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Article (Published Version)

Rowntree, Jennifer K, Fisher Barham, David, Stewart, Alan J A, Hartley, Sue E and Watling, Jenny (2014) The effect of multiple host species on a keystone parasitic plant and its aphid herbivores. Functional Ecology, 28 (4). pp. 829-836. ISSN 0269-8463

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The effect of multiple host species on a keystone parasitic plant and its aphid herbivores

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Summary

1. The exploitation of shared resources by diverse organisms underpins the structure of ecological communities. Hemiparasitic plants and the insect herbivores feeding on them both rely, directly and indirectly, on the resources supplied by the parasite’s host plant. Therefore, the identity and number of host plant species providing these resources is likely to be critical for parasite and herbivore performance.

2. We tested the effect of single and multiple host species on the biomass of the generalist parasitic plant Rhinanthus minor and the abundance of its aphid (Aphis gossypii) herbivores.

3. Parasite biomass was proportional to the number of haustorial connections to host roots and was determined by host species identity rather than host functional group. Host species identity was also an important influence on aphid population size, and parasites attached to Lotus corniculatus experienced a considerable reduction in aphid herbivory.

4. The effects on the parasite attaching to multiple hosts depended on the combination of species present. However, host mixtures generally benefitted aphids by diluting the negative effects of particular host species.

5. Our findings suggest that the specificity of host attachment alters the impact of this keystone parasitic plant on its own herbivores and, potentially, on the wider plant and herbivore community.

Key-words: Aphis gossypii, direct and indirect effects, herbivory, Lotus corniculatus, mixed hosts, Rhinanthus minor

Introduction

Parasitic plants are present, often in high abundance, in many ecosystems where they affect not only their hosts, but indirectly impact on many other organisms. They have been shown to have major effects on the structure and function of ecological communities (Press & Phoenix 2005), including changing plant community diversity and structure (Joshi, Matthies & Schmid 2000; Ameloot, Verheyen & Hermy 2005), influencing nutrient cycling (Quested, Press & Callaghan 2003; Fisher et al. 2013), altering soil microbial communities (Bardgett et al. 2006) and affecting the performance of invertebrate herbivores (Marvier 1996; Ewald, John & Hartley 2011).

Rhinanthus minor is a generalist hemiparasitic plant (Gibson & Watkinson 1989). It is a widespread component of grasslands throughout Europe and North America (Westbury 2004) and exerts much of its influence by dramatically reducing host plant biomass (Cameron et al. 2005). Like many other parasitic plants, R. minor establishes cellular continuity with the xylem stream of its hosts via specialized organs (haustoria), thereby extracting nutrients (Kuijt 1969; Riopel & Timko 1995). The severity of the impact of R. minor on its hosts, and its consequent community level effects, depends, at least in part, on host species identity and the ability of the host to tolerate or resist infection (Gibson & Watkinson 1991; Cameron, Coats & Seel 2006; Rowntree, Cameron & Preziosi 2011).

Just as R. minor has differential effects on host species, hosts can be more, or less, beneficial to the parasite. Variation in the performance of R. minor appears to result largely from how well the host plant can defend its xylem stream from the hemiparasite (Cameron, Coats & Seel 2006; Cameron & Seel 2007), and to variation in the types and amounts of solutes that the hemiparasite can remove from its host (Seel, Cooper & Press 1993; Press 1995), although host growth rate is also a determining factor (Hautier et al. 2010). In general, legumes and grasses are
thought to be ‘good’ hosts for *R. minor* while non-leguminous forbs are regarded as ‘poor’ hosts (Seel, Cooper & Press 1993; Seel & Press 1993; Cameron, Coats & Seel 2006), but thus far, only a limited range of potential host species has been tested in terms of impact on parasite performance.

Host and parasitic plants do not interact with each other in isolation. Host plants can indirectly influence parasite herbivores (e.g. Marvier 1996; Adler 2002), while parasitic plants indirectly affect host plant herbivores (Ewald, John & Hartley 2011). The performance of invertebrate herbivores is dependent on the nutritional quality of the plant on which they are feeding (Douglas 1993), as well as the plant’s physical (Hanley et al. 2007) and chemical (Bennett & Wallsgrove 1994) defences. As the biomass and nutritional quality of hemiparasites depends on the host species they are attached to (Seel, Cooper & Press 1993; Seel & Press 1993), it follows that the parasite’s host plant may also determine the success of parasite-feeding herbivores (Adler 2002).

