the Beals' index distribution for occupied sites (this reduces the effect of outliers; see Botta-Dukát 2012); (ii) the threshold for a given species was computed separately for each of the phytosociological classes in which it occurs (i.e. for one species, there are multiple thresholds of suitability, one for each of the phytosociological classes in which it occurs). This is based on the fact that Beals' indices are frequency-dependent and species occurring in multiple phytosociological classes are not present with the same frequency in each of them. The suitable and occupied plots in individual phytosociological classes were finally pooled to specify a single set of plots defining the habitat of each species. For rare or moderately rare hemiparasitic species that occur in fewer than 50 vegetation plots in the database, we included phytosociological classes with more than one occupied plot in the niche computation. For the common hemiparasites, which occur in more than 50 plots, we included only vegetation classes with more than four plots. These restrictions reduce the effects of outliers caused by the transitional random occurrence of species at unsuitable sites or possible misidentification of species.

Habitat model descriptors

The habitat model descriptors are the positions of occupied and suitable sites on gradients of climate, EIVs and functional traits (disregarding other variables). Such habitat models for the root hemiparasites and the whole of the vegetation in the Czech Republic (represented by the complete data set of the stratified database) were compared. Thus, we plotted the positions of occupied and suitable sites of each of the root hemiparasites against the site scores for the whole database (or their interquartile range in the case of boxplots).

In addition, we quantified the proportions of the variation in the habitat models explained by individual groups of descriptors (climate, EIVs, LHS traits). This was done by fitting sets of generalized binomial models, separately for each species of root hemiparasites, using the classical variation partitioning approach (Borcard et al. 1992). Models contain suitability of habitats as a response (unsuitable = 0; occupied or suitable = 1) and groups of descriptors (both linear and quadratic trends of each descriptor were included) as predictors. Partial proportion of variability in a habitat suitability model accounted by a descriptor group was computed as deviance explained by a model containing climate+ EIVs+traits minus deviance explained by a model containing all the other descriptor groups (e.g. for climate, this model contained EIVs+traits). For example R^2(climate) = R^2(climate+EIVs+traits) – marginal R^2(EIVs+traits). For partial shared effects of two predictor groups (overlap of effects), we subtracted the deviance explained by the third predictor group and partial explained deviance of each of the predictor groups for which the shared effect is computed from the deviance explained by the full model. For example partial R^2(climate+traits) = R^2(climate+EIVs+traits) – partial R^2(climate) – marginal R^2(EIVs) – partial R^2(traits). The proportion of deviance explained not attributable to any individual descriptor or shared effects of pairs of descriptors was considered to be accounted for by the combination of all three descriptors. The proportions of explained deviance in variation partitioning do not sum up to 100% as there is always a certain amount of residual variance not attributable to any of the descriptors or their combinations. R software (version 3.1.1; R Core Team 2014) was used for all computations.

Despite their correlative nature, the habitat suitability models present a basis for identifying the ecological niches of species. This is based on the biological meaning of the habitat suitability model descriptors, which indicate the principal factors limiting plant performance in natural communities including below-ground resources (soil nutrients and water), disturbance and competition for light (Grime et al. 1997). The below-ground resources can be indicated by the EIVs in a straightforward manner, while intensity of disturbance and competition can be estimated from the LHS traits, EIVs and proportion of annual species. Disturbance can be indicated by annual species with small seeds and low canopy height not attributable to scarcity of below-ground resources (Westoby 1998). In contrast intense above-ground competition can be indicated by high canopy height coupled with high SLA, high nutrient and moisture EIVs (Grime et al. 1997, Westoby 1998) and in some cases (competition from the tree layer) also by low EIV for light. In addition, proportions of variation in suitability accounted for by individual habitat descriptor groups are key parameters indicating their significance for defining the niches of individual species.

Results

Habitats of species of root hemiparasites and their phytosociological classification

The occurrence of root hemiparasites in different types of vegetation differed for the different species (Electronic Appendix 2). Nevertheless, some general trends are evident. High or moderately high incidence of hemiparasitic species (13.7–30.1% plots with hemiparasites) combined with high species numbers was recorded in open semi-natural and natural types of vegetation, many of them with limited availability of primary macronutrients (*Molinio-Arrhenatheretea*, *Festuco-Brometea*, *Calluno-Ulicetea*, *Scheuchzerio palustris-Caricetea nigrae*). The *Mulgedio-Aconitetea* and *Elyno-Seslerietea* vegetation classes probably also belong here but they are rare types of vegetation for which there are few plots in the database, which prevents drawing a definitive conclusion. High incidence of hemiparasites (9.3–48.7% plots with hemiparasites) underlain, however, by the occurrence of only one or two species is typical of forest/scrub, often also on macronutrient-poor soils (*Carpino-Fagetea*, *Quercetea robori-petraeae*, *Quercetea pubescentis*, *Vaccinio-Piceetea*, *Erico-Pinetea*, *Roso pendulinae-Pinetea mugo*), which host various *Melampyrum* species (*M. pratense*, *M. sylvaticum*, *M. nemorosum*). A similar pattern of occurrence of hemiparasites (14.3–38.5% plots with hemiparasites) is present in habitats stressed by low macronutrient availability combined with high water level (*Oxycocco-Sphagnetea*), extreme climatic conditions (*Loiseleurio-Vaccinietea*; low number of plots) or high concentrations of salts (*Festuco-Puccinellietea*). The first two are habitats of *Melampyrum pratense* and *Odontites vernus* occurs in the latter. Despite the very low percentage of occupied plots (4.0% plots with hemiparasites) in annual vegetation of arable fields and heavily disturbed sites (*Stellarietea mediae*), this habitat hosts *Odontites vernus*, *Rhinanthus alectorolophus* and *Melampyrum arvense*, and is even the most common type of habitat for the first two. Rarely (less than 5% of plots with hemiparasites) do the hemiparasitic species occur in vegetation in wet mesotrophic to eutrophic places (*Phragmito-Magno-Caricetea*, *Montio-Cardaminetea*, *Bidentetea tripartitae*), disturbed eutrophic habitats (*Galio-Urticetea*, *Epilobietea angustifolii*, *Artemisietea vulgaris*), periodically flooded habitats (*Isoëto-Nano-Juncetea*, *Littorelletea uniflorae*), extremely dry and stressed (*Asplenietea trichomanis*), dry and disturbed (*Koelerio-Corynephoretea*), cold and stressed (*Juncetea trifidi*) or strongly disturbed habitats (*Polygono arenastri-Poëtea annuae*). Similarly, the incidence of species of hemiparasites in eutrophic wet forests and scrub with intense competition in the understory (*Alnetea glutinosae*, *Rhamno-Prunetea*) is very low. Hemiparasites are absent from aquatic habitats (*Lemnetea*, *Potametea*, *Charetea*) and some of saline (*Crypsietea aculeatae*, *Thero-Salicornietea strictae*) and stressed and disturbed habitats (*Cymbalario muralis-Parietarietea judaicae*, *Festucetea vaginatae*, *Thlaspietea rotundifolii*).

Habitat models

Positions of occupied and suitable plots along gradients of annual precipitation and mean annual temperature describe the habitat models in relation to climate. The two climate parameters are closely correlated in the Czech Republic (Fig. 3). Due to the intrinsic dependence of climate on altitude, the habitat models for species can also be described by their ranges in terms of altitude (Electronic Appendix 3). Most of the models for hemiparasitic species indicate relationships with climate (Fig. 3). The habitats of *Euphrasia stricta*, *Melampyrum arvense*, *M. cristatum*, *M. nemorosum*, *Odontites luteus* and *Thesium linophyllon* are located at the dry and warm end of the gradient. The habitats of the *Odontites vernus* group, *Rhinanthus alectorolophus* and *R. major* occupy similar positions but the pattern is less distinct. In contrast, the habitats of *Bartsia alpina*, *Rhinanthus riphaeus* and to some extent also *Thesium alpinum* appear to be associated with a cold and wet climate. All of these three species, however, are rare. The habitats of *Euphrasia officinalis*, *Melampyrum pratense*, *M. sylvaticum*, *Pedicularis palustris*, *P. sylvatica* and *Rhinanthus minor* extend along the whole climatic gradient; although there are higher densities of some species at certain positions on the gradient (e.g. *Melampyrum sylvaticum* grows mostly but not exclusively in cold and wet areas).

The gradients in soil moisture and primary macronutrient availability indicated by the Ellenberg indicator values show a more complex, two dimensional picture of the habitats (Fig. 4). The root hemiparasites are generally absent at sites with high values of both EIVs (except the *Odontites vernus* group and in part also *Pedicularis palustris*). Apart from this rule, it is also possible to distinguish the typical habitats of several groups of species. *Euphrasia officinalis*, *Melampyrum pratense*, *Rhinanthus major* and *R. minor* share a niche which extends from moderately dry to moderately moist macronutrient-poor conditions (extending further towards mesotrophic in the drier part of the gradient). *Euphrasia stricta*, *Melampyrum arvense*, *M. cristatum*, *M. nemorosum*, *Odontites luteus*, *Rhinanthus alectorolophus* and *Thesium linophyllon* occur in dry (to moderately dry) places with low to moderate macronutrient availability. In contrast, *Bartsia alpina* and both *Pedicularis* species prefer wet sites with generally low macronutrient availability. Sites included in the habitats predicted for almost all hemiparasitic species have high EIVs for light (Fig. 5). Exceptions to this are *Melampyrum* species, the predicted habitats of which are located in slightly to heavily shaded areas. EIVs for soil reaction identified four species restricted to alkaline soils (*Melampyrum arvense*, *M. cristatum*, *Odontites luteus*, *Thesium linophyllon*). In contrast, habitats of *Melampyrum sylvaticum*, *Pedicularis sylvatica* and *Rhinanthus riphaeus* are mostly characterized by acidic soils.

The habitat models descriptions obtained using LHS and lifespan traits are summarized by comparing the gradients of community weighted means of the sites included in the models with the median and interquartile range of the whole database (Fig. 6). All of the species of root hemiparasites occur at sites with a low mean canopy height with *Euphrasia stricta*, *Odontites luteus*, *Pedicularis sylvatica* and *Rhinanthus pulcher* displaying the strongest trend in this direction. Similarly, most of the species occur in vegetation with a low mean SLA. *Bartsia alpina*, *Euphrasia stricta*, *Odontites luteus*, *Pedicularis palustris* and *Thesium linophyllon* display the strongest trend in this direction. In contrast, *Melampyrum nemorosum* and to some extent also *M. pratense* show the opposite trend. There is no clear trend in relation to CWM of seed weight across the whole

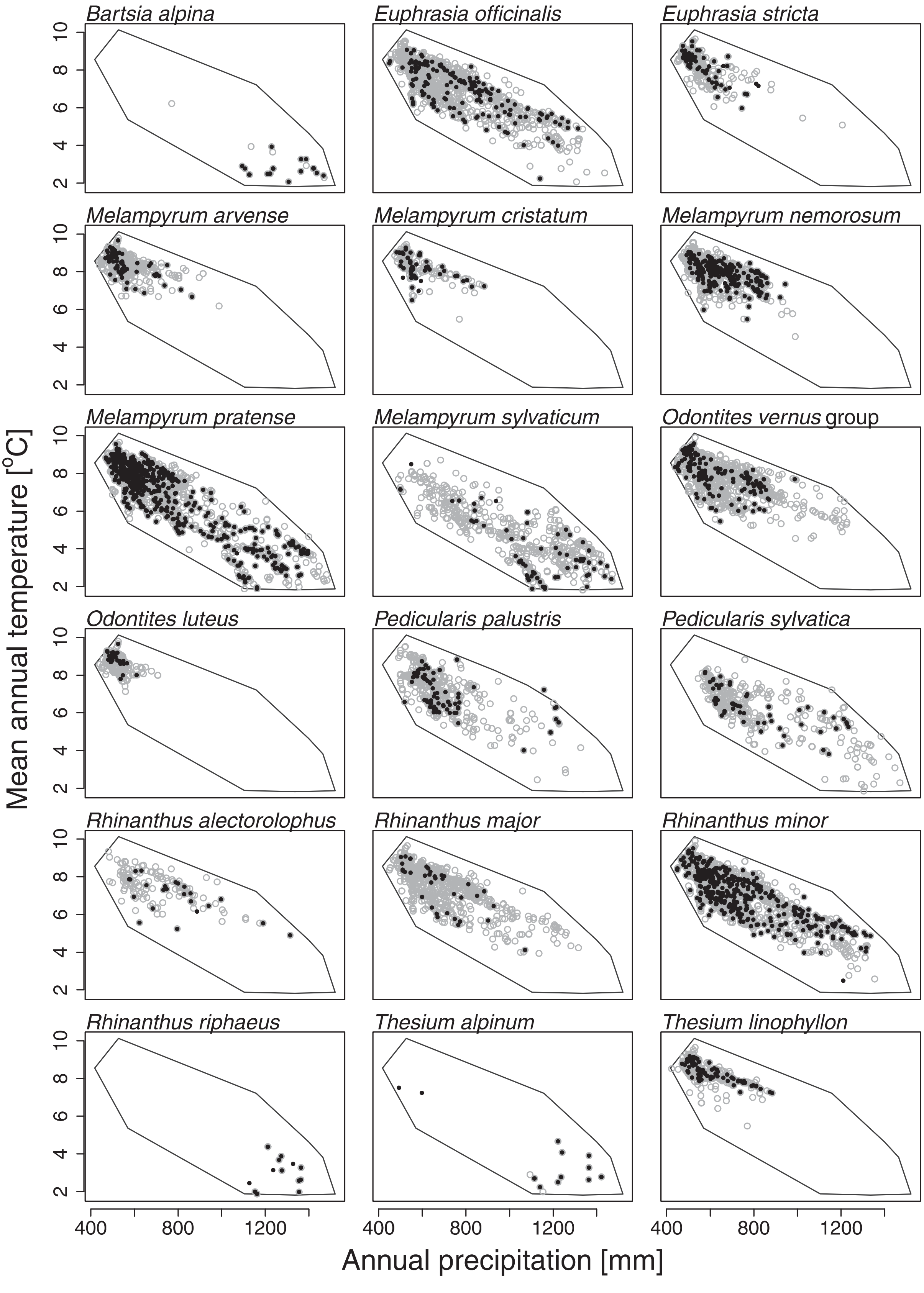

Fig. 3. – Scatterplots of mean annual precipitation and temperature based on data from all the vegetation plots in the database (displayed by the envelope). Suitable sites are displayed for each species of hemiparasite by grey circles. Occupied sites are indicated by black dots.

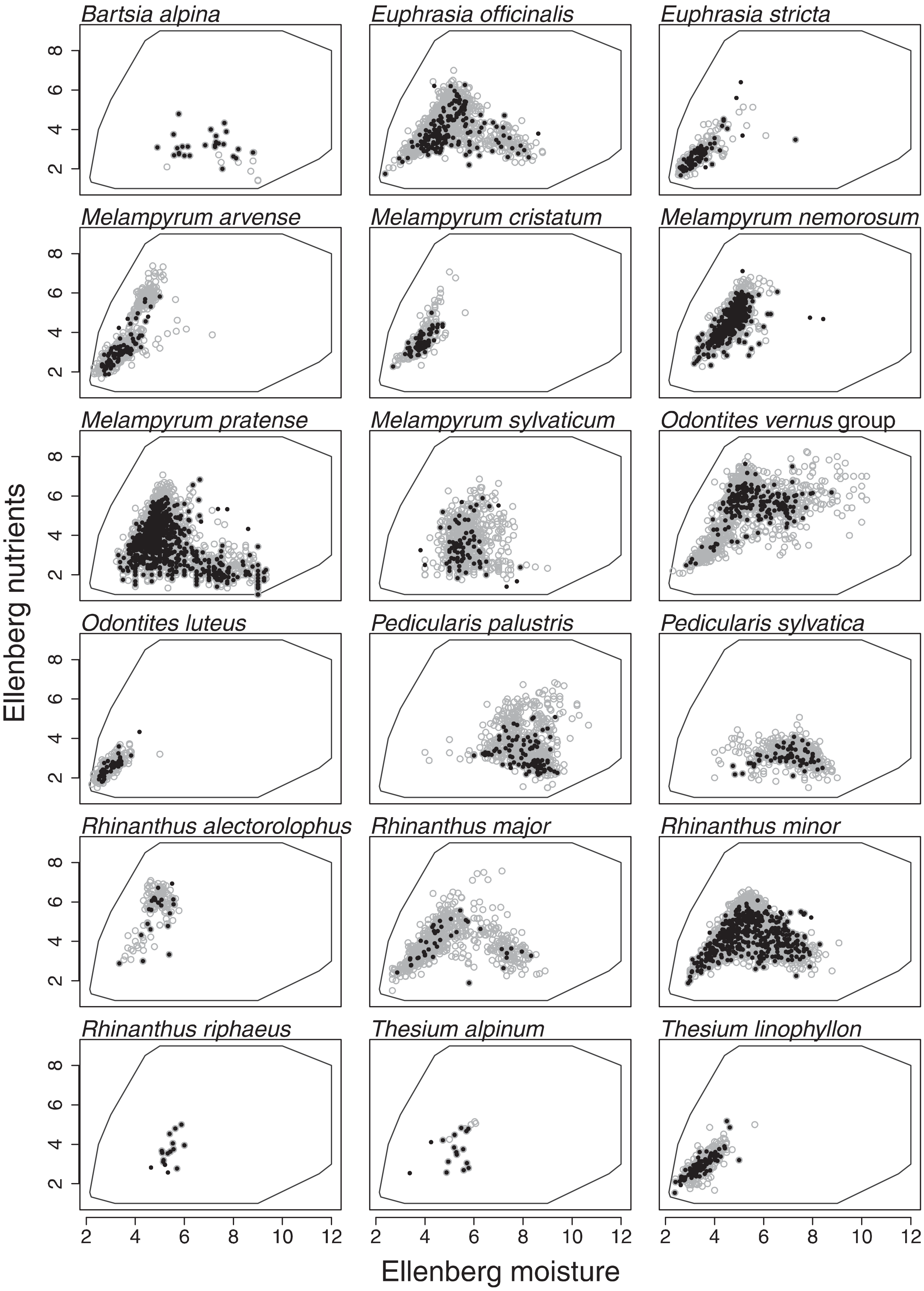

Fig. 4. – Scatterplots of mean Ellenberg indicator values for mineral nutrients and soil moisture based on data from all the vegetation plots in the database (displayed by the envelope). Suitable sites are displayed for each species of hemiparasite by grey circles. Occupied sites are indicated by black dots.

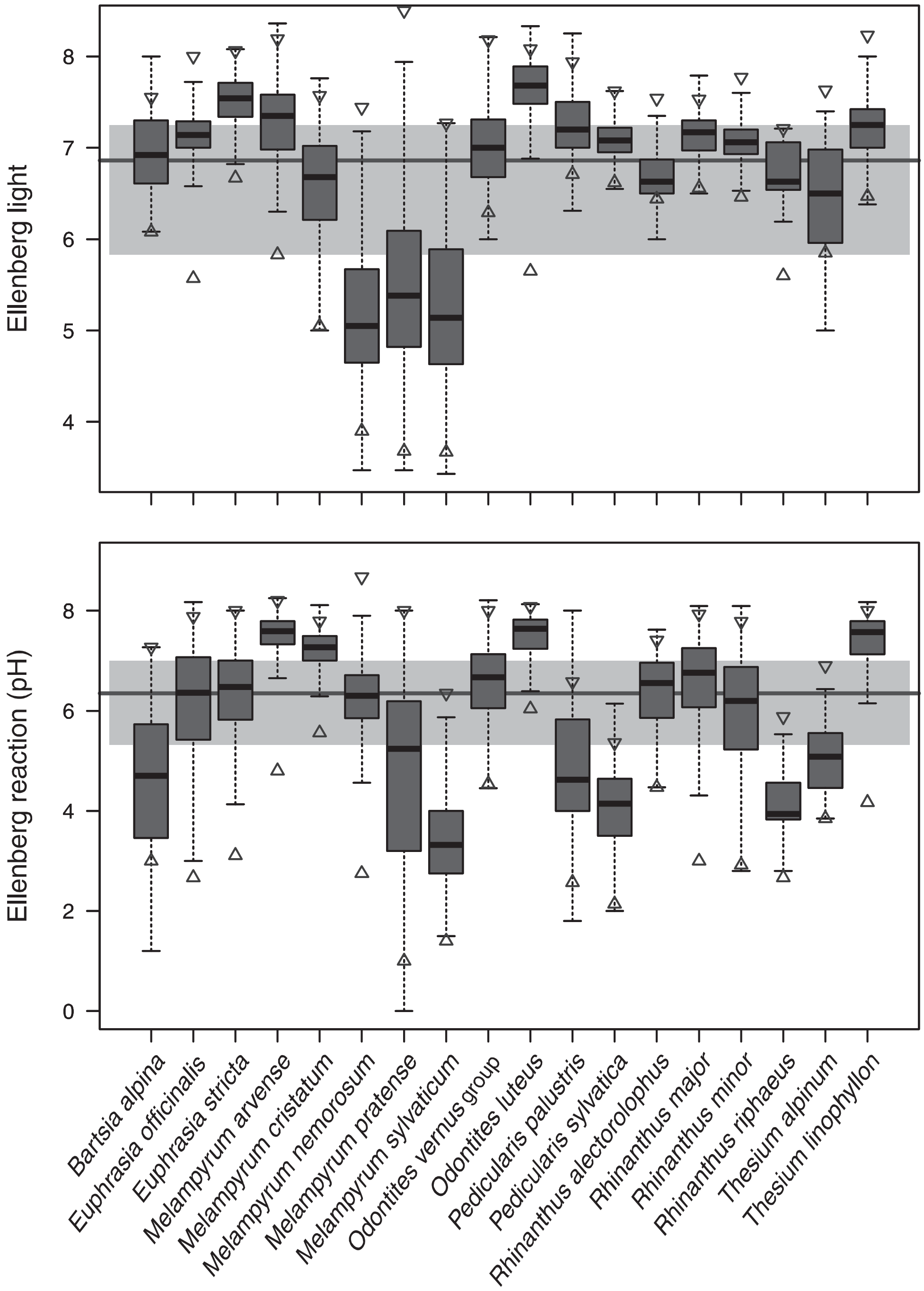

Fig. 5. – Positions of occupied and suitable sites for the root hemiparasites along gradients of Ellenberg indicator values for light and soil reaction. Median, quartiles and non-outlier ranges are displayed. Dark-grey line and grey belt display the median and inter-quartile range of the whole database. Up- and down-pointing triangles display the range of values at occupied sites.

series of hemiparasites with some species being associated with a high and others with a low value. All species of root hemiparasites grow in vegetation largely dominated by perennials (Fig. 7), except *Melampyrum arvense*, *Odontites vernus* group and *Rhinanthus alectorolophus*, which also occur in agroecosystems with numerous annual species.

The analysis of variation in the predicted habitats using sets of descriptors identified EIVs as the most correlated variables followed by climatic variables and LHS traits

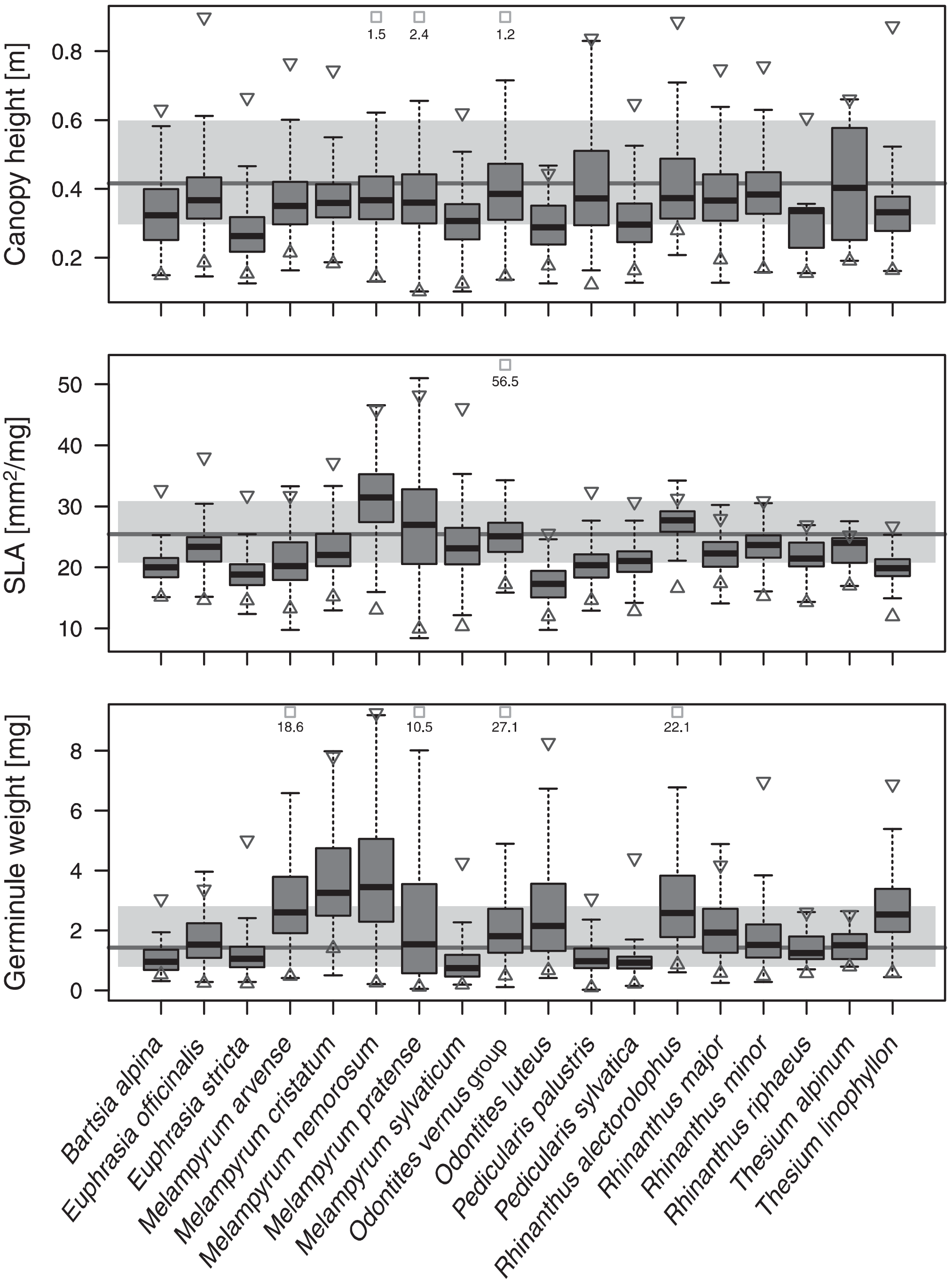

Fig. 6. – Positions of occupied and suitable sites for the root hemiparasites along gradients of CWMs of functional traits canopy height, SLA and seed weight. Median, quartiles and non-outlier ranges are displayed. Dark-grey line and grey belt display the median and inter-quartile range of the whole database. Up- and down-pointing triangles display the range of values of occupied sites. Boxes display extremes that are outside of the axis ranges and their values are indicated by numbers.

