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Effects of abiotic factors on hemiparasitic plants

Ph.D. Thesis

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Annotation

This thesis focuses on the ecophysiology of hemiparasitic plants. In its introduction, I review our understanding of abiotic factor effects on root and stem hemiparasites and highlight gaps in our knowledge which would be interesting to explore in future. The following four chapters are first author articles that investigate responses of selected root hemiparasites from the rhinanthoid clade of Orobanchaceae to varying availability of abiotic factors such as light, water, and mineral nutrients.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

České Budějovice, 25.4.2018

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Petra Světlíková

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

I. 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 (1), 61–68 (IF = 3.982).

PS participated in the experimental design, plant cultivation, physiological measurements, data analysis, and drafted the manuscript.

II. Světlíková P., Blažek P., Mühlsteinová R., Těšitel J., 2016. Tracing nitrogen flow in a root-hemiparasitic association by foliar stable-isotope labelling. Plant Ecology and Evolution 149(1), 39–44 (IF = 1.012).

PS participated in the plant cultivation, isotopic labelling, data collection and analysis, and the manuscript drafting.

III. Světlíková P., Hájek T., Těšitel J., 2018. A hemiparasite in the forest understorey: photosynthetic performance and carbon balance of *Melampyrum pratense*. Plant Biology 20 (1), 50–58 (IF_{2016} = 2.106).

PS participated in the experimental design, physiological measurements, additional data collection, data analysis, and drafted the manuscript.

IV. Světlíková P., Hájek T., Těšitel J., 2018. Water-stress physiology of *Rhinanthus alectorolophus*, a root-hemiparasitic plant. (under revision in PLoS ONE)

PS cultivated the plants, participated in the experimental design, conducted physiological measurements, data collection, and analysis, and drafted the manuscript.

Co-author agreement

Jakub Těšitel as the supervisor of the Ph.D. thesis and co-author of all presented papers fully acknowledges the contribution of Petra Světlíková.

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Contents

Introduction

Ecophysiology of hemiparasitic plants

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Abstract

Hemiparasitic plants are green plants that simultaneously acquire resources via own photosynthesis and heterotrophically by stealing them from the xylem sap of host plant species. They are mostly generalists that establish haustorial connections to the roots (root hemiparasites) or stems (stem hemiparasites) of numerous host species. Being recognized as an important plant functional group, hemiparasites shape key ecosystem processes and some of them are even considered major pests of crops. Their physiology and the interaction with hosts are well understood and the attention was also paid to the effects of abiotic factors on these parasites. However, many intriguing questions related to these effects remain unanswered. Here, I review the knowledge on the effects of abiotic factors, namely light, CO_2, belowground resources (water and nutrients), and soil salinity on root and stem hemiparasites. The current knowledge on their fundamental functional traits is also summarized. I identify the gaps in the literature regarding the effects of abiotic factors on hemiparasites and suggest future perspectives.

Parasitism as a successful strategy within flowering plants

Parasitism is a life strategy, in which the parasitic species exploits another species, its host, without providing corresponding payoff. Although the term parasite usually refers to animal species as helminths and nematodes, a number of plant species are also parasites. Parasitic plants acquire essential resources from other plant species by specialized invasive organs called haustoria (Heide-Jørgensen 2013). Hereby, all parasitic plants obtain water with diluted mineral nutrients from their hosts (Irving and Cameron 2009), but the extent to which they depend on hosts for heterotrophic carbon varies. Partial parasites or hemiparasites conduct their own photosynthesis that provides them with a significant amount of autotrophic carbon (Press et al. 1988). They are therefore less dependent on their hosts for carbon, but still obtain from them a considerable amount of their carbon budget (Marshall and Ehleringer 1990; Těšitel et al. 2010a). On the other hand, full parasites or holoparasites lack the ability of photosynthesis and are fully dependent on host species for their nutrition (Lambers et al. 2008). They also differ in the connection to host vascular bundles in the haustoria (Heide-Jørgensen 2013). While hemiparasites connect only to the host xylem, haustoria of holoparasites comprise mostly both xylem and phloem connections (Těšitel 2016).

Parasitism can be seen as a successful strategy within flowering plants with species distributed in a wide range of habitats across all climatic zones (Heide-Jørgensen 2008a). At least 12 independent origins of plant parasitism can be tracked in the evolution of angiosperms, giving rise to more than 4000 species from over 20 dicotyledonous families (Bell et al. 2010; Westwood et al. 2010). Most lineages are entirely holoparasitic (e.g. Cynomoriaceae and Rafflesiaceae), some entirely hemiparasitic (e.g. Krameriaceae and Misodendraceae), and some encompass the whole range of trophic strategies from fully autotrophic to fully heterotrophic (e.g. Orobanchaceae and Santalales) (Heide-Jørgensen 2008a; Westwood et al. 2010). Whether to consider a species hemiparasitic or holoparasitic is sometimes questionable, since transitional forms between these exist, such as xylem-only feeding holoparasites from Orobanchaceae (Těšitel 2016). How these plants become parasitic has not been fully revealed yet, however, genes concerned with parasitic functions were suggested to arise from plant genes having different functions in non-parasites (Joel et al. 2013).

About 90% of parasitic plant species are hemiparasites which establish their haustoria on either host roots – root hemiparasites – or shoots – stem hemiparasites (Heide-Jørgensen 2008a). Root hemiparasites are assumed to be ancestors of most other parasitic plant forms (except for parasitic vines). Present-day root hemiparasites include diverse life forms as herbs, shrubs, trees and lianas, that originated within the Santalales order and the Orobanchaceae and Krameriaceae families. They are common in open grassland biomes as well as in closed forests. Stem hemiparasites are a largely heterogenous functional group of parasitic plants that can be found in several families of Santalales (mistletoes) and in the *Cassytha* genus of Lauraceae (hemiparasitic vines) (Těšitel 2016). They parasitize mostly woody species in tropical, subtropic, and temperate biomes. Both root and stem hemiparasites are recognized as important functional groups of plants affecting key ecosystem processes and some even acting as keystone species (Davies et al. 1997; Joshi et al. 2000; Watson 2001; Phoenix and Press 2005; Press and Phoenix 2005; Bardgett et al. 2006; Grewell 2008; Watson

et al. 2011; Watson and Herring 2012). Other root-hemiparasitic plants such as the *Striga* species and *Alectra vogelii* are major pests progressively decreasing yield of subtropical and tropical crops (Parker 2013).

The parasitic life strategy makes these plants very interesting in terms of their physiological functioning. Since hemiparasites combine autotrophic and heterotrophic resource acquisition pathways, which interact with each other and are additionally shaped by host species, interesting and non-trivial outcomes can be expected when examining the effects of abiotic factors on these parasites. Here, I review the knowledge on the effects of abiotic factors, namely light, CO_2, belowground resources (water and nutrients), and soil salinity on root and stem hemiparasites. I also introduce the current knowledge on their fundamental functional traits as these are crucial in order to understand the effects of abiotic factors.

Key physiological features of hemiparasitic plants

Germination and establishment of haustorial connection

More than 2000 species of root hemiparasites germinate and grow during their initial ontogenetic life stage of several days to weeks independently of the presence of host species (Masselink 1980; Westbury 2004). This germination strategy is retained from ancestral non-parasitic plants, which also germinated only in response to favorable environmental conditions breaking their seed dormancy. Later, lateral or secondary haustoria start to appear on seedling roots and their attachment to host roots is facilitated by chemical signals from host roots (Yoshida et al. 2016). Self-germinating hemiparasites such as species from the *Rhinanthus* and *Santalum* genera, and *Nuytsia floribunda* are generalists in respect to host range with hundreds of secondary haustoria (Gibson and Watkinson 1989; Radomiljac 1998; Calladine 2000; Cameron et al. 2005; Holá et al. 2017). About 100 root-hemiparasitic species of the Orobanchaceae (e.g. *Striga*, *Alectra*, and *Tozzia* alpina) germinate and develop haustoria only when they receive host-derived chemical stimulants (i.e. strigolactones) (Cook et al. 1966; Visser et al. 1987; Hauck et al. 1992). These species generally form a single terminal or primary haustorium and are often highly host-specific (Weber 1987; Hood et al. 1998). Compared to them, stem hemiparasites germinate independently of hosts, but being epiphytes all of them depend on hosts for attachment and further growth. Mistletoes of Misodendraceae and Viscaceae form only single primary haustoria (Heide-Jørgensen 2008a).

Resource uptake from hosts

When the haustorial connection with the host is successfully established, hemiparasites begin to withdraw resources from the host xylem sap. Besides virtually all water and mineral nutrients, adult hemiparasites acquire some phytohormones and organic carbon mainly in the form amino- and organic acids, and possibly also sugars by mass flow of xylem sap (**Figs 1 and 2**; Cameron and Seel 2007; Těšitel et al. 2010b; Westwood 2013). The resource uptake is often non-selective (but not in *Olax phyllanthi* and *Santalum album* (Tennakoon and Pate 1996; Tennakoon and Cameron 2006)). This is the case for root hemiparasites possessing a xylem-to-xylem continuity with hosts (Pageau 2003; Jiang et al. 2003, 2004a), but also for most of mistletoes connected to the host xylem via

interfacial parenchyma cells (Pate et al. 1991). As a result, the xylem sap of hemiparasites highly resembles that of their hosts. This can be advantageous for taking up host secondary compounds such as alkaloids protecting the hemiparasite against herbivory (Marko and Stermitz 1997). On the other hand, lack of selective ion uptake may lead to the accumulation of excessive amounts of ions (Seel and Jeschke 1999; Loveys et al. 2001; Chen et al. 2013), which need to be coped with in order to prevent ion imbalance (Volkmar et al. 1998; Chaves et al. 2009).

The continuity of resource uptake into hemiparasites is maintained by a water potential gradient between them and their hosts. Hemiparasites lower their water potential to highly negative values relative to hosts (**Figs 1 and 2**; Ehleringer and Marshall 1995). This is achieved by high content of osmotically active compounds such as sugar alcohols or inorganic ions (Fer et al. 1993; Ehleringer and Marshall 1995; Loveys et al. 2001) and high rates of transpiration, which can be several times higher than that measured in hosts (**Figs 1 and 2**; Press et al. 1988; Jiang et al. 2003). Even when transpiration is reduced, water potential gradient was shown to remain steep due to the difference between haustorial and shoot resistance to water flow (Davidson and Pate 1992; Ackroyd and Graves 1997). In addition, stomata of some root hemiparasites seem to be irresponsive to abscisic acid and remain open under severe water stress (Smith and Stewart 1990; Jiang et al. 2003). Their stomata close when the turgor pressure of guard cells is lost by dehydration, which may even happen after leaf wilting. The host stomatal closure might precede both of these processes (Světlíková et al. 2018 (under revision)). An additional trait that supports the resource flow into hemiparasites are specialized hydathode trichomes documented for several species from the rhinanthoid clade of Orobanchaceae (Renaudin and Garrigues 1967; Govier et al. 1968; Weber 1975). Two types of these hydathode trichomes actively secrete water from the surface depressions of abaxial leaf sides surrounded by elevated areas with stomata (**Fig 1**; Těšitel and Tesařová 2013; Světlíková et al. 2015).

Hemiparasitic carbon acquisition

Traditionally, hemiparasitism was hypothesized to be mainly a strategy of water and mineral nutrient acquisition, as xylem contains only low concentration of organic carbon. According to the N-parasitism hypothesis, it is the elevated transpiration rate that provides hemiparasites with sufficient nitrogen uptake from hosts (Schulze and Ehleringer 1984; Bannister and Strong 2001). Although the first evidence of heterotrophic carbon transfer into a hemiparasite was provided already by Govier et al. (1967) for *Odontites vernus*, its significant contribution to carbon budget of hemiparasites was quantified much later. Root and stem hemiparasites were repeatedly documented to obtain heterotrophically up to 80% (Press et al. 1987; Graves et al. 1990; Ducharme and Ehleringer 1996; Tennakoon and Pate 1996; Těšitel et al. 2010b, 2011) and up to 87% (Marshall and Ehleringer 1990; Schulze et al. 1991; Marshall et al. 1994; Richter et al. 1995; Wang et al. 2008) of overall carbon, respectively. The importance of heterotrophic carbon acquisition for hemiparasites seems to greatly fluctuate not only among species, but also within species in response to own photosynthetic rates, host quality, and environmental conditions (discussed in the next section) (Richter et al. 1995; Ducharme and Ehleringer 1996; Tennakoon and Pate 1996; Těšitel et al. 2010b, 2011).

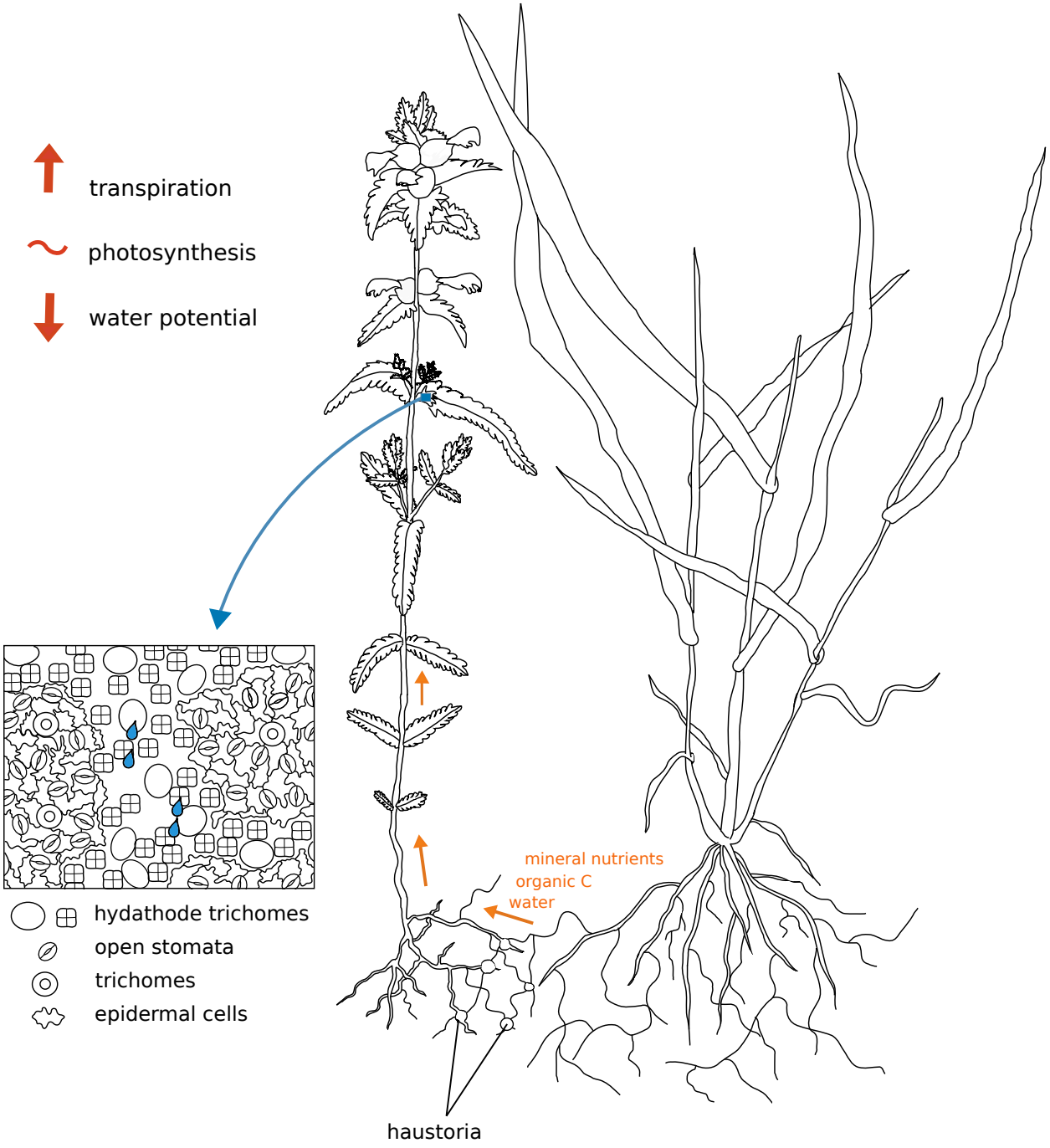

Fig 1. Water-wasting physiological strategy of hemiparasitic *Rhinanthus alectorolophus* parasitizing a wheat host. Compared to the host, the hemiparasite possesses elevated transpiration rate and highly negative water potential, which enable the uptake of the host resources (highlighted in orange). The resources, water with diluted mineral nutrients and organic carbon, are acquired via haustorial connection from the host xylem. The hemiparasite further facilitates the resource withdrawal by active water secretion from specialized hydathode trichomes on the abaxial leaf sides (indicated in inset). In addition to host-derived carbon gain, *Rhinanthus* exhibits a similar photosynthetic rate as the host.

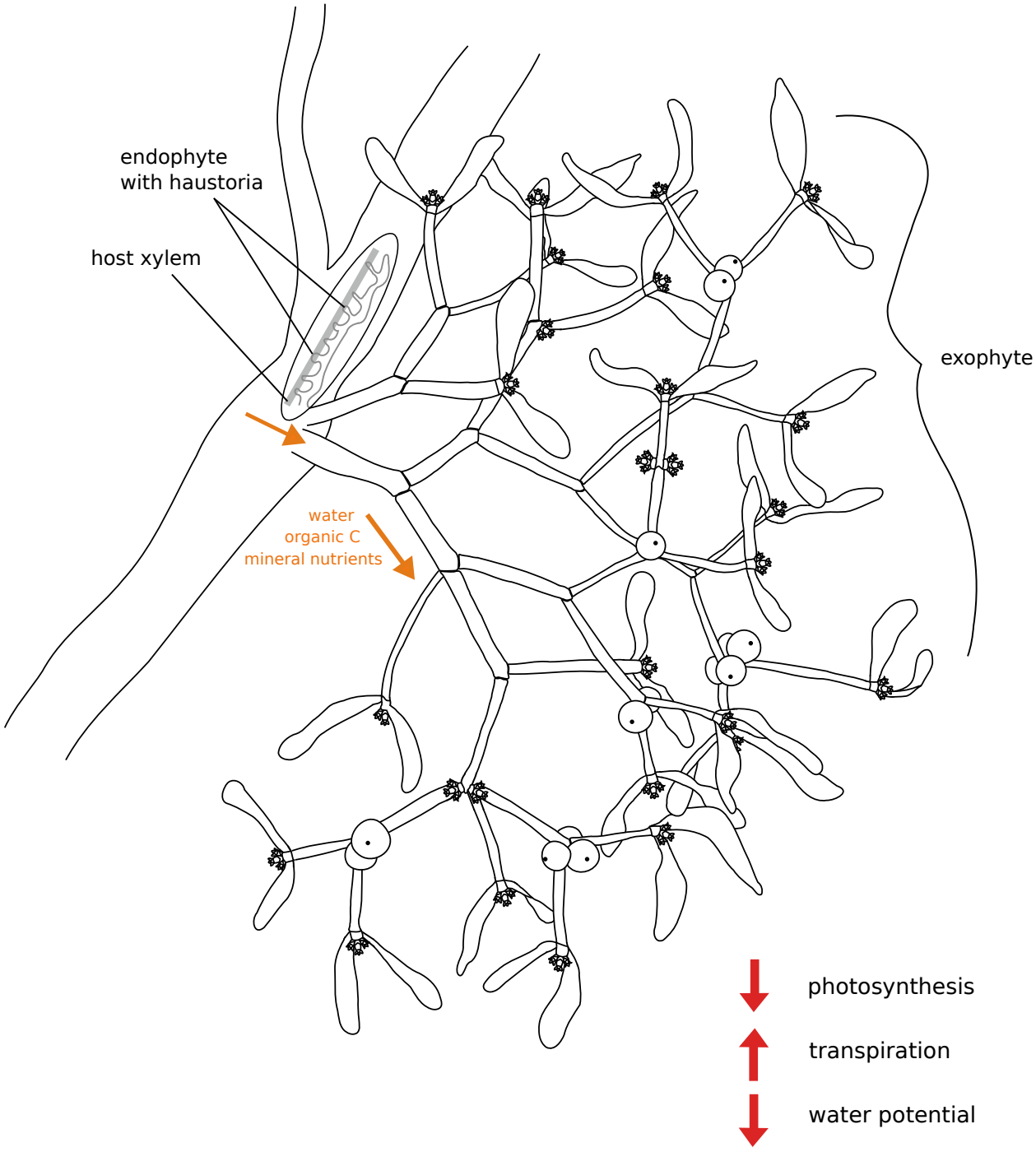

Fig 2. Water-wasting physiological strategy of hemiparasitic *Viscum album* attached to a branch of its tree host. The mistletoe acquires the resources, water with diluted mineral nutrient and organic carbon, from the host xylem (highlighted in orange) by maintaining comparatively higher transpiration rate and markedly lower water potential. Leaves and stems of the exophyte are photosynthetically active, but the rate of photosynthesis is generally lower than that of the host.

Photosynthetic rate of some hemiparasites is lower than that measured in their hosts (**Fig 2**), but many hemiparasites have comparable photosynthetic rates (**Fig 1**). Mistletoes generally behave as shade plants exhibiting low rate of light-saturated photosynthesis (Goldstein et al. 1989; Küppers et al. 1992; Marshall et al. 1994; Strong 2000). The rate is far more decreased in phylogenetically derived species from the Viscaceae family (von Willert and Popp 1995; Logan et al. 2002) and drops to negative values in the phloem-feeding *Arceuthobium* species (Bickford et al. 2005). Photosynthetic rate of root hemiparasites has been suggested to be the lowest among C_3 plants (Press et al. 1987, 1988; Press 1989). This is true only for some of them, such as *Striga* species, *Olax phylanthi*, and *Santalum acuminatum* (Graves et al. 1990; Pate et al. 1990; Tennakoon et al. 1997a; Loveys et al. 2001). More recent findings reported numerous root hemiparasites from Orobanchaceae to have photosynthetic rate comparable with non-parasites (Press et al. 1993; Ducharme and Ehleringer 1996; Těšitel et al. 2011; Světlíková et al. 2018). Autotrophic carbon gain might thus contribute to better performance of these species, especially when mineral nutrients and light are not limited (Těšitel et al. 2015b).

Effects of abiotic factors on hemiparasites

Similarly as in non-parasitic plants, the performance and physiology of parasites are affected by abiotic factors to a great extent (**Tables 1 and 2**). Many field and glasshouse studies dealt with the effect of mineral nutrient availability on root hemiparasites. They focused mainly on the model genera from Orobanchaceae (*Rhinanthus* and *Striga*), that can be easily cultivated on crops. The effects of abiotic factors on mistletoes as well as of other abiotic factors on root hemiparasites were addressed less frequently.

Light

Light is essential energy source in the life of hemiparasites as well as non-parasites and hemiparasites compete for it with all co-occurring species, host and non-host (Fibich et al. 2010; Těšitel et al. 2011). Although they acquire a substantial portion of carbon heterotrophically, having own photosynthetic ability is crucial for both their growth and reproduction (Santos-Izquierdo et al. 2008; Těšitel et al. 2015b). This is further reflected by the retention of plastid genes related to photosynthesis in photosynthetically active hemiparasites (Frailey et al. 2018) and also in parasitic vines of the genus *Cuscuta* with greatly reduced ability of photosynthesis (McNeal et al. 2007). As a consequence of being light demanding plants, many hemiparasites prefer open sunny habitats and avoid deep shade, which is particularly true for the root-hemiparasitic Orobanchaceae (ter Borg 1985). By contrast, some hemiparasites are shade-tolerant, for example several *Melampyrum* species from Orobanchaceae (Masselink 1980; Dalrymple 2007) or tropical trees and lianas from Opiliaceae (Hiepko 1979). A root-hemiparasitic tree, *Okoubaca aubrevillei*, uses different strategy to survive in deep-shaded tropical forests. This parasite likely utilizes its parasitic strategy to drastically reduce the growth of the hosts and even kill them in order to have better access to light (Veenendaal et al. 1996).