As a parasite with the ability to infect and utilize numerous host species simultaneously, it is unlikely that *R. minor* will be attached to a single host species in the field (Gibson & Watkinson 1989). Parasites attached to multiple host species can gain different nutritional components from each host (Govier, Nelson & Pate 1967) and may also receive protection against multiple environmental stressors (Pate et al. 1990), including herbivores (Marvier 1998). Therefore, a mixed host or ‘generalist’ strategy has the potential to benefit the parasite, particularly in the presence of herbivores. However, the extent of such benefits has not been tested, and to our knowledge, this is the first experimental study where the effects of different multiple host combinations on the performance of the parasite and its herbivores have been identified. Investigating the effects of these complex multitrophic interactions on the performance of the organisms concerned will enable us to understand more fully the key role that parasitic plants can play in structuring terrestrial communities and regulating their dynamics.

We used a series of glasshouse experiments to understand the consequences of a generalist host strategy by a keystone parasitic plant, *R. minor*, for itself and other associated organisms. We used a range of host species alone, and in combination, to test the effects of host identity on the performance of the parasite and an aphid herbivore feeding upon it. First, we investigated the effect of individual host species on the parasitic plant and predicted that host functional group would be more important than host species identity in determining the performance of the parasitic plant. Next, we investigated the effect of single host species on the performance of aphid herbivores feeding on the parasite and predicted that host identity would indirectly influence the success of the parasite’s aphid herbivores. Finally, using the results from the first two experiments to inform the choice of host species used, we investigated the effects of the parasite attaching to multiple host species on its aphid herbivores. We predicted that aphid performance would depend on the combination of host species used, with the cumulative effect on aphids reflecting the balance of ‘good’ and ‘bad’ hosts within the mixture.

**Materials and methods**

**EXPERIMENT 1: EFFECT OF HOST SPECIES ON RHINANTHUS MINOR**

We collected and dried at room temperature *R. minor* L. seed from Castle Hill National Nature Reserve in East Sussex (UK OS grid ref: TQ 370 070; WGS84 Lat-long: 50°50’ 46-7126”, −0°3’ 19-2953”). We purchased host plant seeds from Emorsgate Seeds, King’s Lynn, Norfolk. Host plant species were selected on the basis of their presence at Castle Hill and fall into three functional groups: legumes (*Lotus corniculatus* L., *Ononis repens* L. and *Trifolium pratense* L.); non-leguminous forbs (*Achillea millefolium* L., *Plantago lanceolata* L. and *Sanguisorba minor* Scop.) and grasses (*Briza media* L., *Dactylis glomerata* L. and *Holcus lanatus* L.).

We surface-sterilized *R. minor* seeds using 5% household bleach for 60 s, rinsing four times with sterile water. Seeds were placed onto 9 cm Petri dishes containing damp sterile filter paper and capillary matting, which were sealed with parafilm and placed at 4 °C for 84 days. Host plants were germinated on damp vermiculite 28 days after sterilization of the *R. minor* seed. After a further 14 days, we transplanted host plant seedlings into 9 cm pots (one per pot) containing six parts sand and one part John Innes No. 2 compost.

Forty-two days after transplanting host plants, we added five germinating *R. minor* seeds into each pot. Hemiparasite seedlings were thinned to one per pot when the majority showed signs of attachment (see Klaren & Janssen 1978 for details) approximately 21 days after transplanting. This gave us one host plant and one parasitic plant in each replicate pot. All plant material was grown in glasshouses at the University of Sussex (15–25 °C with supplementary lighting (16 : 8 light : dark). Experimental pots were supplied with tap water *ad libitum* and treatments arranged randomly within blocks on benches. We harvested above-ground plant material when the first parasitic plants began to show signs of senescence (at 110 days). All plant materials were dried at 60 °C for 2 days and weighed. Roots were washed, and the number of haustoria counted using a binocular dissecting microscope. Fifteen replicates were set up for each species (*n* = 135), but not all *R. minor* plants survived until the end of the experiment. See Table S1 (Supporting information) for final numbers of replicates.