(Table 1) for most species. Large proportions of the variation in the predicted habitat were also accounted for by the shared effects of EIVs and LHS traits, and EIVs and climate. Climate alone or in combination with EIVs was, however, the best descriptor for some of the species, namely *Bartsia alpina*, *Rhinanthus riphaeus*, *Thesium alpinum* and *Melampyrum sylvaticum.*

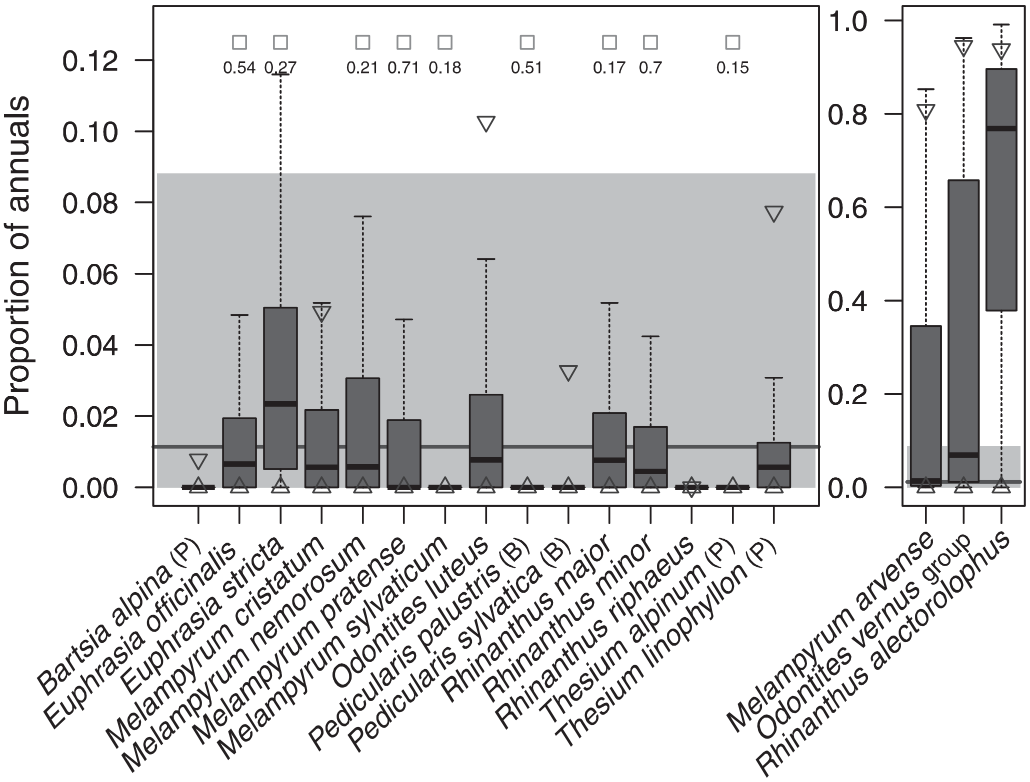

Fig. 7. – Proportion of annual species in communities at occupied and suitable sites for the root hemiparasites. Life span of perennial (P) and biennial/monocarpic perennial (B) hemiparasitic species is indicated. The other species are annuals. Median, quartiles and non-outlier ranges are displayed. Dark-grey line and grey belt display the median and inter-quartile range of the whole database. Up and down triangles display the range of values at occupied sites. Boxes display extremes that are outside of the axis ranges and their values are indicated by numbers.

Table 1. – Percentages of variation in habitat suitability explained by individual habitat descriptor groups and their shared effects (overlap; e.g. climate+EIVs corresponds to overlap of effects of climate and EIVs, not to their interactions).

Species	Climate	EIVs	LHS traits	Climate +EIVs	EIVs +traits	climate +traits	climate +EIVs +traits
Bartsia alpina	36.0	21.4	1.9	0.6	4.7	0.0	8.3
Euphrasia officinalis	2.0	30.0	1.7	1.1	24.4	0.1	0.0
Euphrasia stricta	0.2	20.0	2.9	5.3	24.0	0.1	1.7
Melampyrum arvense	0.8	17.0	3.4	6.9	21.4	0.1	5.4
Melampyrum cristatum	1.5	30.2	3.1	1.2	19.1	0.5	2.5
Melampyrum nemorosum	1.6	33.6	1.5	3.7	17.6	0.3	0.8
Melampyrum pratense	2.5	43.5	1.7	0.0	2.9	0.5	0.0
Melampyrum sylvaticum	7.4	14.7	1.5	18.4	5.5	0.3	7.7
Odontites vernus group	0.8	15.5	6.6	1.9	5.0	0.0	0.0
Odontites luteus	1.8	9.4	2.3	6.3	34.8	0.3	11.9
Pedicularis palustris	1.1	26.9	4.2	3.4	15.4	0.1	0.0
Pedicularis sylvatica	0.9	30.8	0.9	7.3	20.1	0.1	3.2
Rhinanthus alectorolophus	2.7	19.4	5.5	0.0	8.7	0.3	0.9
Rhinanthus major	1.6	16.3	1.5	1.3	23.0	0.0	0.0
Rhinanthus minor	0.7	30.4	2.4	2.4	26.5	0.0	0.0
Rhinanthus riphaeus	17.7	15.6	5.3	19.8	6.9	1.0	0.4
Thesium alpinum	30.1	20.6	4.5	12.5	9.8	0.0	0.0

Discussion

Characteristics of habitats favourable for hemiparasitic plants

Several trends shaping the niches of root-hemiparasitic species are apparent from their occurrence in different types of vegetation and the descriptions of their habitats in terms of EIVs and functional traits. The types of vegetation favourable for most species of root hemiparasites are open ones in which there is a high availability of light in the herb layer, which is dominated by low (low canopy height) and slow-growing species (low SLA). Favourable sites are subject to moderate levels of disturbance (e.g. mowing and grazing) and in which there are moderate levels of water and macronutrients. Suitability of the habitats of the three most frequent species of root hemiparasites, *Melampyrum pratense*, *Rhinanthus minor* and *Euphrasia officinalis*, is clearly co-limited by abundance of water and mineral macronutrients (Fig. 4). Most hemiparasitic species, however, are not recorded at heavily disturbed places and sites severely limited by both a scarcity of water and mineral macronutrients. Exceptions are *Odontites vernus* and *Pedicularis palustris* with ecological niches that include macronutrient-rich and wet sites, and *Odontites luteus* and *Thesium linophyllon* that grow at dry oligotrophic sites.

Interpretation of habitats in terms of niches

The favourable habitats for most of the species of hemiparasites studied provide strong support for the hypothesis that there is a strong and interactive effect of water and mineral macronutrient availability on the performance of root hemiparasites (Těšitel et al. 2015). This hypothesis, based on the results of manipulative experiments, suggests that the performance of root hemiparasites should be best at sites where water and mineral macronutrients are available in moderate amounts, or if one of these is abundant, the other is scarce. Simultaneous abundance of both diminishes the hemiparasite's benefit based on efficient, yet low-cost access to these resources (Irving & Cameron 2009) and results in their competitive exclusion from the community (Van Hulst et al. 1987, Matthies 1995, Hellström et al. 2004, Mudrák & Lepš 2010, Hejcman 2011, Těšitel et al. 2013). In contrast, simultaneous scarcity of these resources may reduce hemiparasites' shoot growth to such extent that it reduces their ability to derive resources from host root xylem (Těšitel et al. 2015) as also suggested by a mathematical model (Fibich et al. 2010). This substantial update of the resource-limitation hypothesis that states that hemiparasitism is most beneficial in low-productive habitats where these resources are scarce (Matthies 1995, Borowicz & Armstrong 2012).

The abundance of the below-ground resources has other effects on hemiparasites beside the increase in competitive pressure. Hemiparasites can benefit from abundant mineral macronutrients to a similar extent as non-parasitic plants by improving the efficiency of physiological processes, especially photosynthesis (Phoenix & Press 2004, Těšitel et al. 2015). This results in a more vigorous growth and greater fecundity of established individuals (Van Hulst et al. 1987, Mudrák & Lepš 2010, Těšitel et al. 2013). Some of the hemiparasitic species are able to inflict extensive damage on their hosts by inducing strong water stress at dry macronutrient-rich sites, which possibly reduces competitive pressure from the host community (Těšitel et al. 2015). Moreover, Demey et al. (2015) have demonstrated that root hemiparasites may prefer to parasitize clonal plants, which may be higher quality hosts due to resource withdrawal from the whole clonal network. In addition, damage inflicted on the clonal hosts might decrease competitive pressure of these frequently dominant competitors, thereby improving establishment success of hemiparasite seedlings (Lepš & Těšitel 2015). In general, hemiparasites are able to suppress their hosts to a variable extent (Těšitel et al. 2015) and reduce total community productivity (Ameloot et al. 2005) and thus the intensity of above-ground competition. This is one of the principal factors underlying persistence of competitively inferior, mostly annual or short-lived monocarpic perennial hemiparasites (Electronic Appendix 2) in perennial-dominated grassland communities (Strykstra et al. 2002; Fig. 7). Moderate disturbance such as grazing and mowing of meadows is another key factor reducing competition and litter production, which strongly facilitates survival of hemiparasite seedlings (Mudrák et al. 2014). In contrast, strong disturbance occurring during a hemiparasite's growth period may have fatal consequences for a population (Mudrák et al. 2014) since the regenerative ability of most species of hemiparasites is very limited (Klimešová & de Bello 2009).

Odontites luteus and *Thesium linophyllon* are the only hemiparasites of all the species studied that conform to the resource limitation hypothesis and occur in low-productive habitats stressed by macronutrient and water deficiency. In contrast to the other species studied, they are, however, unable to colonize less extreme habitats. This may be due to a trade-off between host resource conservation in extreme habitats and host suppression in more productive habitats. Although there are no ecophysiological data for either of these species, *Santalum acuminatum*, which is distantly related to *T. linophyllon* and grows in macronutrient-poor semi-desert habitats, is known to display such a hemiparasitic resource conservation strategy (Tennakoon et al. 1997).

The *Odontites vernus* group and *Pedicularis palustris* present exceptions to the resource-competition niche hypothesis that suggests sites with simultaneous high macronutrient and water availability are unsuitable. The high proportion of annuals and moderate canopy height and SLA values of habitats favourable for the *O. vernus* group indicate they are subject to high levels of disturbance, which decreases the competitive pressure (Grime et al. 1997) and allows establishment of this small-seeded (Těšitel et al. 2010b) annual hemiparasite. This was also demonstrated by Gilhaus et al. (2013) who revealed a strongly positive association between grazing and *O. vernus* dominance in floodplain meadows. In contrast, *P. palustris* grows at wet and frequently waterlogged sites where productivity might be limited by oxygen stress (Schulze et al. 2002). In addition, *P. palustris* is able to suppress tall sedges (e.g. *Carex acuta*), its principal hosts but also strongest competitors in its habitat (Decleer et al. 2013). *Melampyrum sylvaticum*, *M. pratense* and *M. nemorosum* are exceptional in their ability to grow in shaded habitats in forest understory (Fig. 5, Electronic Appendix 2). This is probably because they germinate in autumn and hibernate in epicotyl dormancy, which enables them develop quickly in spring, and have a long lifespan (for an annual), which enables them to exploit resources throughout the whole growing season (Průšová et al. 2013). Ecophysiology of forest-understory hemiparasites, in particular their energy budget, however, remains a challenging question for further research.

The mechanistic interpretation of other distinct patterns in habitat suitability is less straightforward than those dependent on below-ground resources and light availability. The suitability of sites with a cold climate for *Bartsia alpina*, *Rhinanthus riphaeus* and

Thesium alpinum might fit within the competition framework as a short growing season imposes stress on communities, which reduces competitive pressure. However, all of these species are rare and their habitat models are not robust enough to present a solid basis for mechanistic interpretations. Apparent suitability of sites with high soil pH for *Melampyrum arvense*, *M. cristatum*, *Odontites luteus* and *Thesium linophyllon* (Fig. 5) is another distinct pattern. While soil pH is one of the strongest factors affecting species richness and composition of plant communities in central Europe (Ewald 2003), its ecophysiological effect on hemiparasites remains unclear. Therefore, the association with high soil pH in these species might in fact reflect the habitat suitability of communities occurring at calcareous sites in the Czech Republic underpinned by factors (low competition, low primary macronutrient availability, low water availability) other than soil pH.

Implications for ecological restoration and conservation

The identification and description of properties of favourable habitats is crucial for restoration projects that use hemiparasites as ecosystem engineers (Pywell et al. 2004, Westbury et al. 2006, Westbury & Dunnett 2007, Hellström et al. 2011). It can facilitate decisions such as when to use hemiparasites as ecosystem engineers, which species to choose and which additional measures to apply to ensure their establishment. For instance, more intense mowing (twice per season) is likely to be necessary for establishing *Rhinanthus* species and reducing the negative effect of litter at productive restored sites close to the suitability limit (Fig. 4). However, in such cases, the first mowing should always be scheduled for after the seeds of *Rhinanthus* ripen (Blažek & Lepš 2015). In addition, our results suggest possible use of *Pedicularis palustris* and *Odontites vernus* as ecosystem engineers at wet sites with high macronutrient availability where *Rhinanthus* species cannot establish due to competition. While an ecosystem engineering role is established for *P. palustris* (Decleer et al. 2013), it has never been considered in the case of *Odontites*.

Another important message comes from the ratio between numbers of occupied and suitable sites (Electronic Appendix 2). The low values ranging between 0.05 and 0.3 recorded for many species suggest that species of root hemiparasites do not occupy most of the suitable sites. This can be ascribed to limitations on dispersal as many of the species have large seeds and have no efficient means of seed dispersal (Těšitel et al. 2010b), demographic effects limiting fitness in small populations (Schmalholz & Kiviniemi 2007, Kiviniemi 2008) and the high demographic stochasticity of hemiparasite populations (Ameloot et al. 2006). The implication for restoration projects is that an introduction of hemiparasites by sowing can be successful even at sites not occupied by any hemiparasitic species (Mudrák et al. 2014), whereas spontaneous colonization is unlikely.

The majority of the root-hemiparasitic taxa that are reported to occur or have occurred in the Czech Republic are listed in the national Red List (Grulich 2012). Some of these threatened species are listed due to their general rarity caused by the rarity of their habitats (*Bartsia alpina*, rare subalpine species of *Euphrasia*, *Pedicularis sudetica*). Most hemiparasites are Red-Listed due to a recent decline, which is probably connected with landscape-level eutrophication, intense large-scale disturbances and intensification of agriculture during recent decades. This is the case for the meadow species *Euphrasia*, *Melampyrum arvense*, *M. cristatum*, *M. nemorosum* var. *praecox*, *Odontites luteus*, *O. vernus* subsp. *vernus*, *Pedicularis palustris*, *P. sylvatica*, *Rhinanthus alectorolophus*, *Rhinanthus riphaeus* and most *Thesium* species. Both causes underlying their threatened status (rarity and decline) may be valid for some species (*Odontites luteus*, some *Thesium* species). Conservation of the former group of endangered hemiparasites requires only protection of the habitat from destruction and usually no specific management measures are needed. In contrast, the second group is dependent on the existence of sites with limited below-ground resource availability and a moderate disturbance regime, which decreases competition and promotes seedling establishment. Such sites may be fairly variable, ranging from mesic and dry grasslands (favourable for most *Orobanchaceae*) to more disturbed habitats (e.g. several *Thesium* species).

Assessment of the habitat suitability modeling approach

Our study is not the first attempt to explore plant species habitats and ecological niches using vegetation databases, functional traits and EIVs. For example, Hölzel (2003) studied the ecological niches of floodplain-meadow violets. In our study, we further developed this approach by incorporating a distinction between habitat models and ecological niches (Kearney 2006) and the species-pool concept (Eriksson 1993, Zobel et al. 1998, Münzbergová & Herben 2004). This resulted in a three-step approach consisting of building the habitat models, their description and mechanistic interpretation (Fig. 2). The distinction between these steps is crucial. The habitat models are based on an analysis of 'hard' data on species co-occurrence available in a vegetation-plot database using appropriate statistical techniques. Few compromises in the requirements of these techniques need to be made when using a stratified set of vegetation plots (Roleček et al. 2007). In the second step, the nature of the descriptions of the habitat models does not allow the use of formal statistical testing due to non-independence of the data (Zelený & Schaffers 2012). Therefore, only a graphical representation of the patterns is reported here. From this perspective, the partitioning of variation in the habitat models explained by descriptor groups might appear in conflict with the recommendation of Zelený & Schaffers (2012) not to combine individual EIVs (or functional traits) as predictors in a single analysis. However, this is not a classical statistical analysis in which predictors compete to be included in (or omitted from) the model during the model-building procedure. The final step of our approach, mechanistic interpretation of the described models aims to identify niches of species by incorporating knowledge of the ecological interactions of these species with the environment and co-occurring vegetation. Such information cannot be derived from the data in the database. Therefore, results of other experimental studies (such as those of Mudrák & Lepš 2010, Těšitel et al. 2011, 2013, Demey et al. 2015 in case of the current study) are needed to provide this essential information required for identifying their ecological niches.

See www.preslia.cz for Electronic Appendices 1–3

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Souhrn

Kořenoví poloparaziti představují specializovanou funkční skupinu rostlin. Jsou to zelené fotosyntetizující rostliny, které ale paraziticky získávají živiny z kořenů ostatních rostlin. Někteří kořenoví poloparaziti výrazně ovlivňují biotické vztahy v temperátních travinných společenstvech, zatímco jiní patří mezi druhy ohrožené vyhynutím. V naší studii jsme na základě dat z České národní fytocenologické databáze vytvořili modely vhodnosti biotopů pro kořenové poloparazity vyskytující se v České republice. Tyto modely, založené na formalizované klasifikaci vegetace, vzájemné závislosti výskytu druhů ve vegetačních třídách a aktuálním výskytu poloparazitů ve snímcích v databázi určily pro každý poloparazitický druh soubor snímků, v nichž by se tento druh mohl vyskytovat. Tento soubor snímků byl následně popsán pomocí klimatického modelu, Ellenbergových indikačních hodnot a funkčních vlastností zastoupených druhů, což umožnilo charakterizovat vlastnosti vegetace příhodné pro jednotlivé poloparazitické druhy. Díky znalostem ekofyziologických principů poloparazitismu z experimentálních studií bylo možné interpretovat vlastnosti vhodné vegetace jako faktory určující ekologickou niku zkoumaných druhů. Jednotlivé typy vegetace se svou vhodností pro různé druhy kořenových poloparazitů značně liší. Zároveň je ale téměř každý široce vymezený typ vegetace České republiky (s výjimkou vodní vegetace) ekologicky příhodný pro alespoň některý z poloparazitických druhů. Pro poloparazity je vhodná zejména vegetace přirozených nebo polopřirozených trávníků, kde se vyskytuje i největší počet poloparazitických druhů. Podobně hojní jsou poloparaziti i v lesích, ale počet druhů je zde podstatně menší a prakticky omezený pouze na druhy rodu *Melampyrum*. Většina poloparazitických druhů není schopna růst v extrémních biotopech s intenzivním stresem nebo disturbancemi. Stejně tak živinami bohatá a dostatečně vlhká místa, kde dominují rychle rostoucí, konkurenčně silné druhy, nejsou vhodná pro výskyt poloparazitů. Tato omezení vyplývají z podstaty poloparazitizmu, jehož hlavní výhodou je parazitický zisk podzemních zdrojů. Aby bylo možné tuto výhodu využít, je třeba dostatek světla a alespoň relativně příznivé podmínky pro růst. To platí zejména pro jednoleté poloparazitické druhy, které převažují v květeně ČR. Tyto ekologické nároky poloparazitů byly dříve předpovězeny matematickými modely a prokázány ve skleníkových ekofyziologických pokusech, ale naše studie ukazuje jejich platnost v krajinném měřítku. Kromě nich ale mohou jednotlivé poloparazitické druhy vykazovat různé další nároky na podmínky prostředí a růst v různých typech vegetace.

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Root hemiparasitic plants are associated with high diversity in temperate grasslands

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Abstract

Questions: Is the incidence of root hemiparasitic plants in non-forest vegetation associated with high diversity? Are root hemiparasites more associated with species-rich vegetation than other species?

Location: Czech Republic.

Methods: Plot size-corrected species richness, Shannon diversity and Pielou's evenness were computed for a representative set of 18 101 vegetation plots representing all main types of terrestrial open (non-forest) habitats of the country. Null models of species richness assuming occurrence of a random species with given occurrence frequency, reflecting higher incidence probability in species-rich plots, were constructed for 16 common root hemiparasitic species. The null model distribution of species richness was subsequently compared with the actual mean species richness of plots containing the respective root hemiparasites. Median values of plot Shannon diversity and evenness were computed for each species in the database. Values obtained for plots containing individual root hemiparasites were compared with distribution of values for other species in the database.

Results: The occurrence of 11 of 16 root hemiparasites studied was associated with high species richness significantly more than under random expectations; three species were negatively associated. Three root hemiparasites were among the top 5% of all species associated with high species richness and Shannon diversity, and eight were among the top 25%. Almost 50% of the top 1% most species-rich plots contained at least one root hemiparasitic species.

Conclusions: We demonstrated a positive association between the incidence of most root hemiparasites and diversity of non-forest terrestrial plant communities at a broad landscape scale. This finding scales up the results of experimental studies that showed some root hemiparasites act as ecosystem engineers, increasing vegetation diversity. Root hemiparasites should be regarded as important biodiversity indicators and potential drivers of biodiversity. As such, conserving their wild populations or promoting their establishment should become a goal of nature conservation and ecological restoration.

Introduction

Biological diversity has become a central topic in ecology. Vegetation ecologists are particularly interested in factors influencing differences in the number of species between sites and ecosystems (Palmer & White 1994). At a fine scale, temperate grasslands are the most species-rich plant communities globally (Wilson et al. 2012; Chytrý et al. 2015), but mechanisms allowing for the co-existence of so many species within a small area, and their relative importance, are still the subject of lively debate (Wilson 2011). Among these, niche differentiation is probably the most often cited. To support their growth and survival, various plant species use the same essential resources, which limits the chance of niche differentiation. However, there are specialized functional groups of plants with different mechanisms of resource acquisition. These include, for example, the ability to fix atmospheric N through symbiotic bacteria in plants of the *Fabaceae*, or uptake of resources from other species in parasitic plants. Existence

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of these functional groups and the interactions of their members create opportunities for niche partitioning in the plant community.

The effects of specialized enemies have long been considered as a method to support species co-existence (Janzen–Connell effects; Janzen 1970), although mainly in the tropics. Recently, it was demonstrated that these effects are common and sufficiently strong to also support species co-existence in temperate grasslands (Petermann et al. 2008). They are mostly considered in connection with fungal infections and insect herbivores (e.g. Bagchi et al. 2014), but in principle can be associated with any consumer group showing at least some degree of specialization, including root hemiparasitic plants.

Root hemiparasites form a specialized functional group of green plants that attach to roots of other plant species in order to take up resources from their xylem (Sivicek & Taft 2011; Spasojevic & Suding 2011). Some root hemiparasitic species are even considered to be ecosystem engineers (Bardgett et al. 2006; Decleer et al. 2013). This is based on their ability to harm their host, alter competitive hierarchies in the community, affect mineral nutrient cycling and create regeneration gaps in the sward (Gibson & Watkinson 1989; Phoenix & Press 2005; Mudrák & Lepš 2010; Demey et al. 2015; Lepš & Těšitel 2015; Těšitel et al. 2015a). Individual host plant species may differ in their sensitivity to hemiparasite infection, and in their ability to exploit the nutrients mobilized and regeneration gaps created by hemiparasites. These differences can increase niche partitioning and promote species co-existence and community diversity. Although hemiparasites are not highly specialized, they might also support species co-existence by preferential use of productive and dominant hosts, similar to Janzen–Connell effects. It has also been demonstrated that the "luxury" use of resources by hemiparasites (Seel & Press 1994) can lead to a significant decrease in total community biomass in the presence of hemiparasites in comparison with analogous communities without them. Thanks to the well-known negative dependence of species richness on productivity in fertile temperate grasslands (particularly at biomass values >500 g m^{-2} dry mass; Al-Mufti et al. 1977; Grace 1999; Crawley et al. 2005), hemiparasites might support species diversity by limiting the total biomass in grassland communities.