The importance of light availability for hemiparasites varies with their developmental stage and ability to obtain host-derived resources. Self-germinating root hemiparasites from open habitats such as *Rhinanthus* do not depend on light for germination (Masselink 1980; ter Borg 2005). Light availability is, however, essential for survival of their initial seedling stage (Keith et al. 2004; Těšitel et al. 2011) when seedlings are not attached to hosts and obtain resources only via own inefficient photosynthesis and poorly developed roots (Seel et al. 1993; Jiang et al. 2004a, 2005). Seedlings are often under strong competition for light from surrounding vegetation and were demonstrated to benefit from heterotrophically gained carbon shortly after attachment (Těšitel et al. 2011). Light availability can thus determine the proportion of heterotrophic carbon in the biomass of hemiparasites (**Table 1**), which was also reported for other mixotrophic plants such as green orchids (Preiss et al. 2010) and green pyroloids (Zimmer et al. 2007; Hynson et al. 2012). Compared with *Rhinanthus* seedlings, young and adult hemiparasites can cope with shading better than seedlings. They weaken hosts by acquiring their resources and then invest them into own superior performance and competitive ability (Těšitel et al. 2011, 2013, 2015b).

The role of light availability also varies during the life of hemiparasites from closed habitats, but not necessarily in similar manner as for species from open habitats. Root hemiparasites from the *Melampyrum* genus such as *M. pratense* and *M. sylvaticum* present interesting examples of annual hemiparasites growing preferably in forest understories (Heinken 2004; Dalrymple 2007). Similarly to *Rhinanthus* species, they need light for initial growth after germination. With light becoming much less available with the canopy closure in late spring, the hemiparasites probably rely more on the uptake of host-derived carbon (Světlíková et al. 2018). Increased dependence on host-derived carbon during seed production was also suggested for *Castilleja linariifolia* (Ducharme and Ehleringer 1996). These findings contrast with adult root hemiparasites from open habitats, which most likely rely more on own photosynthesis for their overall performance (Těšitel et al. 2010b, 2015b).

Light is an important abiotic factor affecting the development of root hemiparasites that require a host signal for germination (e.g. *Striga* or *Alectra*). They do not depend on light as much as self-germinating root hemiparasites, which might be derived from their host-induced germination strategy and limited photosynthetic abilities (Graves et al. 1990; Cechin and Press 1993a). Moreover, *S. asiatica* can even reproduce in darkness (Rogers and Nelson 1962). The germination and establishment of these hemiparasites is, though, supported by light, as increased light intensity and duration were documented to positively affect production of strigolactones by host species (Weerasuriya et al. 1993; Koltai et al. 2011).

Light was in addition found to have a positive effect on the photosynthetic CO_2 assimilation and biomass production of mistletoes and hemiparasitic vines (**Table 1**), but this topic still remains understudied. Light was also shown to positively affect of transpiration and stomatal conductance of the mistletoe *Amyema micquelii* (**Table 1**). Some mistletoes may prefer shady conditions that protect them from excess light, while still provide them with enough light for photosynthesis saturation (Strong 2000). What makes them extraordinary within the flowering plants is the green endosperm, which is present already in fruits and helps mistletoes to survive initial life stages

before the establishment of primary haustoria (Heide-Jørgensen 2008b). Adult plants gather light energy not only by leaves and stems, but also by photosynthetic endophytic parts with chloroplasts growing inside host branches (**Fig 2**). The energy provided by endophytes may be used for example to maintain osmotic gradient driving the mass flow of xylem sap (Fineran 1995; Heide-Jørgensen 2008c).

Carbon dioxide

Rising atmospheric CO_2 is certainly going to affect hemiparasites, but are they going to be affected in a similar way as non-parasites? They could be, because both of them are autotrophs. On the other hand, hemiparasites also acquire heterotrophic resources and further interact with host, and therefore the effect of elevated CO_2 on hemiparasites might be more complex. To date, only a couple of experimental studies have investigated the effect of elevated atmospheric CO_2 on the ecophysiology of hemiparasites and all of them have been aimed at root hemiparasites of Orobanchaceae (**Table 1**). They consistently showed increased photosynthetic rates of hemiparasites grown and measured under elevated than ambient CO_2, but no significant differences in transpiration rate and stomatal conductance (**Table 1**; Watling and Press 1997, 1998; Hwangbo et al. 2003). These results indicate increased photosynthetic capacity of hemiparasites under elevated CO_2 and that the transpiration-driven resource uptake from the host is independent of CO_2 concentration.

Table 1. The effects of light and carbon dioxide on the physiological traits of hemiparasitic plants. Significantly positive and negative effects of abiotic factors on respective traits are denoted by green and red. Non-significant effects are denoted by white and/or ns. Effects not studied are denoted by grey. A: photosynthetic rate; E: transpiration; WUE: water-use efficiency; g_s: stomatal conductance; Ψπ: osmotic potential; DM: dry mass/growth rate; het C: heterotrophic carbon; R_d: dark respiration. Only the studies comparing at least 2 levels of relevant abiotic factor are included.

Abiotic factor	Parasitic plant	Host plant	A	E	WUE	g_s	Ψπ	DM	het C	Additional traits	Reference
light	*Amyema miquelii*	*Eucalyptus behriana*									(Küppers et al., 1992)
	Rhinanthus alectorolophus	wheat, maize									(Těšitel et al., 2011)
	Rhinanthus minor	*Poa pratensis*		ns				ns			(Hwangbo and Seel, 2002)
	Cassytha pubescens	*Leptospermum myrsinoides*						ns		leaf N	(Cirocco et al., 2016a)
	Cassytha pubescens	*Ulex europaeus*								leaf N	(Cirocco et al., 2016a)
	Pedicularis canadensis	many (field)						ns			(Borowicz and Armstrong, 2012)
	Melampyrum pratense	many (field)								R_d	(Světlíková et al., 2018)
CO_2	*Rhinanthus minor*	*Poa pratensis*		ns						total biomass N and C	(Hwangbo et al., 2003)
	Striga hermonthica	*Eragrostis pilosa*				ns		ns	ns	shoot soluble sugars; parasite emergence	(Watling and Press, 1998)
	Striga hermonthica, *S. asiatica*	*Sorghum bicolor*				ns				phenology	(Watling and Press, 1997)
	Melampyrum sylvaticum	*Picea abies*									(Hättenschwiler and Körner, 1997)
	Rhinanthus alectorolophus	*Lolium perenne* /*Medicago sativa*						ns		number of flowers; phenology; leaf N	(Matthies and Egli, 1999)
CO_2 × N	*Rhinanthus alectorolophus*	*Lolium perenne* /*Medicago sativa*								number of flowers; phenology; leaf N	(Matthies and Egli, 1999)

While the response of their photosynthesis to elevated CO_2 is similar as in non-parasites, non-parasites grown at elevated CO_2 concentration generally down-regulate their stomatal conductance. The only observation of any CO_2 effect on stomatal conductance that might indicate any similar down-regulation in hemiparasites was done by Watling and Press (1997) in *Striga hermonthica* infecting *Sorghum bicolor.* They measured significantly increased stomatal conductance at elevated CO_2 compared to ambient CO_2, but only in plants grown at ambient CO_2 concentration. In contrast to these results, the authors found no effect of CO_2 growth conditions on *Striga*'s photosynthesis and stomatal conductance when grown on *Eragrostis pilosa* (Watling and Press 1998). Its photosynthesis was, however, positively affected by elevated CO_2 during measurements regardless of the growth conditions.

Unlike gas exchange, biomass production and growth of hemiparasites were affected by elevated CO_2 inconsistently: positively or non-significantly for rhinanthoid Orobanchaceae (Hättenschwiler and Körner 1997; Matthies and Egli 1999; Hwangbo et al. 2003) and non-significantly or negatively for *Striga* species (**Table 1**; Watling and Press 1997, 1998). This possibly reflects a complex interaction between various hemiparasites and hosts and is hard to generalize. However, the negative effect of elevated CO_2 on total biomass of the *Striga* plants might be due to the increased number of hemiparasites per pot and therefore decreased availability of light and host resources for individual plants. Furthermore, the difference in the biomass response of rhinanthoid hemiparasites and *Striga* to elevated CO_2 was hypothesized to be related to comparatively low photosynthesis and high dark respiration of *Striga* (Hwangbo et al. 2003). In addition, the phenology of these hemiparasites might be differentially influenced by elevated CO_2 concentration (**Table 1**; Watling and Press 1997, 1998).

It is difficult to predict the response of hemiparasites on elevated atmospheric CO_2, as it may be affected by additional factors such as nutrient availability, interaction with host species, and host species performance. For example, the growth of parasites may be enhanced under elevated CO_2 only in plants with high nutrient supply, whereas it might slightly decrease in nutrient-limited plants (**Table 1**; Matthies and Egli 1999). These authors also showed that the effect of elevated CO_2 changes with the host species parasitized. In summary, elevated CO_2 stimulates photosynthesis and sometimes growth of hemiparasites more than in non-parasitic plants (Hättenschwiler and Körner 1997; Matthies and Egli 1999). Interestingly, the parasite-induced reduction in host biomass production and uptake of heterotrophic carbon seem to remain similar at ambient and elevated CO_2 (Watling and Press 1997, 1998; Matthies and Egli 1999; Hwangbo et al. 2003).

Water and mineral nutrients

Hemiparasites compete with hosts for light, but also steal resources from their xylem. The host xylem sap is thus the only source of non-photosynthetically gained resources for stem hemiparasites and a major source for root hemiparasites (Tennakoon et al. 1997b; Jiang et al. 2004a; Cameron et al. 2008). Root hemiparasites may also acquire a small portion of resources by their own roots (Pate et al. 1990; Seel et al. 2006). The resource uptake and performance of hemiparasites is affected by the composition of hosts' xylem (Seel et al. 1993), which is, in turn, influenced by water and mineral nutrients available in soil. The effect of mineral nutrient availability or ecosystem

productivity on the host–hemiparasite association has been widely investigated, particularly in model species of root hemiparasites (**Table 2**). In contrast, experimental studies focusing on the effect of water availability or a simultaneous effect of water and nutrient availability on hemiparasites are rare (**Table 2**).

Due to easy access to host below-ground resources, hemiparasitic life strategy may be assumed as particularly beneficial when these resources are rare, i.e. under arid and low-nutrient conditions. Regardless of that, hemiparasitic plants are distributed across diverse habitats in terms of below-ground resource availability (Těšitel et al. 2015a). Root hemiparasites adapted to dry and low-productive conditions are uncommon, but can be found in Krameriaceae and Santalales (Heide-Jørgensen 2008a). Mistletoes and hemiparasitic vines frequently occur under such conditions, where they parasitize xerophytic trees and shrubs (Weber 1981; Ullmann et al. 1985). Moreover, some mistletoes (*Phoradendron californicum, Kunkeliella subsucculenta,* and *Korthalsella salicornioides*) have lost leaves and evolved succulency as an adaptation to arid environmental conditions (Fineran 1995; Heide-Jørgensen 2008b).

The effect of water availability on hemiparasites

Water availability shapes many traits of hemiparasites such as their germination rate, seedling survival, growth, reproduction, and physiological traits (**Table 2**). Germination and flowering rate of root hemiparasites were shown to be reduced by high water availability (ter Borg 1972; Ducarme and Wesselingh 2009), while their seedling survival can be reduced either by flooding (ter Borg 1972) or drought (van Hulst et al. 1987; Ameloot et al. 2006; Světlíková et al. 2018 (under revision)). Sufficient water availability has a positive impact on reproduction of the *Rhinanthus* hemiparasites such as number of flowers and seeds per plant (Ducarme and Wesselingh 2009). The water availability effect on growth of hemiparasites is not consistent among studies (**Table 2**), suggesting that other environmental factors may be involved.

Physiology of hemiparasites seems to be affected by water availability similarly as that of non-parasites (Grassi and Magnani 2005; Flexas et al. 2006; Yan et al. 2016). Their transpiration and stomatal conductance decrease with osmotic potential under water shortage (**Table 2**). However, while root hemiparasites such as *Rhinanthus* likely do not close their stomata actively (using ABA signal) (Světlíková et al. 2018 (under revision)), mistletoes regulate their water loss through stomata much more effectively. This is in a stark contrast to early investigations that suggested unlimited transpiration and stomatal closure only after turgor loss in mistletoes (Vareschi and Pannier 1953; Härtel 1956).

Moreover, not only succulent mistletoes mentioned above, but also other hemiparasites from dry areas (*Olax phyllanthi, Santalum album,* and *Lysiana exocarpi*) strongly control their water loss and exhibit a conservative water-use strategy (Ullmann et al. 1985; Pate et al. 1990; Tennakoon et al. 1997a). This strategy of economical transpiration was demonstrated to be correlated in *L. exocarpi* with the stomatal responses of hosts in order to ensure their survival of long-term severe droughts (Ullmann et al. 1985). Similar physiological functioning was observed in mistletoes from saline areas (see “Salinity” section below). Furthermore, such a relationship between host–hemiparasite stomatal conductance was observed even in temperate mistletoes where water is easily accessible

for most of the year (Bannister and Strong 2001). As documented for *Cassytha* (Cirocco et al. 2016b), hemiparasites can be even more conservative in water use than their hosts.

Table 2. The effects of below-ground resources – water and nitrogen – on the physiological traits of hemiparasitic plants. Significantly positive and negative effects of abiotic factors on respective traits are denoted by green and red. Non-significant effects are denoted by white and/or ns. Not studied effects are denoted by grey. A: photosynthetic rate; E: transpiration; WUE: water-use efficiency; g_s: stomatal conductance; Ψπ: osmotic potential; DM: dry mass/growth rate; het C: heterotrophic carbon; F_v/F_m: maximum quantum yield; ETR_{max}: maximum electron transport rate; R_d: dark respiration; RWC: relative water content; SLA: specific leaf area; $\delta^{13}C$ and ^{18}O: stable isotope composition of carbon and oxygen in biomass; * some results statistically significant. Only the studies comparing at least 2 levels of relevant abiotic factor are included.

Abiotic factor	Parasitic plant	Host plant	A	E	WUE	g_s	Ψπ	DM	het C	Additional traits	Reference
water	*Lysiana exocarpi*	*Acacia victoriae*									(Ullmann et al., 1985)
	Amyema nestor	*Acacia grasbyi*	ns							R_d	(Hellmuth, 1971)
	Tapinanthus oleifolius	*Acacia nebrownii*		ns						R_d	(Von Willert and Popp, 1995)
	Viscum rotundifolium	*Acacia nebrownii*								R_d	(Von Willert and Popp, 1995)
	Cassytha pubescens	*Ulex europaeus*	(F_v/F_m)					ns*		stem Na, $\delta^{13}C$ & N	(Cirocco et al., 2016b)
	Striga hermothica	*Sorghum bicolor*	ns							leaf RWC; R_d ; stomatal density & aperture	(Inoue et al., 2013)
	Rhinanthus major, R. minor	many (pots)								phenology; number of flowers and seeds/plant	(Ducarme and Wesselingh, 2009)
	Rhinanthus alectorolophus	wheat, maize	(F_v/F_m)					ns	ns		(Těšitel et al. 2015b)
	Rhinanthus alectorolophus	wheat								seedling survival; leaf $\delta^{13}C$ & $\delta^{18}O$; stomatal density	Světlíková et al. 2018 (under revision)
N	*Phoradendron californicum*	*Acacia greggii*								fruit production	(Schulze and Ehleringer, 1984)
	Phoradendron juniperinum	*Juniperus osteosperma*								fruit production	(Schulze and Ehleringer, 1984)
	Striga hermonthica	*Sorhum bicolor*								germination; seedling survival	(Cechin and Press, 1993b)
	Striga hermonthica	*Sorhum bicolor*								phenology	(Cechin and Press, 1993a)
	Striga hermonthica	*Sorhum bicolor*								emergence; Chl content; R_d; N:C ratio	(Simier et al., 2006)
	Striga asiatica	*Sorhum bicolor*								germination	(Raju et al., 1990)
	Cassytha pubescens	*Ulex europaeus /Acacia paradoxa*						ns		stem N; ETR_{max}	(Cirocco et al., 2017)
	Rhinanthus minor	many (field)								seedling survival; number of plants; number of flowers	(Mudrák and Lepš, 2010)
	Rhinanthus minor	many (field)									(Hejcman et al., 2011)
	Rhinanthus minor	many (field)									(Van Hulst et al., 1987)
	Rhinanthus alectorolophus	wheat, maize	(F_v/F_m)							survival- attached plants	(Těšitel et al., 2015)
	Pedicularis canadensis	many (field)									(Borowicz and Armstrong, 2012)
	Melampyrum sylvaticum	*Picea abies*						ns			(Hättenschwiler and Körner, 1997)
	Melampyrum pratense	many (field)									(Gauslaa 1990)
	Rhinanthus alectorolophus	*Lolium perenne /Medicago sativa*								number of flowers; phenology; leaf N	(Matthies and Egli, 1999)
	Rhinanthus alectorolophus	wheat		ns						R_d; guttation drops	(Světlíková et al., 2015)

Photosynthesis of hemiparasites is also expected to decrease under water-stress conditions as suggested by Ducharme and Ehleringer (1996). However, this was supported only by two pot experiments for *Cassytha pubescens* and *Rhinanthus alectorolophus* (**Table 2**). The photosynthesis of *Striga hermonthica*, grown in another pot experiment, decreased non-significantly (**Table 2**), probably due to short-term water stress. Field reports on the negative effect of water stress on photosynthesis of hemiparasites are missing. To the best of my knowledge, only one hemiparasite, *Amyema nestor*, was subjected to field gas-exchange measurements under optimal water supply and water stress (**Table 2**). Daily net photosynthesis of this mistletoe was surprisingly unchanged by water shortage, seemingly due to only a minor reduction of stomatal opening that did not affect CO_2 uptake. This may be understood as water-wasting strategy of the mistletoe in the arid climate of Western Australia.

The effect of nutrient availability on hemiparasites

The presence and abundance of root hemiparasites in natural ecosystems, particularly *Rhinanthus* species, has been repeatedly shown to be governed by available nutrients. Establishment of these hemiparasites is reduced in high productive grasslands, where they are under strong competition for light leading to an increased mortality of seedlings and attached plants (**Table 2**; van Hulst et al. 1987; Hejcman et al. 2011; Mudrák et al. 2013; Těšitel et al. 2015b). In spite of that, the growth and reproduction of adult *Rhinanthus* individuals were reported to be facilitated by nutrient availability (**Table 2**; Mudrák and Lepš 2010; Těšitel et al. 2013). This is in agreement with documented preferences of herbaceous and woody root hemiparasites for N-fixing legume hosts (Tennakoon et al. 1997a; Jiang et al. 2008). Unlike adults, unattached seedlings are not able to take advantage of high nutrient availability, potentially due to poor ability to uptake soil mineral nutrients (Press et al. 1993; Seel et al. 2006). Similarly to light, nutrients thus also have contrasting effects on hemiparasites of various life stages (Těšitel et al. 2013).

Nutrient supply also markedly impacts early development of root hemiparasites with host-induced germination. Glasshouse experiments with *Striga hermonthica* and *S. asiatica* cultivated on *Sorghum bicolor* revealed a negative effect of mineral nutrient addition on germination, emergence, haustoria establishment, seedling growth, and seedling survival (**Table 2**). Lower germination of *Striga* is likely caused by decreased exudation of strigolactones, germination stimulants from *Sorghum* roots, under high nutrients (Raju et al. 1990; Cechin and Press 1993b; Yoneyama et al. 2007). Furthermore, elevated nutrient availability improves photosynthetic capacity and increases dark respiration in *Striga*, which leads to a decrease of their dependence on heterotrophic carbon as shown by Cechin and Press (1993a). In spite of that *S. hermothica* was evidenced to reduce its growth at higher mineral nutrient concentrations (**Table 2**), even though it might exhibit more haustorial connections to host roots (Boukar et al. 1996).

Positive correlation of mineral nutrient supply with photosynthesis and dark respiration, and lower importance of host-derived carbon acquisition were observed in *Rhinanthus alectorolophus* grown in N-rich substrate (**Table 2**; Těšitel et al. 2015b). Increased growth and reproduction under high nutrients were consistently documented not only for self-germinating root hemiparasites as mentioned above, but also for *Phoradendron* mistletoes (**Table 2**). In

comparison to root hemiparasites, the influence of nutrient availability on mistletoes was investigated only by field measurements, because they cannot be easily cultivated under controlled conditions.

An appropriate way of investigating the effects of mineral nutrient availability on mistletoes is to compare ecophysiological traits of mistletoes infecting N-rich hosts (N_2-fixers) with N-poor hosts (N_2-non-fixers) (Schulze and Ehleringer 1984; Bannister and Strong 2001; Wang et al. 2008). These independent studies demonstrated that water relations of some mistletoes depend on nutrient concentration in the host xylem. Mistletoes attached to N-fixers had lower transpiration and higher water-use efficiency, which is consistent with the N-parasitism hypothesis predicting more conservative water-use strategy under high nutrient availability. In contrast, the water-wasting strategy of *Rhinanthus* seems to be irresponsive to nutrient availability (Seel et al. 1993; Světlíková et al. 2015). This might be true only for some species of the rhinanthoid clade of Orobanchaceae, since transpiration and stomatal conductance of *Melampyrum pratense* were observed to decrease at high nutrient levels (**Table 2**).

Interactive effects of water and nutrient availability on hemiparasites

The responses of hemiparasites to varying nutrient availability can be modulated by change in the availability of water. Water availability promotes mineralization of soil organic matter and mineral nutrients becomes more accessible (Mazzarino et al. 1991; Paul et al. 2003). Therefore, hemiparasites grown at high water supply might have higher foliar nitrogen and SLA than those grown at the same nutrient availability but low water supply (Press et al. 1993). Interactive effect of mineral nutrients and water on plant traits was reported for non-parasites (Ibrahim et al. 1998; Clay et al. 2001; Lower and Orians 2003; Elazab et al. 2016) as well as for parasites (Boukar et al. 1996; Těšitel et al. 2015b). For example, increased nutrient supply increased the number of haustoria and emerged *Striga* plants cultivated at low water availability, while it had no effect on their emergence under high water availability (Boukar et al. 1996). The authors attributed that to generally better performance of *Striga* under moderately dry conditions, which is in agreement with what was observed for *Rhinanthus*. The below-ground resources had an interactive effect on the biomass production and heterotrophic carbon gain of *Rhinanthus alectorolophus*, and on the damage inflicted to the host (Těšitel et al. 2015b). *Rhinanthus* performed the best under contrasting availability of the resources, when its biomass production and damage inflicted to the host were the highest due to efficient photosynthesis and resource acquisition. The water-wasting physiological strategy of *Rhinanthus* seems to be thus the most beneficial under contrasting availability of water and mineral nutrients or as suggested by Těšitel et al. (2015b) when both resources are moderately available.

Salinity

Plants growing in areas with saline soils are generally strongly impacted by stress conditions of high salinity. They have to decrease their water potential in order to uptake water highly enriched in ions (namely Na^+, Cl^-, Mg^{2+}, SO_4^{2-}). Consequently, they have to cope with ion imbalance and even toxicity. Salinity stress can be therefore considered as water stress combined with ionic stress (Schulze et al. 2005; Chaves et al. 2009). Salt tolerance, a key

mechanism dealing with salt excess in plants, has evolved probably multiple times independently (Flowers et al. 2010) and has been recognized for more than 220 years (Flowers et al. 1986). Salt tolerance is induced by a chain of physiological, cellular, and molecular responses that maintain ion and osmotic stability and allow plants to survive salinity stress (Schulze et al. 2005; Lambers et al. 2008; Chaves et al. 2009).