**EXPERIMENT 2: EFFECT OF HOST SPECIES ON RHINANTHUS MINOR APHID HERBIVORES**

Host and parasitic plant sources and preparation were as in Experiment 1. We collected the generalistmelon aphid *Aphis gossypii* from plants of *R. minor* previously grown at the University of Sussex. We reared cultures of aphids on non-experimental *R. minor* plants grown in trays containing two of the potential hosts (*T. pratense* and *H. lanatus*), providing the aphids with prior indirect exposure to these hosts. Seventy days after the germinating *R. minor* seeds were introduced to the pots, we added 20 adult aphids onto each of the hemiparasites and covered these with a cage. Cages were constructed from a transparent plastic pipe (20 cm height by 4.5 cm diameter) with a window (4 cm by 2 cm) cut in the side to facilitate airflow. The ends of the tubes and the windows were sealed with a fine mesh. Cages allowed the aphids...
to move freely around the parasitic plants, but prevented them from colonizing the host plants. Aphids were left to multiply for 14 days and counted. Then, we harvested the above-ground portions of all plants. All plant material was dried at 60 °C for 2 days and weighed. We determined nitrogen-to-carbon (N : C) ratios of the above-ground biomass of *R. minor* from approximately 1.5 mg of ground, homogenized plant material using an elemental combustion system (Costech Instruments, Milan, Italy) and calibrated against a standard compound (C$_{60}$H$_{30}$N$_{20}$O$_{30}$S). We planted 20 replicates per treatment (*n* = 180), but not all *R. minor* plants survived until the end of the experiment. See Table S1 for final replicate numbers. We calculated N : C ratios rather than the natural log transformed prior to analysis. Data were analysed using a linear mixed model (REML) with *R. minor* biomass as the response variable; block as a random factor; haustorial number as a continuous factor; host functional group and host species nested within host functional group as fixed factors. Differences among host species were explored with post hoc Tukey tests with significance values set at *P* = 0.05. Host biomass was not included in the model as initial analysis showed it to have no significant effect, and the model fit was improved with its removal (AICc 283–95 vs. 286–98).

**Experiment 2**

Aphid abundance and *R. minor* biomass were natural log transformed prior to analysis. Data were analysed using a linear mixed model (REML) with aphid abundance as the response variable; block as a random factor; *R. minor* biomass as a continuous factor; and host species as a fixed factor. N : C ratio (Table S3, Supporting information) and host functional group were not included in the model as initial analyses showed them to have no significant effect and model fit was improved with their removal (AICc 327–17 vs. 333–75). Differences among host species were explored with post hoc Tukey tests with significance values set at *P* = 0.05.

**Experiment 3**

For each host functional group – *L. corniculatus* combination, *R. minor* biomass was natural log transformed prior to analysis. Aphid abundance data were analysed using separate linear mixed models (REML) with aphid abundance as the response variable; block as a random factor; *R. minor* biomass as a continuous factor; and host combination as a fixed factor. *Rhinanthus minor* biomass data were analysed using separate linear mixed models (REML) with *R. minor* biomass as the response variable; block as a random factor; and host combination as a fixed factor. Differences among host combinations were explored with post hoc Tukey tests with significance values set at *P* = 0.05. All statistical analyses were carried out using *SAS* Institute Inc., Cary, NC, USA, 1989–2007).

**Results**

**Experiment 1**

The fitted model explained 58% of the variation in the *R. minor* biomass data (*R*$_{adj}^2$ = 0.58). Of this, 0.07% of the variation was attributed to block. There was a significant positive relationship between haustorial number and *R. minor* biomass (*F*$_{1,95.52}$ = 47.07, *P* < 0.0001; Fig. 1a). Biomass was also significantly affected by host functional group (*F*$_{2,38.34}$ = 5.47, *P* = 0.0057) and by host species nested within functional group (*F*$_{6,88.54}$ = 5.00, *P* = 0.0002). On average among functional groups, biomass of *R. minor* grown on legumes was significantly greater than when the plants were attached to non-leguminous forbs (*P* < 0.05), but there were no significant differences between grass hosts and legumes or grass hosts and non-leguminous forbs (*P* > 0.05; Fig. 1b). There was considerable variation in the quality of species as hosts for *R. minor* that did not reflect the average response of the functional groups. Although the highest biomass of *R. minor* was achieved when attached to the legume *L. corniculatus*, the only hosts on which the parasite biomass was significantly lower than these were the grass...
B. minor and the non-leguminous forb P. lanceolata ($P < 0.05$). The lowest biomass of R. minor was achieved by the parasites with P. lanceolata as a host, but there were no significant differences between these plants and those attached to the legumes O. repens and T. pratense, the grass B. media, or the non-leguminous forb A. millefolium ($P > 0.05$; Fig. 1c).