Results of manipulative experiments testing the effects of hemiparasitic species on species diversity have been mixed, with positive (Mizianty 1975; Pywell et al. 2004; Westbury et al. 2006), negative (Gibson & Watkinson 1992) and neutral (e.g. Mudrák & Lepš 2010) effects being reported. Non-target effects, namely facilitation of weedy species (Joshi et al. 2000; Wagner et al. 2011) or support for resistant dominant species (Mudrák & Lepš 2010), are observed occasionally. In addition, data on long-term effects of hemiparasites on community diversity are lacking. Despite this uncertainty and ambiguity, hemiparasites have been suggested and used in nature conservation as a tool for increasing grassland species richness (e.g. Smith et al. 2000; Pywell et al. 2004; Westbury et al. 2006), although there is a risk of establishment of species-poor vegetation dominated by hemiparasites and hosts tolerating the infection.

Here we aim to complement previous experimental studies through analysis of the association between the incidence and diversity of hemiparasitic species of open (non-forest) terrestrial plant communities at the country scale. Specifically, we test the following hypotheses: (1) root hemiparasitic species occur more frequently in species-rich vegetation than would be expected by chance; and (2) root hemiparasites are more strongly associated with high vegetation diversity than other plant species.

Methods

Vegetation data

Data on the association between the occurrence of root hemiparasitic plant species and plant species diversity were obtained from the Czech National Phytosociological Database (Chytrý & Rafajová 2003), which contains records of vegetation plots (relevés) from the Czech Republic. We used a stratified subsample of the database following the resampling criteria used by Chytrý et al. (2005) in order to reduce differences in sampling intensity among areas and vegetation types. This resulted in a set of 31 512 plots with 2006 species, covering all the main types of vegetation in the country. From this data set, we selected 18 101 plots representing non-forest and non-aquatic vegetation (hereafter 'all plots' or 'database'). For each plot in the database, percentage cover–abundance of all vascular plants present (derived from original records on the Braun-Blanquet or Domin scale) and assignment to vegetation type (phytosociological classification into vegetation classes; Chytrý 2007–2013) were available.

We computed three diversity indices for each plot in the database: species richness (i.e. number of vascular plant species, α-diversity, S), Shannon index of diversity ($H' = -\Sigma p_i \ln p_i$, where p_i is the proportional cover-abundance of species i) and Pielou's evenness ($e = H'/\ln S$) for all plots in the database (natural logarithm was applied in all computations). The number of species was standardized by the plot size using the species–area curve to account for variable plot size in the database. This was done by fitting a species–area model:

$$S = cA^z \quad (1)$$

where c and z are parameters from non-linear least-squares fitting and A is plot size in m^2. The number of species in each plot was then standardized to the same plot size (16 m^2 as a common size of non-forest plots):

$$\text{corrected}\, S = S(16/A)^z \tag{2}$$

The corrected number of species is used in all analyses. Shannon diversity and evenness are not size-corrected because their dependence on area is very weak in the range of plot sizes used (1.04–100.00 m^2).

Association of root hemiparasites with diversity

First, we described species richness patterns of plots containing at least one of the 16 common root hemiparasites (those with at least 20 occurrences in the database) by fitting the plot richness values using negative binomial distribution. Subsequently, we tested statistical significance of the association between the incidence of individual hemiparasites and species richness of the plots. Such analysis must consider the fact that most species are more frequent in plots with a higher number of species than is the average for the whole data set. Therefore, we used null models including frequency of individual hemiparasites (n) and species richness of the plots to account for this effect. For each hemiparasite, we randomly chose n plots from the whole database, with probability weighted by their species richness, and computed the mean species richness of this sample. This step was repeated 999 times to generate the null distribution of species richness. Actual mean species richness of the plots containing individual hemiparasites was then compared with the null distributions, and P-values were determined from the null distribution quantiles. Further, we compared associations with diversity between the 16 root hemiparasites and other species in the database. All root hemiparasites studied were members of the Orobanchaceae, with the exception of *Thesium linophyllon* (Santalales). Species with at least 20 occurrences (n = 1039) in the database were considered in the analysis. For each species, we computed median values of Shannon diversity and evenness of the plots where it was present. Thus, we obtained measures of associations of each species with community diversity, which are independent of species frequency ($|r|$ of all relations between abundance and diversity indices were <0.0008). Finally, we compared the values of median diversity indices for root hemiparasites with the distribution of median diversity indices of all the other species.

Statistical analysis used R v 3.2.3 (R Foundation for Statistical Computing, Vienna, AT) and the R package 'vegan', v 2.3–5 for computation of diversity indices.

Results

The distribution of species richness across plots followed a negative binomial distribution (Fig. 1a). Plots containing individual root hemiparasites were notably shifted towards higher species richness in comparison with the richness distributions of all plots (Fig. 1a, Appendix S1). The minimum species richness recorded in plots containing a root hemiparasite was mostly between 15 and 20, which is close to the median of the overall species richness (Fig. 1). Major parts of these species richness distributions were located within the upper quartile of the overall species richness. Several root hemiparasites (*Rhinanthus major, Rhinanthus minor, Melampyrum cristatum, Thesium linophyllon*) occurred in the most species-rich plots in the database. Moreover, 88 of 182 positive outliers of species richness in all plots included a hemiparasitic species (Fig. 1b). Null model comparisons identified significant positive associations between species richness and incidence of 11 of 16 hemiparasitic species (Fig. 2). *Melampyrum nemorosum* and *Pedicularis palustris* did not show any significant associations, whereas *Bartsia alpina, Melampyrum pratense* and *Melampyrum sylvaticum* were significantly associated with species-poor vegetation (Fig. 2).

Most root hemiparasites had median values of species richness higher than the median of other species in the database (Fig. 3a, Appendix S2). *Melampyrum cristatum, Rhinanthus major* and *Thesium linophyllon* were among the top 5% of species associated with high species richness. The proportion of hemiparasites in the top 5% was significantly higher than that of non-hemiparasites (2 × 2 contingency table, $\chi^2 = 4.264$, $P = 0.039$). In addition, *Rhinanthus minor, Euphrasia officinalis* and *Odontites luteus* were close to the top 5% limit. In contrast, the association with high species richness was negative for *Bartsia alpina, Melampyrum pratense* and *M. sylvaticum*. A very similar pattern was observed for Shannon diversity (Fig. 3b). Associations with high evenness values were, in general, lower than those with species richness and Shannon diversity. Nevertheless, 50% of root hemiparasites were in top 25% of the other species (Fig. 3c).

Discussion

We have demonstrated significant positive associations between incidence of many root hemiparasites and high α-diversity of local plant communities using a data set extending over a broad landscape and geographic scale. The most notable diversity patterns include (1) absence of most hemiparasitic species from species-poor vegetation, (2) high frequency of hemiparasites in extremely species-rich plots, and (3) stronger association with species-rich

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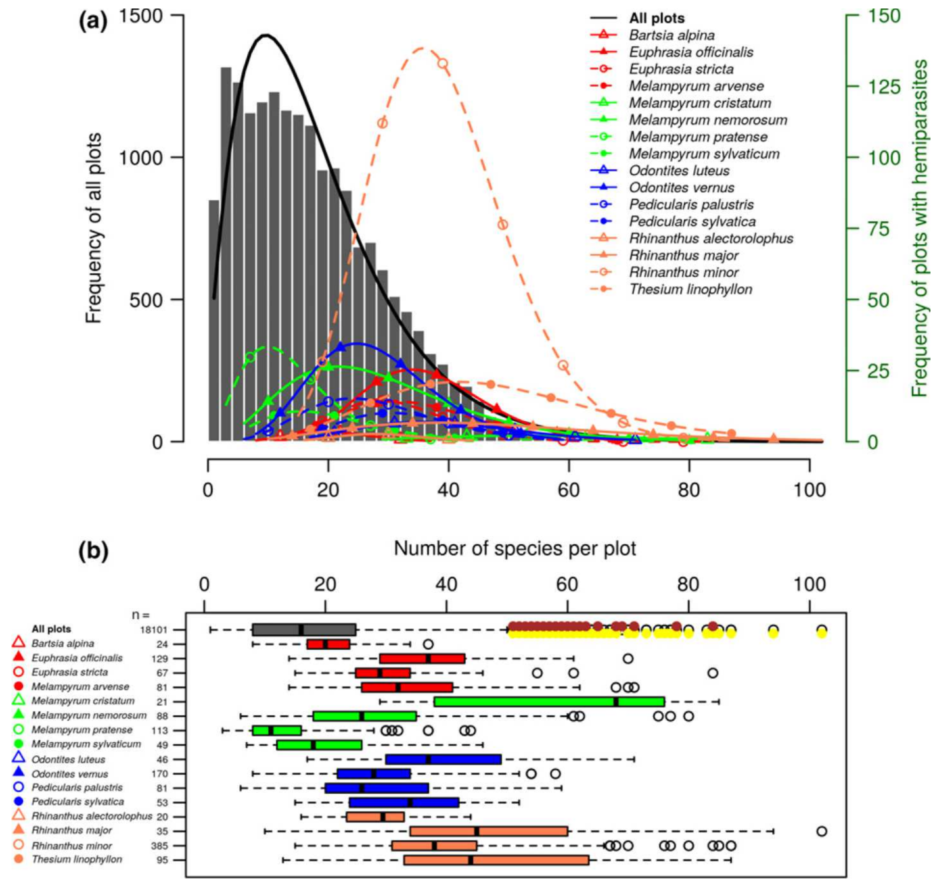

Fig. 1. Distributions of species richness in all plots and plots with individual hemiparasitic species in the database. Lines correspond to fitted negative binomial distribution for all plots and fitted negative binomial probability density scaled by hemiparasite frequency. The histogram is shown for richness in all plots, whereas histograms for individual root-hemiparasitic species are presented in Appendix S1. Yellow and brown dots close to outliers for all plots correspond to plots containing and not containing hemiparasitic species, respectively. *n* corresponds to the number of all plots or plots containing a hemiparasitic species.

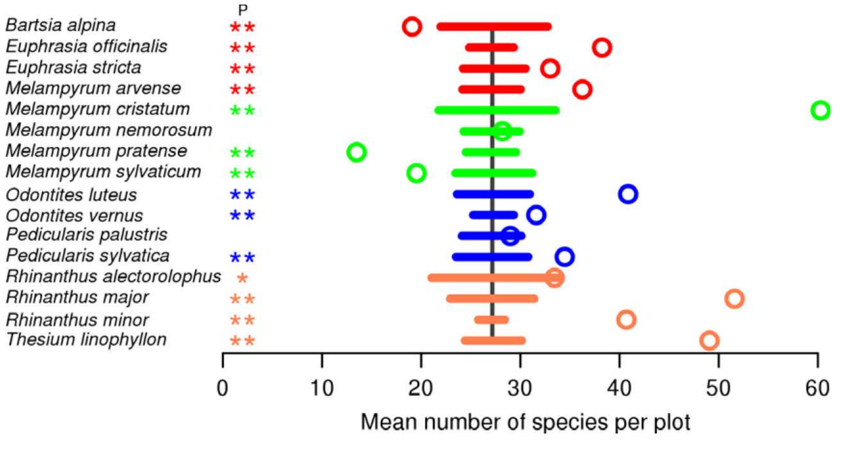

Fig. 2. Comparison between the mean observed species richness across plots containing individual root hemiparasitic species and species richness distributions in the null models. Lines represent the 2.5%–97.5% quantile ranges of the mean species richness under the random expectation. Circles correspond to the mean observed species richness of plots with hemiparasites. Vertical line denotes mean species richness in the database. Asterisks denote significance of associations with species richness: *$P < 0.05$, **$P < 0.001$.

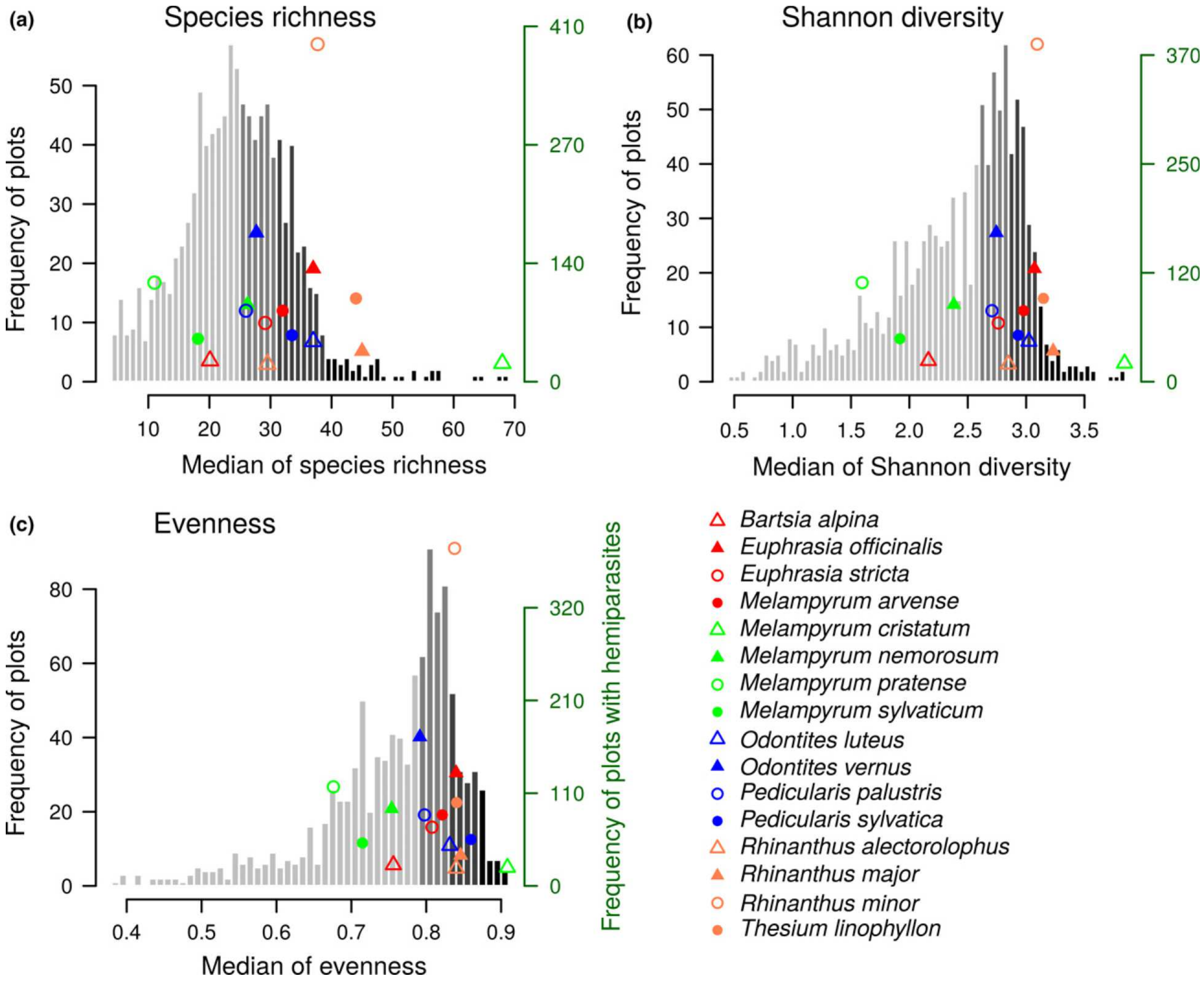

Fig. 3. Comparisons between medians of diversity indices, including (a) species richness, (b) Shannon diversity and (c) evenness, of plots containing the 16 root hemiparasites (colour symbols) and corresponding median distributions of plots containing all the other species in the database (n = 1039; gray bars). 50%, 75% and 95% quantiles of the distributions are depicted by different gray levels. The scores of the symbols on the right-hand (green) y-axis indicate frequencies of the hemiparasitic species in the database.

vegetation for many hemiparasitic species than for the rest of the flora. Three of the hemiparasitic species, *Bartsia alpina, Melampyrum pratense* and *M. sylvaticum*, were exceptions to these patterns, being associated with species-poor vegetation. An explanation of these exceptions is not straightforward. Restricted regional species pool size of some of the habitats where these species occur (e.g. bogs, springs, acidic heathlands; Sádlo et al. 2007) may account for these exceptions, at least in part.

We suggest that the positive association between incidence of most hemiparasites and community diversity identified in our study could correspond to three mechanisms. First, hemiparasites might grow in habitat types with a large species pool (species pool effects). Second, species-rich vegetation may be suitable for hemiparasites, especially due to the low intensity of above-ground competition which limits persistence of hemiparasite populations (habitat suitability effects; Těšitel et al. 2013). And third, hemiparasites may facilitate species co-existence and thus promote biodiversity (ecosystem engineering effects).

It is likely that all three mechanisms influence the association of hemiparasites with diversity. *Rhinanthus major, Thesium linophyllon* and *Melampyrum cristatum*, three species associated with the highest species richness and Shannon diversity, grow mostly in dry grasslands (Těšitel et al. 2015a), a habitat having a large regional species pool (Sádlo et al. 2007). High frequency of hemiparasites in extremely species-rich plots may thus be attributable, to a large extent, to the species pool effects. Absence of most root hemiparasites from species-poor vegetation,

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however, cannot be explained by the species pool effect. Low species richness occurs either in stressed low-productivity habitats or in high-productivity habitats where competitive exclusion prevents co-existence of competitively subordinate species (Grime 1979; Keddy 2005). Hemiparasites perform poorly under either of these extremes (Těšitel et al. 2015b) and consequently rarely occur in such conditions (Těšitel et al. 2015a). This habitat suitability effect can be quite strong and might explain both the absence of hemiparasites from species-poor vegetation and their general preference for diverse vegetation. The ecosystem engineering effects, i.e. increase in community diversity driven by hemiparasites, would be the most interesting of all these mechanisms, but more experimental evidence is needed to confirm this at a landscape scale. Published evidence suggests that ecosystem engineering effects may also positively affect habitat suitability for hemiparasites. Most grassland hemiparasites occur mainly at sites of intermediate productivity and nutrient availability (Fibich et al. 2010; Těšitel et al. 2015a). Under such conditions, they benefit most from the parasitic uptake of resources and their rapid transformation into growth and fitness attributes (Těšitel et al. 2015b). High nutrient availability and productivity are associated with high intensity of competition for light, which strongly reduces both diversity (Hautier et al. 2010) and population density (Těšitel et al. 2011, 2013) of hemiparasites. Hemiparasites are known to decrease community biomass production, especially in productive vegetation (Ameloot et al. 2005), which may increase both diversity and habitat suitability for hemiparasites. However, many hemiparasitic species require at least a moderate abundance of mineral nutrients (Těšitel et al. 2015b). Their nutrient-rich litter may enhance nutrient cycling and possibly increase productivity in nutrient-poor, low-productive habitats (Quested 2008; Spasojevic & Suding 2011; Demey et al. 2013). At such sites, an increase in productivity might be associated with an increase in diversity, following the humped-back productivity–diversity relationship (Keddy 2005; Fraser et al. 2015).

The hemiparasitic species for which we documented a positive association with diversity can be regarded as biodiversity indicators, regardless of the mechanism underlying their association with high diversity. Such an indicator role was suggested for hemiparasites of the genus *Castilleja* in North American grasslands (Sivicek & Taft 2011). Here, we expand this to a whole series of hemiparasites occurring in Central European grasslands and other types of open terrestrial vegetation. Given available experimental evidence (Mizianty 1975; Smith et al. 2000; Pywell et al. 2004; Westbury et al. 2006), it is likely that at least some of the hemiparasites influence community properties in a way that affects community diversity. Such species, in particular *Rhinanthus* spp., could be considered as biodiversity drivers, although their ecosystem engineering effects on diversity might differ depending on habitat type and might be related to community diversity itself. Given the general positive association with diversity and lack of species-poor plots containing these hemiparasites, their use as facilitators of grassland diversification can be considered as a safe method for restoration ecology, with minimum risk of non-target effects that might threaten biodiversity.

Conclusion

Our study demonstrates an association of the functional group of root hemiparasitic plant species with high local diversity of plant communities across a large region and many non-forest terrestrial vegetation types. However, we show that individual root hemiparasites display contrasting biodiversity associations. This highlights the significance of biological differences even among species belonging to the same functional group, a pattern repeatedly shown to affect parasitic interactions between plants (Rowntree et al. 2014; Demey et al. 2015).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Distributions of species richness in plots containing individual hemiparasitic species in the database.

Appendix S2. Medians of diversity indices and their corresponding quantiles considering all species in the database.

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COMMENTARY

Root hemiparasites in productive communities should attack competitive host, and harm them to make regeneration gaps

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Abstract

Demey et al. (2015, this issue) present evidence that root hemiparasitic plants can affect their host community through selectively parasitizing the species within it (with large differences between host species within functional groups), and by promoting seedling establishment through creating gaps. The hemiparasite needs to maintain the host for its water and nutrient supply, but also limit competition for light.

Hemiparasites are vascular plants that are able to photosynthesize, but obtain water and nutrients from the xylem of their host plant. Root hemiparasites connect through haustoria to the xylem of roots of their host and access water and inorganic nutrients, but also, to varying extents, organic carbon. Although the uptake of organic carbon can be very helpful in overcoming critical life stages or stressful conditions, the fitness of hemiparasites generally depends on their own photosynthesis, the efficiency of which is greatly increased by mineral nutrients (Těšitel et al. 2015). This makes their relationship with their host unique – whereas below-ground it is a parasitic interaction, above-ground it is pure competition for light. These two components of the host–hemiparasite interaction, together with the provision of litter of considerably higher quality than its host community (Spasojevic & Suding 2011; Demey et al. 2014), result in a non-trivial ecological behaviour of hemiparasites and underlie their function as ecosystem engineers.

Demey et al. (2015) demonstrate that the suppression of competitively strong species and the creation of gaps lead to increased establishment of seedlings of some species in the community. However, creating gaps for seedling establishment is probably most crucial for the hemiparasites themselves. The vast majority of species in the host communities of Demey et al. (2015) are polycarpic perennials, whereas the hemiparasites are monocarpic species (and the same is true for many other communities and for most other rhinantoid hemiparasites).

The species of the host community are not harmed evenly due to host preferences by hemiparasites. These have generally been assumed to be at the level of 'functional groups', but Demey et al. (2015) demonstrate much larger differences at the species level. Even intraspecific genotypes have been demonstrated to be of different quality as hosts (Rowntree et al. 2011). Accordingly, there is no unequivocal general pattern for the effect of a hemiparasite on the diversity of its host community. The final effect depends on the combination of relative susceptibility of individual host species and their position in the community competitive hierarchy – the presence of a highly-resistant dominant in a community (e.g. *Plantago lanceolata*) can lead to its increased dominance in the presence of a hemiparasite.

For most ordinary parasites (not only plant parasites), the somatic growth of their host means more resources for the parasite, but the growth of the shoots of the hemiparasite's host means increased competition for light. Thus, whereas for ordinary parasites the best strategy is to minimize harm to its host, medium host suppression (to the level which still provides enough water and nutrients, but limits the competition for light) might be the best strategy for a hemiparasite.

The fact that hemiparasitism is most harmful to hosts with clonal traits, as shown by Demey et al. (2015), might be underpinned by several interrelated mechanisms. The first of them is the possible clonal integration effect suggested by Demey et al. (2015), who stress the possibility of resource withdrawal not only from the infected ramet but from the whole clonal network. The effect of this mechanism would not be simple source–sink, since an increase of nutrient gain should increase the hemiparasite growth, which in turn should increase its harmful effect on the host. This makes a positive feedback loop that can result in extensive damage to the clonal host, on the one hand, and vigorous growth of the hemiparasite on the other. In

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addition, plants possessing clonal traits tend to be locally abundant (Herben et al. 2014), which indicates a relatively strong competitive ability. Competition for light has been identified as the factor strongly limiting hemiparasite seedling establishment in highly and moderately productive environments (Těšitel et al. 2013, 2015; Mudrák et al. 2014). Therefore, selecting a strong competitor as host in the community would also increase hemiparasite fitness by increasing the survival of its offspring. Moreover, hemiparasites tend to suppress the growth of host shoots more than roots, decreasing the host's above-ground competitive ability while keeping functional root systems that provide them with nutrients (Těšitel et al. 2015). The host resource depletion is also prevented by at least partial regeneration after the dieback of short-lived annual or biennial hemiparasites, which might be supported via fertilization by good-quality hemiparasite litter (Spasojevic & Suding 2011; Demey et al. 2014). This also provides a likely explanation for the fast response of clonal hosts to hemiparasite removal observed by Demey et al. (2015) because the ramet multiplication would probably require more than one season. In summary, a preference for competitive clonal hosts seems to be an advantageous strategy for short-lived hemiparasites in natural communities. Given the significance of genetic variability for host preferences (Rowntree et al. 2011), such a strategy appears likely to be under positive selection pressure in natural hemiparasite populations.