Several hemiparasitic plants grow in saline areas, being affected by elevated salinity. Only a few root hemiparasitic species seem to tolerate elevated salinity, such as the species of the *Odontites vernus* group occurring on temporarily flooded coastal meadows and continental salt mashes in Europe (Schneider 1964; Snogerup 1982, 1983; Koutecký et al. 2012) or several North American species of the genus *Cordylanthus* (Grewell 2008). Mechanisms allowing these species to deal with excess salt remain unknown, as their physiology has never been investigated. However, taking into consideration high transpiration rate of hemiparasites (Press et al. 1988; Stewart and Press 1990; Jiang et al. 2003) and accumulation of inorganic ions frequently reported from their biomass (Richter and Popp 1992; Popp et al. 1995; Loveys et al. 2001), they are also expected to uptake and accumulate excessive amounts of salts. Sugar alcohols, which are found in hemiparasites at high concentrations (Ehleringer and Marshall 1995; Jiang et al. 2005), may be accumulated to alleviate salinity-related stress as summarized by Lambers et al. (2008). If not sugar alcohols, water accumulation and ion sequestration in older leaves may avoid stress related to ion imbalance, as it does in the succulent mistletoe *Tapinanthus oleifolius* (Popp et al. 1995).

Contrary to root hemiparasites, mistletoes can be commonly found in salty areas, mainly on mangrove species growing in highly saline intertidal zones. Several field studies have been dedicated to the gas exchange and water balance of mistletoes infesting mangroves (Goldstein et al. 1989; Orozco et al. 1990; Chen et al. 2013). All of them demonstrated a low rate of photosynthesis of mistletoes, which was found to be limited mainly by stomatal and mesophyll conductance under elevated salinity (Chen et al. 2013). Trends in transpiration rate and stomatal conductance results were, however, not consistent among the studies. Higher transpiration rates and stomatal conductance in the mistletoes than in the hosts measured by Goldstein et al. (1989) and Orozco et al. (1990), and frequently observed also in root hemiparasites can be explained by the N-parasitism hypothesis. However, under elevated salinity this strategy would result in elevated uptake of salts and their toxic effects in such elevated concentrations due to non-specific mass flow of xylem sap from the host to the hemiparasite.

Hence, there might be a trade-off between the uptake of mineral nutrients and maintaining salt concentration in cells at low levels. Under saline as well as dry conditions, mistletoes might lower their transpiration rate in order to save water, which was also shown by Küppers et al. (1992) in the Australian mistletoe *Amyema miquelii* parasitizing *Eucalyptus behriana*. This might be a convenient strategy when the availability of nutrients in the host xylem sap is low and not worth loosing water (Küppers et al. 1992), but also when the availability of nutrients in

the host xylem sap is high and there is no need to further maintain such high transpiration rate (Schulze and Ehleringer 1984; Chen et al. 2013).

Future perspectives

Although certain effects of abiotic factors on hemiparasites are already well understood, many of them still remain to be uncovered. More attention should be given to shade-tolerant root hemiparasites (*Melampyrum* or the Opiliaceae species) and their carbon budgets which might help to explain evolutionary stability of hemiparasitism in such environments. Effects of light availability and elevated atmospheric CO_2 on the ecophysiology of mistletoes are also yet to be examined. Regarding elevated CO_2 concentration, it would be desirable to reveal its effect on the interaction between various parasite–host pairs. In addition, water and salinity stress might markedly affect the life of hemiparasites with ongoing climate change and thus studying their effects should become more important. For example, little is known about the acclimation mechanisms of hemiparasites to water stress and their recovery from it. This might bring interesting outcomes, since both root hemiparasites and mistletoes were observed to heavily wilt under water stress to a “flaccid appearance” and recover fast without any damage (Glatzel 1983; Jiang et al. 2004b). Furthermore, there are other interesting questions, e.g. what is the role of sugar alcohols and ABA in hemiparasites, what is the mechanism of their salt tolerance, and what is the relationship between water and nutrient availability and water-wasting or water-conservation strategy of mistletoes? To answer such questions, it is necessary to investigate the interactive effects of multiple abiotic factors on hemiparasites.

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Chapter 1

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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

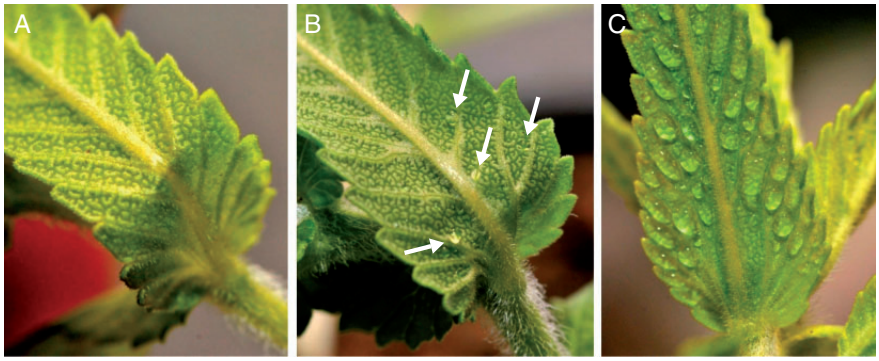

Fig. 1. The density and size of drops on the leaves of *Rhinanthus alectorolophus* (55 d after sowing) classified on an ordinal scale: (A) no drops (0), (B) small drops (0·5), (C) large drops (1). The plant was cultivated under (A) low irrigation and nutrient treatment, (B) low irrigation and high nutrient 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 ($P < 0·05$) are highlighted in bold.
Non-significant terms ($P > 0·05$) 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 ($P < 0·05$) 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 ($z = 2·076$, $P = 0·038$) and lower in flowering plants ($z = –2·311$, $P = 0·021$). 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).

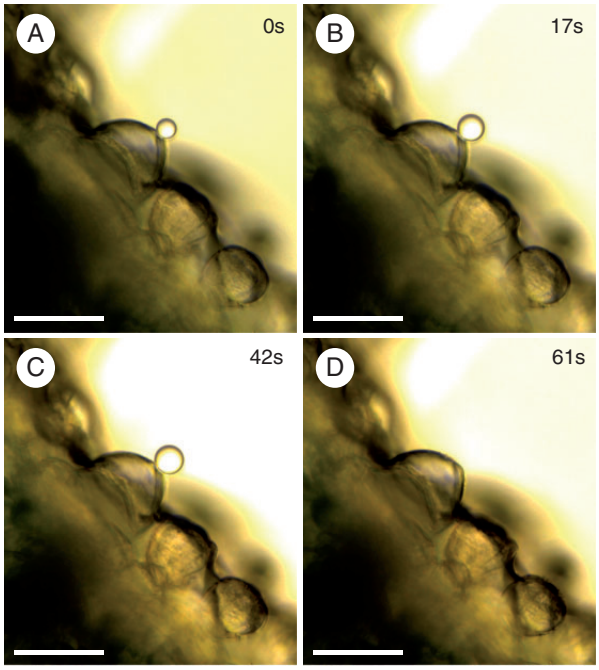

FIG. 2. Micrographs showing secretion from sessile hydathode trichomes on the abaxial leaf surface of *Rhinanthus alectorolophus*. The secretion was observed in oil shortly after immersion of the sample (0s, A) and in the time series as indicated (B–D). The drop of liquid finally detached from the trichome 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 ($t_{38} = –3·613$, $P < 0·001$). *Rhinanthus* cultivated under the N+ treatment displayed a higher dark respiration rate ($t_{38} = 3·172$, $P = 0·003$). Regardless of the significant effect of the water treatment on the RWC in pots (Welch two sample t-test: $t_{32.3} = 3·005$, $P = 0·005$), 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

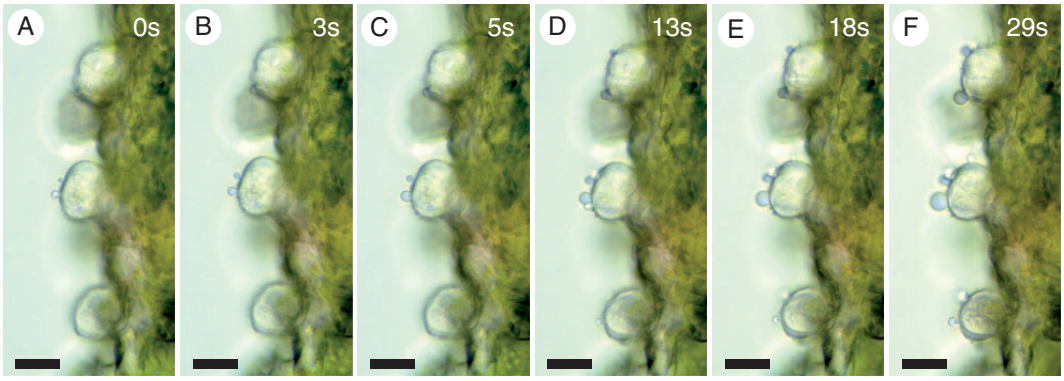

FIG. 3. Micrographs showing secretion from stalked hydathode trichomes on the abaxial leaf surface of *Rhinanthus alectorolophus*. The secretion was observed in oil shortly after immersion of the sample (0s, A) and in the time series as indicated (B–F). The scale bars indicate 25 µm.

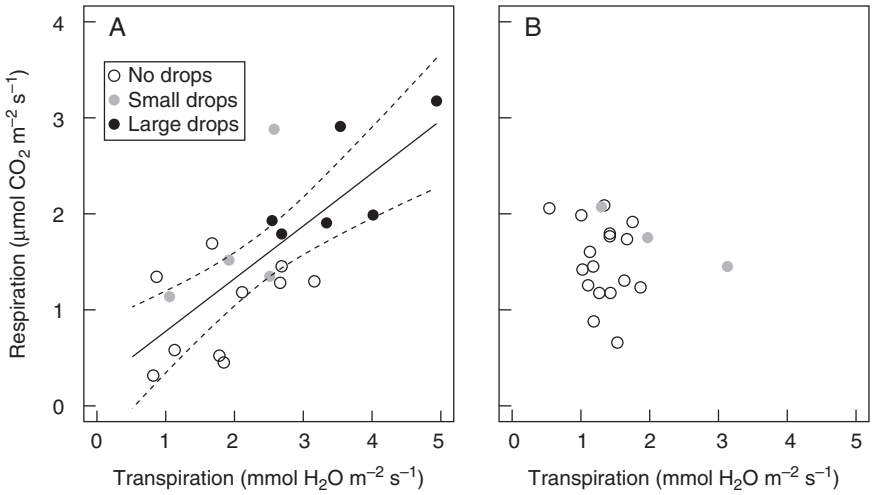

FIG. 4. The relationship between the night-time rates of respiration and transpiration in (A) non-flowering and (B) flowering *Rhinanthus alectorolophus*. Each circle relates to one individual plant. The size of drops observed on the leaves of examined plants immediately before the physiological measurement 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;

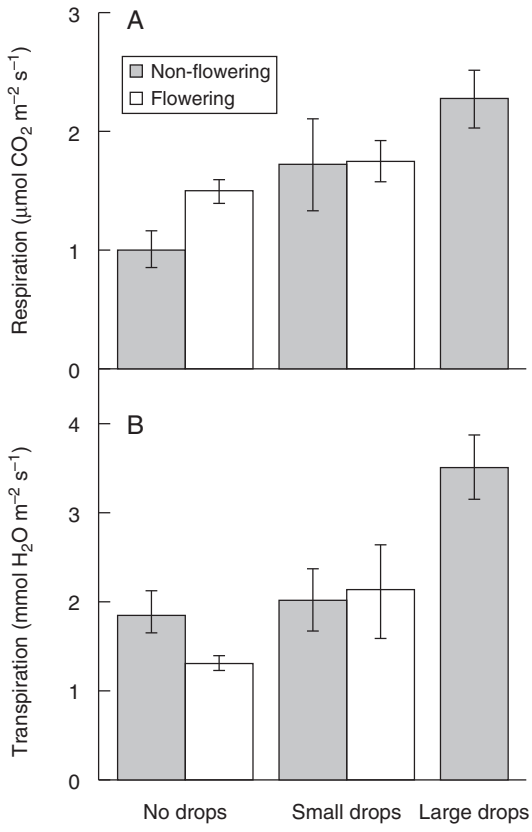

FIG. 5. Rates of respiration (A) and transpiration (B) measured on the leaves of *Rhinanthus alectorolophus* with various sizes of water drops at the two developmental stages of the plants. Means and standard errors are presented. Non-flowering plants (55 d after sowing) and flowering plants (73 d after 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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Chapter 2

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REGULAR PAPER

Tracing nitrogen flow in a root-hemiparasitic association by foliar stable-isotope labelling

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Background and aims – The resource flows in the host-hemiparasite association have been frequently studied by applying stable isotope techniques. However, these methods of artificial labelling required sophisticated equipment preventing their application to field experiments. Here, we aimed to test the applicability of the $^{15}N^{13}C$-urea foliar brushing method in tracing the resource flows between a root hemiparasite, *Rhinanthus major*, and a host, *Triticum aestivum*. In addition, the dynamics of the label movement was examined in order to provide an estimate of the most appropriate harvesting time.
Methods – Double-labelled urea (98 atom % ^{15}N, 99 atom % ^{13}C) solution (2 g dm^{-3}) was applied on host plants grown with hemiparasites by a single foliar brushing. Above- and belowground biomass of both species was harvested 3, 7, and 14 d after host labelling and its isotopic composition was analyzed. Final isotopic enrichment of biomass was expressed as the atom percent difference between labelled samples and the mean of corresponding controls.
Key results – Our results showed that a single leaf-brushing with $^{15}N^{13}C$-urea provided sufficiently ^{15}N-labelled plant material, but it was insufficient to shift the natural abundance of ^{13}C in both species. Similar ^{15}N values were found for the host and hemiparasite biomass already 3 d after labelling, but the ^{15}N enrichment of attached hemiparasite significantly increased in time. Within a week, ^{15}N-label gradually dispersed into the host tissues and was simultaneously transferred into the hemiparasite via the root connections.
Conclusions – We present foliar brushing by ^{15}N-urea as a simple and precise labelling method, which can be widely applied in both greenhouse and field experiments to examine the nitrogen flows between root hemiparasites and their host species. The transfer of nitrogen to the hemiparasite is fast and thus an experimental period of 7 d seems largely sufficient for field studies where the equilibrium state of labelling is of interest.

Key words – Haustorium, leaf, nitrogen flow, Orobanchaceae, hemiparasitic plant, *Rhinanthus*, stable isotope, *Striga*, tracer.

INTRODUCTION

Root hemiparasites are parasitic plants that attach belowground to roots of other plants, withdrawing resources from host vascular bundles and performing their own photosynthesis at the same time (Press 1989). Root hemiparasitism is one of the most common life strategies among parasitic plants (Heide-Jørgensen 2008). Many root hemiparasites have been demonstrated to play important roles in the ecosystem by altering nutrient cycling (Press 1998, Quested et al. 2005, Bardgett et al. 2006, Demey et al. 2014) or changing competitive relations in plant communities (Gibson & Watkinson 1991, Pywell et al. 2004). Others, namely several species of the genus *Striga*, have been extremely harmful weeds causing enormous economical losses in dry tropical and subtropical regions (Parker 2009).

Physiology of the hemiparasite-host association is of central importance when studying the biology of root hemiparasites (Těšitel et al. 2015). The association basically involves two autotrophic plants connected by a unidirectional flow of resources (Jiang et al. 2003, 2004). Quantitative and qualitative analyses of this resource flow as well as the detection of its effect on the physiology of both partners have been the main goals of many physiological studies on root hemiparasites. The application of stable isotope techniques is a frequently used methodological approach in these physiological studies (Ducharme & Ehleringer 1996, Pageau et al. 1998, Pate & Bell 2000, Aflakpui et al. 2005, Cameron & Seel

2007, Těšitel et al. 2010). The use of isotope tracing techniques requires a contrast in stable isotopic composition of hosts and hemiparasites, which can be based either on their natural abundances, e.g. the use of C_4 hosts in the studies of carbon translocation (Ducharme & Ehleringer 1996, Pageau et al. 1998, Pate & Bell 2000, Santos-Izquierdo et al. 2008, Těšitel et al. 2010) or artificial labelling (Pageau et al. 2003, Aflakpui et al. 2005, Cameron & Seel 2007).

Isotope labelling of the host plant is the first step of any study using artificial labelling. Various methods can be used to produce plants enriched in ^{15}N and/or ^{13}C stable isotopes. However, the vast majority of these methods require sophisticated equipment comprising gas-tight chambers and other system components necessary for precise labelling. Moreover, different ^{15}N labelling methods can vary in their effectiveness and depend on a focal species (Hertenberger & Wanek 2004). A relatively new and much more feasible method for in situ ^{15}N and ^{13}C labelling of plants is based on foliar feeding of plants with a double-labelled urea solution. This method was firstly introduced by Schmidt & Scrimgeour (2001), who simultaneously enriched a plant tissue in N and C stable isotopes by daily foliar misting, extending the application of the method to C translocation studies. The method was later modified by Putz et al. (2011), who replaced foliar misting by brushing, which prevents the contamination of soil and co-occurring plants. Leaf-brushing by a double-labelled urea solution has been suggested as a straightforward, low-cost and technically easy way of controlled isotope labelling of plants.

In addition to the leaf-misting and leaf-brushing labelling methods, another feasible labelling method has been widely used both in greenhouse and field to study nutrient flow between plants. This method, developed by Ledgard et al. (1985), introduces ^{15}N by immersion of a leaf in a ^{15}N-enriched urea solution and mostly serves to detect and further examine nitrogen transfer between legumes and neighbouring plant species (Ledgard et al. 1985, McNeill et al. 1997, Gylfadóttir et al. 2007, Pirhofer-Walzl et al. 2012) and below-ground N deposition from legumes in the soil (McNeill et al. 1997, Hertenberger & Wanek 2004, Gasser et al. 2015).

Here, we tested the applicability of the $^{15}N^{13}C$-urea foliar brushing method in tracing the resource flows between a host and root hemiparasite. Not only did we aim to demonstrate the flow of nitrogen and carbon, but also the dynamics of the label movement. This is crucial for practical use as it provides a guideline regarding the length of the period between labelling and sampling for stable isotope analysis. The study used a model root-hemiparasitic association between hemiparasitic *Rhinanthus major* L. (= *R. angustifolius, R. serotinus*; Orobanchaceae) and a host, *Triticum aestivum* L. (common wheat).

METHODS

Cultivation and stable isotope analysis

Rhinanthus major seeds were collected from a natural population occurring on the Čertoryje meadows, Bílé Karpaty Mts., Czech Republic. Seeds of common wheat were obtained from the school farm of the Faculty of Agriculture, University of South Bohemia, České Budějovice, Czech Republic.

Seeds of *Rhinanthus* were germinated for 86 days on Petri dishes padded with moist filter paper at 4°C to break seed dormancy. Seeds of common wheat were germinated on Petri dishes with moist filter paper for four days at room temperature. Seedlings of both the parasite and host were planted to 0.8 dm^3 pots filled with a 1:1 (v/v) mixture of universal gardening compost and sand. The distance between the parasite and host was 3–4 cm. Plants were cultivated in a growth chamber at the Department of Botany, Faculty of Science, University of South Bohemia, Czech Republic under following conditions: 12:12 h light:dark cycle, PAR intensity 400–500 µmol m^{-2} s^{-1}, and 23°C/18°C day/night temperature.

Labelling of host by $^{15}N^{13}C$-urea (98 atom % ^{15}N, 99 atom % ^{13}C; obtained from Sigma-Aldrich Corporation, St. Louis, USA) was conducted after 44 days of growth. The concentration of double-labelled urea in the labelling solution was 2 g dm^{-3}, which corresponds to 62.2 mmol ^{15}N dm^{-3} and 31.1 mmol ^{13}C dm^{-3}. The labelling solution and a drop of detergent were applied by brush on 5-cm long sections of host leaves (3 leaves per host plant). The labelled sections were marked by paper stickers for a permanent identification

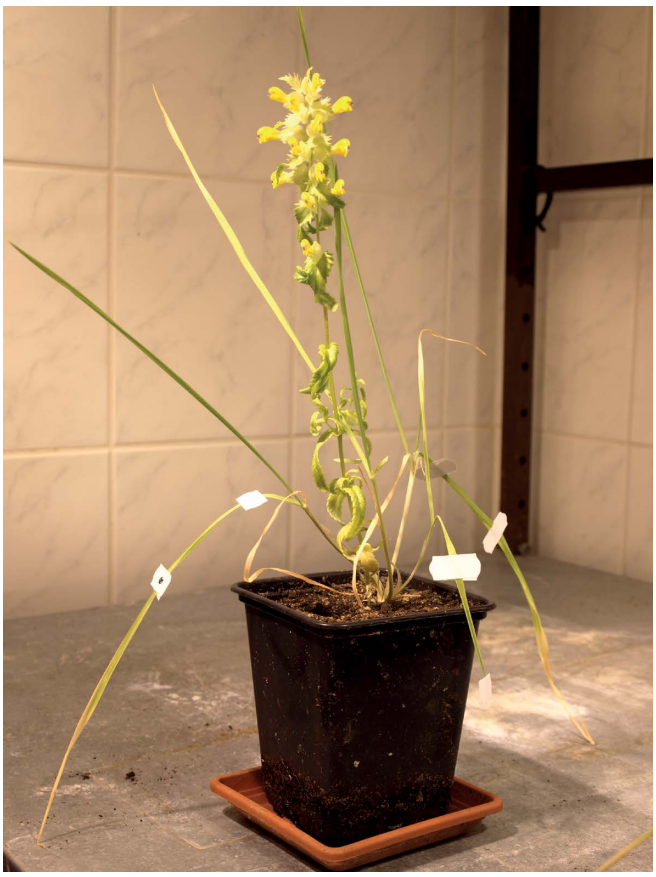

Figure 1 – $^{15}N^{13}C$-urea labelled host plant, *Triticum aestivum*, and attached hemiparasite, *Rhinanthus major*. Note the paper stickers used to mark the leaf sections where the labelling solution was applied. The picture was taken at the time of harvest (14 d after labelling).

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Table 1 – Analysis of variance table of linear models.
The table summarizes the effects of time, plant part (shoot vs. root), and their interaction on the ^{15}N atom percent excess in the biomass of $^{15}N^{13}C$-urea labelled hosts (unlabelled sections) and attached hemiparasites. Significant effects ($p < 0.05$) are marked in bold.

Effect		Hemiparasite			Host		
	DF	SS	F	p	SS	F	p
Time of harvest	1	**0.00714**	**5.638**	**0.024**	0.00024	0.075	0.786
Plant part	1	0.00018	0.143	0.708	0.00639	2.020	0.166
Time × Part	1	0.00072	0.565	0.458	0.00057	0.180	0.675
Residuals	30	0.03799			0.09419		

(fig. 1). There were seventeen labelled pots and nine unlabelled control pots in total. The pots were positioned at random in the growth chamber at a distance to prevent contact between plants in different pots.

Five to seven experimental and three control pots were harvested 3, 7, and 14 d after host labelling. Above- and below-ground biomass samples of each host and hemiparasite were collected in separate paper bags and dried at 80°C for 48 hours. The host leaf sections on which the labelling solution was applied were processed separately. Dried biomass was homogenized and a subset of it was embedded in tin capsules for stable isotope analysis.

The stable isotope analysis was conducted with a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the Stable Isotope Facility at UC Davis (University of California, Davis, CA, USA). The N and C isotopic compositions of the biomass samples was expressed as ^{15}N and ^{13}C atom percent relative to the international standards, Air and V-PDB (Vienna PeeDee Belemnite), respectively.

Data analyses

The ^{15}N and ^{13}C atom percent data of each sample type (host/hemiparasite, root/shoot biomass, harvesting time, labelled/control pots) were plotted as boxplots to illustrate the isotopic composition of the samples. Since a substantial enrichment of the experimental pots in heavy isotopes was observed only for ^{15}N, the ^{13}C data were not further analyzed. ^{15}N atom percent excess was calculated by subtracting the mean atom percent of corresponding control sample from each labelled sample value. Linear models were used to test the effect of time (days after labelling), plant part (shoot vs. root), and their interaction on the ^{15}N atom percent excess of the labelled pot samples of host and parasite separately. All statistical analyses were conducted in R, version 3.0.1 (R Core Team 2013).