**EXPERIMENT 2**

The fitted model explained 60% of the variation in the aphid abundance data ($R^2_{adj} = 0.60$). Of this, 10% of the variation was attributed to block. There was a significant positive relationship between aphid abundance and

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**Fig. 1.** (a) Rhinanthus minor biomass plotted against haustorial number for all plants. Natural log scaled axes are used to demonstrate the relationship between the model response variable and covariate. The analysis showed a significant positive relationship between R. minor biomass and haustorial number ($P < 0.0001$) (b) Back-transformed least squares means of R. minor biomass when grown without aphids on non-leguminous forb [F, filled bars], grass [G, hatched bars] and legume [L, open bars] hosts. Error bars are 95% confidence intervals. Different letters on the bar graphs denote significant differences among treatments calculated from post hoc Tukey tests ($P < 0.05$).

**Fig. 2.** (a) Aphid abundance per Rhinanthus minor plant plotted against R. minor biomass. Natural log scaled axes are used to demonstrate the relationship between the model response variable and covariate. The analysis showed a significant positive relationship between aphid abundance and R. minor biomass ($P < 0.0001$). (b) Back-transformed least squares means of aphid abundance per R. minor plant grown on nine species of host plants. Filled bars show the non-leguminous forbs Achillea millefolium [AM], Plantago lanceolata [PL] and Sanguisorba minor [SM]. Hatched bars show the grasses Briza media [BM], Dactylis glomerata [DG] and Holcus lanatus [HL]. Open bars show the legumes Lotus corniculatus [LC], Ononis repens [OR] and Trifolium pratense [TP]. Error bars are 95% confidence intervals. Different letters on the bar graphs denote significant differences among treatments calculated from post hoc Tukey tests ($P < 0.05$).
R. minor biomass ($F_{1,138} = 47.59$, $P < 0.0001$; Fig. 2a). There was also a significant effect of host species on aphid abundance ($F_{8,123.9} = 13.94$, $P < 0.0001$). The main species level effect was caused by L. corniculatus, as aphid abundance was significantly reduced when this was the host of R. minor compared with all other host species ($P < 0.005$). Aphid abundance on R. minor attached to H. lanatus and A. millefolium was also significantly reduced compared with numbers on the parasites with O. repens hosts ($P < 0.05$; Fig. 2b).

Colour changes in the picrate papers confirmed the presence of HCN in the L. corniculatus plants, but no HCN was detected from the R. minor attached to these hosts.

**EXPERIMENT 3**

When the legume O. repens was the partner species to L. corniculatus, the model fitted to aphid abundance explained 41% of the variation in the data ($R^2_{adj} = 0.41$). Of this, 4-6% of the variation was attributed to block. There was a significant positive relationship between aphid abundance and R. minor biomass ($F_{1,42.06} = 9.70$, $P = 0.003$; Fig. 3a) and a significant effect of host combination ($F_{2,33.41} = 1.99$, $P < 0.0001$). Aphid abundances on R. minor were significantly different between all host combinations ($P < 0.05$), with the highest number of aphids on the R. minor growing only on O. repens and the least number of aphids on the R. minor growing only on L. corniculatus (Fig. 3b). The model fitted to R. minor biomass explained 34% of the variation in the data ($R^2_{adj} = 0.34$). Of this, 21% of the variation was attributed to block. There were no significant effects of host combination on R. minor biomass ($F_{2,27.96} = 2.17$, $P = 0.13$; Fig. 4a).

When the grass D. glomerata was the partner species to L. corniculatus, the model fitted to aphid abundance explained 70% of the variation in the data ($R^2_{adj} = 0.70$). Of this, 21% of the variation was attributed to block. There was no significant relationship with R. minor biomass ($F_{1,48.36} = 1.56$, $P = 0.22$; Fig. 3c), but there was a significant effect of host combination ($F_{2,34.54} = 40.47$, $P < 0.0001$). Removing R. minor biomass did not improve the model fit. Aphid abundances on R. minor were significantly different between all host combinations ($P < 0.05$) with the highest number of aphids on the R. minor growing only on D. glomerata and the least number of aphids on the R. minor growing only on L. corniculatus (Fig. 3d). The model fitted to R. minor biomass explained 41% of the variation in the data ($R^2_{adj} = 0.41$). Of this, 13% of the variation was attributed to block. There was a significant effect of host combination on R. minor biomass ($F_{2,25.45} = 10.93$, $P = 0.0002$). Biomass for the mixed host combination was significantly greater than for the single