Similarly, as there is no consistent effect at the level of functional groups, the effects of individual hemiparasite species differ among themselves. The vast majority of studies are based on *Rhinanthus* spp. (including ours, e.g. Mudrák et al. 2014). Demey et al. (2015) nicely demonstrated that the effect of *Pedicularis sylvatica* could be rather different and less harmful to the community in terms of the decrease in total productivity. Moreover, Spasojevic & Suding (2011) have demonstrated that hemiparasitic *Castilleja occidentalis* (Orobanchaceae) can even increase local productivity via nutrient-rich litter, compensating for the effect of parasitism. Hemiparasites growing in productive communities, where competition limits plant performance, need to be harmful to create gaps and increase the survival of their seedlings. In contrast, for hemiparasites growing in vegetation limited by nutrient deficiency or climatic constraints, as with *Pedicularis sylvatica* and *Castilleja occidentalis*, respectively, it might be more advantageous to conserve host resources. Nevertheless, very few hemiparasite species have been experimentally studied, and for any generalization we need data on more species from a range of environmental conditions.

Hemiparasites are unique in that their complex relationship with their host includes both parasitism and competition. Also, due to their selectivity (but not too strict specialization to a single or a very limited number of species), they are an ideal model group for studying mechanisms of community assembly and competitive hierarchies in plant communities. We maintain that this opportunity is still under-used. The paper of Demey et al. (2015) is a good attempt at filling this gap.

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Suppressing competitive dominants and community restoration with native parasitic plants using the hemiparasitic *Rhinanthus alectorolophus* and the dominant grass *Calamagrostis epigejos*

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Summary

1. Dominance of native or alien competitive plants causes competitive exclusion of subordinate species and represents a major mechanism reducing biodiversity following land-use changes. The successful competitive strategies may, however, be interfered with by parasitic plants, which withdraw resources from other plants' vasculature. Parasitism may strongly reduce the growth of the dominants, which may facilitate regeneration of other species and consequently trigger restoration of natural communities of high diversity.

2. Here, we aim to provide robust empirical evidence demonstrating this restoration potential of parasitic plants. We present a case study testing suppressive effects of hemiparasitic *Rhinanthus alectorolophus* on competitive grass *Calamagrostis epigejos*. In recent decades, *C. epigejos* has invaded many high-nature-value semi-natural grasslands of Central Europe, which is one of the prominent factors causing their biodiversity decline.

3. We conducted three manipulative field experiments testing the effect of sowing of *R. alectorolophus* in different vegetation types infested by *C. epigejos*. *Rhinanthus* sowing was compared to different mowing treatments recommended as the 'best practice' management at respective sites.

4. *Rhinanthus alectorolophus* established itself in most *C. epigejos*-dominated plots where sown. *Calamagrostis epigejos* was virtually exterminated in 2 years in two of the experiments (dry meadow and industrial area). In the wet-meadow experiment, the suppressive effect was variable as a result of uneven establishment success of *Rhinanthus*. In this experiment increased mowing intensity had an additional suppressive effect on *C. epigejos*. *Rhinanthus* also increased regeneration potential of other species by a temporary reduction of vegetation density. Restoration of target vegetation composition was, however, dependent on community context.

5. *Synthesis and applications*. We demonstrated that hemiparasitic *Rhinanthus alectorolophus* is an accessible and efficient tool for targeted biological control of *Calamagrostis epigejos*, with a great potential to restore infested grassland vegetation. The strong effect of *Rhinanthus* is caused by interference with the underground storage and clonal growth strategy of *Calamagrostis epigejos*, which are both traits that underlie its competitive ability. The potential of native parasitic plants should be considered in restoration management of sites infested by competitive dominants, either alien or native.

Key-words: clonal plant, competition, diversity decline, dominance, ecological restoration, ecosystem engineering, land use change, parasitic plant, rhizome, semi-natural grassland

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Introduction

Dominance of competitive plant species is associated with low community diversity (Wisheu & Keddy 1992). An increase of dominance is a major mechanism causing biodiversity decline following land-use change (Lepš 2014). Many competitive dominants are alien invasive species (Gioria & Osborne 2014) but native dominants may have comparable effects on plant diversity (Somodi, Virágh & Podani 2008; Lepš 2014). Regulation of competitive dominants and restoration of infested communities is a serious issue in nature conservation. Optimal restoration measures should suppress competitive dominants and simultaneously support spontaneous recovery species-rich communities such as high-nature-value (HNV) grasslands. From this perspective, drastic mechanical (e.g. topsoil removal) or chemical (herbicide application) measures do not represent a desirable solution.

Parasitic plants, which take up resources directly from their host's vascular bundles may be used as biocontrol agents suppressing the competitive dominants. This parasitic mechanism interferes with the resource translocation mechanism of vascular plants. As a result, parasitic plants can display high growth rates while strongly reducing growth of their hosts (Shen *et al.* 2005, 2010; Prider, Watling & Facelli 2009; Těšitel *et al.* 2015). Many parasitic plants preferentially attack hosts of high nutrient status (Kelly 1992) or grow vigorously when attached to faster-growing (Hautier *et al.* 2010) or clonal hosts (Demey *et al.* 2015; Lepš & Těšitel 2015). Parasitic plants may thus inflict disproportional harm to host plant species displaying fast growth, efficient nutrient use or clonality, that is, traits connected to competitive ability and local dominance (Herben, Nováková & Klimešová 2014). Empirical support for such suppressive potential has recently emerged, e.g. in the case of *Pedicularis palustris* L. (Orobanchaceae). This root hemiparasite suppressed dominant sedge *Carex acuta* L., which consequently facilitated transformation of species-poor tall sedge fens into species-rich transition mires (Decleer, Bonte & van Diggelen 2013). Similarly, stem-parasitic *Cuscuta campestris* Yuncker (Convolvulaceae) has been demonstrated to suppress invasive *Mikania micrantha* H.B.K. in South China (Yu *et al.* 2008). Extensive research is being conducted on stem-parasitic *Cassytha pubescens* R.Br (Lauraceae) as a native biocontrol of invasive leguminous shrubs in Australia (Prider, Watling & Facelli 2009; Shen *et al.* 2010; Cirocco *et al.* 2015). While these studies indicate a general ability of plant parasites to decrease vitality of some competitive dominants (including alien invasives), their use as a targeted measure in ecological restoration remains to be evaluated.

Here, we examine the potential of plant parasitism, namely root-hemiparasitic species *Rhinanthus alectorolophus* (Scop.) Pollich (Orobanchaceae), as a targeted restoration measure for biocontrol of the range-expanding grass *Calamagrostis epigejos* (L.) Roth. *Calamagrostis epigejos* is a perennial rhizomatous species widespread across the whole of temperate Eurasia. It uses the guerrilla strategy of clonal growth (Rebele & Lehmann 2002) to spread rapidly in previously unoccupied communities diversity of which is consequently reduced by competitive exclusion of subordinate species (Somodi, Virágh & Podani 2008; Rebele 2014). The competitive success of the *C. epigejos* lies in its ability to store and translocate resources below ground (Rebele & Lehmann 2001; Kavanová & Gloser 2005; Gloser, Košvancová & Gloser 2007) and high nutrient use efficiency (Yuan *et al.* 2005). Despite the fact that it is relatively slow-growing (Holub *et al.* 2012), the canopy height of *C. epigejos* still reaches up to 100 cm and the grass accumulates a large amount of standing biomass over the growth season (Rebele & Lehmann 2001). *Calamagrostis epigejos* efficiently translocates nutrients from senescing shoots to its roots at the end of the growth season. As a result, its litter is nutrient-poor, decomposes slowly and accumulates in a thick layer, which reduces growth and establishment of other species (Mudrák *et al.* 2013).

Unlike most other competitive meadow grasses (e.g. *Arrhenatherum elatius* (L.) J. Presl et C. Presl, *Lolium perenne* L., *Dactylis glomerata* L.), *C. epigejos* does not require high soil nutrient availability to spread and attain dominance in the community. Instead, it benefits from low nutrient availability in HNV grasslands and their low-intensity conservation management (Háková, Klaudisová & Sádlo 2004; Kleijn *et al.* 2009). Moreover, many European HNV grasslands of low productivity (and high diversity) have been abandoned (Lepš 2014), which further facilitates *C. epigejos* to attain dominance. *Calamagrostis epigejos* is difficult to suppress by re-establishment of low-intensity land use (e.g. single-cut mowing) since this usually does not result in its substantial decrease in the short term (Lehmann & Rebele 2002; Házi *et al.* 2011). More intense restoration measures (e.g. intense mowing) may be harmful for valuable species still remaining in the community (Somodi, Virágh & Podani 2008). In summary, the expansion of *C. epigejos* represents a major threat to the biodiversity of Central European semi-natural HNV grasslands, some of which are remarkable due to the globally unparalleled species-richness at the small spatial scale (Chytrý *et al.* 2015). Conventional land-use practices (mowing, grazing) applied in low intensities (otherwise favourable for maintenance of species-rich grasslands) are inefficient in terms of suppressing *C. epigejos* as the species is well adapted to non-frequent above-ground disturbance by mobilising its below-ground resources. The below-ground resource storage could, however, be the 'Achilles heel' of the successful *C. epigejos* strategy if susceptible to infection by a root parasitic plant. A recent pilot experiment demonstrated the ability of root-hemiparasitic *R. alectorolophus* to establish in *C. epigejos*-dominated vegetation (Mudrák *et al.* 2014) and form functional haustorial connections to its roots (Fig. S1, Supporting Information).

Here, we follow up this pilot study by three manipulative field experiments to demonstrate the potential of *R. alectorolophus* to restore grasslands overgrown by *C. epigejos*. Specifically, we test three hypotheses: (1) *Rhinanthus* has the capacity to suppress *C. epigejos* rapidly when sown into its stand and established, (2) *Rhinanthus* opens gaps in the sward (community features known to be crucial for seed or bud bank regeneration; Fibich *et al.* 2013), and (3) *Rhinanthus* can increase community diversity and drive community composition towards semi-natural HNV grasslands. Sowing of *Rhinanthus* was the main and identical treatment in all experiments. We compare its effects to mowing treatments corresponding to recommended 'best practice' management of respective vegetation types at individual experimental sites (Háková, Klaudisová & Sádlo 2004).

Materials and methods

GENERAL EXPERIMENT LAYOUT AND SEED SOURCE

Three experiments were established in different habitats in 2012 to test the effect of sowing of *R. alectorolophus* on grassland vegetation dominated by *C. epigejos*. Baseline data was collected prior to any experimental treatments. Further monitoring was done annually for the following 3 years (2 years in the case of experiment 3, see below). Monitoring was conducted in late June/early July to match the phenology of *C. epigejos* which peaks at this time. At that time, *Rhinanthus* plants were senescent, mostly in the fruiting phase with some leaves having already fallen off. The recorded *Rhinanthus* cover/biomass values thus may be lower than its peak standing crop. All plots were free of any hemiparasitic species at the start of the experiments. Seed origin of *R. alectorolophus* and the sowing approach was identical in all experiments. The seeds were collected in a wild population located close to Huslenky, Vsetínské vrchy Mts., Czech Republic (49°18′58″N, 18°05′39″E, 600 m a.s.l.) and sown in the corresponding experimental plots in October 2012; no additional sowing was conducted in experiments 2 and 3. In addition, experiment 1 also comprised two mowing treatments. Experiments 2 and 3 comprised sowing of legumes as an additional treatment, which was motivated by local field observations indicating a possible supportive effect on *Rhinanthus* establishment. However, no significant effect of legumes on any of the monitored parameters was detected. Therefore, the plots where these were sown were removed from the data and the results are not reported.

Experiment 1

Experiment 1 was established on an abandoned meadow close to Švihov, Železné hory Mts., Czech Republic (49°50′08″N, 15°51′44″E, 440 m a.s.l.). The meadow consisted of a mosaic of intermittently wet meadows (*Molinion*) and oligotrophic submontane grasslands (*Violion caninae*; Chytrý 2007–2013) that had been unmanaged for at least a decade. Most of the site was overgrown by *C. epigejos* with an admixture of other grasses (*Alopecurus pratensis* L., *Deschampsia cespitosa* (L.) P. Beauv.) but species typical of local species-rich meadows, such as *Carex hartmanii* Cajander, *Betonica officinalis* L., *Sanguisorba officinalis* L., *Solidago virgaurea* L., and *Viola canina* L. were still scarcely present in the community.

The experimental layout consisted of six blocks each composed of four 3 m × 3 m plots. Whole blocks were mown in summer (after the vegetation composition data sampling and biomass collection) which is a standard management practice of this vegetation type. Two experimental treatments were combined in a full factorial design: (i) sowing of *R. alectorolophus* (500 seeds m^{-2}) and (ii) an additional mowing in October (including litter removal and gentle moss layer disturbance by raking, a treatment known to potentially improve *Rhinanthus* establishment; Mudrák *et al.* 2014). This resulted in four treatment combinations within each block: (i) summer mowing only (control; corresponds to recommended conservation management), (ii) summer and autumn mowing (corresponds to conventional restoration management of *C. epigejos*-heavily infested sites), (iii) summer mowing + *Rhinanthus*, (iv) summer and autumn mowing + *Rhinanthus*. Sowing (using fresh seeds) and all treatments were repeated every year. Vegetation composition of the central 2 m × 2 m square of each of the plots was monitored every year in late June/early July by a visual estimate of cover (%). In addition, above-ground biomass of *C. epigejos* was harvested from a 1 m × 1 m permanent square located within the central square to determine its dry-weight.

Experiment 2

Experiment 2 was established on a dry meadow in the northern part of the White Carpathian Mts. (49°6′41″N, 18°03′00″E, 410 m a.s.l.). The meadow had been abandoned for approximately 40 years and regular summer mowing had restarted 8 years prior to the experimental layout. The plant community was dominated by *C. epigejos* and *Brachypodium pinnatum* (L.) P. Beauv., but many dicotyledonous forbs (*Centaurea jacea* L., *Knautia arvensis* (L.) J. M. Coult., *Pulmonaria mollis* Hornem., *Trifolium medium* L.) persisted on the site. The site was fenced to prevent browsing of roe deer.

The experimental layout included five blocks each composed of two 2 m × 2 m plots. *Rhinanthus alectorolophus* was sown (500 seeds m^{-2}) onto one of the plots while the other was a control without any sowing. Whole blocks were mown in summer (after the vegetation composition data sampling and biomass collection), which follows the 'best practice' conservation management of this vegetation type. Vegetation composition of the central 1 m × 1 m square was monitored annually at the end of June using the calibrated weight-estimate method (Tadmor *et al.* 1975). Species biomass estimate was based on its estimated proportion in the community multiplied by the community biomass, which was harvested from the same square and its dry weight was determined.

Experiment 3

Experiment 3 was established on an abandoned site located on the campus of the University of South Bohemia and the Biology Centre of the Czech Academy of Science in České Budějovice (48°58′33″N, 14°26′47″E, 390 m a.s.l.). The site was unmanaged for c. 20 years and was largely overgrown by *C. epigejos*. The site was fenced which prevented access of larger wild mammals such as roe deer or wild boars.

The experimental layout included four blocks each composed of two 3 m × 3 m plots. *Rhinanthus alectorolophus* was sown

(500 seeds m^{-2}) onto one of the plots while the other plot was a control without any sowing. Whole blocks were mown twice a year – in summer (July) and autumn (October). Following the autumn mowing, the litter was removed and gentle raking was applied as in experiment 1. This mowing treatment corresponds to the conventional restoration management suitable for sites. Vegetation composition of the central 2 m × 2 m square of each of the plots was monitored every year in late June/early July by a visual estimate of cover (%). Biomass of *C. epigejos* was not sampled in this experiment; therefore the percent cover was used as a dominance measure. The experimental site was destroyed in the autumn of 2014 due to establishment of an arboretum. Therefore, the monitoring could not extend to 2015.

DATA ANALYSIS

We analysed the following variables in each experiment: a measure of dominance of *C. epigejos* (biomass dry weight or its estimate or cover), which was used to test hypothesis 1, herb layer cover relevant to hypothesis 2, and cover of *Rhinanthus* as an indicator of *Rhinanthus* establishment. To test hypothesis 3, we computed the Shannon diversity index (*H*) from the vegetation composition data using natural logarithms of species abundances. In addition, we computed dissimilarity of vegetation composition of each sample to the corresponding potential target vegetation. Vegetation composition of reference grassland types was obtained from the Czech National Phytosociological Database (Chytrý & Rafajová 2003) from which we selected plots of intermittently wet meadows and submontane oligotrophic grasslands (*Molinion, Violion caninae*), semi-dry suboceanic grasslands (*Bromion*), and mesic meadows (*Arrhenatherion*; Chytrý 2007–2013) as targets for experiments 1, 2 and 3, respectively. The dissimilarities were subsequently computed as Bray–Curtis dissimilarity between composition of individual vegetation samples and the most similar plot in the corresponding reference set. The dissimilarities were based on the square-root of cover (experiments 1, 3) or square-root of biomass estimate values (experiment 2).

Mixed-effect linear models were used for all univariate statistical analyses. Biomass and cover data were log-transformed prior to analysis. A saturated model containing a year of sampling (recoded as a year since the start of the experiment), all treatments and all possible interactions as fixed effects and plot identity nested within a block as random effects was first fitted for each response variable. Minimum adequate models were subsequently selected by sequential removal of non-significant fixed-effect terms or their interactions. We retained non-significant terms, of which interactions were significant. Significance tests of individual regression coefficients of the final models are reported in the text. All analyses were conducted in R, version 3.2.2 (R Core Team 2015) using R package *nlme*, version 3.1 (Pinheiro *et al.* 2015). We also conducted multivariate constrained ordination analyses (Šmilauer & Lepš 2014) of the community composition presented in Appendix S1.

Results

EXPERIMENT 1

In the wet meadow overgrown by *C. epigejos*, *R. alectorolophus* established in all sown plots in 2013 but its cover was variable (Fig. S2.1 in Appendix S2). *Rhinanthus* decreased slightly in 2014 followed by a steep decline in 2015. Flowering *Rhinanthus* plants were regularly damaged by grazing roe deer which nibbled the inflorescences, but this damage never occurred before flowering and mostly affected a minor proportion of the plants. Deer damage was only apparent on *Rhinanthus*; other plants were left intact.

Biomass of *C. epigejos* was significantly reduced by both sowing of *Rhinanthus* and mowing twice per season (Fig. 1; Table 1). These negative effects (*Rhinanthus* × year interaction $t_{69} = -4{\cdot}33$, $P < 0{\cdot}001$; mowing twice × year interaction $t_{69} = -2{\cdot}17$, $P = 0{\cdot}033$) were additive (Table 1); therefore the largest reduction of *C. epigejos* biomass was observed in the plots where both treatments were applied. Reduction of *C. epigejos* was variable among the blocks, which was correlated with the abundance of *Rhinanthus*. More than 90% decline of *C. epigejos* was generally observed only in plots where *Rhinanthus* cover reached 20% at least once during the experimental period (Fig. 2). The experimental block where *Rhinanthus* failed to reduce *C. epigejos* was co-dominated by *A. pratensis*, a fast-growing tall grass, which *Rhinanthus* apparently did not parasitise and which probably prevented its better establishment.

In addition to the significant suppression of *C. epigejos, Rhinanthus* significantly reduced herb layer cover 2 years after its sowing (Fig. 1; *Rhinanthus* × 2014 vs. 2012 interaction $t_{66} = -4{\cdot}70$, $P < 0{\cdot}001$) and increased Shannon index (Fig. 1; *Rhinanthus* × year interaction $t_{70} = 2{\cdot}88$; $P = 0{\cdot}005$). Both sowing of *Rhinanthus* and mowing twice a year significantly decreased dissimilarity (i.e. increased similarity) to target vegetation (*Rhinanthus* × year interaction $t_{69} = -2{\cdot}64$, $P = 0{\cdot}010$; mowing twice × year interaction $t_{69} = -2{\cdot}20$, $P = 0{\cdot}031$). Significant directional effect of *Rhinanthus* on community composition was identified also by a community ordination analysis (Appendix S1).

EXPERIMENT 2

In the dry meadow, *R. alectorolophus* established itself in all sown plots in 2013 (Fig. S2.2 in Appendix S2). The establishment success was rather even among the blocks. *Rhinanthus* abundance did not substantially change in the following year but a marked decline was observed in 2015 (Fig. S2.2 in Appendix S2).

Calamagrostis epigejos was strongly suppressed by the sowing of *Rhinanthus* (Fig. 3, Table 1; *Rhinanthus* × year interaction $t_{28} = -4{\cdot}68$, $P < 0{\cdot}001$). The suppression was considerable already in the first year after sowing but even more pronounced in the successive years, when the abundance of *C. epigejos* declined close to zero in all plots with *Rhinanthus* (Fig. 3).

Rhinanthus significantly decreased herb layer cover in the year following its sowing (Fig. 3; *Rhinanthus* × 2013 vs. 2012 interaction $t_{24} = -2{\cdot}58$, $P = 0{\cdot}016$) and in the successive year (*Rhinanthus* × 2014 vs. 2012 interaction $t_{24} = -2{\cdot}56$, $P = 0{\cdot}017$). The herb layer cover increased

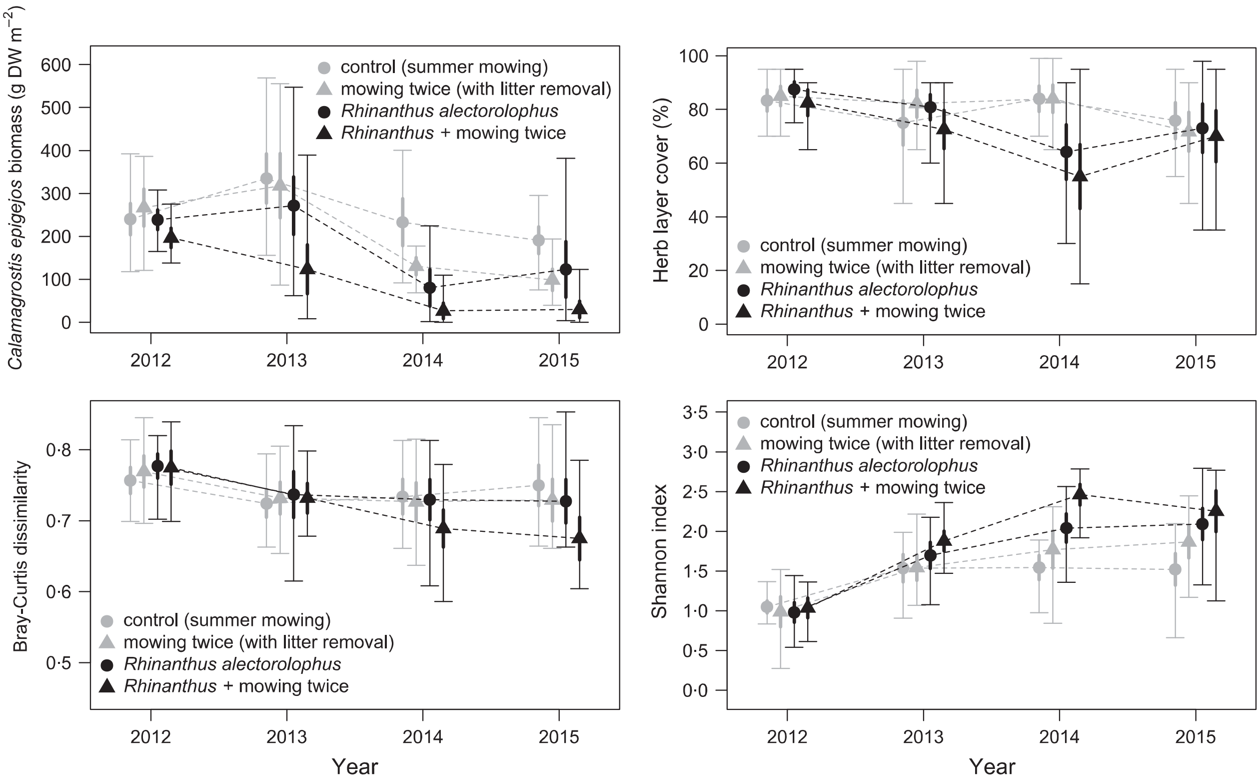

Fig. 1. Effects of the experimental treatments on *Calamagrostis epigejos* above-ground biomass, herb layer cover, dissimilarity of vegetation composition to target vegetation and Shannon index in the experiment 1. Means, one standard error intervals, and data ranges are displayed by points, bold lines, and whiskers, respectively.

Table 1. Summaries of minimal adequate models describing the effects of individual predictors on response variables in experiments 1–3

Exp.	Response	Model structure (fixed effects)†
1	*Calamagrostis epigejos* biomass	Mowing twice + *Rhinanthus* + Year + Mowing twice × Year* + *Rhinanthus* × Year***
1	Herb layer cover‡	*Rhinanthus* + Year + *Rhinanthus* × Year***
1	Distance to target vegetation	Mowing twice + *Rhinanthus* + Year + Mowing twice × Year* + *Rhinanthus* × Year*
1	Shannon index	Mowing twice* + *Rhinanthus* + Year*** + *Rhinanthus* × Year**
2	*Calamagrostis epigejos* biomass estimate	*Rhinanthus* + Year + *Rhinanthus* × Year***
2	Herb layer cover‡	*Rhinanthus* + Year + *Rhinanthus* × Year*
3	*Calamagrostis epigejos* cover	*Rhinanthus* + Year** + *Rhinanthus* × Year***

†Only models containing at least one significant fixed-effect predictor are listed.
‡Year was used as a categorical predictor in these models.
*$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$; non-significant regression coefficients are displayed in grey.

again in the last year of the experiment (Fig. 3). No significant effects of *Rhinanthus* on the Shannon index, dissimilarity to target vegetation or a directional change of community composition (Appendix S1) were observed.