RESULTS AND DISCUSSION

All samples from labelled pots were substantially enriched in ^{15}N compared to the controls and the ^{15}N isotopic composition of labelled and control pots did not overlap (fig. 2, electronic appendix 1). The samples from labelled pots largely varied in ^{15}N atom percent, while control pot samples showed almost no variation (electronic appendix 1). The parts of host leaves where the label had been applied were highly ^{15}N enriched 3 and 7 d after labelling when compared with non-labelled parts of host leaves, but this difference decreased after 14 d (electronic appendix 1). Similar ^{15}N values were found for the host and hemiparasite biomass already 3 d after labelling, but the enrichment in ^{15}N of *Rhinanthus* attached to a labelled host significantly increased in time ($F_{1, 30} = 5.64$, $p = 0.024$; table 1, fig. 2). The ^{15}N enrichment further increased between 3 and 7 d after labelling, but not between 7 and 14 d, suggesting that the major transfer of the label to the hemiparasite occurred during the week after host labelling (fig. 2). We found a similar pattern for the host biomass, but

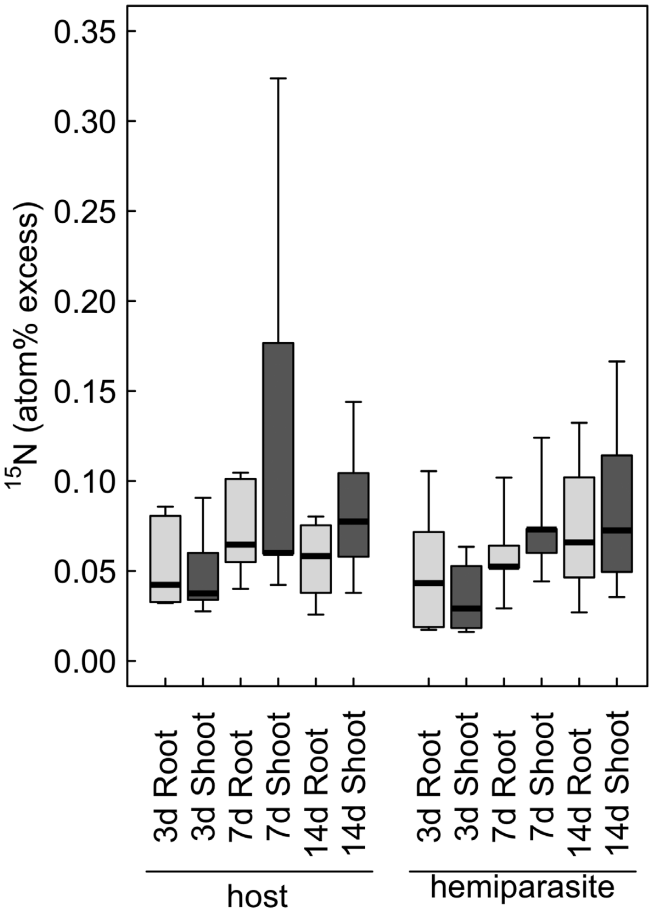

Figure 2 – Distribution of ^{15}N atom percent excess in roots and unlabelled sections of shoots of the hemiparasite, *Rhinanthus major*, and the host, *Triticum aestivum*, harvested 3, 7, and 14 d after host labelling. Medians, quartiles, and ranges are displayed. $n = 7$ for labelled samples collected on day 14, $n = 5$ for other labelled samples, and $n = 3$ for control samples.

its increase in time was not significant (table 1) which was probably caused by great variation in ^{15}N of host shoots 7 d after labelling (fig. 2). These results indicate a gradual translocation of the label into the host tissue and its immediate transfer into the hemiparasite via the root connections. Consequently, the harvesting period should be shifted to earlier dates, e.g. 1 to 7 d instead of 3 to 14 d, in order to examine the dynamics of the label movement in the host-hemiparasite association in more detail. However, an experimental period of 7 d seems largely sufficient for field studies where the major transfer of the label is of interest.

None of the other tested effects comprising hemiparasite plant material and its interaction with time significantly differed in ^{15}N atom percent (table 1). Moreover, ^{15}N atom percent of the host was not significantly affected by any of the tested predictors (table 1). This is in contrast with other studies reporting lower isotopic enrichment in roots due to the preferential storage of absorbed N in shoots (Below et al. 1985, Ledgard et al. 1985, McNeill et al. 1997, Schmidt & Scrimgeour 2001, Putz et al. 2011). The hemiparasite may alter this relationship in host species by supporting the preferential translocation of absorbed tracer to host roots, from which it is acquired by the hemiparasite resulting in no significant differences in the tracer between host roots and shoots. However, a comparison with non-infected hosts would be needed to confirm such a possibility.

In contrast to ^{15}N, the samples of labelled and control pots displayed very small differences in ^{13}C atom percent (appendix 2). Isotope labelling had a significant effect on ^{13}C composition of the host and hemiparasite tissues 3 d after labelling ($F_{1, 13} = 10.61$, $p = 0.006$; $F_{1, 13} = 24.41$, $p = 0.0003$, respectively). This initial enrichment diminished already 7d after labelling which was clearly caused by the dilution of the labelled carbon by newly produced assimilates. Additionally, roots of the hemiparasite had significantly higher ^{13}C atom percent than its shoots ($F_{1,13} = 109.6$, $p < 0.0001$). Despite being statistically significant, the absolute size of the differences was too small to be interpreted or further discussed. However, the shift in ^{13}C composition of the hemiparasite following host labelling presents a qualitative evidence on the uptake of host-derived carbon by root-hemiparasitic plants. As such it complements the previous studies based on radioisotope tracing (e.g. Govier et al. 1967), natural abundance of carbon stable isotope (e.g. Press et al. 1987, Těšitel et al. 2010), and composition analyses of simultaneously collected host and hemiparasite xylem sap (e.g. Seel & Jeschke 1999).

The lower enrichment of plants in ^{13}C than in ^{15}N was also found in other studies using leaf-brushing or spraying labelling with $^{15}N^{13}C$-urea solution (Schmidt & Scrimgeour 2001, Putz et al. 2011). According to Putz et al. (2011), it might be a consequence of the atomic structure of urea containing two atoms of N per one atom of C, and it might result from the loss of ^{13}C through respiration. Another reason for the lower enrichment in ^{13}C might be the N over-supply of the plant decreasing carbohydrate accumulation. We can exclude an exchange of ^{13}C between labelled and unlabelled plants by photorespiration and photosynthesis, as we did not detect this in a previous experiment (Těšitel et al. 2010).

Therefore, a single leaf-brushing labelling by a $^{15}N^{13}C$-urea provided sufficiently ^{15}N-labelled plant material. A single foliar application of ^{15}N-urea was also recently validated as a new method of studying seed dispersal and seedling recruitment (Castellano & Gorchov 2013). By contrast, the single brushing was insufficient for C labelling. The application of more concentrated $^{15}N^{13}C$-urea solution or repeated labelling might provide plants that are sufficiently enriched in both stable isotopes. That might, however, affect the plants by providing a significant N-supply. Repeated labelling by a low-concentrated urea solution would be more appropriate, as the application of more concentrated urea (> 5 g dm^{-3}) was shown to cause an N over-supply or leaf burning in crop plants (Hinsvark et al. 1953, Bremner 1995).

Experimental studies on hemiparasites frequently used ^{15}N isotope tracers to elucidate the host-hemiparasite nutrient translocation (Pageau et al. 2003, Cameron & Seel 2007) or the functional role of hemiparasites in ecosystems (Ameloot et al. 2008, Demey et al. 2013, 2014). However, urea/doubly-labelled urea leaf-feeding has never been employed as labelling method in these studies. To trace the resource transfer to the hemiparasite, Pageau et al. (2003) and Cameron & Seel (2007) subjected roots of the host species to a $K^{15}NO_3$ labelling solution. Although the method provided evidence about a non-specific transfer of nutrients through transpiration stream of *Striga* (Pageau et al. 2003) and high effectiveness of resistance mechanisms of two forb species (Cameron & Seel 2007), it required a highly sophisticated pot design, not applicable to field tracing experiments. In contrast, the field studies by Ameloot et al. (2008) and Demey et al. (2013) used spraying to apply ^{15}N labelling solution onto their experimental plots. Although this method is simple and provided a large amount of data on N turnover in the experimental communities, it is largely unspecific and cannot be used to study the individual host-hemiparasite interaction.

The leaf-immersion method might also be applied to examine nitrogen flows at the hemiparasite-host interface, although it seems to be not so simple and easy to do compared to leaf brushing. Similarly to other shoot-labelling techniques, even leaf-immersion can introduce some artifacts leading to over- or underestimation of transferred nitrogen (Pirhofer-Walzl et al. 2012, Chalk et al. 2014). For example, the direct leakage of applied label or transfer of the absorbed label to the soil result in the overestimation of transferred nitrogen (McNeill et al. 1997, Khan et al. 2002, Gylfadóttir et al. 2007). Thus caution must be taken when interpreting the results provided by shoot labelling.

Our results confirmed the applicability of single foliar brushing by $^{15}N^{13}C$-urea (of ^{15}N-urea) in tracing nitrogen flow between the host and root hemiparasite. Repeated labelling by low-concentrated urea would probably be necessary to track carbon flow in the host-hemiparasite association. The main advantage of the foliar brushing method is its simplicity on the one hand and specificity on the other hand. Thus, leaf brushing by ^{15}N-urea can be widely applied in both greenhouse and field experiments in order to examine the nitrogen flows between root hemiparasites and their various host species. If applied in the field, rainy conditions should be definitely avoided to prevent direct root uptake of the label. Using doubly labelled urea for monitoring both ni-

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trogen and carbon would, however, require further optimization of the labelling protocol.

SUPPLEMENTARY DATA

Supplementary data are available in pdf at *Plant Ecology and Evolution*, Supplementary Data Site (http://www.ingenta-connect.com/content/botbel/plecevo/supp-data), and consist of: (1) ^{15}N atom percent in shoots and roots of the hemiparasite and host harvested 3, 7, and 14 d after host labelling by $^{15}N^{13}C$-urea in labelled and control pots; and (2) ^{13}C atom percent in shoots and roots of the hemiparasite and host harvested 3, 7, and 14 d after host labelling by $^{15}N^{13}C$-urea in labelled and control pots.

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Chapter 3

plant biology

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RESEARCH PAPER

A hemiparasite in the forest understorey: photosynthetic performance and carbon balance of *Melampyrum pratense*

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ABSTRACT

- *Melampyrum pratense* is an annual root-hemiparasitic plant growing mostly in forest understorey, an environment with unstable light conditions. While photosynthetic responses of autotrophic plants to variable light conditions are in general well understood, light responses of root hemiparasites have not been investigated.
- We carried out gas exchange measurements (light response and photosynthetic induction curves) to assess the photosynthetic performance of *M. pratense* in spring and summer. These data and recorded light dynamics data were subsequently used to model carbon balance of the hemiparasite throughout the entire growth season.
- Summer leaves had significantly lower rates of saturated photosynthesis and dark respiration than spring leaves, a pattern expected to reflect the difference between sun- and shade-adapted leaves. However, even the summer leaves of the hemiparasite exhibited a higher rate of light-saturated photosynthesis than reported in non-parasitic understorey herbs. This is likely related to its annual life history, rare among other understorey herbs. The carbon balance model considering photosynthetic induction still indicated insufficient autotrophic carbon gain for seed production in the summer months due to limited light availability and substantial carbon loss through dark respiration.
- The results point to potentially high importance of heterotrophic carbon acquisition in *M. pratense*, which could be of at least comparable importance as in other mixotrophic plants growing in forests – mistletoes and partial mycoheterotrophs. It is remarkable that despite apparent evolutionary pressure towards improved carbon acquisition from the host, *M.* pratense retains efficient photosynthesis and high transpiration rate, the ecophysiological traits typical of related root hemiparasites in the Orobanchaceae.

INTRODUCTION

The forest understorey is an environment characterised by constantly changing light conditions, with long periods of low light intensity interrupted by short flashes of high irradiance, sunflecks (Chazdon 1988; Pearcy 1990; Chazdon & Pearcy 1991). Sunfleck dynamics, including their duration, intensity, quantity and position, varies with weather on short time scales and with the stage of canopy closure and solar angle on longer time scales (Lambert 1970; Tang *et al.* 1988; Pearcy 1990; Chazdon & Pearcy 1991; Way & Pearcy 2012). This variation affects the amount of light available for understorey herbs at the forest floor. While delayed canopy development allows a considerable portion of light to reach the forest floor in spring, irradiance decreases with leaf expansion (Hutchison & Matt 1977), which highlights the importance of sunflecks in summer. Sunflecks have been repeatedly shown to significantly contribute to total carbon gained through photosynthesis in understorey plants (Chazdon 1988; Pfitsch & Pearcy 1989; Pearcy 1990; Chazdon & Pearcy 1991; Pearcy & Pfitsch 1995).

The efficiency of sunfleck utilisation by an understorey plants depends on induction state of the leaves (*e.g.* Chazdon & Pearcy 1986; Chazdon 1988; Barradas & Jones 1996). The induction state reflects the readiness of light-induced processes such as photosynthetic enzyme activation and stomatal opening (Kirschbaum & Pearcy 1988). The rate of photosynthesis in a plant exposed to a sunfleck is limited through biochemical processes such as Rubisco activation for a short initial period; later it becomes limited by stomatal opening (Kirschbaum *et al.* 1988; Sassenrath-Cole & Pearcy 1992; Way & Pearcy 2012; but see Roden & Pearcy 1993). The light-induced state with maximum photosynthesis is reached after a certain sunfleck duration, depending on species (Chazdon 1988). After a sunfleck, photosynthesis decreases and the plant gradually looses its induced state in low light (Chazdon 1988).

While the ecophysiology of autotrophic plants living in sunfleck environments is a well-established topic (*e.g.* Chazdon 1988; Hull 2002; Montgomery & Givnish 2008; Way & Pearcy 2012), no study has hitherto investigated this aspect in root-hemiparasitic plants. Root hemiparasites are typical representatives of a mixotrophic strategy (Selosse *et al.* 2017); *i.e.* they acquire carbon both autotrophically from photosynthesis, and also heterotrophically from the host roots *via* specialised organs called haustoria. Most root hemiparasites occur in open

vegetation (grasslands, alpine or semiarid habitats), while closed-canopy forests mostly contain more specialised parasitic plant forms – root holoparasites and mistletoes (Těšitel 2016). Several species of the root-hemiparasitic genus *Melampyrum* (Orobanchaceae), however, occur in holarctic temperate forests (De-Yuan 1983; Heide-Jørgensen 2008) and thus represent a remarkable exception to the habitat requirements of parasitic plants. All *Melampyrum* species are annual herbs that acquire a significant part of their resources parasitically through a root connection with multiple host plants (Hodgson 1973; Press 1989; Dalrymple 2007; Heide-Jørgensen 2008; Těšitel *et al.* 2010). Similar to many other root hemiparasites, they exhibit elevated transpiration rates and stomatal conductivity, which facilitates resource uptake from the host species (Ehleringer & Marshall 1995; Lechowski 1996).

Melampyrum pratense, the model species used in our study, is a widespread herb occurring across western Eurasia (Meusel *et al.* 1978). The species is one of the most common root hemiparasites on the continent and grows mostly in forests (Těšitel *et al.* 2015). It is considered a generalist in term of host species that might prefer woody and shrub hosts (ter Borg 1985), but host range and the functionality of such haustorial connections have not yet been studied. Its life cycle starts by hypocotyl germination in autumn, continues with epicotyl germination and cotyledon emergence in spring, and ends with flowering and seed production in summer (Masselink 1980; Dalrymple 2007). In forest environments, the major part of a hemiparasite's life cycle takes place under a closed canopy. The annual nature means that plants have to complete their whole life cycle within a single season. This ecological constraint also makes it possible to monitor the autotrophic energy balance of *M. pratense* individuals across their whole lifespan.

We conducted gas exchange measurements in spring and summer leaves of *M. pratense* to assess its photosynthetic performance in the forest understorey. Light conditions on the forest floor were monitored throughout the entire growth season to characterise changes, including sunfleck dynamics. Subsequently, we used these data to develop models estimating the autotrophic carbon balance of the hemiparasite in the forest understorey. We aimed to answer the following questions: (i) Do spring and summer leaves differ in their physiological characteristics relevant for photosynthesis; (ii) Is *M. pratense* able to utilise sunflecks efficiently; and (iii) Does photosynthesis contribute sufficient autotrophic carbon for *M. pratense* growth and reproduction in the forest understorey?

MATERIAL AND METHODS

Study site

Photosynthetic responses of *M. pratense* were examined in its natural environment in an acidophilous oak forest near Mokré, České Budějovice, Czech Republic (48°57′40″ N, 14°24′28″ E; 430 m a.s.l.) in spring and summer 2013.

Light dynamics

Light dynamics on the forest floor were monitored with a Minikin datalogger (EMS, Brno, Czech Republic). Photosynthetic photon flux density (PPFD, $\mu mol \cdot m^{-2} \cdot s^{-1}$) was recorded every 2 min from 1 May to 29 August.

Physiological measurements

Physiological measurements were performed with a Li-6400 portable photosynthesis system (Li-Cor, Lincoln, NB, USA) in spring (5–6 May; Figure S1a) and summer (23–24 July; Figure S1b) 2013. The youngest fully expanded, intact leaf was enclosed in a 6-cm^2 leaf chamber at ambient temperature (20–25 °C), ambient relative air humidity (60–75%) and controlled CO_2 concentration (400 $\mu mol \cdot mol^{-1}$). Different individual plants were measured in each season as the plants are frequently damaged by herbivores in summer (Průšová *et al.* 2013).

Light responses of five individual plants in each season were measured at consecutively decreasing PPFD of 1500, 1000, 800, 500, 250, 100, 50, 0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ using the LI-6400-02 LED light source. Gas exchange was recorded at each PPFD after reaching a steady state (60–120 s). Before initiation, measured leaves were stabilised at a saturating irradiance of 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for at least 10 min. In addition, ten zero PPFD values per plant were logged at the end of measurements to increase precision of dark respiration measurement.

Photosynthetic induction was examined in five and seven individual plants in spring and summer, respectively. Plants were covered with a cardboard box for at least 1 h prior to measurements, acclimated at low light (10–20 $\mu mol \cdot m^{-2} \cdot s^{-1}$) corresponding to non-sunfleck radiation in the forest understorey. Leaves were subsequently placed in the chamber and rate of gas exchange logged at 10-s intervals at 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (1 min) and after an increase of PPFD to 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (until steady state; approx. 30 min). Transpiration rate was also recorded as a part of light response and photosynthesis measurements.

Data analysis

Light responses were fitted to non-linear regression models using the following equation:

$$A = (\varphi I + A_{max} - \{(\varphi I + A_{max})^2 - 4\theta\varphi I A_{max}\}^{0.5}))/2\theta - R_d$$

where A = rate of photosynthesis, φ = apparent quantum yield, I = irradiance, A_{max} = maximum photosynthesis, θ = curvature factor, R_d = dark respiration.

Dark and light-saturated transpiration rates were measured as a part of light responses as were low light and light-saturated transpiration rates as a part of photosynthetic induction to test for inter-seasonal differences using linear mixed-effects models, with plant identity as a random effect.

Induction data were fitted using generalised Michaelis-Menten kinetics:

$$A = A_{max} t^d / (t^d + t^d_{1/2})$$

where A = rate of photosynthesis, A_{max} = maximum photosynthesis, t = time, d = exponent determining curve shape, $t_{1/2}$ = time needed to reach ½ A_{max}.

Fitted light response and induction curve parameters were tested for seasonal differences using linear models. Overall

model significance was tested with *F*-tests (Table 1). Direction of significant effects is reported by displaying results of *t*-tests of corresponding regression coefficient. To better illustrate differences in induction parameters, mean induction curves were normalised by dividing actual A by corresponding A_{max}. All analyses were conducted in R, version 3.1.3 (R Core Team 2013).

Carbon balance modelling

The carbon balance of the hemiparasite was modelled throughout the growth season by calculating CO_2 balance per unit leaf area at each measured PPFD using the measured photosynthetic responses. PPFD ≤ 40 μmol·m^{-2}·s^{-1} was considered non-sunfleck conditions; *i.e.* carbon balance was estimated from light response only; PPFD > 40 μmol·m^{-2}·s^{-1} was considered as sunflecks, *i.e.* carbon balance estimated from the product of light response and normalised induction curves. This means that under sunfleck conditions, the estimated rate of photosynthesis corresponding to a given PPFD was further multiplied by a value between 0 and 1 derived from the induction curve and time since sunfleck start. As a result, the estimate was reduced or not affected in periods short or long sunfleck start time, respectively. This allowed carbon balance per unit leaf area to be estimated every 2 min throughout the entire growth season. These data were finally multiplied by 120, assuming stable PPFD during 2-min intervals, and summed to obtain daily values. As a reference, we also constructed a carbon balance model based on light response only, ignoring the inductive state of a modelled plant (hereafter steady-state model).

The models were developed according to season: mean spring and summer parameters were used for the periods 1–6 May and 23 July–29 August, respectively. Within the period from 7 May to 22 July, parameters were estimated for each day using weighted averaging of spring and summer parameters and temporal proximity to the two gas exchange measurement dates.

Individual plant carbon balance was estimated from the carbon balance per unit leaf area. We used biomass growth data on *M. pratense* from Průšová *et al.* (2013), collected at the same site, to model exponential leaf area growth throughout the growing season. Initial leaf area was set to 15 cm^2 based on our observations of young plants. This model was validated by comparing predicted leaf area and the product of maximum vegetative dry mass of an average plant individual from Průšová *et al.* (2013) and known specific leaf area (320 cm^2·g^{-1}) obtained from the LEDA trait database (Kleyer *et al.* 2008). We assumed leaf mass represents 2/3 of total vegetative biomass.

Our models represent simplifications of a possibly very complex reality. The precision of these models might have been increased by incorporation of induction responses of *Melampyrum* to dynamic sunflecks and shorter interval of PPFD measurements. However, the effect of these improvements would be relatively low compared with current models taking into account only moderate differences between outcomes of the models, considering and ignoring photosynthetic induction. Therefore, we assume that the current photosynthetic induction model for *M. pratense* carbon balance is a reasonable approximation of reality.

RESULTS

Light dynamics

Light conditions on the forest floor varied with time of day and time of year because of progressive canopy closure (Figs 1 and 2). The understorey received the largest amount of light in May, mainly irradiance values >40 μmol·m^{-2}· s^{-1} (Figs 1 and 2). The average daily PPFD integral was lower in summer (June–August) than in May. This was caused by a strong decrease in the sunfleck integral (>40 μmol·m^{-2}·s^{-1}), while low-irradiance integrals (≤40 μmol·m^{-2}·s^{-1}) were slightly higher compared with May (Fig. 1). PPFD in June was lower than in July probably due to a higher number of cloudy days in June. The total daily PFFD in the understorey was between 1.5 and 3.87 mol·m^{-2}·day^{-1}.

Physiological parameters

Spring and summer leaves of *M. pratense* responded differently to simulated irradiance. Summer leaves had significantly lower A_{max} and R_d ($t_8 = -5.58$, $P = 0.001$; $t_8 = -2.50$, $P = 0.037$; Fig. 3, Table 1). There were no further differences in other parameters (φ and θ) defining the shape of the light curves (Table 1). The same difference in A_{max} between spring and summer leaves was revealed in induction curves ($t_{10} = -3.05$, $P = 0.012$; Fig. 4). The parameter d determining the shape of induction curves was significantly lower in summer ($t_{10} = -3.00$, $P = 0.013$), which indicates faster photosynthetic induction in spring leaves. Photosynthetic induction from

Table 1. Parameters of light response and induction curves (mean ± 1SE) and summaries of linear models testing their seasonal differences.

	light curve parameters				induction curve parameters		
	A_{max}	R_d	φ(phi)	θ(theta)	A_{max}	d	$t_{1/2}$
Spring	19.71 (±1.35)	1.90 (±0.22)	0.081 (±0.010)	0.62 (±0.04)	16.32 (±1.81)	1.10 (±0.10)	61 (±5.7)
Summer	11.8 (±0.43)	1.20 (±0.17)	0.079 (±0.004)	0.69 (±0.06)	11.37 (±0.53)	0.82 (±0.04)	111 (±24.0)
R^2	0.77	0.37			0.43	0.42	
F	31.10	6.23			9.30	8.99	
df	1,8	1,8	No significant difference		1,10	1,10	No significant difference
P	0.00052	0.03721			0.01228	0.01337	

A_{max}, maximum photosynthesis (μmol·m^{-2}·s^{-1}); R_d, dark respiration during photosynthesis (μmol·m^{-2}·s^{-1}); φ, apparent quantum yield; θ, curvature factor; $t_{1/2}$, time needed to reach 1/2 of A_{max} (s); d, exponent determining curve shape.