![Fig. 3](image-url) (a, c, e) Aphid abundance plotted against Rhinanthus minor biomass. The x-axis is natural log scaled to demonstrate the relationship between the model response variable and covariate. Different host combinations are shown by circles [a] Ononis repens; (c) Dactylis glomerata; (e) S. minor, crosses [mixed hosts] and triangles [Lotus corniculatus]. Analyses showed a significant positive relationship between aphid abundance and R. minor biomass for host O. repens (a; $P = 0.003$), no significant relationship between aphid abundance and R. minor biomass for host D. glomerata (c; $P = 0.22$) and a significant negative relationship between aphid abundance and R. minor biomass for host S. minor (e; $P = 0.03$). (b, d, f) Least squares means of aphid abundance per R. minor plant when grown on single or mixed host treatments. Host plants are (a, b) O. repens [O] and L. corniculatus [L]; (c, d) D. glomerata [D] and L. corniculatus [L]; (e, f) Sanguisorba minor [S] and L. corniculatus [L]. Error bars are 95% confidence intervals. Different letters on the bar graphs denote significant differences among treatments calculated from post hoc Tukey tests ($P < 0.05$).
Fig. 4. Back-transformed least squares means of *Rhinanthus minor* biomass when grown on single or mixed host treatments. Host plants are (a) *Ononis repens* [O] and *Lotus corniculatus* [L]; (b) *Dactylis glomerata* [D] and *L. corniculatus* [L]; (c) *Sanguisorba minor* [S] and *L. corniculatus* [L]. Error bars are 95% confidence intervals. Different letters on the bar graphs denote significant differences among treatments calculated from post hoc Tukey tests ($P < 0.05$).

Discussion

This study is the first assessment of how attachment to different combinations of multiple host species affects both *R. minor* and its associated aphid herbivores. Our data show that when attached to a single host, *R. minor* biomass was best explained by the number of haustorial attachments to the host rather than the size, or biomass, of the host plant. While it is known that not all haustoria produce functional attachments (Cameron & Seel 2007), our results demonstrate that the investment in such structures by the parasite reflects the quality of the host. They also support the idea that differential resistance among a variety of host species across functional groups (Cameron, Coats & Seel 2006; Cameron & Seel 2007) is related to the number and effectiveness of haustorial connections in addition to any effects of host growth rate (Hautier et al. 2010). Certainly, haustorial connections were a more important determinant of host quality for the parasitic plant in our study, since host biomass had no influence on *R. minor* performance.

There was considerable variation in host suitability at the species level, which was not predictable from the species’ functional groups. For example, although when analysed at the level of functional group, legumes and grasses were better hosts for *R. minor* than non-leguminous forbs (see also Seel, Cooper & Press 1993; Seel & Press 1993); when species level effects were considered, *R. minor* actually performed no worse on the non-leguminous forbs *S. minor* and *A. millefolium* than on the best host the legume *L. corniculatus*. In fact, the poor performance of *P. lanceolata* as a host, which has been previously well documented (Cameron, Coats & Seel 2006; Cameron & Seel 2007), is the predominant factor in reducing the performance of the non-leguminous forbs as hosts overall. Similarly, the performance of the functional group legumes as hosts is predominantly influenced by the high biomass attained when *R. minor* is growing on *L. corniculatus*, while the other legumes, *O. repens* and *T. pratense*, were no better hosts than *P. lanceolata*. The grasses *H. lanatus* and *D. glomerata* were as good hosts as *L. corniculatus*, but overall, the grasses were no better as
hosts than the non-leguminous forbs and no worse than the legumes. In light of this species level variation, we caution against making generalizations of *R. minor* host performance based only on information about plant functional group.