EXPERIMENT 3

At the abandoned site, *R. alectorolophus* established itself in all sown plots in 2013 and retained its dominance also in the following year (Fig. S2.3 in Appendix S2). As a result of seed dispersal, few *Rhinanthus* plants were established at some of the control plots in 2014 (Fig. S2.3 in Appendix S2).

Calamagrostis epigejos declined in plots of all treatments throughout the experiment (Fig. 4; year main effect $t_{14} = -3{\cdot}89$, $P = 0{\cdot}002$). However, the decline was significantly steeper in plots with *Rhinanthus* (Fig. 4; *Rhinanthus* × year interaction $t_{14} = -5{\cdot}12$, $P < 0{\cdot}001$). *Calamagrostis epigejos* cover approached zero in the second year after sowing of *Rhinanthus*. There was no significant effect of *Rhinanthus* on herb layer cover, the Shannon index, dissimilarity to target vegetation or a directional change of community composition (Appendix S1).

Discussion

All three experiments support hypothesis 1 by demonstrating the potential of *R. alectorolophus* to substantially and rapidly (in 1 or 2 years) suppress *C. epigejos*. The

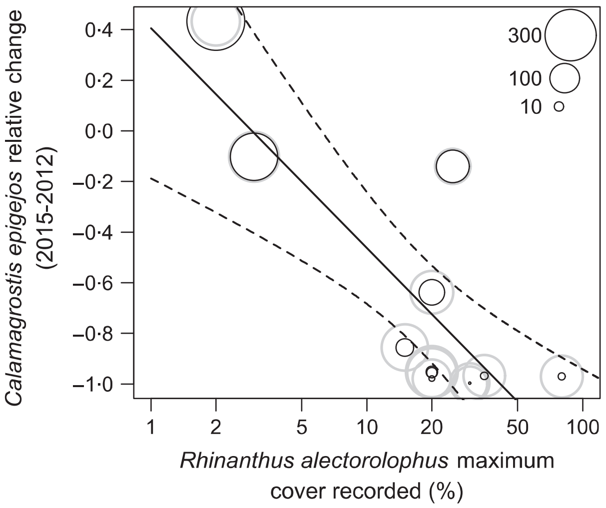

Fig. 2. Effect of maximum cover of *Rhinanthus alectorolophus* on the relative change of *Calamagrostis epigejos* above-ground biomass between the start and the end of experiment 1. Each experimental plot with *Rhinanthus* is represented by two concentric circles. Grey circles and black circles indicate *C. epigejos* above-ground biomass in 2012 and 2015, respectively. Size of the circles is proportional to the dry mass in g m^{-2} as indicated by the legend. Note the log-scale of the *x*-axis. Regression line with confidence intervals is displayed ($r^2 = 0{\cdot}65$, $F_{1,10} = 18{\cdot}43$, $P = 0{\cdot}0016$).

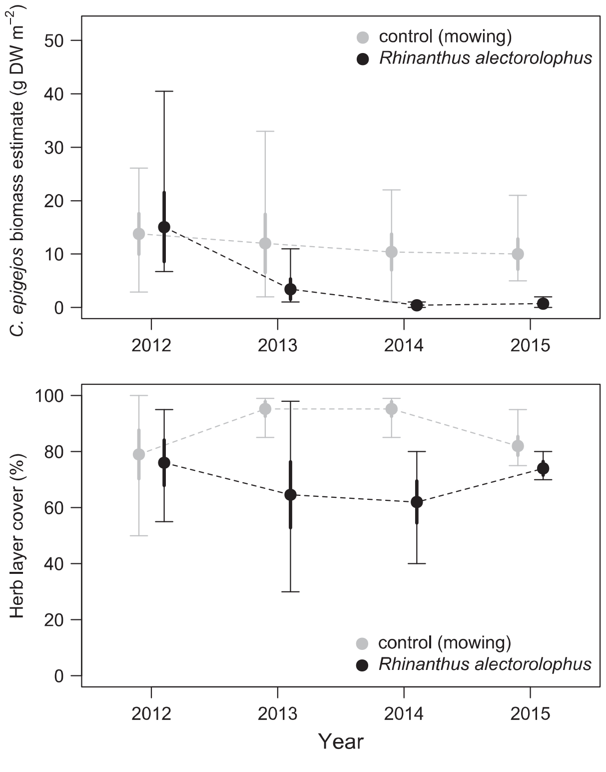

Fig. 3. Effect of *Rhinanthus alectorolophus* on *Calamagrostis epigejos* above-ground biomass estimate and herb layer cover in comparison with single mowing control in experiment 2. Means, one standard error intervals, and data ranges are displayed by points, bold lines, and whiskers, respectively.

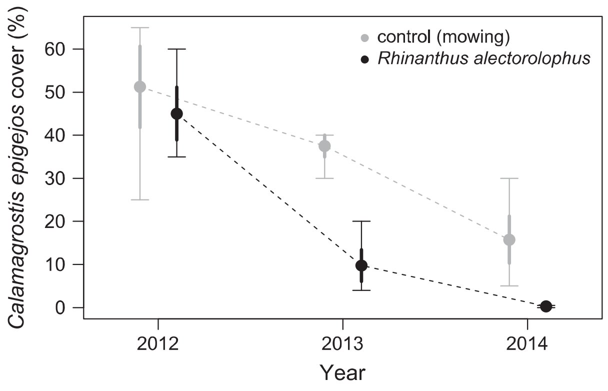

Fig. 4. Effect of *Rhinanthus alectorolophus* on *Calamagrostis epigejos* cover in comparison with mowing-twice control treatment in experiment 3. Means, one standard error intervals, and data ranges are displayed by points, bold lines, and whiskers, respectively.

level of suppression is tightly linked with the establishment success of *Rhinanthus*. Successful application requires a minimum threshold abundance of the hemiparasite (20% cover in the case of experiment 1). *Rhinanthus* establishment in favourable environmental conditions can be impeded by competition for light during its early development (before or shortly after the attachment to the host; Těšitel *et al.* 2011). Therefore, co-dominance of fast-growing dominants with early phenology may be a limiting factor for *Rhinanthus* as indicated by one block of experiment 1 co-dominated by fast-growing *A. pratensis*. This limitation due to competition for light from fast-growing dominants can be expected to be strongest at sites that are simultaneously moist and nutrient-rich where the relative advantages provided by hemiparasitism decrease as does the suppressive effect on the host (Těšitel *et al.* 2015).

The rapid decline of *C. epigejos* following the establishment of *Rhinanthus* was comparable to the effect of a selective herbicide. The initial rapid increase of the hemiparasite causing the rapid decline of *C. epigejos* followed by the decline of *Rhinanthus* in successive years indicates a relatively strong specificity of the hemiparasitic interaction. The specificity is furthermore supported by the moderate effect of *Rhinanthus* on the community composition in experiment 1 and the lack of directional effects in experiments 2 and 3. Such strong and specific interaction may seem surprising given the widely shared view on *Rhinanthus* species as generalists with preferences for grasses and legumes (Cameron & Seel 2007). However, this view has recently been challenged by Rowntree *et al.* (2014) who demonstrated host species identity was more important than functional group membership for the outcome of the hemiparasitic interaction. From this perspective, *C. epigejos* may be a host with traits that make it exceptionally susceptible to hemiparasite infection. Its extensive resource storage located in the roots (Kavanová & Gloser 2005) can be directly accessed by the hemiparasites.

Root-parasitism thus interferes with the key trait underlying *C. epigejos* growth and competitive strategy, which inflicts intense stress on the grass. Its relatively slow growth and late phenology decrease the above-ground competitive effects on hemiparasite seedlings (provided the slowly decomposing litter layer is removed or not too thick). Moreover, *Rhinanthus* growth and the massive harm inflicted to *C. epigejos* were likely further increased by the host clonality as suggested by recently formulated clonal integration hypothesis (Demey *et al.* 2015; Lepš & Těšitel 2015). Experiments 1 and 2, however, indicate that the hemiparasite population may collapse after depleting the clonal host resources. This is crucial for the practical use of the hemiparasites as biological control and restoration agents because it diminishes the possibility of their long-lasting dominance. However, it also means that the suppressive effect on *C. epigejos* is rather short-term and restoring its dominance must be prevented by standard methods of conservation management (e.g. mowing once a year). On a larger spatial scale than just a few square metres, *Rhinanthus* can be expected to establish patch dynamics within a site, which may result in a continuous and spatially heterogeneous suppressive effect on *C. epigejos* population.

The community effects of *R. alectorolophus* corresponding to hypotheses 2 (sward opening) and 3 (a directional community composition change and an increase of diversity) were only observed in two or one of the experiments, respectively. A temporary decrease of herb layer cover induced by *Rhinanthus* was observed in experiments 1 and 2, that is, the semi-natural HNV grassland sites. Such sward opening indicates potential for community composition change towards the target vegetation type. However, realisation of such potential differed between experiments 1 and 2. In addition, experiment 1 has demonstrated that the effect of the hemiparasites may be synergic with increased mowing intensity. This variability of the community effects was probably caused by differential community contexts. The vegetation in experiment 1 was largely overgrown by *C. epigejos*. Most other species had disappeared from the experimental plots but were still scarcely present at the site, in the seed bank or in dormant underground stages. Their re-appearance or re-establishment after opening the canopy increased species-richness up to 42 species 4 m^{-2} which approaches the maximal recorded species-richness (51 species 4 m^{-2}) of wet meadows in the region (Horník *et al.* 2012). In experiment 2, *C. epigejos* was less dominant than at the start of the other two experiments due to re-establishment of management 8 years before. Therefore, plant diversity had been preserved because the competitive exclusion of subordinate species occurs only after increasing dominance of *C. epigejos* (Somodi, Virágh & Podani 2008). Experiment 3 might have been too short-lasting to observe sward opening, although substantial gaps not captured by the monitoring must have occurred in late summer following the death of the *Rhinanthus* annuals. The context dependency seems typical of *Rhinanthus* biodiversity effects as indicated by previously observed positive (Pywell *et al.* 2004; Westbury *et al.* 2006) or neutral (Westbury & Dunnett 2007, 2008; Mudrák & Lepš 2010) effects of *Rhinanthus minor* on community diversity despite almost universal reductions of standing crop biomass and relative proportion of grasses.

Root hemiparasites are recognised as a functional group with profound community and ecosystem effects (Press & Phoenix 2005; Watson 2009; Demey *et al.* 2015). Species of the genus *Rhinanthus* have been demonstrated to decrease community productivity (Ameloot, Verheyen & Hermy 2005), alter competitive relations in the communities (e.g. Davies *et al.* 1997; Westbury & Dunnett 2007; Mudrák & Lepš 2010; Hellström, Bullock & Pywell 2011; Demey *et al.* 2015) and affect nutrient cycling (Fisher *et al.* 2013; Demey *et al.* 2014). These effects may facilitate temperate grassland diversification. Specifically, native *R. minor* has been demonstrated to suppress grasses and increase subordinate forbs, an effect extensively used in grassland restoration in the United Kingdom (e.g. Pywell *et al.* 2004; Westbury *et al.* 2006; Hellström, Bullock & Pywell 2011). However, this application of the hemiparasites concerns the re-creation of semi-natural grasslands on ex-arable land or restoration of grasslands degraded by high land-use intensity (fertiliser application mainly), that is, ecosystems in which biodiversity has been negatively affected by human activity. The underlying community effect of the hemiparasites is largely non-specific as it reduces standing crop and the grass:forb ratio (i.e. relation between broadly defined functional groups). By contrast, complete eradication of the competitive dominant and pronounced decrease of herb layer cover inflicted by *R. alectorolophus* in our study, represent extreme forms of these ecological effects of root hemiparasites.

Several studies testing the impacts of other (hemi)parasitic plants on particular competitive dominants revealed similarly drastic and at least partially specific effects (Yu *et al.* 2008; Prider, Watling & Facelli 2009; Shen *et al.* 2010; Decleer, Bonte & van Diggelen 2013; Cirocco *et al.* 2015). Such suppressive effects suggest that native (hemi)parasitic plants may be considered as potential biological controls of competitive dominants possibly including invasive species. However, any such application must be underlain by detailed knowledge of both host and parasite biology and experimental field assays to ensure its efficiency and minimise risks of possible adverse side-effects.

Given the profound effects of many parasitic plants on ecosystems (Press & Phoenix 2005; Watson 2009) extensive damage may be incurred by an alien parasitic plant; therefore, only native parasitic plants may be considered. Local propagule sources should be used preferably to conserve local genetic resources. However, the lack of a clear phylogeographic pattern in *Rhinanthus* species in Europe (Vrancken, Brochmann & Wesselingh 2009, 2012) suggest a lower importance of this rule in this particular case. A more pragmatic approach to seed origin may be considered; e.g., the recently suggested genetic diversity approach, which involves sowing a mixture of seeds originating from multiple sources (Kettenring *et al.* 2014).

Conclusions and applications

We demonstrated the potential of *R. alectorolophus* to act as a largely specific native biological control of competitive dominant *C. epigejos*. Depending on the community context, *Rhinanthus* may also facilitate restoration of biodiversity of *C. epigejos*-infested grasslands. Our study is one of the pioneering works to demonstrate native parasitic plants as promising tools to control problematic, mainly competitive, plant populations across the globe. The parasites' interference with the growth strategy of the targeted competitive species underlies the strong suppressive effect. Such targeted use of (hemi)parasitic plants complements their current generic use to facilitate diversification and increase forb abundance in HNV semi-natural grassland restoration.

Authors' contributions

J.T., J.M. and J.H. conceived the ideas and designed the methodology. J.T., J.M., J.H., T.T. and V.A. collected the data. J.T. and L.T. analysed the data. J.T. led the writing of the manuscript. All authors contributed to the drafts and gave final approval for publication.

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Data accessibility

Vegetation composition and *Calamagrostis epigejos* biomass data are available at Dryad Digital Repository https://doi.org/10.5061/dryad.4r390 (Těšitel *et al.* 2017).

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Supporting Information

Details of electronic Supporting Information are provided below.

Fig. S1. Anatomical section of haustorium of *Rhinanthus alectorolophus* on the root of *Calamagrostis epigejos*.

Appendix S1. Community ordination analysis.

Appendix S2. Relative dominance of *Rhinanthus alectorolophus*.

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DNA barcoding as a tool for identification of host association of root-hemiparasitic plants

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Abstract Root hemiparasites are green plants which attach to roots of other plants and extract solutes from the host-root xylem parasitically. They frequently act as keystone species by affecting competitive relations in plant communities and altering mineral nutrient cycling. Understanding their ecology has been hindered by the difficulty to identify host associations of hemiparasitic plants growing in natural conditions. Direct examination of host associations is only possible at sites with specific soil types (e.g. sandy substrates) that allow host roots to be traced to the shoot to identify the host species. We investigated the possibility of using a DNA barcoding-based approach to identify host associations of root hemiparasites growing naturally. Host associations of *Melampyrum nemorosum*, *Rhinanthus major* and *Rhinanthus minor* were investigated. Their root systems were washed free of soil to reveal haustorial connections. Host-root fragments were subjected to DNA extraction and the plastid DNA *trn*L intron was sequenced. The functioning of haustoria was examined in semi-thin anatomical sections. The analysis of the DNA of host roots yielded a ca 60% success rate. The *Rhinanthus* species were confirmed to attack mostly grasses and legumes. By contrast, *Melampyrum nemorosum* attached mainly to plants of Rosaceae and Asteraceae. In addition, both conspecific and heterospecific connections between the hemiparasites were frequently observed. Our study demonstrates the usefulness of the DNA barcoding approach for identifying host associations of root hemiparasites at sites where tracing host roots to their shoot is not possible due to soil structure or high rooting density.

Keywords DNA barcoding · haustorium · hemiparasite · host spectrum · *Melampyrum nemorosum* · Orobanchaceae · *Rhinanthus major* · *Rhinanthus minor* · *trn*L intron

Introduction

Root hemiparasites are green photosynthetic plants which parasitize other species by attacking their roots (Irving and Cameron 2009; Těšitel 2016). They use specialized root organs called haustoria to attach to the roots of other plants and withdraw mineral nutrients, water and a variable amount of organic assimilates from their hosts (Irving and Cameron 2009; Těšitel et al. 2010; Těšitel et al. 2015). Nevertheless, root hemiparasites are generally dependent on their own photosynthesis for most organic carbon, which makes the hemiparasite-host relationship a unique ecological interaction. It is a parasitic interaction below ground, while above ground it is pure competition (Matthies 1995a; Mudrák and Lepš 2010; Těšitel et al. 2013;

Plant nomenclature vascular plants – Danihelka et al. (2012)

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Těšitel et al. 2015). Root-hemiparasitic plants mainly of the family Orobanchaceae were demonstrated to affect competitive relations (Matthies 1996; Press and Phoenix 2005; Lepš and Těšitel 2015) and nutrient cycling (Spasojevic and Suding 2011; Demey et al. 2013; Fisher et al. 2013). A major part of the knowledge on the ecology and ecosystem effects of root-hemiparasitic plants originates from field manipulative experiments (e.g. Pywell et al. 2004; Ameloot et al. 2005; Mudrák and Lepš 2010; Borowicz and Armstrong 2012; Demey et al. 2015). However, the inference power of all these studies is limited by the fact that the host associations of the hemiparasites are not investigated directly but rather guessed on the basis of the reactions of individual species on hemiparasite sowing/removal.

Root hemiparasites generally tend to have wide ranges of suitable host species (based on direct identification, see paragraph below), e.g. 50 host species were identified for *Rhinanthus minor* (Weber 1976; Gibson and Watkinson 1989), 68 host species for *Santalum spicatum* (Woodall and Robinson 2003), 80 host species for *Pedicularis canadensis* (Piehl 1963). Selectivity is not consistent within one population, and even between plants from different parts of the same population (Gibson and Watkinson 1989). In addition, various pot experiments demonstrate that some species higher-quality host plants than others and are more preferred; i.e. hemiparasites form more haustoria on their roots and show a better growth if attached to them (Gibson and Watkinson 1991; Matthies 1996; Helton et al. 2000). Traditionally, grasses and legumes have been considered more frequent and favourable hosts of temperate root hemiparasites of Orobanchaceae (Weber 1976; Gibson and Watkinson 1989; Cameron et al. 2006). The sole presence of the haustorium attached to a host root does not necessarily mean that the host is suitable. Some of the potential hosts are able to impregnate their cells (with lignine or suberine) or cause a tissue fragmentation and thus prevent the hemiparasite from tapping their xylem vessels. The functionality of the connection can, however, be examined through its anatomical structure (Cameron et al. 2006; Cameron and Seel 2007; Rümer et al. 2007).

The excavation of haustoria and tracing the host roots to the shoot for species identification presents the only current method of direct identification of the host spectra of root-hemiparasitic plants under natural conditions (e.g. Piehl 1963; Gibson and Watkinson 1989; Suetsugu et al. 2008; Dostálek and Münzbergová 2010). This approach is extremely labourious and only applicable in some types of substrate (e.g. sandy soils). Here, we aim to test the applicability of the DNA barcoding approach for the identification of the host associations of hemiparasitic plants. This method allows the host species to be identified using a short DNA sequence which represents a standardized position in the genome (Hebert et al. 2003). Sequencing of DNA of a small part of the root to which the haustorium is attached would thus allow to identify the host species without the need for tracing the host root to the shoot. Host associations of three root hemiparasites of the family Orobanchaceae were examined by washing the roots free of soil, the haustorial connections were identified, and DNA barcoding was consequently applied to fragments of host roots. In addition, the anatomy of haustoria was checked to ascertain whether the haustoria were functional.

Materials and methods

Site description and sampling

The field sampling was conducted on the Čertoryje meadow, Bílé Karpaty Mts., Czech Republic (48°51′26″ N, 17°25′07″ E) in the first week of June 2010. The Čertoryje meadow is recognized for its high vascular plant species richness which reaches world record values at plot sizes of 25 m^2 and 49 m^2 (Chytrý et al. 2015). The bedrock consists of folded flysh with a predominance of clay soils. Wet clay soils are heavy and sticky but when dry, they become a hard, concrete-like crust which is difficult work with. Thus, the high number of possible host species, high plant rooting density and the soil type create very difficult conditions for studying host associations of hemiparasitic plants and the traditional direct analysis of host communities based on host root tracking is therefore not feasible.

Host associations of three root-hemiparasitic Orobanchaceae were studied: *Melampyrum nemorosum*, *Rhinanthus major* and *R. minor*. All three species are herbaceous annuals and occur abundantly on meadows throughout the study site. Naturally established plants in the Čertoryje meadow were sampled. Two plots (4 × 4 m) with intermixed populations of all three studied species (plot 2 and 3) and one plot with only *M. nemorosum* and *R. major* were established. ten individuals of each species under study were sampled at each plot including the root systems. The hemiparasite individuals for the study were sampled in an approximately regular spatial arrangement over the sampling plot. The root system of hemiparasites was sampled by a soil core (5 cm in diameter; 10 cm deep with the studied hemiparasite in its middle). The resulting core size is sufficient to cover most of the root system of the hemiparasitic species, since the root systems of these plants are rather small (Matthies 1995a, Matthies 1995b; Press 1998). In addition, a list of all species with their respective cover-abundances was recorded in each of the three studied plots (Table S1 in the Electronic Supplementary Material). A voucher specimen was collected for each plant species recorded in the three plots and a small piece of its leaf was dried and stored in silica gel. This material was also used for later building the reference DNA barcode library. All herbarium vouchers are stored in the herbarium of the Faculty of Science, University of South Bohemia (CBFS).

The cores were immediately examined for haustorial connections using a stereo-microscope. The soil was gently washed by a stream of water to expose the haustorial connections. As the heavy clayish soils are present in the Čertoryje and the haustorial connections are quite brittle, many haustoria were found detached from the host root and therefore not used in the study. Only attached haustoria were fixed for further examination of haustorial anatomy. They were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH = 7.2) for at least 24 hours (allowing the fixation solution to penetrate the sample). The maximum possible length of connected host roots (the average length was only about 0.5 cm, because they were fragile and can be easily shred) were taken, dried and stored in silica-gel for DNA extraction.

Host identification

The root fragments of the host plants were identified by the DNA barcoding. To distinguish the host species we used the chloroplast *trn*L (UAA) intron. This barcode allows the differentiation of closely related or even congeneric species, is suitable for analysis of highly degraded DNA (Taberlet et al. 2007), offers an extensive set of host reference sequences in the GenBank database and is well established.

DNA extraction, PCR and DNA sequencing

For host roots (Fig. 1), REDExtract-N-Amp Plant PCR kit (Sigma-Aldrich, Missouri, USA) was used to extract and then amplify the DNA following the manufacturer's manual. The *trn*L (UAA) intron was amplified with primers *c* 5'–CGAAATCGGTAGACGCTACG–3' and *d* 5'–GGGGATAGAGGGACTTGAAC–3' (Taberlet et al. 1991). PCRs were performed in a reaction mixture containing 2 μL of genomic DNA, 5 μL Extract-N-Amp PCR ReadyMix, 1.2 μL of each primer and 0.6 μL of sterile water to make up a final volume of 10 μL. Amplifications (touch-down PCR) were performed with an initial denaturation of 4 min at 94°C; followed by 40 cycles of denaturation (30 s at 94°C), annealing (30 s at the temperature gradually decreasing by 0.5°C from 61°C to 56°C), elongation (2 min at 72°C), and a final extension of 10 min at 72°C. Samples with no PCR product after touch-down PCR were once more amplified using a semi-nested PCR protocol to higher the chance of getting PCR product. First, touch-down PCR (see above) was employed to enrich target *trnL* intron DNA with primers *c* and *f* 5'–ATTTGAAC TGGTGACACGAG–3', and the PCR product was used

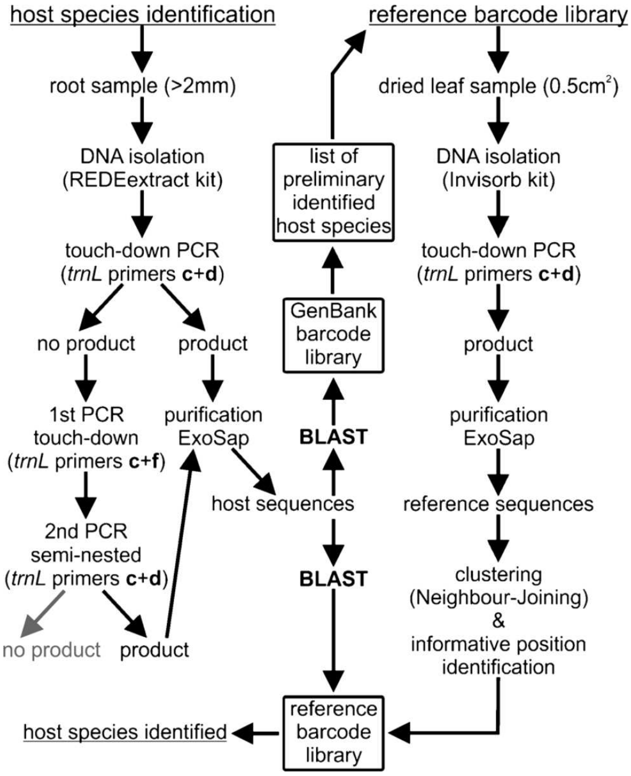

Fig. 1 Schematic diagram of host identification.

as a template for second, regular PCR with primers *c* and *d*. The protocol of the second PCR was as follows: initial denaturation of 4 min at 94°C; followed by 40 cycles of denaturation (1 min at 94°C), annealing (1 min at 53°C), elongation (2 min at 72°C) and a final extension of 10 min at 72°C. Resulting PCR products were purified using ExoSap (Exonuclease I and FastAP Thermosensitive Alkaline Phosphatase; Thermo Fisher Scientific, Lithuania) and sequenced (Biology centre ASCR, České Budějovice, Czech Republic). The length of high quality readout host root sequences ranged from 240 to 652 bp, matching *trnL* lengths published by Taberlet et al. (2007).