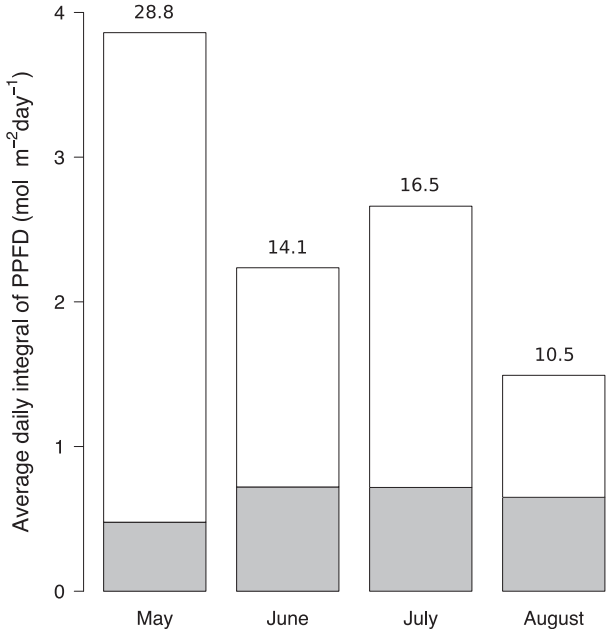

Fig 1. Average daily integrals of irradiance (PPFD) recorded in the forest understorey for *M. pratense* from 1 May to 29 August 2013. Integrals of irradiance values ≤40 and >40 μmol·m^{-2}·s^{-1} are displayed in grey and white bars, respectively. Numbers above columns corresponds to time period (in %) with PPFD > 40 μmol·m^{-2}·s^{-1}.

diffuse light was reached in <12 min in spring leaves and <20 min in summer leaves (Fig. 4). No significant difference was identified in $t_{1/2}$ (Fig. 5, Table 1). Spring data showed more variation between individual plants compared with summer measurements (Figs 3 and 4, S2, S3). Light-saturated transpiration rates were significantly higher than dark and low-light transpiration rates recorded as a part of light responses and photosynthetic induction ($t_{85} = 19.43$, $P < 10^{-4}$; $t_{1413} = 52.96$, $P < 10^{-4}$; Fig. 6, Table 2). Transpiration rates differed significantly between seasons only for induction data (Table 2).Transpiration rates were slightly higher in spring plants than summer plants ($t_{10} = 2.04$, $P = 0.069$). Transpiration was significantly affected by the interaction between season and light (Table 2).

Carbon balance

The modelled daily carbon balance of the hemiparasite fluctuated highly throughout the growing season (Fig. 7). Values were positive in the first half of May and the middle of July, but were negative in late May, June and August (Fig. 7). The overall carbon balance modelled per unit leaf area throughout the entire growth season was positive both when ignoring (2000 mmol·m^{-2}) and considering (681 mmol·m^{-2}) plant inductive state. The overall carbon balance modelled per individual plant was positive (9.2 mmol) when the inductive state was not considered, but was negative (−8.5 mmol) when the inductive state was considered. The average daily carbon balance per unit leaf area for models without and with induction was 16.4 and 5.6 mmol·m^{-2}·day^{-1}, respectively. Monthly autotrophic carbon balance per unit leaf area decreased throughout the growing season (except June), in agreement with recorded PPFD data. Calculated per day, it was positive in May and July (54.4 and 26.1 mmol·m^{-2}·day^{-1} without induction; 49.5 and 8.4 mmol·m^{-2}·day^{-1} with induction) and negative in June and August (−7.2 and −9.3 mmol·m^{-2}·day^{-1} without induction; −17.0 and −20.1 mmol·m^{-2}·day^{-1} with induction). The average daily autotrophic carbon balance per individual plant was also positive in May and July (0.067 and 0.412 mmol·day^{-1} without induction; 0.054 and 0.124 mmol·day^{-1} with induction) and negative in June and August (−0.028 and −0.160 mmol·day^{-1} without induction; −0.09 and −0.38 mmol·day^{-1} with induction).

DISCUSSION

Melampyrum pratense exhibits efficient photosynthesis in the forest understorey but its autotrophic carbon gain is insufficient, especially in summer

Light response curves showed inter-seasonal acclimation in leaves of *M. pratense* to changing light conditions in the forest understorey. Spring leaves acted as sun leaves with comparatively higher A_{max} and R_d than summer leaves adapted to more shade (Fig. 3, Table 1; Larcher 2003; Lambers *et al.* 2008). Nevertheless, summer leaves still had high values of A_{max} and R_d, which were not comparable with values for shade-acclimated plants. Shade-acclimated herbs from the forest understorey typically have *ca.* 50% of A_{max} measured in *M. pratense* (Hull

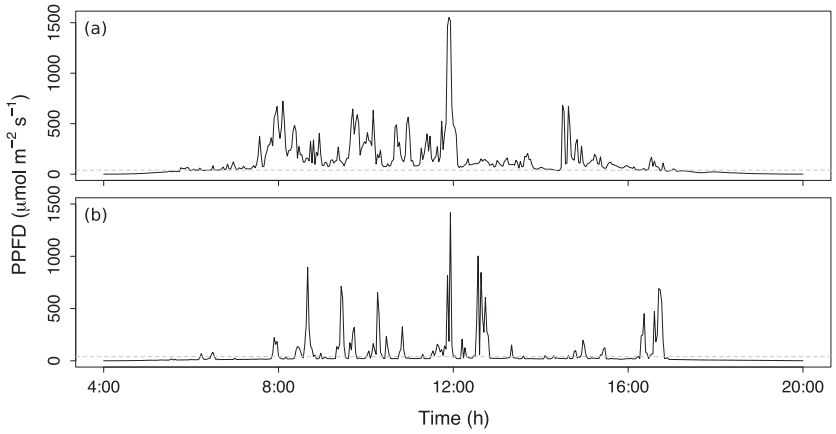

Fig 2. Light conditions (PPFD) recorded in the forest understorey for *M. pratense* on sunny days in spring (9 May 2013; a) and summer (17 July 2013; b). High peaks correspond to individual sunflecks. Irradiance of 40 μmol·m^{-2}·s^{-1} (considered the threshold between diffuse irradiance and a sunfleck start) is indicated by dashed lines.

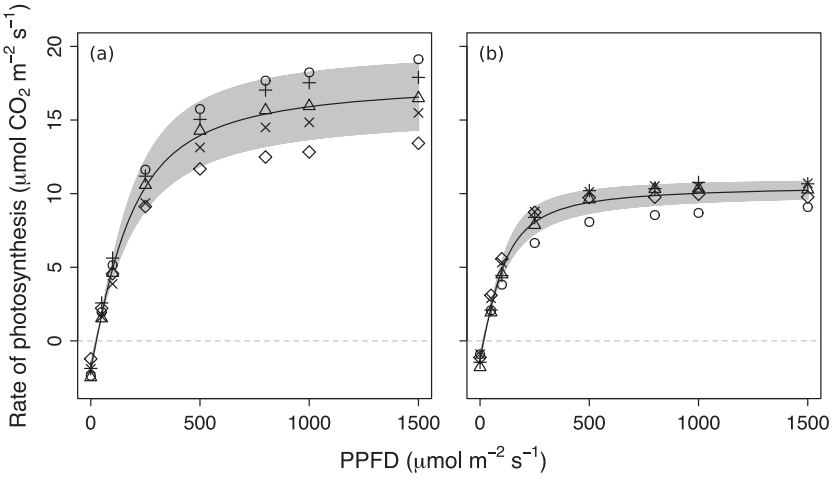

Fig 3. Photosynthetic response curves of *M. pratense* in spring (a) and summer (b). Five individual plants (replicates) for each season are represented. Fitted mean nonlinear regression curves and 95% confidence intervals are indicated by solid lines and shaded areas, respectively.

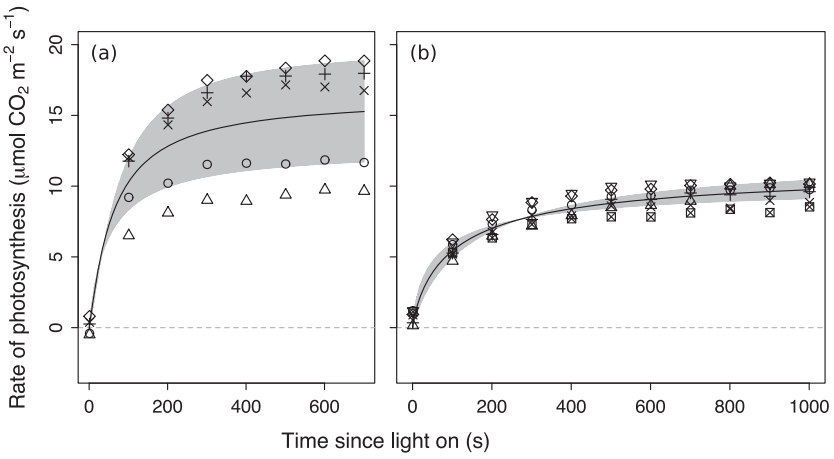

Fig 4. Induction responses of *M. pratense* to simulated sunfleck (1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in spring (a) and summer (b). Individual plants (n = 5 and n = 7) are represented by symbols. Fitted mean generalised Michaelis-Menten curves and 95% confidence intervals are indicated by solid lines and shaded areas, respectively. Time intervals needed for photosynthesis stabilisation are displayed. Every tenth data point from the time series with 10-s intervals is depicted.

2002). This might result from annual life history of *M. pratense*, in contrast with most perennial understorey herbs and forcing the hemiparasite to complete its life cycle under a closed canopy in a single growing season and with no resources available from previous years (except seed reserves).

The rate of photosynthetic induction from diffuse light was average or slightly faster than times required to reach 90% A_{max} in other dark-measured understorey plant species (Chazdon & Pearcy 1986; Roden & Pearcy 1993; Allen & Pearcy 2000; Rijkers *et al.* 2000; Leakey *et al.* 2005). Faster induction rates in spring compared to summer leaves (Figs 4 and 5) might be explained by higher transpiration rates due to higher stomatal conductance. Higher rates of transpiration in low light and faster stabilisation of photosynthesis rate in spring leaves indicate that there was no delay caused by stomata opening compared with summer leaves. However, values of stomatal conductance cannot be calculated from transpiration in *M. pratense* and several related root hemiparasites from Orobanchaceae as they have specialised hydathode trichomes that secrete water from leaves (Fedorowicz 1915; Govier *et al.* 1968; Weber 1973; Kubát & Weber 1987; Těšitel 2011; Těšitel & Tesařová 2013; Světlíková *et al.* 2015). Measured transpiration rates thus reflect not only stomatal conductance, but also evaporation of guttation water from trichomes. Therefore, this inter-seasonal difference in induction rate, which is opposite to that in non-parasitic plants (*e.g.* Chazdon & Pearcy 1986; Valladares *et al.* 1997; but see Naumburg & Ellsworth 2000; Rijkers *et al.* 2000; Tausz *et al.* 2005), may be an effect of elevated spring transpiration rather than an adaptation to light availability.

The average daily carbon balance modelled in this study for an entire growing season (16.4 $mmol \cdot m^{-2} \cdot day^{-1}$) corresponds to values predicted in other steady-state models for various understorey plants (*e.g.* Chazdon 1986; Pfitsch & Pearcy 1989; Tang *et al.* 1999; Beaudet *et al.* 2000). Compared with the steady-state model, the modelled carbon balance markedly decreased when the induction state was considered (Fig. 7). Overestimation of carbon uptake in steady-state models and importance of taking the induction state into account have already been emphasised by several authors (Gross *et al.* 1991; Naumburg & Ellsworth 2002; Schulte *et al.* 2003). The negative overall carbon balance per plant suggests that there was not enough autotrophically gained carbon to cover carbon demand, especially in June and August. Such shortage of autotrophically gained carbon might be due to the high rate of dark respiration reported for other hemiparasites (Press *et al.* 1988; Lechowski 1996) and potentially magnified by energy-demanding water excretion from hydathode trichomes, which are most active at night (Světlíková *et al.* 2015).

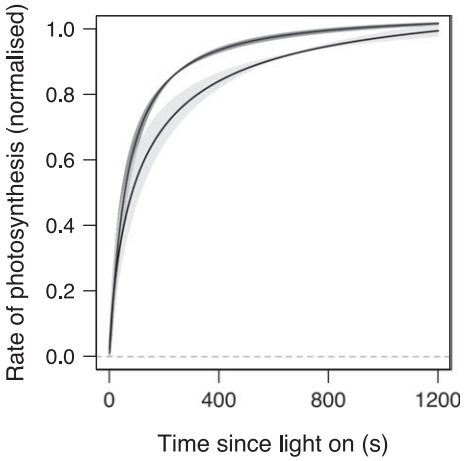

Fig 5. Fitted curves of normalised induction responses (Fig. 4) in spring (dark grey) and summer (grey) leaves of *M. pratense* to simulated sunfleck conditions (1500 μmol·m^{-2}·s^{-1}). Solid lines and shaded areas represent means and 95% confidence intervals.

Melampyrum pratense is extraordinary compared to other mixotrophs from the forest understorey

Compared with other mixotrophic plants from the forest understorey, such as hemiparasitic mistletoes and partial mycoheterotrophs (Selosse & Roy 2009), which have limited photosynthetic capacity (Strong 2000; Julou *et al.* 2005; Serafini *et al.* 2007), *M. pratense* still retains a high photosynthesis rate that is unusual for plants growing in habitats under a closed tree canopy. In spite of this efficiency, the autotrophic carbon gain of *Melampyrum* seems insufficient according to the carbon balance models and is therefore likely to rely on uptake of host-derived or heterotrophic carbon. Heterotrophic carbon was shown to play an important role in the carbon budget of other hemiparasites. Up to 80% of overall carbon in root hemiparasites (Press *et al.* 1987; Graves *et al.* 1990; Ducharme & Ehleringer 1996; Tennakoon & Pate 1996; Těšitel *et al.* 2010, 2011) and up to 87% of overall carbon in mistletoes (Marshall & Ehleringer 1990; Schulze *et al.* 1991; Richter *et al.* 1995; Wang *et al.* 2008) may be of heterotrophic origin. Moreover, significant amounts of heterotrophic carbon have also been repeatedly documented in partial mycoheterotrophic plants, which acquire organic carbon heterotrophically from fungi in addition to their own photosynthesis (Selosse & Roy 2009). Several species of understorey green orchids (Gebauer & Meyer 2003; Julou *et al.* 2005; Abadie *et al.* 2006; Tedersoo *et al.* 2007) and non-orchid pyroloids (Tedersoo *et al.* 2007; Zimmer *et al.* 2007; Hynson *et al.* 2012; Johansson *et al.* 2015) receive up to 85% of their total carbon from mycorrhizal fungi. Although the importance of heterotrophic carbon and its proportion in the biomass of *M. pratense* still remain to be determined, we propose that it might be as high as in other hemiparasites and partial mycoheterotrophs.

Table 2. Summary of linear models testing the effects of season, light, and their interaction on transpiration rates recorded as a part of light response and photosynthetic induction measurements. The effect of light tested the differences between irradiance (PPFD) of 0 and 1500 μmol·m^{-2}·s^{-1} for light responses and between 20 and 1500 μmol·m^{-2}·s^{-1} for photosynthetic induction.

	light responses			photosynthetic induction		
Effect	df	*F*	*P*	df	*F*	*P*
Season	1,8	0.25	0.6338	1,10	15.36	0.0029
Light	1,85	458.64	<0.0001	1,1413	2701.73	<0.0001
Season × Light	1,85	38.16	<0.0001	1,1413	533.40	<0.0001

Heterotrophic carbon might play an important role for successful seed production of *M. pratense* and in the evolution towards holoparasitism

Our results suggest that the dependence of *M. pratense* on heterotrophic carbon gain changes during the growing season with development stage and light availability on the forest floor. High light availability on the forest floor in spring enables seedlings to obtain enough autotrophic carbon for fast vegetative growth. This is in contrast with seedlings of root hemiparasites from open habitats (grasslands), which may experience strong

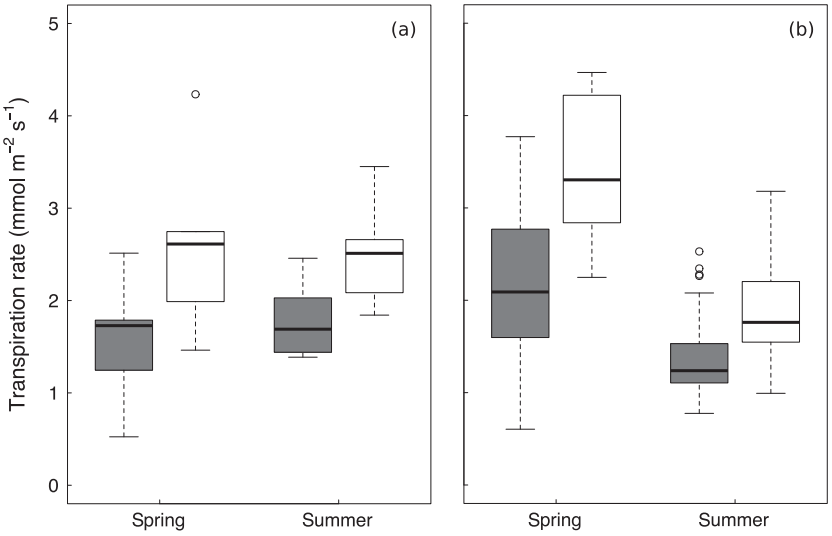

Fig 6. Transpiration rates of *M. pratense* in spring and summer measured as a part of light responses (a) and photosynthetic induction (b). Grey represents dark (a; 0 μmol·m^{-2}·s^{-1}) or low light (b; 10–20 μmol·m^{-2}·s^{-1}) and white represents saturated light conditions (1500 μmol·m^{-2}·s^{-1}). Medians, quartiles and ranges are displayed for measurements on five (a) and seven (b) plants per treatment. Transpiration also includes evaporation of guttation water from hydathode trichomes.

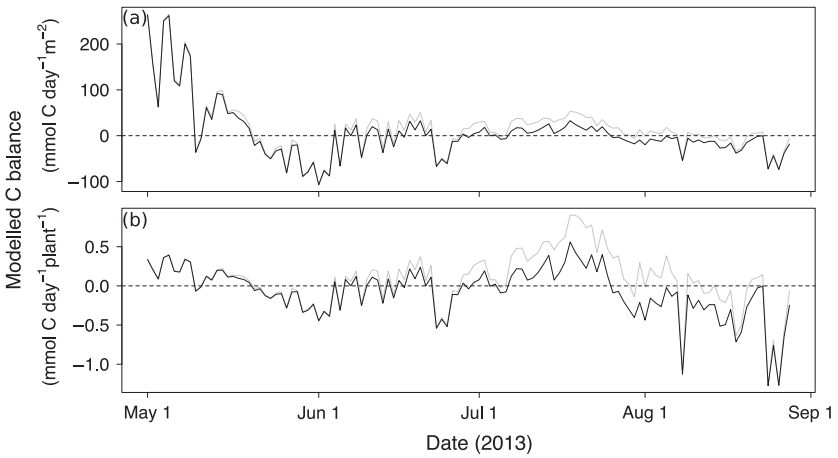

Fig 7. Modelled carbon balance of *M. pratense* in spring and summer 2013 expressed (a) per m^2 leaf area, (b) per mean individual plant. Modelled carbon balance is based on measurements of light conditions on the forest floor and photosynthetic responses. Models both ignoring (grey) and considering (black) induction state are shown. Individual plant balance (b) was estimated on the basis of leaf area balance, growth data from Průšová *et al.* (2013), known SLA and exponential growth model of leaf area.

light competition and partly depend on heterotrophic carbon acquisition for survival in initial life stages (Těšitel *et al.* 2011). As the forest canopy closes, autotrophic carbon might not suffice and *M. pratense* probably becomes more dependent on host-derived carbon gain. Light availability determines the proportion of heterotrophic carbon in the biomass of root hemiparasites (Těšitel *et al.* 2011), green orchids (Preiss *et al.* 2010) and green pyroloids (Zimmer *et al.* 2007; Hynson *et al.* 2012). The shortage of autotrophic carbon in June might correspond to elevated mortality of *M. pratense* recorded in summer from the same population (Průšová *et al.* 2013). Furthermore, the shortage of autotrophic carbon gain in June and August revealed here might support our hypothesis of high dependence of *M. pratense* on heterotrophic carbon gain for the start of flowering at the end of June and, more importantly, for seed production in August.

Such a high dependence of generative reproduction on heterotrophic carbon could be expected to push the hemiparasite to improve acquisition of heterotrophic carbon and thus trigger evolution towards holoparasitism. It is remarkable that despite this evolutionary pressure, *M. pratense* still retains physiological characteristics typical of related root hemiparasites of Orobanchaceae growing in open habitats, *i.e.* own photosynthesis, high transpiration rates and stomatal conductivity that drive acquisition of heterotrophic carbon. This may be because *M. pratense* is a habitat generalist that not only occurs in forests but also in open habitats such as oligotrophic meadows, subalpine grasslands and peat bogs (Těšitel *et al.* 2015), where the autotrophic carbon balance would be much more positive due to higher light availability. In addition, a physiological mechanism allowing highly efficient organic carbon transfer from the host, such as a phloem connection, requires establishment of biochemical compatibility with the host (Irving & Cameron 2009). Evolution of this may be highly complicated and recent research has demonstrated that multiple horizontal gene transfer events might be required (Yang *et al.* 2016).

CONCLUSION

We demonstrated *M. pratense*, a root hemiparasite, has high rates of light-saturated photosynthesis that are uncommon in shade-acclimated herbs of the forest understorey. Such extraordinary physiology may be related to its annual life history and consequent need to gather resources for life cycle completion within a single season, in contrast to the majority of perennial species typical in this habitat. Despite its potential photosynthetic efficiency, the autotrophic carbon balance of *M. pratense* under a closed canopy is negative (or close to neutral at best), especially at the time when seeds are produced. This represents a strong, albeit indirect, indication that *M. pratense* must have access to abundant heterotrophic carbon. This would be expected to induce evolutionary pressure to improve heterotrophic carbon acquisition and consequently trigger evolution to holoparasitism, as in many other parasitic plants from the forest understorey (Těšitel 2016). However, *M. pratense* retains efficient photosynthesis. Further investigations of its biology (*e.g.* resolving the full carbon budget) may thus provide new insight into mechanisms involved in the transition from hemiparasitism (mixotrophy) to holoparasitism (heterotrophy).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. The study species, hemiparasitic *Melampyrum pratense* in spring (May 5, 2013; a) and summer (July 23, 2013; b).

Figure S2. Induction responses of spring *Melampyrum pratense* (n = 5) to simulated sunfleck (1500 μmol·m^{-2}·s^{-1}).

Figure S3. Induction responses of summer *Melampyrum pratense* (n = 7) to simulated sunfleck (1500 μmol·m^{-2}·s^{-1}).