Aphid abundances feeding on *R. minor* attached to a single host increased with *R. minor* biomass, but were not affected by the N : C ratio of the parasite. This suggests that the size of the available resource rather than its quality, at least when expressed in relation to nitrogen, is of greater importance to aphid fitness. Host functional group was not important in determining aphid abundance, but host species was. The aphids used in these experiments had prior exposure to *R. minor* attached to two of our host species (*T. repens* and *H. lanatus*). It is possible, although not necessarily the case (Via 1991), that prior exposure enabled the aphids to better tolerate any negative effects of these hosts. Numbers of aphids were reduced on *R. minor* attached to *H. lanatus* and *A. millefolium* compared with those attached to *O. repens*, but by far, the greatest effect was seen when the parasite was attached to *L. corniculatus*. On these plants, numbers of aphids were considerably reduced compared with all other host species, possibly because of antiherbivore secondary metabolites associated with this species. Previous studies on other parasitic plant species have demonstrated that compounds with antiherbivory properties can be transferred from the host to the parasitic plant (Marvier 1996; Adler & Wink 2001). In *L. corniculatus*, cyanogenic glycosides are the principle toxic metabolites, known to be effective against herbivores (Scriber 1978). These interact with degradation enzymes within the plant to release HCN when plants are under attack. The picrate assay that we used detects the release of HCN as a result of this degradation process, and, while we did detect this compound in *L. corniculatus*, there was no evidence of HCN production in the parasitic plant. We cannot, however, rule out the transfer of cyanogenic glycosides themselves between the host and parasite as it is possible that the compounds were present in *R. minor*, but not degraded to release HCN.

Our third experiment investigated the relationship between host species, in particular *L. corniculatus*, and *R. minor* aphid herbivores, by testing mixtures of species including *L. corniculatus* against single host plantings. For all host combinations tested, aphid abundance decreased as the proportion of *L. corniculatus* plants increased. While this does not reveal the specific mechanisms by which the host plants were influencing the parasite's aphid herbivores, it does demonstrate that *L. corniculatus* confers resistance to herbivores on *R. minor*. The most likely mechanism for this is via the transfer of secondary metabolites across the haustoria (Adler & Wink 2001), although we found no evidence of this mechanism here (see above). These results also suggest that the propensity of *R. minor* to attach to multiple host species in the field is likely to benefit the parasite's herbivores by diluting any negative effects of particular host species.

When attached to multiple host species, the relationship between *R. minor* biomass and aphid abundance breaks down and depends entirely on host species identity. When *O. repens* was paired with *L. corniculatus*, larger *R. minor* plants supported greater numbers of aphids, but when *S. minor* and *L. corniculatus* were paired as hosts, smaller plants supported more aphids. This discrepancy in the impact of host combinations is likely due, in part, to the negative effect of *L. corniculatus* on the aphids, but also its suitability as a host for *R. minor*.

Attachment to multiple host species had little discernible effect on *R. minor* biomass, except when *D. glomerata* was paired with *L. corniculatus*. In this case, the parasite was larger when attached to both host species compared with when it was only attached to the grass. Previous work has produced contradictory conclusions on whether attachment to a single host species (Matthies 1996) or multiple host species (Marvier 1998) is the most beneficial for parasitic plants. Our results suggest that the host identity within mixtures is likely to be critical in explaining these apparent contradictions on *R. minor* performance. Further, the responses of *R. minor* to host mixtures did not predict the response of the aphids since the relationship between *R. minor* biomass and aphid abundance is different for each combination of hosts.

In conclusion, host species identity has considerable effects on the performance of both *R. minor* and its aphid herbivores, and this variation between species cannot be explained solely by plant functional group. Furthermore, this effect holds for interactions with single and multiple host species. Investment in haustorial structures is a good indication of host suitability for the parasite, while parasite biomass is a good predictor of the size of aphid populations feeding on *R. minor*, but only when the parasite is attached to a single host. When attached to multiple hosts, the relationship between host plant and parasite herbivore is more complex. The ‘generalist’ nature of *R. minor* is likely to benefit its aphid herbivores by diluting any negative effects of a single host species thereby significantly influencing herbivore population dynamics. With this work, we demonstrate that the interactions between the keystone parasitic plant *R. minor* and its hosts extend beyond the plants themselves and have significant consequences for the wider ecological community.

**Acknowledgements**

We thank Dr. Richard Harrington at Rothamsted Research for confirming the identity of the aphids and Natural England for permission to collect seeds. We thank Prof. Malcom Press, Dr. Hehn Jones and Prof. Richard Bardgett for comments on earlier drafts of the manuscript. DFB was supported by a NERC PhD studentship (NER/S/A/2006/14074), JKR by a NERC postdoctoral fellowship (NE/H016821/2-3) and SEH by a NERC standard research grant (NE/DO12333/1).

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Received 6 November 2013; accepted 14 March 2014

Handling Editor: Jenny Watling

Supporting Information

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

Table S1. Treatments and replicate numbers for Experiment 1 and 2.

Table S2. Treatments and replicate numbers for Experiment 3.

Table S3. *Rhinanthus minor* N : C values from Experiment 2.