For the reference library (Fig. 1), the total genomic DNA was isolated from 0.5 × 0.5 cm dried leaf by using Invisorb Spin Plant Mini Kit (INVITEK, INC., California, USA) following the manufacturer's protocol. The *trn*L (UAA) intron was amplified with primers *c* and *d* (Taberlet et al. 1991). PCRs were performed in a reaction mixture containing 0.4 μL of genomic DNA, 1.2 μL of each primer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP and 0.5 U Taq polymerase (Top-Bio, Czech Republic) in the manufacturer's reaction buffer, and sterile water to make up a final volume of 10 μL. We used the same touch-down PCR protocol with the same parameters as described for the root samples (see above). PCR products were purified using ExoSap and sequenced (Biology Centre ASCR, České Budějovice, Czech Republic). Only sequences with sufficient length and no ambiguities were used. The length of sequences ranged from 310 to 603 bp. GenBank accession numbers are provided in Table S2 in the Electronic Supplementary Material.

Building the reference barcode library and final host identification

Host root samples were sequenced first and preliminarily assigned to the species or genus by comparing them with the *trnL* sequences in the GenBank database using the BLAST algorithm with default setup (Fig. 1). Then we built a reference barcode library from the leaf samples of species occurring at the studied plots and best fitting the preliminary identified host species. All reference library sequences were hierarchically clustered using the Neighbour-Joining method to identify informative positions (bases, indels) within each species sequence.

Definitive host species identification was based on comparisons of root sequences with the reference barcode library using the BLAST algorithm. A 97% similarity cut-off value was set, which is a similarity threshold commonly used (e.g. Hiiesalu et al. 2012). 10% of analysed sequences with similarity lower than 97%, caused by lower sequence quality and consequent presence of ambiguities mostly in non-informative positions, were included in the results because BLAST in combination with hierarchical clustering (neighbour-joining method) allowed the unequivocal identification of the host species. It was possible to use the time-consuming combination of BLAST and hierarchical clustering due to a small number of species and samples in the dataset. The programme Geneious 7.06 was used for the BLAST comparisons and neighbour joining analyses (Kearse et al. 2012). GenBank accession numbers of root sample sequences that were successfully identified are available in Table S3 in the Electronic Supplementary Material.

Anatomy of haustoria

Anatomical sections of the haustoria were used to examine the functionality of the parasite-to-host connection. Larger parts of host roots, which were connected to haustoria, were taken to DNA analysis and their haustorial connections were used for anatomical analysis. The samples of haustoria fixed in glutaraldehyde were washed in 4% glucose solution in 0.1 M phosphate buffer three times (15 min each) and post-fixed in a 1% solution of osmium tetroxide. The samples were then washed three times in 0.1 M phosphate buffer, dehydrated by a graded acetone series (30, 50, 70, 80, 90, 95 and 100%) with 15 minutes for each step and embedded in Spurr resin (Sigma-Aldrich, Missouri, USA). The embedded samples were cut to semi-thin sections 400 nm thick, stained by toluidine blue and examined under a light microscope for xylem to xylem connection. The connections were considered functional either the secondary xylem of hemiparasite and/or the direct contact of xylem vessels of the host and the hemiparasite could be observed (Fig. 2). The sections displaying a dark-staining layer and lacking both the secondary xylem of hemiparasite and xylem contact between host and hemiparasite were classified as a non-functional connection (Fig. 2).

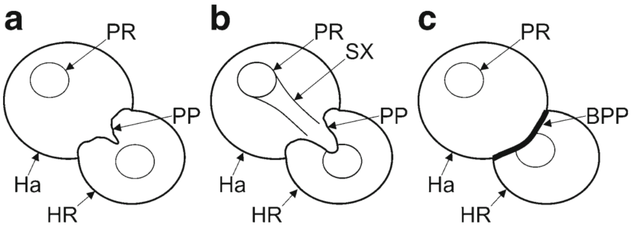

Fig. 2 Schematic diagram showing haustoria formed by hemiparasites on potential hosts. BPP – blocked penetration peg (lignified region), Ha – haustorium, HR – (potential) host root, PP – penetration peg (endophyte), PR – parasitic root, SX – secondary xylem of hemiparasite.

Statistical analysis

The data on haustorial connections between the root hemiparasites and their hosts were pooled across the three sampling plots. Unique combinations of host species and hemiparasite individuals (i.e. soil core sample centred around an individual hemiparasitic plant) are considered independent observations in the data analysis; however, raw counts of haustoria are also presented.

Rarefaction (Hurlbert 1971) was used to assess the species richness of communities of potential hosts of the root-hemiparasitic species. This technique estimates the expected number of species in a sample of given sample size based on species frequencies in the dataset. Expected numbers of species can be computed for each sample size from 1 to the size of the whole dataset, which may be plotted as a rarefaction curve. Consequently, diversity can be compared between the datasets on the basis of differences between the rarefaction curves. The shape of the curves also allows the completeness of the communities to be assessed because the initial slope of the curve is steep and decreases with the completeness of the community.

In addition to the host community we compared the frequency of haustorial attachments to cover abundances of plant species in the community. Unfortunately, this was not possible at the species level as the sample size was insufficient given the high species richness of the studied vegetation. Therefore, we performed this analysis at the family level only. A goodness-of-fit test was employed in this analysis. Expected counts of haustorial attachments under the null hypothesis assuming attachment frequencies proportional to family cover abundances were computed as family proportion in the vegetation multiplied by the total number of haustoria of respective hemiparasite. Such calculation of the expected numbers of haustoria based on the relative above-ground dominance of the plant species means that the revealed deviations cannot be interpreted in terms of chance to attach a root of a species if a hemiparasite is given an option to select. Instead they involve also the effects caused by variability of root traits among potential host species. That is, some species may form more extensive root system or produce more roots in such depth where hemiparasites forage for host roots. Such species would come out as parasitized more frequently than expected despite the probability of producing a haustorial connection to them may not differ or may be even lower than other species. This measure is thus indicative of the relations between below-ground and above-ground community context of the hemiparasitic species habitat.

All statistical analyses were computed in R v. 3.1.2 (R Core Team 2014). The rarefaction analysis was conducted using the R package vegan v. 2.2 (Oksanen et al. 2014).

Results

Host identification

We excavated 92, 103 and 81 haustorial connections of *Melampyrum nemorosum*, *Rhinanthus major* and *R. minor*, respectively. The amplification and subsequent host identification rates yielded 64, 50, and 62% success for *M. nemorosum*, *R. major* and *R. minor*, respectively. In total, we detected 17, 14 and 16 species of host plants on the basis of 58, 51 and 50 haustorial connections in *M. nemorosum, R. major*, *R. minor*, respectively. The rarefaction analysis produced similar saturation curves for the three species. The saturation curves did not reach plateaus but displayed a notable decrease of slopes (Fig. 3). This indicates that a substantial proportion of the host community was sampled.

Despite growing in the same host community, the host associations of the respective hemiparasitic plants differed. *Melampyrum nemorosum* was most frequently attached to forb species *Fragaria viridis, Inula salicina* and *Filipendula vulgaris*. *Rhinanthus major* parasitized mainly legumes, mostly *Medicago falcata*, and *R. minor* attached to roots of grasses, particularly *Festuca rupicola* (Table 1). Large proportions of haustorial connections of both *Rhinanthus* species were found to be attached to hemiparasites, either *M. nemorosum* or *Rhinanthus* spp. In the latter case, both conspecific and

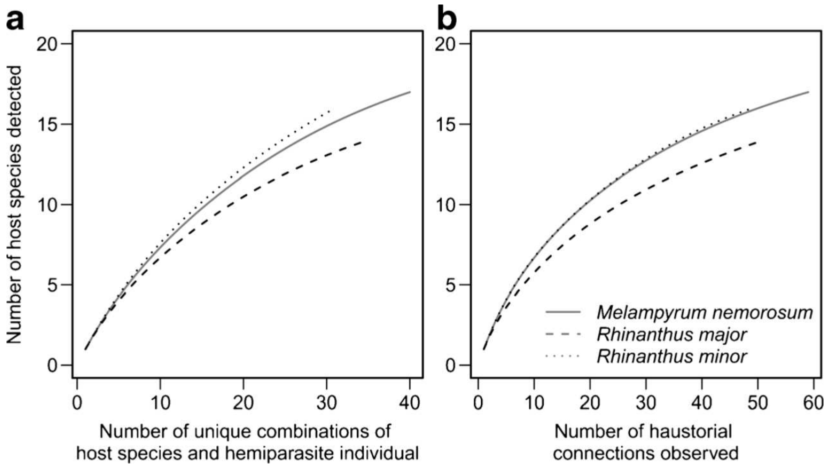

Fig. 3 Rarefaction curves of potential host species of the three root hemiparasites under study. The curves indicate expected number of species in number of individuals or samples analysed. The rarefaction curves were computed across unique combinations of host species and hemiparasite individual (a) and across individual haustorial attachments (b).

heterospecific connections were found, while only conspecific connections were observed in *M. nemorosum*. No haustoria of this species attached to *Rhinanthus* spp. were observed. Most of the potential hosts can be considered as subdominants of the stands sampled (i.e. their cover is at least a few percent). Haustorial attachments to some of the dominants, namely *Colchicum autumnale*, *Geranium sanguineum* and *Potentilla alba,* were not observed (Table S1 in the Electronic Supplementary Material).

At the family level (Table 2), *Melampyrum nemorosum* was most frequently attached to members of Asteraceae, Orobanchaceae and Rosaceae. Significantly more haustorial connections were identified to Asteraceae, Plantaginaceae, Rosaceae, Rubiaceae than expected from above-ground abundance and significantly less to Geraniaceae, Lamiaceae and Poaceae. *Rhinanthus major* was most frequently attached to Fabaceae and Orobanchaceae. Its haustoria were significantly more frequently attached to Orobanchaceae and Rubiaceae than expected from above-ground abundance but significantly less to Geraniaceae, Poaceae and Rosaceae. *R. minor* was most frequently attached to Orobanchaceae and Poaceae. The number of its haustorial connections to Geraniaceae was significantly lower than expected from above-ground abundance.

Haustorial connection functionality

Good quality anatomical sections were obtained for ca 50% of hemiparasite to host species connections. Many samples were lost due to failure of fixation (possibly caused by decay of old haustoria) and insufficient removal of the clay particles which obstructed the ultramicrotome cutting. Sections (Table 1; Figs 1–3 in the Electronic Supplementary Material) indicate that the connections between the hemiparasites and most of the host species, in which good quality anatomical sections were obtained, are functional. Only few species appeared to be able to prevent the haustorium from penetrating and tapping their xylem vessels, namely *Centaurea jacea* and *Melampyrum nemorosum*, parasitized by *M. nemorosum*, and *Asperula tinctoria* and *M. nemorosum*, parasitized by *Rhinanthus major.* In all cases a dark staining layer on the contact of the haustorium peg with the host root was visible (Figs 1, 2 in the Electronic Supplementary Material).

Haustorial connections between hemiparasites varied in their functionality. Haustoria of *Rhinanthus major* attacking *Melampyrum nemorosum* were not classified as functional, while those of *R. minor* attacking *M. nemorosum* appeared to be functional. *Melampyrum nemorosum* appeared to attack only the roots of other individuals of *M. nemorosum*. However, this particular connection seems to be dysfunctional (Figs 1–3 in the Electronic Supplementary Material).

Discussion

The DNA barcoding approach based on the *trn*L intron was found to be a generally suitable tool for studying host associations of root-hemiparasitic plants in particular in conditions where other methods are not feasible (e.g. direct identification of the species to which the hemiparasites are attached. On average, the amplification rates yielded 60% success, which is acceptable, considering the varying quality of the plant material

Table 1 Number of haustorial connections and their functionality evaluated by anatomical sections (+ connection between host species and hemiparasite is functional; – connection between host species and hemiparasite is not functional; NA, it was not possible to study due to failure of sample preparation, usually due to decay of the haustorium).

Species group	Host species	*Melampyrum nemorosum*			*Rhinanthus major*			*Rhinanthus minor*		
		Number of haustorial connections	Number of hemiparasite individuals connected	Haustorium connection functionality	Number of haustorial connections	Number of hemiparasite individuals connected	Haustorium connection functionality	Number of haustorial connections	Number of hemiparasite individuals connected	Haustorium connection functionality
Grasses	*Arrhenatherum elatius*	1	1	NA	0	0	.	0	0	.
	Bromus erectus	0	0	.	1	1	NA	2	1	+
	Festuca pratensis	0	0	.	0	0	.	1	1	NA
	Festuca rupicola	0	0	.	2	2	NA	6	3	NA
	Poa pratensis	0	0	.	0	0	.	2	2	NA
	Trisetum flavescens	0	0	.	0	0	.	2	1	NA
Legumes	*Lathyrus pratensis*	1	1	+	2	2	+	3	2	+
	Medicago falcata	2	2	NA	6	4	+	0	0	.
	Medicago lupulina	1	1	NA	0	0	.	0	0	.
	Trifolium alpestre	0	0	.	0	0	.	1	1	+
	Trifolium pratense	1	1	NA	0	0	.	0	0	.
	Vicia tenuifolia	2	2	+	1	1	+	1	1	NA
Forbs	*Asperula tinctoria*	3	1	NA	3	2	–	0	0	.
	Centaurea jacea	2	2	–	0	0	.	0	0	.
	Cruciata verna	2	2	NA	0	0	.	0	0	.
	Euphorbia cyparissias	0	0	.	0	0	.	1	1	NA
	Filipendula vulgaris	5	5	+	0	0	.	2	2	NA
	Fragaria viridis	9	2	+	0	0	.	0	0	.
	Galium pumilum	0	0	.	1	1	NA	0	0	.
	Inula salicina	6	3	+	1	1	+	1	1	+
	Plantago lanceolata	3	3	NA	0	0	.	2	1	NA
	Salvia pratensis	0	0	.	2	2	NA	0	0	.
	Serratula tinctoria	3	2	+	1	1	NA	0	0	.
	Valeriana stolonifera	1	1	NA	0	0	.	0	0	.
Hemiparasites	*Melampyrum nemorosum*	16	10	–	5	4	–	11	5	+
	Rhinanthus major	0	0	.	21	11	NA	3	2	NA
	Rhinanthus minor	0	0	.	4	2	NA	10	6	NA
	*Rhinanthus minor/ major**	0	0	.	1	1	NA	2	2	NA

*Species identification not possible due to low sequence quality used. The root fragments of the host plants were often very small (thin and 2 mm to several centimetres long) and contaminated with soil particles, which may have impeded the DNA extraction process. The chloroplast *trn*L intron represents a good target region for the DNA barcoding procedure. Its sequences have been used for identifying plant species (Ronning et al. 2005) even in phylogenetic studies among closely related species (Gielly and Taberlet 1996). Despite the popular use of the *trn*L intron for plant DNA barcoding, its application on roots has been rather restricted (but see Hiiesalu et al. 2012; Tedersoo et al. 2008). In our study, *trn*L intron allowed the differentiation between closely related or even congeneric species. Still, a potential bias might be introduced by differential DNA extraction efficacy and the quality of DNA in some species. Also secondary metabolites, such as polyphenols, tannins and polysaccharides, naturally occurring in some species can inhibit DNA amplification. Although DNA extraction protocol can be adjusted for secondary metabolites removal (Porebski et al. 1997), low DNA concentration especially with presence of secondary metabolites can make amplification very difficult or even impossible. Failure of DNA amplification could, however, be due to more or less random factors such as eventual sampling of dead root fragments (that is not apparent during core dissection), which would not introduce any systematic bias to the resulting host community data.

Inspections of the anatomy of haustoria allows to check their functionality (Cameron et al. 2006). In our study, host species for which anatomy is not available are considered as only potential hosts here. The definitive evidence of host species suitability can be confirmed by a pot-cultivation experiment. Relatively low success of the preparation of anatomical sections of haustoria can be attributed to low quality of the material coming from natural conditions and contamination by soil particles. In our anatomical sections we dealt with the following problems: (A) After obtaining the semi-thin sections, the microscopic anatomical structures proved to be indiscernible in many samples because some of these were scratched with the residual soil particles during the process of cutting, although haustouria and their host roots were properly cleaned; (B) The haustoria got sometimes disconnected from the host root when handled during the process of embedding it in the resin; (C) Haustoria were either not yet fully developed or too senescent (decay) to clearly demonstrate the functionality of the given haustorium. In addition, the process of embedding to the resin and following semi-thin cutting is very laborious and time consuming. Hand-cutting of haustoria with a razor blade under a dissection microscope may be an alternative approach not sensitive to clay particle contamination and avoiding laborious sample preparation. Skills to work precisely with small plant structures are however required. Nevertheless, this approach was used in a recent study (Ebermannová 2015) with a similar success rate to the approach used in our study.

The finding of conspecific (and to some extent also heterospecific) haustorial connections between hemiparasites raises the question whether this could be due to contamination. Although this can never be completely excluded in individual cases, it is not likely that the observed high numbers of connections between hemiparasites (Table 2) would be caused mainly by contamination. Many of such haustorial connections were heterospecific, which means that the likelihood of contamination is the same as for the attachment to a non-parasitic host. In addition, we also directly documented haustorial attachments between individuals of *Rhinanthus major* during examination of its root system in a field sowing experiment nearby (Fig. 4 in the Electronic Supplementary Material). Conspecific (Gibson and Watkinson 1989; Prati et al. 1997; Ebermannová 2015) and heterospecific (Weber 1976) connections among hemiparasites have also been reported in earlier studies. The conspecific haustorial connections may function as an underground network, potentially enabling resource redistribution among hemiparasite individuals (Prati et al. 1997). The functionality of the conspecific and heterospecific connections in natural conditions needs to be verified by anatomy or e.g. using stable isotope labelling techniques (Světlíková et al. 2016) since these connections may not be always functional. However, our study is the first to demonstrate that the connections among hemiparasite individuals can be rather common and as such may have a significant effect on the population ecology of root-hemiparasitic plants.

Our study updates the recognized host ranges of all hemiparasitic plants under study. Together with previous data (Weber 1976; Gibson and Watkinson 1989) 63 potential host species are recently recognized for *Rhinanthus minor* growing in natural conditions (13 of these were detected here for the first time). Twenty-nine potential host species (Heinricher 1909; Heinricher 1924; Oesau 1975; Weber 1976; Matthies 1991) are

Table 2 Statistics of haustorial attachments of the three root hemiparasites at the family-level host resolution. Counts of attachments are based on unique combinations of species and hemiparasite individuals. *P*-values are based on comparisons of the test statistics with the χ^2 distribution with 1 degree of freedom. Up and down arrows indicate species with higher and lower attachment frequencies than expected, respectively. Overall goodness-of-fit tests for individual species: *Melampyrum nemorosum* $\chi^2_8 = 68.31$, $P < 10^{-6}$, *Rhinanthus major* $\chi^2_6 = 58.13$, $P < 10^{-6}$, *Rhinanthus minor* $\chi^2_4 = 13.99$, $P = 0.007$. Only families with observed or expected value higher or equal to 3.0 were included in the statistical test.

Family	*Melampyrum nemorosum*				*Rhinanthus major*				*Rhinanthus minor*			
	Number of haustorial connections	Expected	χ^2	*P*	Number of haustorial connections	Expected	χ^2	*P*	Number of haustorial connections	Expected	χ2	*P*
Asteraceae	11	3.30	17.97	< 0.0001 ↑	2				1			
Euphorbiaceae	0				0				1			
Fabaceae	7	10.4	0.92	0.3377	9	9.7	0.00	0.9802	5	3.74	0.42	0.5156
Geraniaceae	0	5.78	5.78	0.0162 ↓	0	5.22	5.22	0.0223 ↓	0	6.35	6.35	0.0117 ↓
Lamiaceae	0	4.87	4.87	0.0274 ↓	2	4.40	1.31	0.2526	0			
Orobanchaceae	16	11.94	1.38	0.2398	30	10.79	34.18	<0.0001 ↑	24	16.44	3.47	0.0624
Plantaginaceae	3	0.61	9.40	0.0022 ↑	0				2			
Poaceae	1	12.55	10.63	0.0011 ↓	3	11.34	6.14	0.0132 ↓	13	10.77	0.46	0.4975
Rosaceae	14	6.46	8.79	0.0030 ↑	0	5.84	5.84	0.0156 ↓	2	6.69	3.29	0.0698
Rubiaceae	5	1.46	8.58	0.0034 ↑	4	1.32	5.44	0.0197 ↑	0			
Valerianaceae	1				0				0			

now known for *Melampyrum nemorosum* (14 of them were detected here for the first time). The host range of *R. major* with twelve newly identified species was not studied previously. The host ranges differed among the hemiparasitic species, although they grew in the same host community. Only 17% of all parasitized species were common to the studied.

Hemiparasites more importantly, the host ranges did not differ only at the species level, but also at higher taxonomic and functional group levels. Apart from the connection to hemiparasites, *R. minor* attached most frequently to grasses (Poaceae) followed by legumes (Fabaceae), while *R. major* to legumes (Fabaceae) followed by Rubiaceae. These frequencies mostly followed expectations based on high cover-abundances of these families in the vegetation (Table 2). The revealed host associations of both *Rhinanthus* species thus more or less correspond to those reported in the literature and tested experimentally with grasses and legumes as principal hosts (Gibson and Watkinson 1989; Cameron et al. 2006). The differential associations of individual *Rhinanthus* species with these functional groups observed here have never been reported. However, this may actually be a characteristic of particular populations studied rather than the whole species given the existence of genotypic lineages differing in their interactions with genotypes of a single host species (Rowntree et al. 2011). The observed host range of *M. nemorosum* with Rosaceae, Asteraceae followed by Fabaceae and Rubiaceae is largely different from those of either *Rhinanthus* species. However, the associations with Rosaceae and Asteraceae are not exceptional in hemiparasitic Orobanchaceae. They were reported, for example, for the Chinese species *Pedicularis kansuensis* (Bao et al. 2015) and the Arctic species *P. dasyantha* (Gauslaa and Odasz 1990).

In conclusion, the DNA barcoding approach based on the *trn*L intron coupled with anatomy of haustoria was demonstrated here as a viable method of the identification of host associations of generalist root-parasitic plants in natural conditions. Our case study on three generalist root-hemiparasitic plants has demonstrated its usefulness at a site where host identification is extremely challenging due to high species richness of the plant community and clay-rich soil type that obscures root system dissection. Therefore, at least similar performance of the method can be expected in other temperate grasslands that display the highest diversity of root-hemiparasitic plants. The method may be applicable also to root-hemiparasitic Santalales. The fact that they are mostly shrubs or trees complicates the identification of their host associations because their root systems cannot be completely excavated. Still, the DNA barcoding approach would benefit from further methodical refinements. In particular, improvements of the DNA extraction and alternative methods of haustorial anatomy examination facilitating the workflow have a potential to overcome the current bottlenecks.

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SPECIAL FEATURE: ECOLOGICAL RESTORATION

Establishment of hemiparasitic *Rhinanthus* spp. in grassland restoration: lessons learned from sowing experiments

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Keywords
Ecological restoration; Ecosystem engineer; Grassland management; Hemiparasite; Seedling establishment; Species-rich meadows

Abbreviation
DW = dry weight

Nomenclature
Kubát et al. (2002)

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Abstract

Questions: What are the factors limiting the establishment of hemiparasitic *Rhinanthus* spp., ecosystem engineers promoting diversity and stability of communities, in restoration of species-rich meadows? How can these constraints be overcome?

Location: Czech Republic, Central Europe.

Methods: Sowing of *Rhinanthus* seeds in grasslands of different history, biotic and abiotic properties accompanied by a variety of management practices. Followed by consequent monitoring of establishment and population dynamics and synthesis of relevant literature resources.

Results: Appropriate timing of seed sowing is needed for successful *Rhinanthus* introduction. *Rhinanthus* seeds must be sown at latest in November to break their dormancy (valid for Central European populations; might be different e.g. in oceanic regions). *Rhinanthus* is able to establish a viable population mainly in low- to moderately-productive grasslands with biomass production below 500 $g\cdot DW\cdot m^{-2}$. Its establishment is difficult in grasslands of higher productivity due to the effect of competition for light on the seedlings. *Rhinanthus* requires grasslands with appropriate management including mowing and removal of litter, if it accumulates. Otherwise, the litter layer strongly suppresses seedling establishment. Litter removal can allow establishment even in some productive communities such as stands of *Calamagrostis epigejos*. However, early mowing (i.e. mowing conducted before ripening of seeds) can lead to a strong decline or even local eradication of the hemiparasitic annuals. Soil conditions on the site of seed origin and the target site can play an important role, as indicated by failures of establishment of *Rhinanthus* originating from slightly acidic soils on sites with soil pH values around 8. Suitability of a given site for *Rhinanthus* introduction can be estimated on the basis of its species composition using the Beals index or less formal expert knowledge of local floristic associations of *Rhinanthus* spp. In the case of suitable conditions, sowing density of 200–500 $seeds\cdot m^{-2}$ should be sufficient for *Rhinanthus* establishment.

Conclusion: Mowing or grazing, litter removal, proper timing of sowing, and use of the seeds from local seed sources should considerably increase probability of *Rhinanthus* successful introduction. However, stochastic events like adverse weather conditions or damage through herbivory make the prediction of introduction success difficult.