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Chapter 4

Water-stress physiology of *Rhinanthus alectorolophus,* a root-hemiparasitic plant

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Abstract

Root-hemiparasitic plants of the genus *Rhinanthus* acquire resources through a water-wasting physiological strategy based on high transpiration rate mediated by the accumulation of osmotically active compounds and constantly open stomata. Interestingly, they were also documented to withstand moderate water stress which agrees with their common occurrence in rather dry habitats. Here, we focused on the water-stress physiology of *Rhinanthus alectorolophus* by examining gas exchange, water relations, stomatal density, and biomass production and its stable isotope composition in adult plants grown on wheat under two contrasting water treatments. The effect of water stress on the survival of *Rhinanthus* seedlings was also experimentally determined. Water shortage reduced seedling survival as well as the biomass production and gas exchange of adult hemiparasites. In spite of that drought-stressed and even wilted plants from both treatments still considerably photosynthesized and transpired. Strikingly, low-irrigated plants exhibited elevated photosynthetic rate compared with high-irrigated plants of the same water status. This might relate to biochemical adjustments of these plants enhancing the resource uptake from the host. Moreover, low-irrigated plants did not acclimatize to water stress by lowering their osmotic potential, perhaps due to the capability to tolerate drought without such an adjustment, as their osmotic potential at full turgor was already low. Contrary to results of previous studies, hemiparasites seem to close their stomata in response to severe drought stress and this happens probably passively after turgor is lost in guard cells. The physiological traits of hemiparasites, namely the low osmotic potential associated with their parasitic lifestyle and the ability to withstand drought and recover from the wilting likely enable them to grow in dry habitats. However, the absence of osmotic adjustment of adults and sensitivity of seedlings to severe drought stress demonstrated here may result in a substantial decline of the hemiparasitic species with ongoing climate change.

Introduction

Plants rely on water for their structure, maintaining a positive pressure (turgor) against their cell walls [1]. Water shortage induces significant stress in plants; stomatal closure and turgor loss are accompanied by suppression of growth and certain physiological processes such as photosynthesis and transport of assimilates [1,2]. Plant water status is usually described by water potential, a measure of water availability in the system (Ψ; [1,2]). One component of Ψ is osmotic potential (Ψ_π), the water potential of a solution expressing the molar concentration of dissolved substances in the cell. The examination of water relations allows estimating a number of physiological parameters involved in plant adjustment to water stress. In general, plants adjust to water stress by either decreasing Ψ_π via accumulation of osmotically active compounds or increasing the elasticity of their cell walls. While the first strategy leads to the increase of turgor and facilitates water uptake from drier soil, the second strategy enables plants to store more water at full turgor, both of them provide plants with the ability to lose more water without losing turgor [1].

Autotrophic plants acquire water directly and exclusively from the surrounding environment but this is not the case of parasitic and hemiparasitic plants. Root hemiparasites acquire virtually all water and mineral nutrients from their host roots through haustorial connection to their vascular bundles [3,4]. In contrast, organic carbon is acquired partly autotrophically from own photosynthetic activity with the host contributing a variable fraction of organic carbon used by the hemiparasite [5,6]. This mixotrophic resource acquisition strategy [7] is highly efficient in *Rhinanthus* species from the Rhinanthoid clade of the *Orobanchaceae* family as it is based on an open direct xylem-to-xylem connection with hosts [8,9]. It was shown to be driven in particular by comparatively high day- and night- transpiration rates in hemiparasites [10–12], lowering thus their water potential to highly negative values and acting as a strong sink [13]. Similarly to all parasitic plants, *Rhinanthus* spp. accumulate osmotics such as sugar alcohols inside cells to maintain low water potential conditions [13,14], which further facilitates the resource flow through haustoria. Moreover, stomata of some hemiparasites are irresponsive to abscisic acid (ABA) and remain permanently open, even under severe water stress [12,15]. Stomatal transpiration and high content of osmotically active compounds are not the only means by which the solute flux is drawn into hemiparasite. Several species from the Rhinanthoid clade actively secrete excess water from hydathode trichomes present on the abaxial leaf sides [16–18] to make the resource acquisition from the host even more efficient.

The genus *Rhinanthus* comprises at least 25 annual species occurring in northern hemisphere [19]. Some of them are most commonly found and studied root-hemiparasites in Europe, colonizing grassland habitats of low to moderate productivity and water availability [19–22]. The performance of *Rhinanthus* spp. was demonstrated to depend on water availability in a non-trivial way. Depending on the ecological context, established *Rhinanthus* plants may be positively or negatively affected by decreased water availability [6,23]. This is rather surprising considering their water-wasting physiological strategy of resource acquisition based on high transpiration. However, experimental evidence [6,23] and occurrence of stable populations of *Rhinanthus* spp. in dry grasslands [22] indicates that they are able to withstand at least moderate water stress. Moreover, wilted *Rhinanthus* fully recovers from severe water stress within several hours after re-watering [24]. This points to the ability to tolerate water stress, even though their stomata do not close under

increased ABA concentration. Surprisingly, studies evaluating the water-stress physiology of root hemiparasites are missing.

Here, we examined water-stress physiology of flowering *Rhinanthus alectorolophus* (Scop.) Pollich using a manipulative experiment with two irrigation levels and monitoring gas exchange, water relations, stomatal density, and biomass production and its stable isotope composition. We also focused on seedling survival under water stress conditions. We hypothesized that i) the survival of hemiparasite seedlings is negatively affected by drought stress, ii) wilted plants of flowering hemiparasites still considerably photosynthesize and transpire, iii) low-irrigated plants osmotically adjust to long-term water deficiency and therefore iv) their photosynthesis and transpiration are suppressed at more negative Ψ_π than in high-irrigated plants, v) stable C and O isotopes and stomatal density differ between treatments reflecting the acclimation of water-related physiological processes to prolonged water stress.

Materials and Methods

Plant material

Rhinanthus alectorolophus is an annual hemiparasitic plant of the family Orobanchaceae [25,26]. It grows in open habitats such as meadows and road verges where it parasitizes wide range of host species. *R. alectorolophus* reaches an average height of 30 cm and flowers from May to July [27–29]. It used to be considered as an agricultural pest in Central Europe infecting cereal crops [28] and can be easily grown on wheat or maize.

Rhinanthus alectorolophus seeds were collected from a natural population near Nenkovice, Czech Republic (49°0’19.8’’N, 16°59’54.1”E). Seeds of wheat (*Triticum aestivum*), which was used as a host species, were obtained from the Krásná Hora nad Vltavou collective farm, Haklovy Dvory, Czech Republic.

Growth chamber experiments

The experiments were conducted in a growth chamber from January to March 2016. Pre-germinated seeds of wheat were sown to 130 0.8L-pots filled with a mixture of sand and peat (1:1, v:v ratio). All pots contained 0.5 g Osmocote Exact Standard 5–6M fertilizer per liter of substrate and were well watered (200 mL of water/pot). The diurnal light cycle was set to 12 h light/12 h dark. Temperature ranged from 15–17 (dark) to 17–20 °C (light). Metal halide lamps provided photosynthetically active radiation (PAR) flux of 200–600 µmol m^{-2} s^{-1} (depending on spatial position). Young seedlings (3 per pot) of the hemiparasite pregerminated on Petri dishes kept at 4 °C for three months were transplanted to 110 pots two days after wheat planting. Of these, only a single plant was kept for the experiment while excessive seedlings were removed after a week. The pots were randomly relocated within the chamber table once a week to filter out possible heterogeneity in non-treatment cultivation conditions (mainly PAR flux).

Three contrasting water treatments were established in 30 parasitized pots to study the effect of drought on the survival of *Rhinanthus* seedlings. Ten pots (hereafter referred to as A-pots) were

watered only once (after wheat sowing), ten pots (hereafter referred to as B-pots) were watered twice (after wheat sowing and the hemiparasite planting), and ten pots (hereafter referred to as C-pots) were watered as B-pots and every sixth day after that.

The rest of pots (n=100) were used to study the physiological response of *Rhinanthus* adults to long-term water stress. Twenty pots served as a non-parasitized control. Two water regimes were established after *Rhinanthus* attachment to the host (indicated by rapid leaf expansion of *Rhinanthus*; [30]). High irrigation pots (W+) and low irrigation pots (W–) received 200 and 100 mL of water every fifth to seventh day, respectively. The intervals between irrigation events were determined on the basis of visibly dry soil in W– pots and clear marks of wilting of respective plants. Both W+ and W– pots were irrigated for entire course of the experiment by isotopically constant source water to minimize its effect on the proportion of oxygen isotopes in plant final biomass [31].

Seedling survival and soil moisture measurements

Survival of *R. alectorolophus* seedlings in A, B, and C-pots was daily documented for 17 consecutive days. Dry or heavily-wilted seedlings were assumed to be dead. In addition to that, we measured the relative water content (RWC) of soil in the pots using an HH2 Moisture Meter with an SM200 sensor (Delta-T Devices Ltd, Cambridge, UK). Three measurements per pot were taken every day and their averages are presented. Only pots with seedlings which were considered as alive on the previous day were measured.

Physiological measurements

Photosynthetic and transpiration rates were measured 46–63 d after *Rhinanthus* transplant at the irradiance of 500 μmol m^{-2} s^{-1} on intact leaves of flowering hemiparasites (13× W+ and 12× W–) with a Li-6400 Portable Photosynthetic System (LI-COR, Lincoln, USA). The measurements were conducted between 0900 and 1930h on partially dehydrated plants (at least 2 d after the last watering), hereafter referred to as drought-stressed plants. Some of these plants were wilted indicating that they have already undergone turgor pressure loss. Chamber CO_2 concentration and block temperature were set to 400 μmol mol^{-1} and 20°C, respectively. The relative air humidity inside the Li-6400 chamber was controlled at 60–75%. After finishing the measurements, all measured plants were watered and covered with a plastic bag until additional gas-exchange measurements of fully water-saturated plants, hereafter referred to as water-saturated plants, on the following day. These measurements were done in a same way as previous ones and one leaf per plant was sampled for $\Psi_{\pi\ \text{gas-exchange}}$ determination after finishing the measurements. Two plants (1× W+ and 1× W–) did not recover from water stress experienced during the first measurements and were therefore excluded from the data set.

The actual $\Psi_{\pi\ \text{gas-exchange}}$ of sampled plant parts was measured using thermocouple psychrometry [32]. Leaf samples were cut, immediately sealed in a 2-mL syringe, and frozen at –20 °C. The samples were allowed to thaw for maximum 60 min before the start of the measurements. The freeze–thaw cycle disrupted the cell membranes and allowed squeezing the cytoplasm. About 7 μL of the fluid was pipetted onto a cellulose filter paper disc, placed in a 1.25 mm deep sample holder, and enclosed inside the C-52 sample chamber linked to a Wescor HR-33T microvoltmeter (Wescor

Electronics, Logan, UT, USA). The air Ψ in the sample chamber equilibrated within 5 min. Measurements were calibrated using 0.3 M NaCl (Ψ_π = –1.37 MPa).

The second part of parasitized pots (10× W+ and 9× W–) was subjected 48-62 d after *Rhinanthus* transplant to water potential (Ψ) measurements by a pressure chamber (The Plant Water Status Console, Model 3000; Soil Moisture Equipment Corp., Santa Barbara, USA). The pressure-chamber method measures the decline in leaf Ψ with ongoing leaf dehydration [32–34] and enables to construct the pressure–volume (p–v) curves and Höfler diagrams providing detailed information about the water relations of measured plants. Upper part (up to 20 cm) of fully water-saturated plants were gently blotted up with cotton sheets to remove droplets of external water, cut, immediately wrapped in stretch film to prevent water loss via transpiration during measurements, weighed, and sealed into the pressure chamber. The pressure–volume data were collected using a "squeeze method" to prevent damage to the soft herbal tissue. Briefly, the water loss was induced by pressurization of the chamber with synthetic air and the sap squeezed at each balance pressure (steps of about 0.2 MPa) was collected and weighed, while the plant remained enclosed in the chamber [33].

Evaluation of pressure–volume curves

We plotted a p–v curve for each measured plant. The p–v curves showed the relationship between the inverse of the balance pressure and the cumulative volume of cell sap squeezed, which was then replaced by RWC. Using the "squeeze method" instead of repeated pressurizing was the only way how to avoid mechanical damage to the soft herbal tissue; however, the method generated some identifiable artefacts that required further data processing. The values of RWC after turgor loss were slightly overestimated in most samples because not all the water had been squeezed from the plant before switching to higher balance pressure. This overestimation resulted in steeper slope of the linear part of p–v curves (representing Ψ_π) and thus in overestimated intercept with x-axis (RWC) and underestimated Ψ_π at full turgor ($\Psi_{\pi\ FT}$; at RWC=1). The intercept with x-axis denotes the volume of apoplastic water (RWC_{AW}), which usually represents 3–50% of the total volume of water in a leaf [32]. Our values ranged between unrealistically high 35 and 74%, representing unlikely high variability in single species. To reduce these artefacts, we fixed the intercept of all curves at RWC of 23.1% (S1B Fig). This value corresponded to such slopes of the linear parts of all the p–v curves that yield mean $\Psi_{\pi\ FT}$ of –1.38 MPa (*y*-intercept in S1B Fig), which is the mean value measured by thermocouple psychrometry in water-saturated leaf samples collected before pressure-chamber measurements (thermocouple psychrometry measurements were thus used to calibrate the pressure-chamber measurements). Solver module of MS Excel was used to find the slopes. Moreover, as the turgor ceased very slowly (hyperbolically), it was difficult to determine the turgor loss point accurately (S1B Fig). In order to reduce the variability due to this inaccuracy, we defined a corrected turgor loss point (TLP_{cor}) so that ψ_π at TLP_{cor} ($\psi_{\pi\ TLPcor}$) and RWC at TLP_{cor} (RWC_{TLPcor}) corresponded to the values at 10% of full turgor (Fig. S1A). The modulus of elasticity (ε) was defined as a slope of the turgor–RWC relationship (between full turgor and the point preceding TLP_{cor}).

Stable isotope analyses

Above-ground biomass of flowering hemiparasites and parasitized wheat (n=44, 23× W+, 21× W–) were harvested after finishing the measurements, i.e. 48–63 d after *Rhinanthus* planting. Above-ground biomass of control wheat and flowering hemiparasites (n=20, 10× W+, 10× W–) were harvested 62 d after the planting. Biomass samples were dried at 80 °C for 48 hours and weighted. Newly-grown leaves of both species were sampled to separate paper bags from 20 parasitized pots and all controls, dried, ball-milled, and embedded in tin capsules for stable isotope analysis of carbon.

Stable isotopes of oxygen were analyzed from alpha-cellulose isolated from the subset of these samples. The isolation of alpha-cellulose started by placing milled leaves (30–50 mg) in 15-mL plastic centrifuge tubes and washing them in 8 mL of 80% acetone for 15 min. The tubes were then centrifuged (12 min at 4000 ×*g*), the pellet was resuspended in 8 mL of distilled water, and the tubes were placed to water bath at 75 °C. After addition of 80 µL of glacial acetic acid and 160 µL of 25% sodium chlorite, the tubes were incubated for 1 h and the addition of sodium chlorite was repeated. After 2 h, the addition of acetic acid and two subsequent additions of sodium chlorite were repeated and the tubes were incubated once more for 1+2 h. The tubes were vortexed every 30 min during the total 6 h of extraction. Cooled tubes were repeatedly centrifuged and washed in distilled water to get clean holocellulose pellet, which was subsequently resuspended in 8 mL of 4.2 M KOH and kept at 22 °C for 2 h. Finally, the tubes were centrifuged and the pellet of alpha-cellulose was successively washed with 2% HCl, water, and acetone, dried at 50 °C, and weighted to silver capsules.

The stable isotope analyses were conducted with a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the Stable Isotope Facility at University of California, Davis, USA. Isotopic compositions of the biomass samples were expressed as the δ values reflecting the isotopic difference between the sample and relevant international standards, V-PDB (Vienna PeeDee Belemnite) for carbon and SMOW (Standard Mean Ocean Water) for oxygen.

Stomatal density

Leaves (n=14, 9× W+, 5× W–) and bracts (n=16, 9× W+, 7× W–) of the hemiparasite were examined for stomatal density. Stomatal impressions of the adaxial and abaxial leaf and bract sides were taken by transparent nail polish and observed on a slide by an Olympus CX41 Microscope (Olympus Imaging America Inc., Center Valley, Pennsylvania, USA) and INFINITY1-3C 3.1 MP CMOS Color Camera (Lumenera Corp., Ottawa, Canada). Stomata were counted at 200× magnification from 5 microscopic fields per leaf/bract side of a plant. The number of stomata on the area of 0.325 mm^2 corresponding to an examined microscopic field was converted to the number of stomata per mm^2. It should be noted, that stomatal density was hard to analyze, in particular due to the presence of hydathode trichomes on abaxial bract and leaf sides.

Statistical analysis

Seedling survival was analyzed by estimating the survival curves by Kaplan-Meier survival function [35] for each water treatment. Comparison between the treatments was performed by a Mantel–Haenszel test [36]. Biomass and isotope data were analyzed using linear models. The biomass data of *Rhinanthus* adults were fitted by a linear model with day after transplant as a predictor to estimate their biomass 60 d after *Rhinanthus* transplant (i.e. when the control pots were harvested). This estimate was used as a response in further statistical modeling of the hemiparasite biomass to minimize the effect of different harvest dates. We did not apply this correction to parasitized wheat due to low correlation of its biomass with harvest day, presumably caused by differential growth dynamics in individual treatments. Biomass data were logarithmically transformed before analysis. We used linear models to test the effects of irrigation treatment, infection by the hemiparasite and their interaction on above-ground-biomass production and stable-isotopic composition of the wheat host. Linear models were also used to test the effect of irrigation treatment on the same biomass parameters of the hemiparasite. Gas-exchange parameters were tested by linear mixed-effect models containing irrigation treatment, osmotic potential, and their interaction as fixed-effect predictors and plant identity as a random factor. Stomatal densities of the hemiparasite were tested separately for adaxial and abaxial sides by linear mixed-effect models with irrigation treatment, bract vs. leaf sample, and their interaction as a fixed-effect predictors and plant identity as a random factor. The differences in water-relation parameters were analyzed by two-tailed t-tests. All statistical analyses were conducted and visualized in R software [37], R packages *survival* and *nlme* were used for survival analysis and linear mixed-effect models.

Results

Seedling survival

Survival of parasite seedlings differed among pots (χ^2=34.9, df=2, P<0.001). The seedlings from A-pots started to die 8 d after single watering event (6 d after parasite transplant) at average soil RWC of 20.1% and they were not able to survive more than 18 d after single watering event (2 d before the day 0; Fig 1). Compared with A-pots, B-pot seedlings started to die 15 d after the second watering event (indicated by an increase in soil RWC from 0 d to 1 d; Fig 1) at 17.1% of soil RWC. Second watering event delayed the onset of survival decline by 9 d. All seedlings from non-stressed C-pots survived 17 d after their transplant, when the experiment was terminated (Fig 1B). Host plants showed no distinctive signs of water deficiency and all of them survived till the harvesting times.

Biomass

Biomass production of flowering parasites, as well as control and parasitized host from W– pots was considerably lowered by long-term water stress (Table 1; Fig 2; S1 Tab; S2 Tab). Harvest day had no significant effect on dry mass weight of the host (S1 Tab). Parasitism and irrigation treatment markedly affected host biomass (Table 1; S1 Tab). Parasitized hosts visibly suffered from water shortage, especially under W– treatment, causing many of their leaves to dry (S2 Fig).

Table 1. Summary of linear models describing the effects of irrigation treatment and infection by the hemiparasite on host *Triticum aestivum* and hemiparasite *Rhinanthus alectorolophus* above-ground biomass production and their stable-isotopic composition.

	Host				Hemiparasite		
Effect	Biomass (parasitized and control)	Control biomass	$\delta^{13}C$	$\delta^{18}O$	Biomass	$\delta^{13}C$	$\delta^{18}O$
Irrigation treatment	** W+	*** W+	*** W–	*** W–	*** W+	** W–	+ W–
Infected	*** ↓		*** ↑	n.s.			
Treatment × Infected	n.s.		n.s.	n.s.			

*** $P \leq 0.001$; ** $P \leq 0.01$; + $P = 0.051$. Arrows indicate positive (up) and negative (down) relationship between the response variable and related effect. W+/W– indicate the irrigation treatment with higher values of response variables. Factor Infected represents the effect of parasitic infection on host parameters. $\delta^{13}C$ and $\delta^{18}O$ represent the isotopic composition of host overall biomass and hemiparasite biomass. n.s. indicates non-significant terms omitted from the final models. The effects not tested for a particular variable are indicated by light grey. More information in full anova tables (S1 Tab; S2 Tab).

Physiological measurements

Rates of photosynthesis and transpiration were positively correlated with leaf osmotic potential ($\Psi_{\pi\ gas\text{-}exchange}$; $t_{22}=7.18$; $P<0.001$; $t_{22}=6.51$; $P<0.001$, respectively; S3 Tab; S3 Tab; Fig 3). Regardless the irrigation treatment, both processes were significantly lowered in drought-stressed plants (linear models; $t_{22}=–5.60$, $P<0.001$ and $t_{22}=–7.38$, $P<0.001$ for photosynthesis and transpiration, respectively) compared with water-saturated plants (S4 Tab), which had greater $\Psi_{\pi\ gas\text{-}exchange}$ (Table 2). Drought-stressed plants displayed low ψ_π, but still exhibited substantial rates of photosynthesis and transpiration (Table 2). Moreover, photosynthetic rate was higher in the plants grown under W– treatment compared with those of similar ψ_π from the W+ treatment (Table 1; Fig 3; S3 Tab). We did not find such relationship for transpiration rate (Table 1; Fig 3; S3 Tab). Measured plants from contrasting irrigation treatments did not significantly differ in their average $\Psi_{\pi\ gas\text{-}exchange}$ (–1.70 (W+) and –1.76 (W–)).

Table 2. Physiological traits (means ± SE) of hemiparasitic *Rhinanthus alectorolophus* grown under two contrasting irrigation treatments, high (W+) and low (W−).

		Gas-exchange and osmotic potential			Pressure-chamber parameters			
Irrigation treatment	Plant water status	Photosynthesis (μmol CO_2 m^{-2} s^{-1})	Transpiration (mmol H_2O m^{-2} s^{-1})	$\Psi_{\pi\ gas\text{-}exchange}$ (MPa)	$\Psi_{\pi\ FT}$ (MPa)	$\Psi_{\pi\ TLPcor}$ (MPa)	RWC_{TLPcor} (%)	ε
W+	Drought-stressed	6.8 ± 0.9	1.88 ± 0.36	-1.98 ± 0.08	-1.39 ± 0.03	-1.66 ± 0.03	88.9 ± 0.7	12.5 ± 1.1
	Water-saturated	9.1 ± 0.4	2.91 ± 0.27	-1.42 ± 0.06				
W-	Drought-stressed	8.6 ± 1.1	1.05 ± 0.19	-2.08 ± 0.15	-1.37 ± 0.04	-1.66 ± 0.03	87.4 ± 1.0	11.6 ± 1.4
	Water-saturated	12.9 ± 0.9	3.38 ± 0.22	-1.44 ± 0.08				

Gas exchange was measured at the irradiance of 500 μmol m^{-2} s^{-1} in plants under water stress (drought-stressed) and in the same, but fully water-saturated plants (water-saturated). Leaves subjected to gas-exchange were immediately sampled for actual osmotic potential ($\Psi_{\pi\ gas\text{-}exchange}$). Pressure-chamber parameters were calculated from pressure-chamber measurements initiated on fully water-saturated plants. We used a corrected turgor loss point equalling to 10% of full turgor ($\Psi_{\pi\ TLPcor}$), as the RWC decrease in p-v curves (S1B Fig) was hyperbolic and the turgor loss point was hard to determine. $\Psi_{\pi\ FT}$= osmotic potential at full turgor (RWC=100%), $\Psi_{\pi\ TLPcor}$=osmotic potential at corrected turgor loss point, RWC_{TLPcor}=relative water content at corrected turgor loss point, ε=modulus of elasticity. n=13 and 12 for gas-exchange and osmotic potential measurements of W+ and W– plants; n=10 and 9 for pressure-chamber measurements of W+ and W– plants.

Pressure-chamber measurements showed no apparent difference between W+ and W– plants in their water-relation parameters (Table 2; S1 Fig), including $\psi_{\pi\ FT}$, $\psi_{\pi\ TLPcor}$, RWC_{TLPcor}, and ε. These measurements enabled us to determine actual water status of the plants, in which gas-exchange was measured, by projecting $\psi_{\pi\ FT}$ and $\psi_{\pi\ TLPcor}$ into Fig 3. It is clear from the figure that even wilted plants ($\Psi_{\pi\ gas\text{-}exchange} < \psi_{\pi\ TLPcor}$) were still able to carry out photosynthesis and transpiration.

Despite the absence of osmotic adjustments, wilted hemiparasites recovered very fast from severe drought stress after re-watering (S2 Fig). Photosynthetic and transpiration rates of W+ plants increased on average by 34 and 55% approximately 24 h after re-watering, while it was 55 and 222% in W– plants, respectively (Table 2). Osmotic potential of hemiparasites of W+ and W– increased on average by 28 and 31% (Table 2), respectively. Interestingly, there was a significant interactive effect of irrigation treatment and water saturation (S4 Tab), which might indicate an physiological adjustment to water stress in W– hemiparasites.

Stable isotopes

Biomass of the parasite from W– irrigation treatment was significantly enriched in ^{13}C, but only slightly in ^{18}O (Table 1; Fig 4)compared with its biomass from W+ treatment. Biomass of the host from W– treatment was significantly enriched in both ^{13}C and ^{18}O (Table 1; Fig 4). $\delta^{13}C$ of host biomass was in addition positively affected by parasitism (Table 1). Biomass of the parasite was less enriched in ^{13}C and ^{18}O regardless the treatment than the biomass of parasitized hosts (t_{19}=–28.39, P<0.001; t_{19}=–2.14, P=0.046; S5 Tab).

Stomatal density

Plants grown under contrasting water treatments did not significantly differ in the density of stomata on their leaves and bracts (S3 Fig; S6 Tab).

Discussion

Both parts of our experimental work focusing on seedlings and adults of hemiparasitic *R. alectorolophus* brought novel insights into understanding of the water-stress ecophysiology of root-hemiparasitic plants. For the first time we experimentally showed the sensitivity of *Rhinanthus* seedlings to drought. The seedling survival of first two weeks after their transplant was strongly lowered by drought stress as we hypothesized, in contrast to two-days-older wheat hosts. Our observations also suggest that seedlings might be drought-sensitive both before and shortly after attachment to the host if we assume B-pots already host-connected. Drought stress is likely the mechanism behind field observations documented pronounced mortality of seedlings during spring droughts causing frequent population fluctuations [23,38,39]. Frequency and intensity of such fluctuations may increase in future as a result of climate change, which may eventually cause *Rhinanthus* extinction in some areas.

Similarly to *Rhinanthus* seedlings, water stress negatively affected adult hemiparasites in terms of biomass production and physiological functioning. Water shortage inhibited the hemiparasite's gas exchange, but drought-stressed and even wilted plants from both treatments still photosynthesized

and transpired considerably (Table 2; Fig 3). A negative effect of drought on photosynthetic performance is well recognized for parasitic [40,41] and many non-parasitic plants [42–44] and mostly attributed to reduced stomatal and mesophyll conductance, and to a lesser extent to biochemical limitations [42,45,46]. We could not evaluate the importance of these, as we measured only transpiration rate consisting of stomatal conductance and evaporation of guttation water from hydathode trichomes, which cannot be separated from each other. Interestingly, the ability of wilted hemiparasites to carry out photosynthesis of substantial rate indicates that their guard cells were still turgid and stomata open.

The absence of significant differences in the water-relation parameters between the irrigation treatments (Table 2) indicated that low-irrigated plants did not osmotically adjust to long-term water stress which contrasts with our original hypothesis. We expected these plants to lower their osmotic and water potential as frequently observed in non-parasitic plants responding to drought stress [47–49]. The absence of any osmotic adjustment may refer to limited capacity of *Rhinanthus* to acclimatize to water stress or more probably its capability to tolerate drought without a need to adjust. The latter is supported by the fact that *Rhinanthus* of either treatment had rather low osmotic potential at full turgor ($\psi_{\pi\ FT=}$ −1.38 MPa in average; Table 2). Compared to non-parasitic species of similarly dry habitats, e.g. semiarid grassland dicots [50], this value may be low enough to ensure good physiological adjustment to water stress.

The photosynthetic rate of the flowering hemiparasites was affected by the irrigation treatment despite the absence of corresponding osmotic adjustment. The photosynthetic rate in low-irrigated plants (Table 1; Table 2; Fig 3) was elevated compared with W+ plants of the same osmotic potential and these plants could therefore obtain more autotrophic carbon. This result is unexpected and can be related to a differential acclimation of water-stressed plants to repeated cycles of water stress sometimes referred to as stress memory [51,52]. Studies comparing plants acclimated and not-acclimated to drought stress are rare. Recently, Menezes-Silva et al. [53] reported elevated photosynthesis in coffee plants that underwent multiple drought events and attributed it to biochemical adjustments (e.g. increased activity of Rubisco). Similar evidence had earlier been suggested to be associated with the maintenance of higher electron transport rates in plants acclimated to drought stress [49]. Increased photosynthetic rate may also be related to increased chlorophyll [54] and/or Rubisco concentrations in the hemiparasite underpinned by enhanced resource uptake from host root system under drought conditions [6]. This may be caused by delayed stomatal closure in the hemiparasite under these conditions when the hemiparasite acts as a strong sink. Alternatively, allocating more assimilates into roots, the host might facilitate its resource uptake by providing more space for the establishment of haustorial connections and thus enables the hemiparasite to acquire nutrients essential for building up chlorophyll and/or Rubisco molecules.

Lower enrichment of the hemiparasite biomass in ^{13}C and ^{18}O compared with that of host corresponds to higher transpiration and lower water-use efficiency (WUE) of rhinanthoid root hemiparasites associated with their water-wasting physiological strategy. A similar pattern was reported by Cernusak et al. [55], but the isotope proportions seem to highly depend on particular growing conditions. This might be a reason why non-significant differences in ^{13}C were found between *R. alectorolophus/Euphrasia rostkoviana* and wheat [5]. The lack of differences in ^{13}C in root-hemiparasite –C_3 host pair was also reported for *Striga-gesnerioides–Vigna-unguiculata* and *Olax-phylanthi*–multiple-hosts. Similar enrichment in *Olax* and their hosts was explained by their

similar WUE. The evaluation of $\delta^{13}C$ results is further complicated by heterotrophic C uptake of *Rhinanthus*, which might underestimate the differences in WUE between species as suggested by Cernusak et al. [55].

Although the physiological functioning of hemiparasitic and host plants differs in many aspects, both species seem to respond to water stress conditions in a similar way, contrasting to what was previously suggested [6,13]. Hemiparasites and hosts close stomata under severe water stress, increasing their WUE and restricting transpiration and stomatal conductance, which is evident from higher enrichment of both species biomass in ^{13}C and ^{18}O under W– (Fig 4). While stomatal closure is ABA-mediated in the host, it is likely that stomata of the hemiparasite close passively after turgor of guard cells is lost. Interestingly, gas exchange of wilted hemiparasites recorded here (Fig 3) demonstrate that the passive stomatal closure is preceded by leaf turgor loss in *R. alectorolophus*. Passive stomatal closure was reported to prevail active ABA-mediated stomatal closure in ferns and lycophytes [58], and also in woody angiosperms [59,60], but these two processes might operate in the same species together [61]. This is unlikely to happen in *Rhinanthus* since their stomata actively close only in response to extremely high concentration of ABA [12]. Despite the absence of ABA-mediated stomatal regulation in attached *Rhinanthus*, these plants are known to contain unusually high ABA concentration [24]. ABA may thus contribute to the acclimation of the hemiparasites to drought stress via the formation of dehydrins or other drought-protective proteins as suggested by Jiang et al. [14], but its exact mechanism remains unknown.

In summary, we demonstrated that the adult hemiparasites have certain capacity to withstand drought stress. Their physiological traits, in particular generally low osmotic potential associated with the ability to recover from the wilting, are likely crucial for their growth in moderately dry habitats [22,62]. However, most root-hemiparasitic species of temperate grasslands (including those of the genus *Rhinanthus*) display rather prominent limit of their ecological niche at the dry-end of the water availability gradient. The lack of further physiological adjustment to more severe drought demonstrated here may thus cause a substantial decline of the hemiparasitic species under the projected (and recently also observed) climate change-induced increase of temperature and drought events [63]. Nevertheless, more studies on water-relations of root hemiparasites under repeated drought stress are needed to accurately estimate the stability of their populations in future warmer climate.

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Fig 1. Soil relative water content (A, soil RWC) and survival of hemiparasitic *Rhinanthus alectorolophus* 0–17 days after its transplant (B) to the pots with the host, *Triticum aestivum*. Black lines represent pots watered only once (after host planting, A-pots), dark grey represents pots watered twice (after host and parasite planting, B-pots), and light grey represents pots watered regularly. Day averages of four soil RWC measurements per pot ± 1.96 standard error are displayed (A). Dashed lines represent 95% confidence intervals (B). n=10 for each water treatment in the beginning of the experiment.

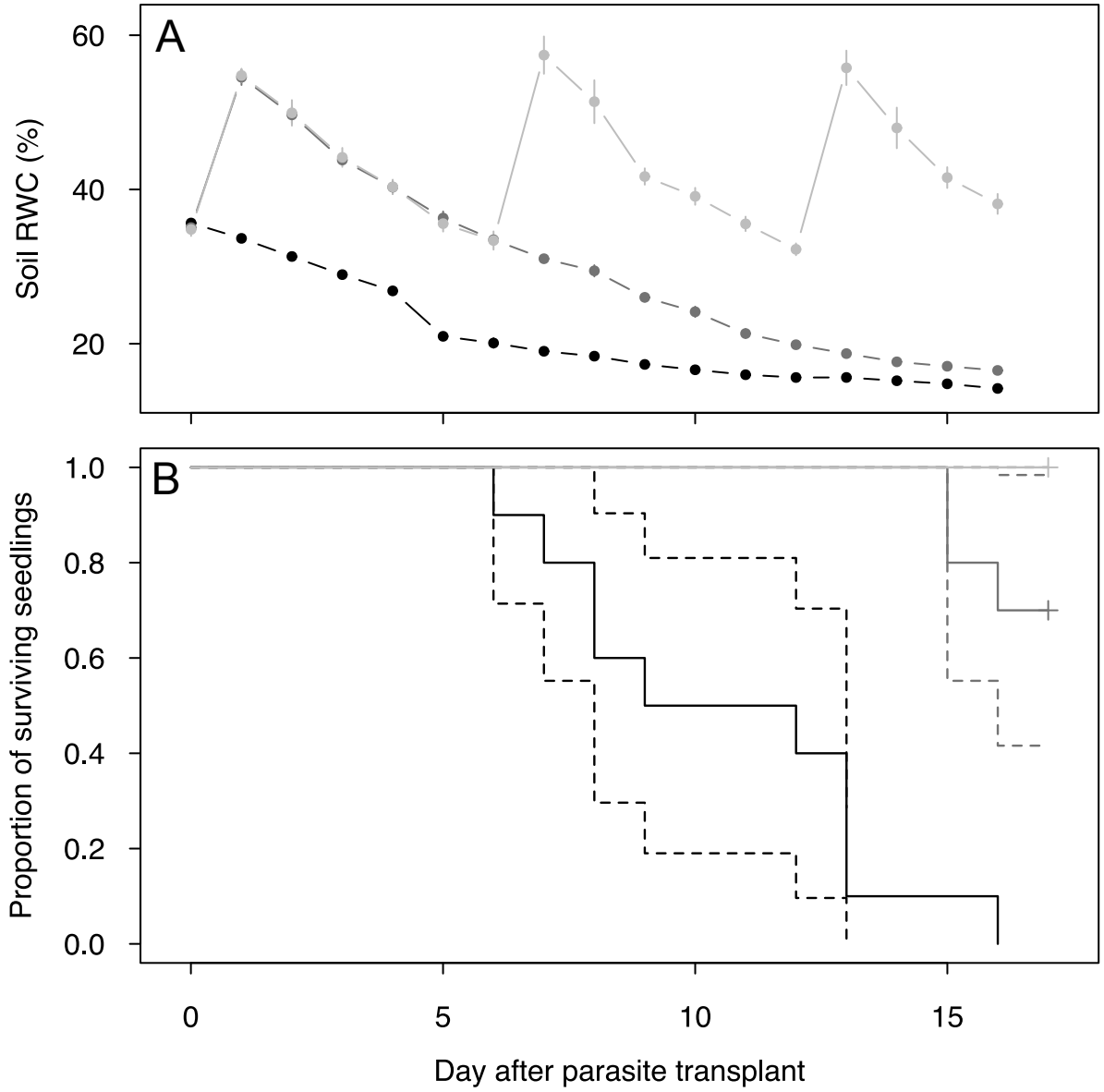


Fig 2. Above-ground biomass of control and parasitized host, *Triticum aestivum*, and the hemiparasite, *Rhinanthus alectorolophus*, grown under high (W+) and low irrigation treatments (W–). n=10 for W+ and W– unparazitized control pots, n=21 (W+) and n=23 (W–) for parasitized pots.

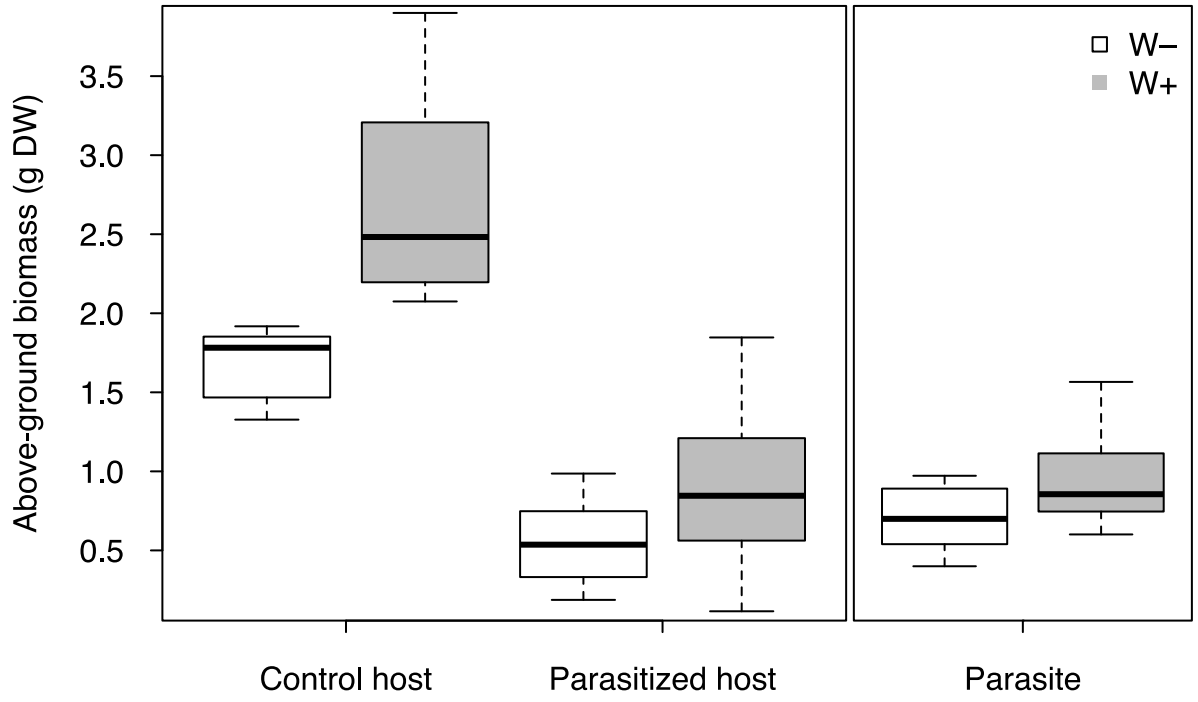


Fig 3. Photosynthetic (A) and transpiration rates (B) at the irradiance of 500 µmol m^{-2} s^{-1} in the leaves of flowering *Rhinanthus alectorolophus* as a function of osmotic potential. Plants were grown under high (W+) and low irrigation treatments (W–). Points correspond to individual plants, n=11 and n=10 for W+ and W–. Each plant was measured twice: when drought-stressed and water-saturated. Linear regression lines are presented by solid and dashed lines (df=5; LR=42.15, P<0.0001 (A); df=4, LR=31.00, P<0.0001 (B)). Grey vertical line relates to average osmotic potential at turgor loss point ($\Psi_{\pi\ TLPcor}$=–1.66 MPa; S1 Fig, Table 1) determined by pressure-chamber measurements.

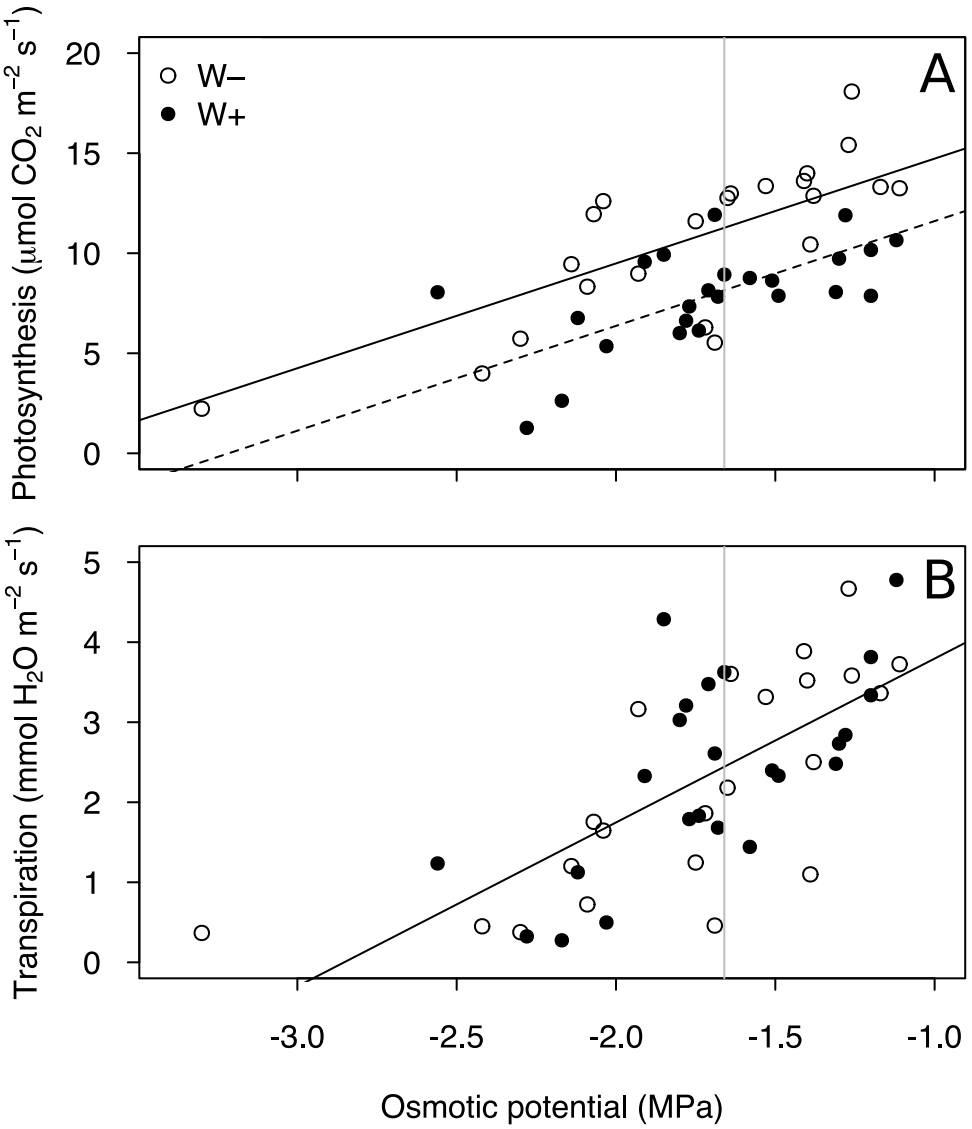
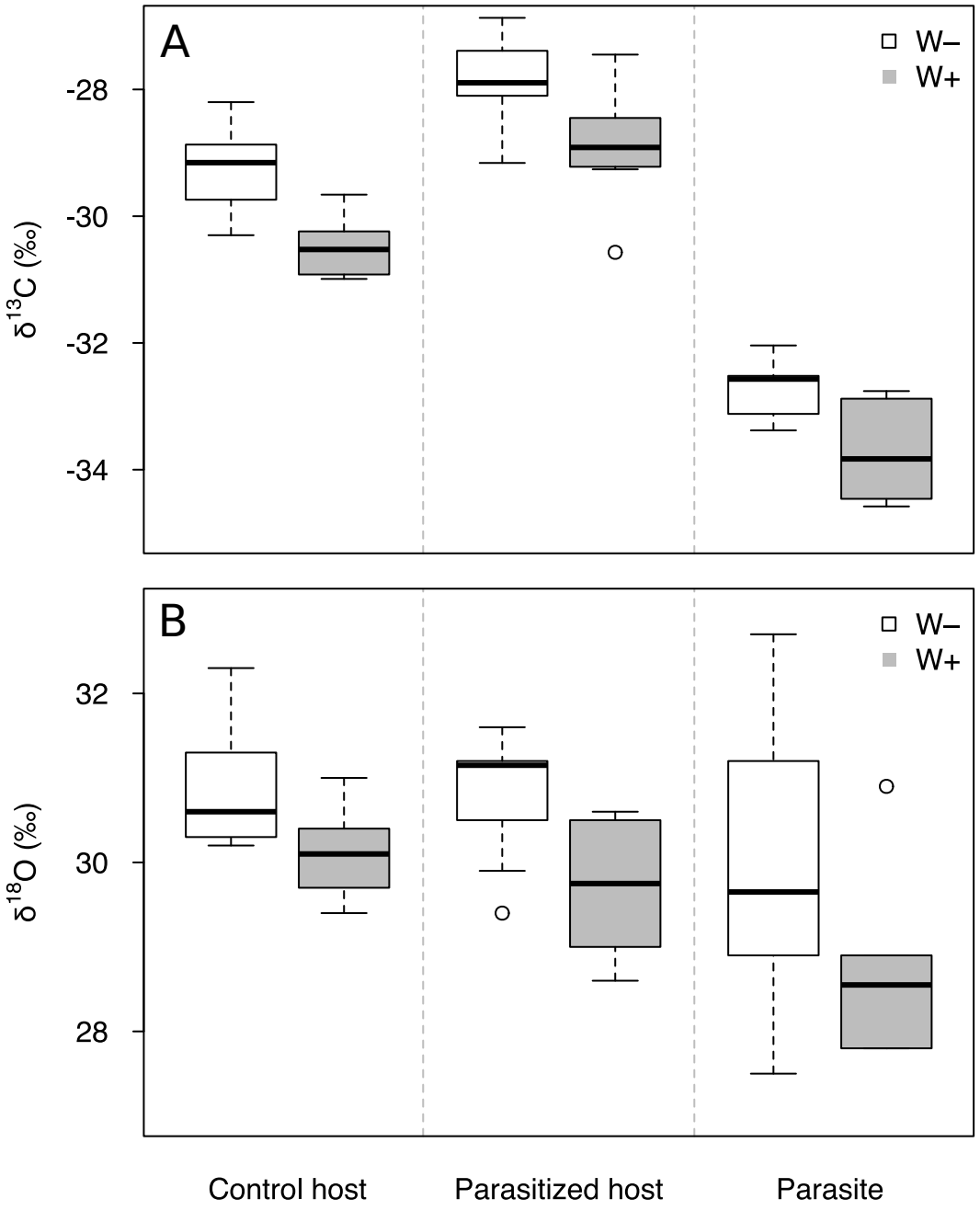
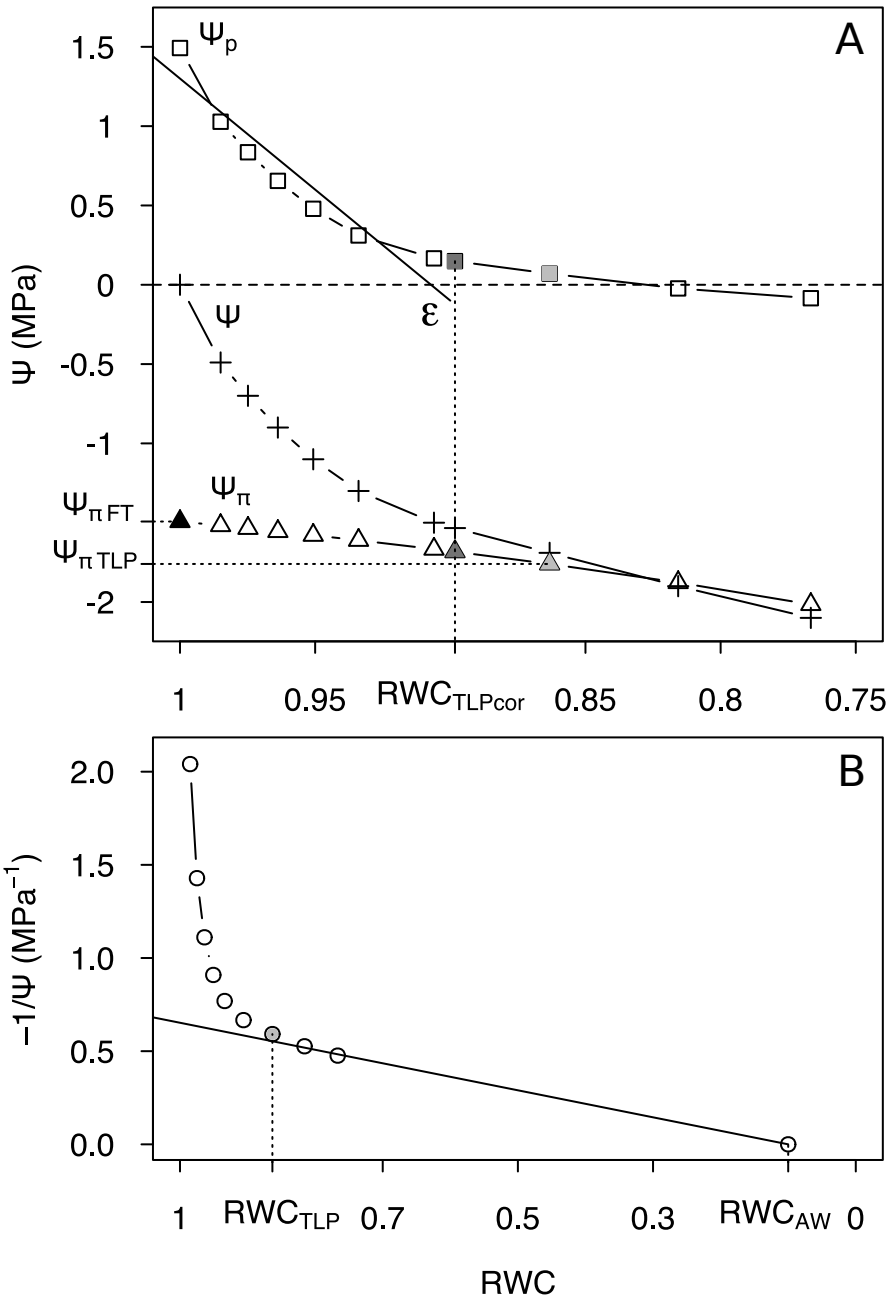