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Introduction

European semi-natural grasslands host one of the most species-rich plant communities. The intensification of agricultural production and abandonment of unproductive or inaccessible areas has led to the loss of this habitat and is a considerable threat to biodiversity (Poschlod et al. 2009). To reverse this negative trend, large effort has been invested into grassland restoration. The main obstacles to the restoration often include lack of propagules of target species and high productivity resulting in dominance of competitively strong grasses, which prevent target species establishment (Walker et al. 2004). While the lack of propagules can be overcome by seed sowing (Jongepierová et al. 2007; Kirmer et al. 2012; Mitchley et al. 2012) or by hay transfer from species-rich meadows (Kiehl & Wagner 2006; Klimkowska et al. 2010), methods of decreasing productivity are less straightforward. High productivity is mainly caused by excessive amounts of nutrients in the soil profile, which are difficult to remove. Removal of nutrient-rich topsoil could be an effective method in this respect; however its feasibility is limited due to its drastic nature and high cost (Hölzel & Otte 2003; Kiehl & Wagner 2006). Productivity reduction by removal of hay from the site represents another option, which, however, does not always bring the desired results even over a long time scale (Smits et al. 2008; Pavlů et al. 2011). Recently, introduction of hemiparasitic species of the genus *Rhinanthus* (Orobanchaceae) to grassland communities has been identified as an effective and relatively cheap option for sward productivity reduction (Bullock & Pywell 2005).

The genus *Rhinanthus* comprises ca. 25 closely related species in Europe (Soó & Webb 1972). All of them are annual, yellow flowering, root-hemiparasitic herbs frequently occurring in grassland communities (Skála & Štech 2000; Chytrý & Tichý 2003). *Rhinanthus* spp. have the ability to substantially reduce total biomass production of the community (even with *Rhinanthus* biomass included) due to their low nutrient and water use efficiency (Phoenix & Press 2005) and damage inflicted to the hosts (Cameron et al. 2008). A reduction of up to 73% (mean value 26%) of total plant community biomass was reported (Ameloot et al. 2005; see this meta-analysis for more details on factors affecting the extent of grassland productivity reduction by *Rhinanthus*). Grasses and legumes are generally preferred hosts of *Rhinanthus* spp., while non-leguminous dicots (forbs hereafter) tend to be hosts of poor quality (Cameron et al. 2006). *Rhinanthus* presence or introduction therefore usually suppresses grasses, while promoting forbs, often including target species of restoration projects (Ameloot et al. 2005). Still, there are species, which do not follow this general pattern. The effect of *Rhinanthus* involves multiple mechanisms such as litter input (Fisher et al. 2013), and knowledge on the host preferences of *Rhinanthus* is still rather poor. Moreover, there might be hitherto unstudied differences among individual *Rhinanthus* species in this respect.

Simultaneously with the general biomass reduction, diversity of the host community often increases (Pywell et al. 2004; Westbury et al. 2006). In some cases, however, the suppression of competitive dominants can be compensated by a dominant forb resistant to parasitism. For example, Mudrák & Lepš (2010) reported that reduction of grasses by *R. minor* promoted *Plantago lanceolata*, which possesses defence mechanisms against hemiparasitism (Cameron et al. 2006). Other forb species remained suppressed, and hence the total biodiversity (expressed as number of species and Shannon–Wiener index) did not change. However, *Rhinanthus* has a positive effect on plant diversity not only because competitive grasses are reduced but also due to effective uptake of nutrients from perennial hosts and their quick release to the environment after death of *Rhinanthus* (occurring mostly at the beginning of July) and rapid decay of its biomass (Bardgett et al. 2006; Ameloot et al. 2008). Its nutrient-rich litter stimulates decomposition, which reduces the litter layer (Quested et al. 2003) and thus promotes seedling establishment in meadows (Špačková & Lepš 2004). Moreover, death of *Rhinanthus* individuals creates gaps in the sward, which can be colonized by seedlings of other plants (Joshi et al. 2000).

The use of *Rhinanthus* in grassland restoration has several advantages. (1) As a natural part of many grassland communities, its introduction is desirable for biodiversity even without further effects on the community. (2) Introduction of *Rhinanthus* represents a relatively cheap and environmentally friendly method of productivity reduction. (3) *Rhinanthus* spp. have only a short-term persistent seed bank and their populations therefore depend mainly on seed production every year. Therefore, if its presence becomes problematic for some reason, the population can be substantially reduced (or even exterminated) by cutting at the time of flowering. (4) *Rhinanthus* spp. accelerate nutrient cycling and usually suppress dominant competitive species, thus supporting functional diversity and stability of plant communities (Quested et al. 2003; Bullock & Pywell 2005; Bardgett et al. 2006; Ameloot et al. 2008).

Sowing experiments with *Rhinanthus* spp. under field conditions resulted in generally successful establishment (at least in a short-term horizon) of *Rhinanthus* populations (Mizianty 1975; Joshi et al. 2000; Matthies 2003; Pywell et al. 2004; Westbury 2004; Westbury et al. 2006; Westbury & Dunnett 2007; Hellström et al. 2011). However, this knowledge is based on published papers, among which positive results have a higher probability of occurrence. In our experience, the establishment of *Rhinanthus* popula-

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tions can be difficult under certain circumstances. We sowed *R. minor* in meadows restored on an ex-arable field in Bílé Karpaty Mts., Czech Republic (Jongepierová 2008) in October 2006. The experimental site was species-poor, mostly dominated by grasses, namely *Festuca rupicola* and *F. rubra*, with biomass production of 270 ± 69 g·DW·m^{-2} (mean ± SD). As grasses, both *Festuca* species can be expected to be preferred hosts (Gibson & Watkinson 1989; Keith et al. 2004; Cameron et al. 2006), and *R. minor* attachment to their roots was indeed observed directly in a natural population nearby (J. Těšitel, unpubl.). Nevertheless, almost no establishment of *R. minor* was observed in the following spring. Only two flowering individuals were recorded out of more than 30 000 seeds sown in ten 2 m × 2 m plots at densities of 500 or 1000 seeds·m^{-2}, which is comparable to other studies (Pywell et al. 2004; Westbury et al. 2006). The establishment failure could have been caused by unusually low precipitation levels in spring 2007, since spring drought was reported to have a detrimental effect on *Rhinanthus* populations (Ameloot et al. 2006). Hence, we repeated the sowing at the same site (but in a simplified design) in October 2007. More than 11 000 seeds were sown on ten plots at a density of 500 seeds·m^{-2}. The establishment of *R. minor* was still negligible, accounting for just 154 *Rhinanthus* individuals. Moreover, most of these individuals were substantially damaged by herbivores, and following year only four individuals were recorded. The weather conditions were quite typical from the long-term perspective in spring 2008, so they could not be expected to be the main reason for the unsuccessful establishment. This failure motivated us to focus more deeply on the mechanisms responsible for successful establishment of *Rhinanthus* in the field. Namely, we were interested in: (1) how the establishment of *Rhinanthus* was affected by the date of sowing; (2) the appropriate grassland management for enhancing *Rhinanthus* establishment; and (3) which biotic and abiotic factors are limiting for *Rhinanthus* establishment.

In this paper, we summarize the available evidence on factors affecting success of *Rhinanthus* establishment. This evidence is primarily based on a series of our own sowing experiments established at various sites across the Czech Republic and an extensive literature survey. Based on a synthesis of these facts, we formulate practical recommendations for introduction of *Rhinanthus* into grassland communities for experimental and restoration purposes.

Methods

Seed dormancy experiment

The experiment was established at four meadows located in the surroundings of České Budějovice town (48°55′15.2″N, 14°39′46.1″E; 48°57′11.5″N, 14°35′34.4″E; 49°00′30.7″N, 14°25′29.1″E; 48°59′58.3″N, 14°22′26.8″E). At each site, 100 *Rhinanthus minor* seeds (seed origin: Hejdlov, Blanský les Mts., Czech Republic) were sown on 30 plots of 0.5 m × 0.5 m (density 400 seeds·m^{-2}) on one of three sowing dates (30 Oct–3 Nov 2009, 14–21 Jan 2010, 25–27 Feb 2010; ten plots per date). Soil temperature dropped below 5 °C on 12 Dec 2009 and rose above 5 °C on 17 Mar 2010 (data from a station of Czech Hydrometeorological Institute in České Budějovice), so the seeds received 14, 8 and 3 wk of cold stratification. We counted and removed the seedlings in the three following springs (mid-May, 2010–2012), so that no emerged plant produced any seeds (Blažek 2011).

Multi-site sowing experiment

A total of 21 sites without *Rhinanthus minor* were selected in the surroundings of České Budějovice town (between 48°55′46″N–48°58′50″N, and 14°18′42″E–14°35′12″E). The sites represent various possible communities potentially suitable for *R. minor* occurrence, i.e. grasslands with various management intensity, abandoned grasslands and fields, paths and forest edges. Three permanent plots of 1.5 m × 1.5 m were established at each site and 500 seeds of *R. minor* (seed origin: Lenora, Šumava Mts., Czech Republic) were sown in each plot (density 222 seeds·m^{-2}) in November 2007 (Blažek 2011).

In the first year after sowing, *Rhinanthus* plants were counted, cover of vascular plant species and height of litter layer was visually estimated in each plot, and slope inclination was measured at each site. *Rhinanthus* plants were also counted in the second year after sowing. In this case, the census included all plots and their surroundings to account for population spread out of the plots. To make the data from both years comparable, we also merged the within-site data from the first year. Based on species composition, we calculated the Shannon–Wiener index of diversity, mean for the Ellenberg indicator value for soil reaction, moisture and nutrients (Ellenberg et al. 1992), and the Beals index (Münzbergová & Herben 2004). The Beals index indicates the probability that a target species (here *R. minor*) will occur in the target relevé. The calculation is based on comparing the co-occurrences of the target species with other species from the target relevé in a reference relevé database. We used a stratified regional subset of relevés from the Czech National Phytosociological Database (Chytrý & Rafajová 2003). The JUICE software, v. 7.0 (Tichý 2002), was used to calculate the mean Ellenberg indicator values and the Beals index.

Passive projection of variables into a non-metric multidimensional scaling (NMDS) ordination space was used for visualization of correlations between all parameters used. As a basis for this analysis, a two-dimensional NMDS was fitted based on the Bray–Curtis distances of vegetation composition, type 1 stress in R package 'vegan' v. 2.0-7 (R Foundation for Statistical Computing, Vienna, AT; http://CRAN.R-project.org/package=vegan), 20 random starts with a maximum of 200 iterations per start. Linear regression was used to extract more details on the relationship of the number of *Rhinanthus* seedlings with the Beals index and on the relationship of the relative *Rhinanthus* population change with the number of *Rhinanthus* seedlings in the first year. The number of *Rhinanthus* seedlings was log-transformed ($\log_{10}(X+1)$) in all analyses.

Mowing experiment 1

The experiment was established on an oligotrophic meadow (nearby České Budějovice town, 48°59′23.8″N, 14°36′27.7″E) of mean productivity 460 g·DW·m^{-2}. Twenty adjacent squares of 1 m^2 were set up on the meadow in 2002. Half of these were mown in the first half of July (continuation of previous management) and the others were left unmown (checkerboard design). In September, 50 seeds of *R. minor* (Záblatí surroundings, Šumava Mts., Czech Republic) were sown on 0.5 m × 0.5 m subplots of the mown and unmown plots. The individuals of *R. minor* were counted in June 2003. In addition, total above-ground biomass of each mown plot was collected, later sorted into living and dead (litter), dried for 24 h at 105 °C and weighed.

Mowing experiment 2

The experiment was carried out at five sites of a long-term, multi-site management experiment running since 2004 in Bílé Karpaty and Javorníky Mts. (SE of the Czech Republic), namely Brumov-Bylnice (49°5′49.9″N, 18°1′46.901″ E), Lopeník (48°56′12.5″N, 17°48′5.0″E), Suchov (48°53′ 46.9″N, 17°34′51.0″E), Francova Lhota (49°13′32.4″N, 18°4′41.5″E), Huslenky (49°17′27.8″N, 18°7′55.1″E). In September 2011, we sowed three *Rhinanthus* species (200 seeds of each; seed origin: *R. alectorolophus* – Francova Lhota surroundings, Javorníky Mts., Czech Republic; *R. major* – Vyškovec roadside, Bílé Karpaty Mts., Czech Republic; *R. minor* – Nedašov meadows, Bílé Karpaty Mts., Czech Republic) on four 1-m^2 subplots at each experimental site. Two of the subplots were placed in long-term mown plots and the other two in fallow plots. Just before sowing we scarified the sward and removed plant litter and moss by raking in one subplot under each of the management treatments. In July 2012, we counted the number of established *Rhinanthus* individuals and measured overall biomass production. Average dry matter biomass productivity of sites ranged from 121 to 317 g·m^{-2}. For further details on the sites see Mládek et al. (2011).

Litter removal experiment

This experiment was designed to test the possibility of *R. alectorolophus* (seed origin: Francova Lhota surroundings, Javorníky Mts., Czech Republic) and *R. major* (seed origin: Čertoryje meadows, Bílé Karpaty Mts., Czech Republic) establishment in a wet meadow overgrown by expansive *Calamagrostis epigejos*. The experiment was located at an abandoned wet meadow near the city of České Budějovice (48°59′23.1″N, 14°26′23.0″E). The experiment consisted of three blocks, each of which included four 2 m × 2 m plots in which either of the species was sown (at a density of 300 seeds·m^{-2}) and litter was or was not removed (combined with raking) at the time of sowing (October 2011). The number of *Rhinanthus* plants was counted in June 2012 in the middle 1 m^2 in order to avoid edge effects.

Ex-arable land sowing experiment 1

In 1996, the experiment was established on a former arable crop field, which was grassed over by three different sowing treatments, i.e. by sowing low-diversity seed mixtures containing seeds of four species and high-diversity seed mixtures containing seeds of 15 species and the natural colonization of the not sown field. The site is located close to the village of Benešov u Kamenice, in the Českomoravská vrchovina highlands (49°19′58.5″N, 15°0′ 10.0″E, 659 m a.s.l). Treatments were applied to 10 m × 10 m plots arranged in five blocks. In each plot, cover of individual species was annually recorded in 12 1 m × 1 m subplots. Mowing was applied at all plots with one mowing machine on the same date, usually in late June, and then in autumn, depending on the regrowth (in dry years with low biomass, autumn mowing was not applied). Biomass production was assessed (in selected years) using 12 (exceptionally six) 0.25 m × 0.25 m plots in each 10 m × 10 m plot. The above-ground biomass was harvested at peak standing biomass, oven-dried and weighed (see Lepš et al. 2007 for more details of the experimental setup). In December 1998, we sowed 200 seeds of *R. minor* (mixture of seeds of multiple populations surrounding the experimental site) to one subplot of each plot. The annual recording of vegetation provided an overview of the spread and establishment of *Rhinanthus* in the plots, except for the 2006 season, when no data were sampled.

Ex-arable land sowing experiment 2

The experiment was established at 12 meadows restored on ex-arable fields situated surrounding the villages Hrubá Vrbka and Malá Vrbka in Bílé Karpaty Mts. region (SE of the Czech Republic). The meadows were restored by sowing species-rich seed mixtures of local origin but differed in abiotic conditions, year of grassing, seed mixture used, number of established species and productivity. To capture these differences, we recorded plant biomass (mean of total above-ground dry matter biomass in three 0.5 m × 0.5 m squares positioned between the experimental plots), floristic composition of experimental plots and cover of individual species, cover of moss layer, cover of litter layer (all cover data were based on visual estimates), and the following soil properties (based on three mixed samples from top 10 cm of soil): loss on ignition, total nitrogen content, plant available phosphorus following Mehlich III protocol (Mehlich 1984) and pH (both in H_2O and KCl, which displayed a tight inter-correlation). These data and samples for laboratory analyses were collected in June 2010. In September 2010, we sowed seeds of *R. minor* and *R. major* (origin of seeds of both species: Čertoryje meadows, Bílé Karpaty Mts., Czech Republic) at a density of 500 seeds·m^{-2} on two 1.5 m × 1.5 m plots in each of the meadows. Number of individuals of both *Rhinanthus* species was counted in the following season to assess the establishment success. Number of flowers was also counted to estimate seed production based on the mean number of seeds per capsule (eight in both species; based on seed counts in 20 per species).

Results

Seed dormancy experiment

In the seeds sown in autumn, an establishment rate usual for field sowing experiments occurred (16.5%), a very low number of seedlings emerged in the second year after sowing (0.175%), and no seedlings emerged in the third year (Fig. 1). Virtually no seedlings emerged from the seeds sown on both winter dates in any of the years, except for a single plant in the second year from January-sown seeds (0.025%; Fig. 1).

Multi site sowing experiment

The number of *Rhinanthus* individuals was positively correlated with mean Ellenberg indicator value for moisture (range: 3.7–6.4; Fig. 2a) and for soil reaction (range: 4.2–6.4; Fig. 2a). By contrast, there was a negative correlation with height of litter layer (range: 1–30 mm) and with slope inclination (range: 0.3°–14.0°), and there was no correlation with Ellenberg indicator value for nutrients (range: 3.5–6.3). All of these correlations were slightly stronger in the first year. In contrast, the positive correlation with the Beals index was stronger in the second year (Fig. 2a). The Beals index was significantly correlated with the number of *Rhinanthus* individuals in the second year only (Fig. 2b). The number of *Rhinanthus* individuals in the first year was not correlated with the relative population change in the second year (linear regression: $r^2 < 0.001$, $F_{1,19} = 0.003$, $P = 0.96$).

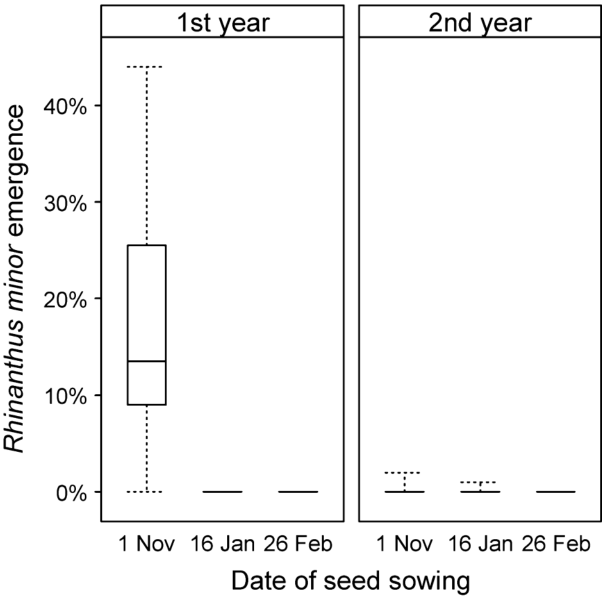

Fig. 1. Results of the seed dormancy experiment examining *Rhinanthus minor* establishment following different sowing dates over autumn and winter (30 Oct–3 Nov 2009, 14–21 Jan 2010, 25–27 Feb 2010). Percentage of emerged seedlings of the total seeds sown in each plot is shown in the diagram. Only data for the first and second year are shown, because there were only zero values in the third year. Middle line – median, box – quartiles, bars – range.

Mowing experiment 1

Rhinanthus minor individuals were observed in all (10) mown plots but only in two unmown plots. This difference in number of individuals between treatments was significant (Fig. 3). In the mown plots, the number of *R. minor* individuals was negatively correlated with the amount of litter (Fig. 3), but no correlation was observed with the living biomass.

Mowing experiment 2

We performed a linear mixed-effect model with log-transformed numbers of *Rhinanthus* individuals as a response variable. *Rhinanthus* species identity, mowing and scarification were factors as fixed effects and subplot nested within site factor as random effect). The model indicated that establishment of *Rhinanthus* was significantly higher in the mown plots ($F_{1,12} = 11.37$, $P = 0.0056$). There were also significant differences among species ($F_{2,32} = 9.59$, $P = 0.0005$), with the highest number of individuals observed for *R. alectorolophus* and the lowest for *R. minor*

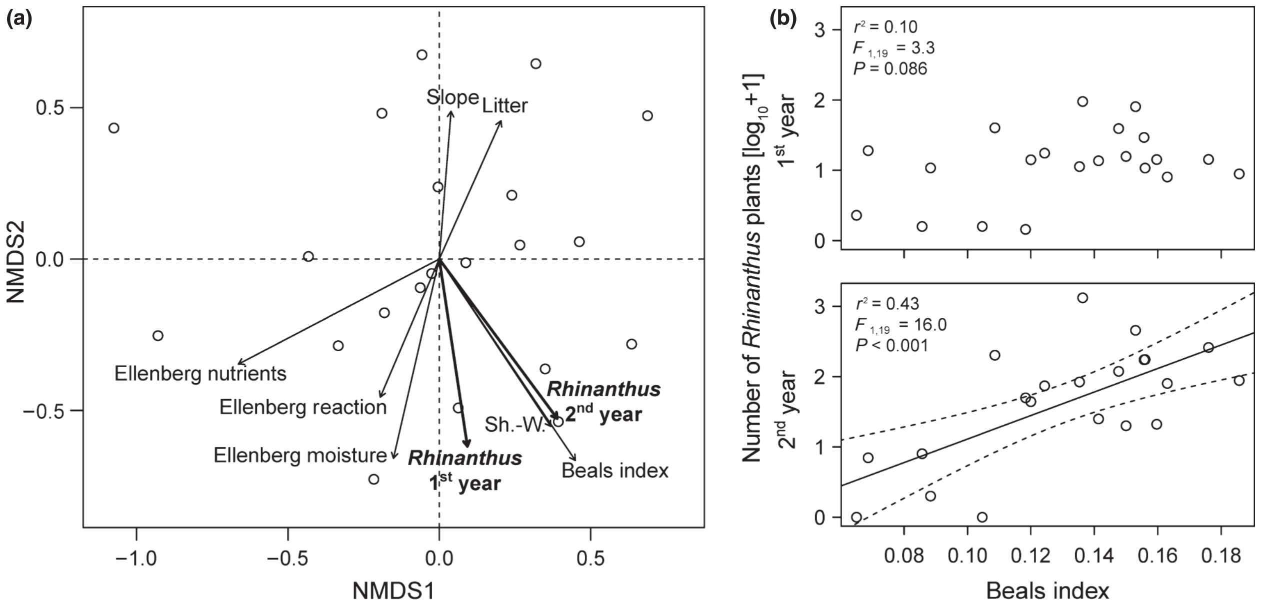

Fig. 2. Results of the multi-site sowing experiment aiming to detect sites suitable for *Rhinanthus minor* establishment. (**a**) Ordination diagram of non-metric multidimensional scaling (NMDS) based on vegetation composition data. Points – sites, Litter – height of litter, Sh.-W. – Shannon–Wiener index of diversity. The variables are passively projected in the ordination space. (**b**) Relationship of the number of *Rhinanthus* plants and the Beals index in the first and second year after sowing. Number of *Rhinanthus* plants was log-transformed. Results of linear regression are shown, dashed line – 95% confidence interval.

(Fig. 4). There was, however, no significant difference in reaction to mowing among the *Rhinanthus* species ($F_{2,32} = 0.62$, $P = 0.05$, interaction of species and mowing) and no significant effect of scarification ($F_{1,12} = 2.17$, $P = 0.17$).

Litter removal experiment

A linear mixed-effect model with blocks as a random factor and *Rhinanthus* species and litter removal as fixed effect terms was used to analyse the log-transformed counts of hemiparasite individuals. We did not detect a significant difference between *Rhinanthus* species ($F_{1,6} = 4.10$, $P = 0.89$), but there was a strong effect of litter removal ($F_{1,6} = 39.35$, $P = 0.0008$). The effect of litter removal was consistent across the species, as indicated by a non-significant interaction of *Rhinanthus* species and litter removal ($F_{1,6} = 1.48$, $P = 0.27$; Fig. 5).

Ex-arable land sowing experiment 1

The seeds sown in December 1998 did not germinate in the following year (1999), but persisted viable and germinated in 2000 in all (except one) sown subplots. *R. minor* established in the sown subplots and persisted over the following 4 yr and then almost disappeared from the records. However, *R. minor* started to spread rapidly again in 2009 (after a 3-yr long gap) and consequently colonized most of the subplots of all restoration treatments within 3 yr, regardless the treatment. Repeated measures ANOVA, where block was used as random effect and sowing treatment as fixed effect, revealed significant effect of the year for both number of subplots occupied by *R. minor* ($F_{10,40} = 60.94$, $P < 10^{-6}$) and cover of *R. minor* in occupied subplots ($F_{10,40} = 4.83$, $P < 10^{-3}$), but neither number of occupied subplots ($F_{2,8} = 0.77$, $P = 0.736$) nor the cover of *R. minor* ($F_{2,8} = 0.93$, $P = 0.556$) differed among the sowing treatments (Fig. 6).

Ex-arable land sowing experiment 2

Soil pH value was the best and the only significant predictor of the number of individuals for both species. The number of established plants decreased with increasing pH in both species (generalized linear model with quasi-Poisson distribution, proportion of explained deviance: 60.1%, $F_{1,10} = 16.8$, $P = 0.002$, model based on mean number of *Rhinanthus* plants per meadow to avoid pseudoreplicates, preliminary analyses did not detect a significant difference between the species). Based on seed production, we classified the established populations into two groups. Populations producing more seeds than sown were considered as potentially viable, while those not reaching this threshold were considered as non-viable. The effects of site condition on probability of establishing a potentially viable population was tested using a binomial generalized linear model.