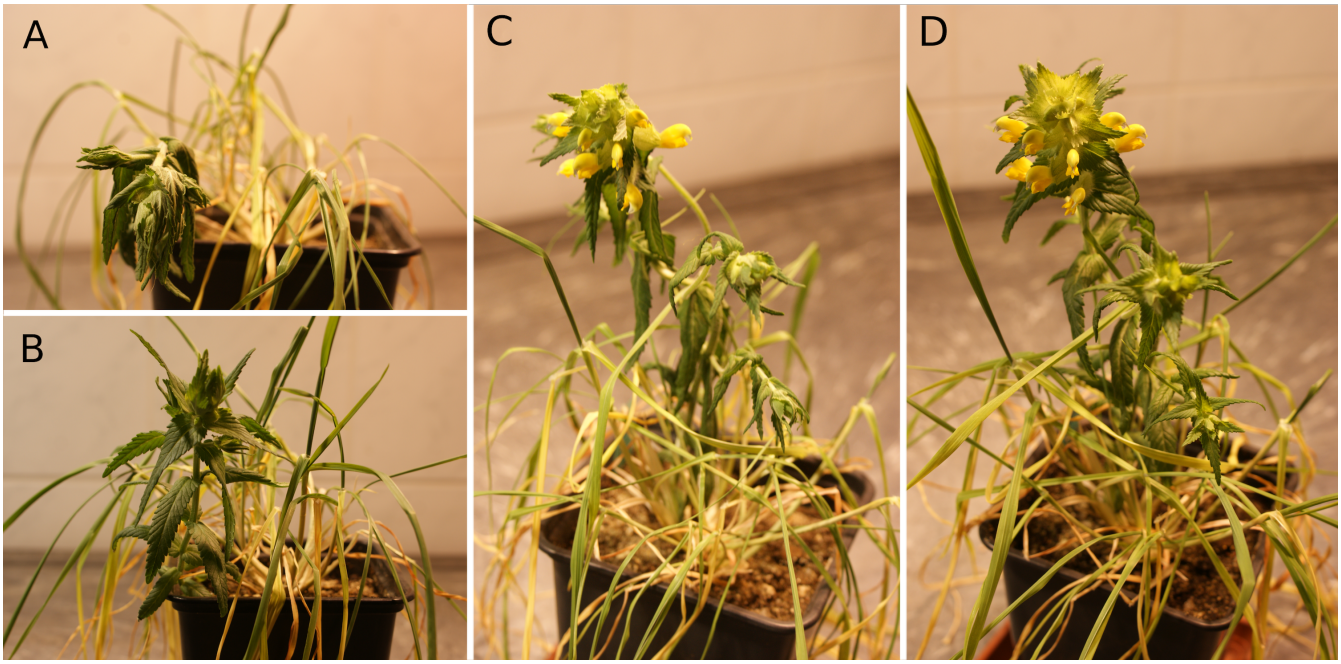
Fig 4. Carbon (A) and oxygen (B) stable-isotopic composition of above-ground biomass of control and parasitized *Triticum aestivum* host and the hemiparasitic *Rhinanthus alectorolophus* grown under high (W+) and low (W–) irrigation treatments. n=10 for both control and parasitized pots and each water treatment.



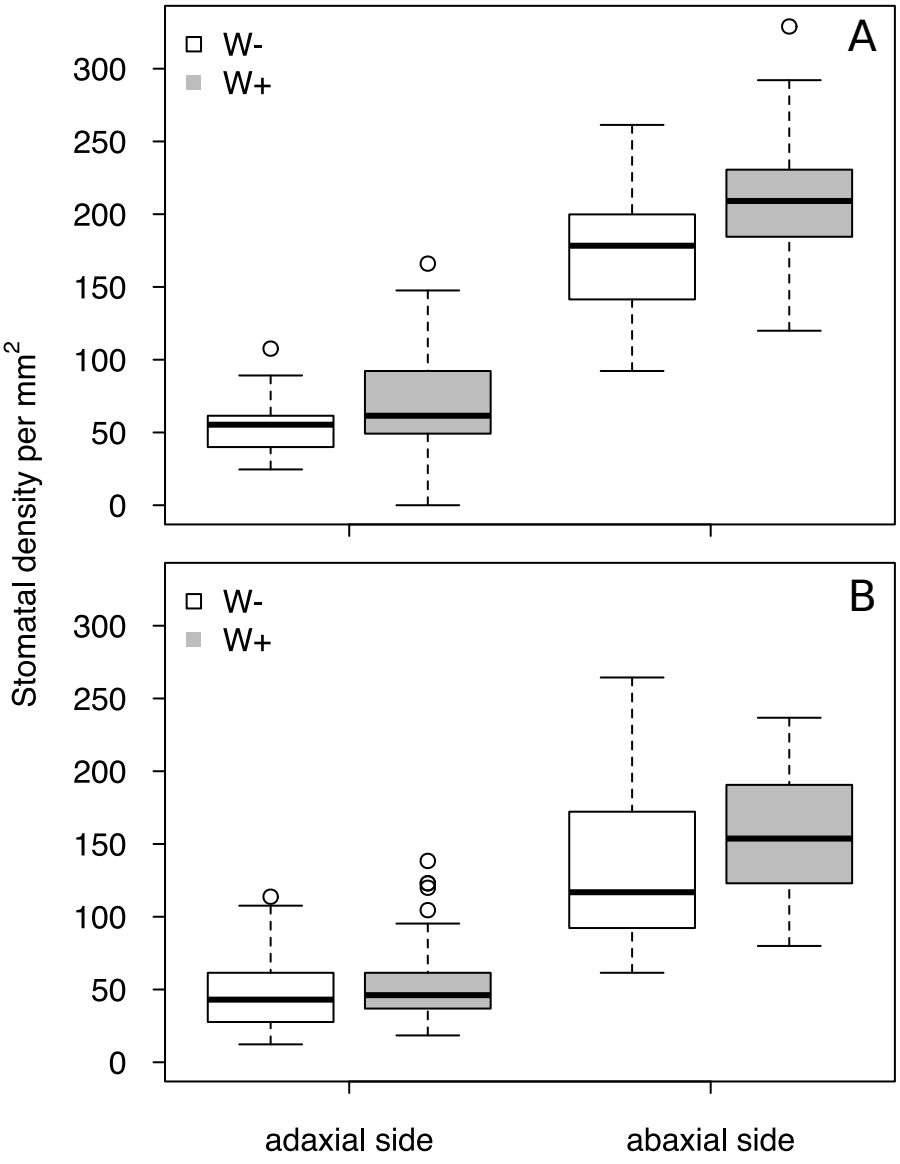
S1 Fig. Höfler plot (A) and pressure–volume curve (p–v curve, B) revealing water relation parameters of flowering *Rhinanthus alectorolophus*. The turgor loss point, which is usually defined as the first point of linearly decreasing segment of a p–v curve (light grey), was hard to determine due to hyperbolic shape of the p–v curve. To reduce the error of the determination, we replaced it by a corrected turgor loss point (dark grey) corresponding to 10% of full turgor (Ψ_p). Additionally, the intercept of the linear segment of the p–v curve with the x-axis was fixed to 23.1% of total RWC (RWC_{AW}) estimated to represent the volume of apoplastic water inside measured plant (B). This enabled us to define the linear segment, i.e. osmotic potential and hence other parameters more precisely. Ψ=water potential, Ψ_p=turgor or pressure potential, Ψ_π=osmotic potential, $\Psi_{\pi\ FT}$=osmotic potential at full turgor (black triangle), $\Psi_{\pi TLP}$=osmotic potential at turgor loss point, RWC= relative water content, RWC_{AW}=fraction of apoplastic water fixed at 23.1% of total RWC, RWC_{TLP}=RWC at turgor loss point, RWC_{TLPcor}=RWC at corrected turgor loss point, ε=the modulus of elasticity.



S2 Fig. The recovery of wilted *Rhinanthus alectorolophus* (A and C) from severe drought stress several hours after re-watering (B and D). One non-flowering and one flowering individuals are shown. Note the effect of drought stress on wheat, which was used as a host species.



S3 Fig. Stomatal density on leaves (A) and bracts (B) of hemiparasitic *Rhinanthus alectorolophus* grown under high (W+) and low irrigation treatments (W–). Both adaxial and abaxial sides are presented. n=9 for W+ leaves, n=5 for W– leaves, n=9 for W+ bracts, and n=7 for W– bracts.



Supplementary Tables

S1 Tab. ANOVA table of linear models describing the effects of irrigation treatment, infection by the parasitic *Rhinanthus alectorolophus*, harvest day, and their interactions on the host (*Triticum aestivum*) above-ground biomass and its stable-isotopic composition.

	Host biomass (parasitized and control)				Host control biomass				Host $\delta^{13}C$				Host $\delta^{18}O$			
Effect	*df*	*SS*	*F*	*P*	*df*	*SS*	*F*	*P*	*df*	*SS*	*F*	*P*	*df*	*SS*	*F*	*P*
Treatment	**1,58**	**2.44**	**9.24**	**0.004**	**1,18**	**1.02**	**29.82**	**3.46×10^{-5}**	**1,36**	**11.69**	**24.90**	**1.55×10^{-5}**	**1,36**	**7.83**	**18.01**	**1.47×10^{-4}**
Infected	**1,58**	**20.94**	**79.34**	**1.9×10^{-12}**					**1,36**	**21.37**	**45.55**	**7.02×10^{-8}**	1,36	0.31	0.70	0.41
Harvest day	1,58	0.02	0.06	0.81												
Treatment × Infected	1,58	0.01	0.02	0.88					1,36	0.04	0.78	0.38	1,36	0.34	0.79	0.38
Treatment × Harvest day	1,58	0.01	0.03	0.86												

Factor Infected represents the effect of parasitic infection on host parameters. $\delta^{13}C$ and $\delta^{18}O$ represent the isotopic composition of host overall biomass. The effects not tested for a particular variable are indicated by light grey. Significant terms (P<0.05) are in bold. df: degrees of freedom; SS: sum of squares; F: F-statistics; p: significance level.

S2 Tab. ANOVA table of linear models describing the effect of irrigation treatment on the above-ground biomass and its stable-isotopic composition of parasitic *Rhinanthus alectorolophus*.

	Parasite biomass				Parasite $\delta^{13}C$				Parasite $\delta^{18}O$			
Effect	*df*	*SS*	*F*	*P*	*df*	*SS*	*F*	*P*	*df*	*SS*	*F*	*P*
Treatment	**1,42**	**0.97**	**13.17**	**7.6×10^{-4}**	**1,18**	**4.78**	**13.31**	**0.002**	1,18	8.06	4.38	0.051

$\delta^{13}C$ and $\delta^{18}O$ represent the isotopic composition of the parasite biomass. Significant terms (P<0.05) are in bold. df: degrees of freedom; SS: sum of squares; F: F statistics; p: significance level.

S3 Tab. ANOVA table of linear models describing the effects of irrigation treatment, osmotic potential of leaves subjected to gas exchange measurements, and their interaction on photosynthetic and transpiration rate of parasitic *Rhinanthus alectorolophus.*

	Photosynthesis			Transpiration		
Effect	*df*	*F*	*P*	*df*	*F*	*P*
Treatment	**1,21**	**13.47**	**0.001**	1,21	0.42	0.52
Osmotic potential ($\Psi_{\Pi\ gas\ exchange}$)	**1,21**	**51.49**	**<0.001**	**1,21**	**40.22**	**<0.001**
Treatment × Osmotic potential ($\Psi_{\Pi\ gas\ exchange}$)	1,21	1.31	0.27	1,21	0.15	0.70

Significant terms (P<0.05) are in bold. df: degrees of freedom; F: F statistics; p: significance level.

S4 Tab. ANOVA table of linear models describing the effects of irrigation treatment, leaf water saturation, and their interaction on photosynthetic and transpiration rate of parasitic *Rhinanthus alectorolophus.*

	Photosynthesis			Transpiration		
Effect	*df*	*F*	*P*	*df*	*F*	*P*
Treatment	**1,21**	**7.07**	**0.015**	1,21	0.32	0.58
Saturated	**1,21**	**35.06**	**<0.0001**	**1,21**	**57.27**	**<0.0001**
Treatment × Saturated	1,21	3.59	0.07	**1,21**	**8.84**	**0.007**

Factor Saturated represents the effect of leaf saturation by water on the hemiparasite parameters. Significant terms (P<0.05) are in bold. df: degrees of freedom; F: F statistics; p: significance level.

S5 Tab. ANOVA table of linear models describing the effects of irrigation treatment, plant species, and their interaction on the stable-isotopic composition ($\delta^{13}C$ and $\delta^{18}O$) of host (*Triticum aestivum*) and parasite (*Rhinanthus alectorolophus)* above-ground biomass.

	$\delta^{13}C$			$\delta^{18}O$		
Effect	*df*	*F*	*P*	*df*	*F*	*P*
Treatment	**1,18**	**766.12**	**<0.0001**	**1,18**	**4.76**	**0.043**
Plant	**1,18**	**12.73**	**0.002**	**1,18**	**8.87**	**0.008**
Treatment × Plant	1,18	0.07	0.80	1,18	1.80	0.20

Factor Plant represents the effect of plant species on isotopic parameters. $\delta^{13}C$ and $\delta^{18}O$ represent the isotopic composition of plant biomass. Significant terms (P<0.05) are in bold. df: degrees of freedom; F: F statistics; p: significance level.

S6 Tab. ANOVA table of linear models describing the effects of irrigation treatment and sampling place (leaf/bract) on the density of stomata on abaxial and adaxial leaf and bract sides of parasitic *Rhinanthus alectorolophus.*

	Stomatal density (adaxial)			Stomatal density (abaxial)		
Effect	*df*	*F*	*P*	*df*	*F*	*P*
Treatment	1,16	0.21	0.65	1,15	3.25	0.09
Leaf/bract	**1,131**	**23.49**	**<0.0001**	**1,116**	**53.85**	**<0.0001**

Factor Leaf/bract represents the effect of sampling place on stomatal density. Significant terms (P<0.05) are in bold. df: degrees of freedom; F: F statistics; p: significance level.

Summary of results

The introduction of this thesis summarized the current knowledge on the effects of abiotic factors on hemiparasitic plants with highlighting the gaps in the literature and suggesting potential future directions in the field. The following first author articles broadened this knowledge by providing new insights into the ecophysiology of the selected rhinanthoid root hemiparasites.

Chapter 1 provided experimental evidence on the role of hydathode trichomes in active water secretion that was suggested a century ago for several rhinanthoid hemiparasites. The hydathodes presented on the abaxial leaf side of *Rhinanhus alectorolophus* were found to be particularly active in the juvenile plants. These plants exhibited positive correlation among water secretion, night-time respiration, and transpiration. The presence and activity of hydathode trichomes thus explain earlier observation of correlated night-time transpiration and respiration rates. Active water secretion from hydathode trichomes can be seen as an additional mechanism decreasing the water potential of hemiparasites and hence facilitating the resource uptake from the host.

The resource uptake from host species can be examined by labelling host leaves and detecting the label transfer in the hemiparasite. In **Chapter 2**, I present simple labelling method that can be used to examine the nitrogen flows between the hemiparasite and host. In this chapter, I found out that a single leaf-brushing of the host leaves with $^{15}N^{13}C$-urea is sufficient to verify the root connection with the parasitic *Rhinanthus major*. I showed that the transfer of nitrogen can be detected in the hemiparasite biomass already 3 d after the host labelling and it stabilizes 7 d after the host labelling. Therefore, I recommended to harvest the biomass a week after the labelling.

I focused not only on hemiparasites from open grassland habitats, but also on shade-tolerant hemiparasites from forest understories, such as *Melampyrum pratense.* In **Chapter 3**, I revealed that summer leaves of *Melampyrum* acclimate to decreased light availability under canopy by lowering their saturated photosynthesis and dark respiration compared to spring leaves. Interestingly, light-saturated photosynthesis of these hemiparasites was much higher than that reported for other understory herbs indicating that *M. pratense* still retains the traits typical of related root hemiparasites from open habitats – efficient photosynthesis and high transpiration. However, the models of carbon balance suggest its autotrophic carbon gain to be insufficient in summer months. Therefore, this annual hemiparasitic plant probably relies on heterotrophic carbon acquisition for seed production.

Taking into account water secretion from hydathodes and overall water-wasting physiological strategy of *Rhinanthus* growing in moderately dry habitats, I decided to dedicate **Chapter 4** to *Rhinanthus* survival and physiology under long-term water stress. I documented the negative effect of water stress on seedling survival of *Rhinanthus alectorolophus* as well as the biomass production and gas exchange of the adult hemiparasites. Surprisingly, the wilted adult hemiparasites were still able of considerable photosynthesis and transpiration and low-irrigated plants had elevated photosynthetic rate compared with high-irrigated plants of the same water status. I also found out that full-turgor osmotic potential of the adults was low in comparison to non-parasitic plants from

similar habitats. This might explain a lack of their acclimation in response to water stress. In contrast to results of previous studies, rhinanthoid hemiparasites seem to close their stomata under severe drought stress. They probably close them passively after turgor is lost in guard cells by dehydration rather than actively via ABA signaling pathway. I highlighted the sensitivity of their seedling to drough stress, but also the ability of the adult *Rhinanthus* plants to withstand drought and recover from wilting.

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Personal Information ——————————————————————————

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Education ——————————————————————————————

Department of Botany, Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic

Since March 2013

PhD student of Botany, thesis title: Ecophysiology of root hemiparasites – variability of physiological traits of hemiparasites on environmental gradients (in English), supervisors: Jakub Těšitel and Tomáš Hájek

October 2011 – January 2015

BSc. student of Applied Mathematics, thesis title: Mathematical modelling of the population dynamics of hemiparasitic plants (in English, 32 p.), supervisor: Luděk Berec

October 2010 – February 2013

MSc. in Botany (Vegetation Ecology), thesis title: Impact of local heat leakage on vegetation and participation of non-native species (in English, 61 p.), supervisors: Karel Prach and Stanislav Mihulka

October 2007 – June 2010

BSc. in Biology, thesis title: Vegetation of heat pipelines and participation of invasive species (in Czech, 62 p.), supervisors: Karel Prach and Stanislav Mihulka

Professional experience ——————————————————————————

Employment:

Since 2012

Research worker, Faculty of Science, University of South Bohemia

Research Interests:

- ecophysiology of parasitic plants
- mathematical modelling of the population dynamics of hemiparasitic plants
- vegetation ecology, non-native plants, and polar ecology

Research stays:

2015

Visiting student, Anna Sala lab, University of Montana, Missoula, USA (13.4.-22.5.)

2016

Visiting Erasmus+ student, Ülo Niinemets lab , Estonian University of Life Sciences, Tartu, Estonia (15.6.-15.9.)

Conferences ——————————————————————————

2013

Third symposium on the biology of non-weedy parasitic plants. “Does elevated stomatal conductance provide a photosynthetic gain benefit to hemiparasitic *Melampyrum pratense* in the forest understory environment with sunflecks?”. Namur, Belgium, 12-15 September 2013. Poster presentation.

2014

PEPG Early Career Scientist Minisymposium. “Ecophysiology of *Melampyrum pratense*, a root-hemiparasite growing in forest understory”. Castleton, UK, 13-15 April 2014. Oral presentation.

2015

The 13th World Congress on Parasitic Plants. “The physiological role of hydathode trichomes in parasitic Orobanchaceae”. Kunming, China, 5-10 July 2015. Oral presentation.

Publications

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 (1), 61–68.

Světlíková P, Blažek P, Mühlsteinová R, Těšitel J., 2016. Tracing nitrogen flow in a root-hemiparasitic association by foliar stable-isotope labelling. Plant Ecology and Evolution 149 (1), 39–44.

Světlíková P., Hájek T., Těšitel J., 2018. A hemiparasite in the forest understorey: photosynthetic performance and carbon balance of *Melampyrum pratense*. Plant Biology 20 (1), 50–58.

Světlíková P., Hájek T., Těšitel J., 2018. Water stress physiology of *Rhinanthus alectorolophus*, a root-hemiparasitic plant. (under revision in PLoS ONE).

Awarded grants

2010

Undergraduate research grant from the Student Grant Agency of the Faculty of Science, University of South Bohemia

Teaching

- Selected exercises from Ecology
- Selected exercises from Biological Laboratory Techniques
- Selected field courses for first-year students (Field Work I, II)

Skills

- Extensive experience with basic ecological and ecophysiological fieldwork (Czech Republic, Germany, Georgia, Turkey, Italian and Slovene Alps, and Svalbard) and planning and conducting outdoor and indoor manipulative experiments (10 years)
- Matematical modeling using Matlab (Matcont and Pplane packages)
- Basic experience in R, Inkscape, Gimp and Canoco
- Languages: English (professional working proficiency), German (elementary proficiency), Czech (native proficiency)

Peer-review

Acta Botanica Brasilica, Functional Plant Biology