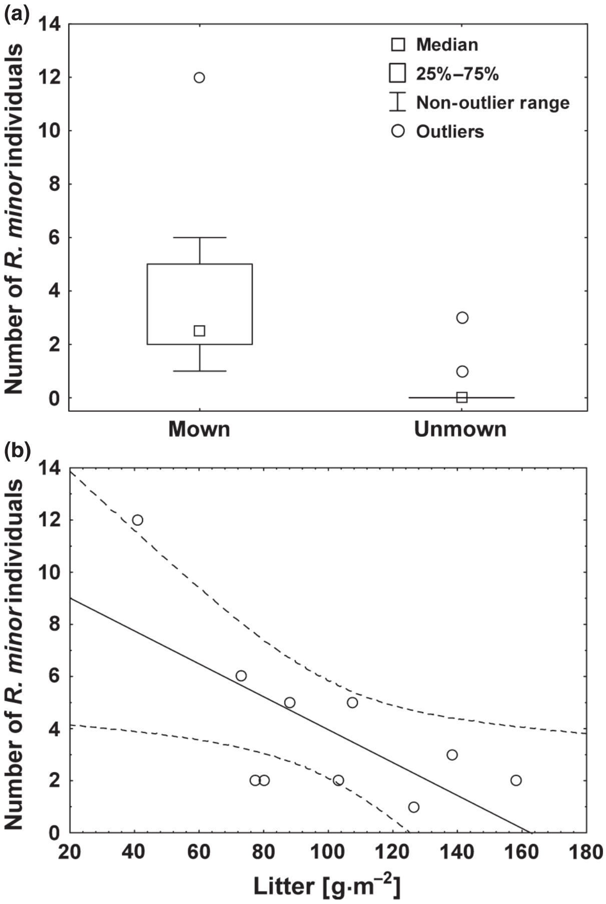

Fig. 3. Results of the mowing experiment 1. (**a**) The number of *R. minor* individuals in mown and unmown plots (two sample *t*-test: $t_{18} = 5.43$, $P < 10^{-4}$). (**b**) Correlation of the number of *R. minor* individuals with the amount of litter in the plot (shown for mown plots only; $R = -0.67$, $P = 0.033$).

This revealed pH as the only significant predictor (proportion of explained deviance: 32.4%, deviance = 10.7, df = 1, $P = 0.001$; Fig. 7b).

Discussion

Seed dormancy

Seed dormancy must be broken by a period of cold stratification for successful *Rhinanthus* germination. The seeds should be exposed to temperatures of about 0–5 °C (up to 10 °C) for 2–6 mo (see ter Borg 2005 for more details). This knowledge is widely used in pot experiments (seeds are germinated in a fridge before planting in pots) but field observations were missing. Our seed dormancy experiment demonstrated that correct timing of seed sowing is essential. A stratification period of 8 wk, which we had supposed to be sufficient to break dormancy, turned out to be still too short for *R. minor*. To ensure successful *Rhinanthus* germination, we suggest sowing seeds in November at the latest.

The seed bank of *Rhinanthus* species is predominantly transient, i.e. all seeds should germinate in the first spring after sowing and a period of cold stratification (Thompson et al. 1997). However, several studies found that some seeds remain viable and germinate in the following years (up to 10% and 20% of seeds germinating in the second year as found by ter Borg 1985 and Pons 1991; respectively; Thompson et al. 1997). In the seed dormancy experiment, we found a short-term persistent seed bank of *R. minor*, even though <0.2% of plants emerged in the second year and no plants emerged in the third year.

Results of laboratory experiments (ter Borg 2005) and some field trials (e.g. in our ex-arable land sowing experiment 1 or in an experiment with *R. alectorolophus*, M. Hejcman, pers. comm.) suggest that the seed bank of *Rhinanthus* can be induced by a short winter (where the seeds dormancy is not broken, seeds can remain dormant until the following stratification period). However, this experience was not confirmed when tested directly in our manipulative seed dormancy experiment.

Seeds of different populations can differ widely in their stratification requirements and germination patterns. This might be an adaptation to local climate, e.g. the length of winter period differs among European regions (ter Borg 2005). Our experiment provided data that can be used when planning sowing experiments in Central Europe. More data is needed to generalize these ideas to other European regions.

Productivity

High productivity of a plant community is considered as one of the main factors limiting *Rhinanthus* establishment. The above-ground biomass production and also sward density is closely connected with soil nutrient levels. High productivity induced by elevated nutrient levels has an ambiguous effect on *Rhinanthus*. It increases seedling mortality due to competition (Matthies 1995; Těšitel et al. 2011), resulting in lower abundance in the community on the one hand, while on the other hand it also increases size and fecundity of the hemiparasites that survive the seedling stage (van Hulst et al. 1987; Mudrák & Lepš 2010; Těšitel et al. 2013).

In general, the life strategy of hemiparasites relies on one unique feature: parasitic acquisition of nutrients substantially reduces the need for investment in the development of the root system. However, at high nutrient levels, non-parasitic plants are also less forced to invest energy in the development of the root system, and the advantage of hemiparasites diminishes (Matthies 1995). Moreover, larger hosts can more easily suppress hemiparasites by

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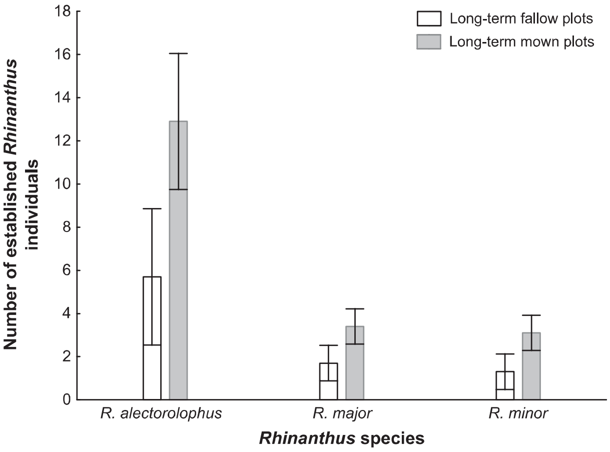

Fig. 4. Results of mowing experiment 2, conducted at five equally designed long-term management experimental sites running since 2004. Bars represent average number of *Rhinanthus* spp. individuals established from 200 seeds of each species sown into the 1-m^2 subplots; Bars and error bars indicate means and SE, respectively.

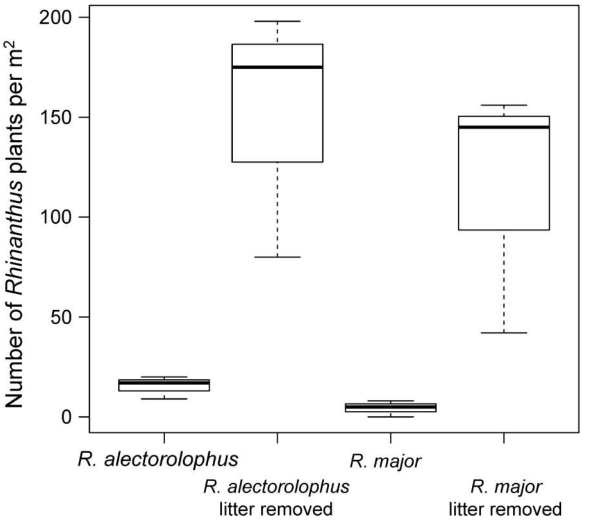

Fig. 5. Results of the litter removal experiment testing the possibility of establishment of *Rhinanthus alectorolophus* and *R. major* in a wet meadow overgrown by *Calamagrostis epigejos*. Displayed are medians, upper and lower quartile and range of the *Rhinanthus* numbers within the treatments.

competition for light, and also host loss of nutrients due to parasitism can be relatively reduced (Jiang et al. 2010). The sap containing dissolved nutrients is acquired from the host through haustoria via mass flow induced by the strongly negative water potential of the hemiparasite (Cameron et al. 2006), but transpiration flow is dependent on the leaf area (and number of stomata). Vigorous hosts with large leaf area (and with a strong transpiration flow) could therefore substantially reduce the flow of xylem sap to the hemiparasite (Jiang et al. 2010).

These competitive components of the hemiparasitic interaction disadvantage *Rhinanthus* on productive sites, where its ability to reduce host and community biomass substantially decreases (Matthies 1995; Matthies & Egli 1999). The vegetation becomes too dense above a certain level of community productivity, making *Rhinanthus* unable to establish or re-establish its population from seed (de Hullu 1985). Hejcman et al. (2011) studied population dynamics of *R. minor* in a long-term fertilization experiment and proposed a threshold productivity of 5 t·ha^{-1} (500 g·m^{-2}) of dry above-ground biomass for a viable *R. minor* population. In an extensive review, Ameloot et al. (2005) demonstrated that *Rhinanthus* is able to establish in both freshly sown and long-term established grasslands, with a productivity ranging from 152 g (*R. alectorolophus*; Joshi et al. 2000) to 866 g·DW·m^{-2} (*R. minor*; Davies et al. 1997). The absolute value of the productivity threshold for a viable *Rhinanthus* population is likely not to be general but dependent on many factors, such as climate, community species composition and *Rhinanthus* species. Nonetheless, it indicates that *Rhinanthus* can be used for species-rich grassland restoration only across a limited range of grassland productivity.

Species composition of the host community

The suitability of a site for *Rhinanthus*, and thus its establishment success in sowing experiments, can be estimated by species composition. Provided a reference database of relevés is available, the Beals index can present a formal way of such estimation, indicating the probability of a species occurring on a given site (Münzbergová & Herben 2004). Technically, it is based on comparison of a given site's species composition with relevés in the reference database (Münzbergová & Herben 2004). Many plant species are good indicators of overall site conditions, so the nature of this index accounts for all aspects of population persistence, which results in a good prediction of long-term establishment success. In contrast, emergence of seedlings in the first year after sowing is affected by different factors than the further completion of their life cycle and might be misleading in predicting future population dynamics. We illustrated this pattern for *R. minor* in our multi-site sowing experiment (Fig. 2). The number of *Rhinanthus* plants in the first year was correlated with environmental conditions that are known to influence *Rhinanthus* establishment, such as moisture or thickness of the litter layer (Ameloot et al. 2006; our litter removal experiment and mowing experiment 1). On the contrary, we found no strong correlation of the number or *Rhinanthus* plants in the second year with environmental conditions, but the correlation with the Beals index was highly significant. In addition, the number of *Rhinanthus* plants in the first year

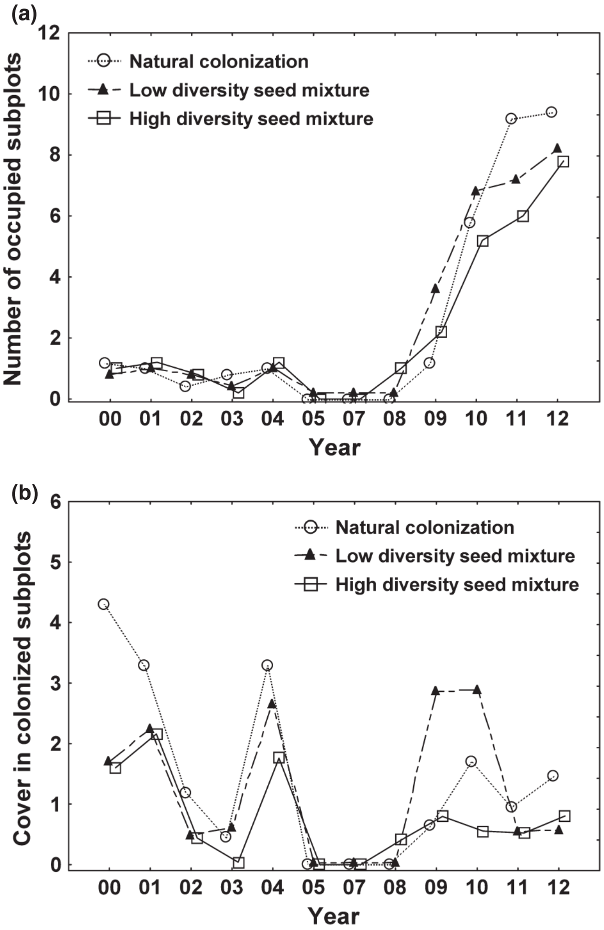

Fig. 6. Dynamics of *Rhinanthus minor* establishment in the ex-arable land sowing experiment 1, where we established grassland on ex-arable land through three different treatments, i.e. by sowing low diversity and high diversity seed mixtures and by natural colonization of the unsown field. (**a**) Mean number of subplots occupied by *R. minor* per plot (12 maximum), (**b**) Mean cover of *R. minor* in occupied subplots.

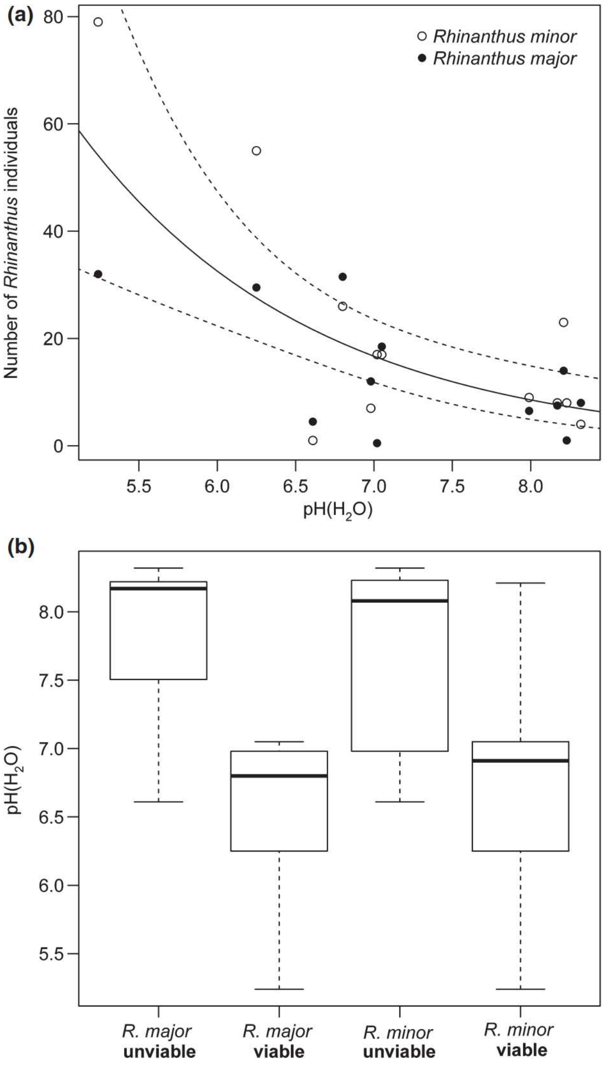

Fig. 7. Results of the ex-arable land sowing experiment 2, established at 12 meadows restored on ex-arable fields. (**a**) Relationship between the number of established *Rhinanthus* individuals (per 1.5 m × 1.5 m square) and pH. Solid line displays predicted values of a quasi-Poisson generalized linear model. Dashed lines indicate 95% confidence limits of the model estimate. The model fit and confidence limits were first computed on the scale of the GLM linear predictor and then back-transformed for plotting purposes. (**b**) Estimated viability of *Rhinanthus* populations (based on number of flowers and mean number of seeds per capsule) as affected by the pH. Displayed are medians, upper and lower quartiles and ranges of pH values.

did not predict its population change. This supports the Beals index as a good predictor of long-term *Rhinanthus* establishment success in sowing experiments, despite that the seedlings in the first year after sowing can behave in a different manner.

Effects of mowing and litter removal on establishment

A large amount of litter has been repeatedly demonstrated to have a harmful effect on many plants, and mainly on their seedlings (Špačková & Lepš 2004; Mudrák et al. 2013). Litter acts as a mechanical obstacle, preventing light penetration and plant establishment (Sydes & Grime 1981; Facelli & Pickett 1991). In addition, it can prevent seeds from reaching the soil surface. Moreover, litter can suppress plants through allelopathy (Ruprecht et al. 2010). *Rhinanthus* has to go through the seedling stage each year, which is a crucial part of its life cycle, when it is most sensitive to competition for light (Těšitel et al. 2011), as are many non-parasitic grassland species (Grubb 1977). The seedlings acquire energy from seed storage components or from their own photosynthesis between germination and attachment to the host. If the seedling fails to penetrate through the litter during

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this time, it dies. Accumulation of litter can therefore be detrimental for *Rhinanthus* populations. This was clearly demonstrated in mowing experiment 1, where *R. minor* emerged reasonably only in the mown plots. Accumulation of the litter was apparently the reason for unsuccessful establishment in the unmown plots, because even in the mown plots the number of flowering *R. minor* individuals correlated negatively with the amount of litter (Fig. 3b).

The suppressive effect of litter on *Rhinanthus* seedling establishment was also demonstrated through a direct manipulation with litter in our litter removal experiment (Fig. 5). The results demonstrated that litter could be a major limiting factor, preventing establishment of annual hemiparasites. In addition, the experiment shows the potential to use hemiparasites for restoration measures in habitats where they cannot occur spontaneously. Such introduction, however, must be accompanied by an appropriate management practice, decreasing the harmful effects of litter on the seedlings and thus facilitating their establishment.

In general, the literature does not refer directly to litter removal, but only to the sward scarification. Despite scarification being primarily done in order to create bare ground, it also removes the litter. However, its effect seems to be site specific. In mowing experiment 2 conducted in low productive grasslands (Fig. 4), scarification had no effect. Similarly Hellström et al. (2011) found a non-significant effect of scarification for the *R. minor* sown in the restored meadows. In contrast, Westbury et al. (2006) found a significant and strongly positive effect of scarification for *R. minor* sown in a freshly established sward of *Lolium perenne*. Due to the scarification, 16% more *R. minor* individuals established, but in the second year of the experiment the effect of scarification on *R. minor* abundance disappeared.

Too intensive meadow management, however, represents a limiting factor for the successful establishment or persistence of *Rhinanthus* in plant communities (Pywell et al. 2004; Westbury 2004). The annual life strategy and absence of a persistent seed bank makes *Rhinanthus* dependent on annual seed production. Although *Rhinanthus* individuals have some ability to regenerate when they are cut or grazed (Huhta et al. 2000), it is rather limited and too frequent or improperly timed mowing or grazing can substantially reduce or exterminate *Rhinanthus* populations (Magda et al. 2004). The appropriate timing of the first mowing to sustain a *Rhinanthus* population is in late June at the earliest. This is however in fact later than optimal if fodder quality is considered (e.g. Mládek et al. 2011, 2012), and consequently, 2–3 wk later than usual in meadows used for fodder production. This delay can provide time for seeds of *Rhinanthus* to ripen. Failure to set seeds before cutting of a major part of meadows is probably one of the causes of *Rhinanthus* general decrease in the landscape. Interestingly, one of the most vigorous populations of *Rhinanthus* that we are aware of occurs in an active scout-like camp close to Frantoly in South Bohemia. The camp is located in an oligotrophic meadow, which is mown just to make the camp ready for children's vacations (starting on the 1 July in the Czech Republic).

Other management effects

Correct management can support the spread of *Rhinanthus* over the locality also through its indirect effects. Strykstra et al. (1997) identified mowing machinery as an important vector for *R. angustifolius* seed dispersal. Haymaking in June (Bullock et al. 2003) or July followed by grazing (Coulson et al. 2001) was found as the management practice effectively supporting *R. minor* dispersal. In both cases, most seeds were transported over a distance up to 10 m. Autumn grazing (applied after seed release) without previous mowing spread *R. minor* seeds over substantially lower distances (<3 m). However, grazing by cattle and sheep usually supports *Rhinanthus* populations in terms of increasing *Rhinanthus* cover (Smith et al. 2000; Hellström et al. 2003).

Similarly, we found hay cut twice a year (June and September) as effective management, supporting *Rhinanthus* establishment and dispersal (ex-arable land sowing experiment 1; Fig. 6). Mowing applied to all plots with a single mowing machine probably contributed substantially to spread of seeds. Interestingly, in the period when *R. minor* was not recorded in the experimental subplots, it found a refuge in the space between the subplots, which was disturbed by trampling during vegetation monitoring. The expansion of *R. minor* cannot be explained by any change in site productivity because the dry biomass of established communities ranged from 194 to 908 $g \cdot m^{-2}$ without any temporal trend. The treatments were quite different in their community species composition, hence, the changes in community species composition cannot be the reason for the expansion. We consider that the pattern of *R. minor* expansion was mainly due to an inner dynamic of *R. minor* populations. First, it increased population density in the most suitable microhabitat, which consequently boosted seed production resulting in the later expansion. Later, the seeds were spread (presumably by the mowing machinery) into the rest of the experiment. It is also interesting to note that *Rhinanthus* remained localized mostly in sown plots for 8 yr (and nearly disappeared at the end of this period), and then started to spread and colonized the whole experimental area within 3 yr. We have had a similar experience from another sowing experiment in a semi-natural meadow, which was abandoned after 3 yr. After an additional 5 yr, *Rhinanthus* started to spread rapidly, and finally occupied most of the locality.

Long-term mowing is important not only as a vector of the *Rhinanthus* seeds but also as a management practice generally improving success of *Rhinanthus* establishment, as indicated by our mowing experiment 2, where mowing had a significant effect in contrast to scarification (Fig. 4). Hence, not only the decrease in shading but also other effects of mowing affected *Rhinanthus* establishment, since no litter was present on the scarified unmown plots. The mown plots were in general slightly warmer (1.17 °C mean difference of soil temperature at 10 cm depth between the mown and fallow plots; $t_4 = 4.05$, $P = 0.015$, paired *t*-test) and drier (−9.2% mean difference of soil v/v humidity between the mown and fallow plots; $t_4 = -4.56$, $P = 0.011$, paired *t*-test). These differences in microclimate could be responsible for differential success of *Rhinanthus* establishment between the treatments, since moisture levels can have a significant effect on *Rhinanthus* germination and performance, as demonstrated in an experiment by Ducarme & Wesselingh (2010).

Chemical properties of soil

In our original experiment in the Bílé Karpaty Mts. (discussed in the Introduction), litter accumulation was presumably an important factor preventing *Rhinanthus* establishment, but was not the only factor. The ex-arable land experiment 2 revealed a high variability in *Rhinanthus* establishment among the restored meadows driven by soil pH. *Rhinanthus* was able to set a viable population only on meadows with pH below 7 (Fig. 7b).

The preference for more acidic soils was unexpected. The two *Rhinanthus* species used in the experiment grow on both alkaline and acidic soils according to local flora (Skála & Štech 2000). Westbury (2004) also concluded that *R. minor* grows on a large variety of substrates, but commonly on alkaline soils. Hellström et al. (2011) did not find pH as significant for *R. minor* establishment, despite pH was in a similar range to our experiment. Nevertheless, long-term application of calcium (which increases pH) was demonstrated to reduce the cover of *R. minor* in a study from Hejcman et al. (2011). Given the reported variability of soil pH on sites with natural *Rhinanthus* occurrence (Skála & Štech 2000; Westbury 2004), it is not likely that the specific results of our experiment (e.g. no establishment on sites with pH > 7) have general validity. On the other hand, the reaction of *Rhinanthus* on pH can have important significance in the context of genotypic variability between populations (Rowntree et al. 2011), possibly limiting establishment success of seeds originating from a population growing on an acidic substrate on a site with alkaline soil. This is furthermore supported by the fact that the natural population used as the seed source of *R. major* seeds grew on a site with soil pH 5.4. Although we did not measure soil pH of the source population of *R. minor*, the two sites were quite similar and of similar geological history, hence values between pH 5.0 and 7.0 could be expected on the basis of extensive soil pH monitoring in the region (Chlubná 2007; Merunková et al. 2012).

Suggestions for *Rhinanthus* successful introduction

For the successful introduction of *Rhinanthus* into the sward, its seeds must be sown before winter to overcome seed dormancy. We suggest sowing the seeds at a density of 200–500 seeds·m^{-2}. The required seed density depends on the availability of suitable microsites. When suitable microsites are available, even very low densities of *Rhinanthus* seeds can lead to successful establishment of a viable *Rhinanthus* population. Such patches often serve as a seed source for later *Rhinanthus* expansion. Grassland communities with biomass production below 500 g·DW·m^{-2} are in general suitable for *Rhinanthus* introduction. In grasslands with higher productivity, the establishment of a long-term persistent *Rhinanthus* population is possible, but would be difficult in most cases unless accompanied by some other management practices. *Rhinanthus* is also likely to have a lower impact on the community in these highly productive grasslands. Sward scarification can substantially help *Rhinanthus* to establish or may be without any effect on establishment but, most importantly, no suppressive effect of sward scarification for *Rhinanthus* establishment has been reported. On the other hand, scarification itself or in a combination with *Rhinanthus* can have a negative impact on the target community, e.g. by supporting weedy forbs (Wagner et al. 2011). Apart from scarification, removal of litter can be recommended on sites with high litter accumulation to promote the hemiparasites and also generative regeneration of other species. The selection of *Rhinanthus* seed sources represents another important factor. Ideally, seeds coming from a local natural population growing on a similar soil type as the target community should be used. Despite following all of these suggestions, establishment of *Rhinanthus* on some sites can be difficult or fail completely. This can be caused by weather conditions or other *a priori* hardly predictable factors (e.g. seed predation by rodents or damage to flowering plants by grazing deer). Pilot experiments testing the feasibility of introduction of hemiparasites are therefore recommended before starting their broad application in particular restoration projects. In this respect, the Beals index and/or less formal knowledge of the natural occurrence of hemiparasites in a local landscape can provide approximate guidelines for selection of suitable sites. Nevertheless, successful introduction of hemiparasites is still a stochastic process to some extent, often requiring longer time scales than just one vegetation season.

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