

# Chapter 5 - Life history evolution, species differences, and phenotypic plasticity in hemiparasitic eyebrights (*Euphrasia*)

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## Abstract

Species delimitation in parasitic organisms is challenging because traits used to identify species are often plastic and vary depending on the host. Here, we use species from a recent radiation of generalist hemiparasitic *Euphrasia* to investigate trait variation and trait plasticity. We tested whether *Euphrasia* species show reliable trait differences, investigated whether these differences correspond to life history trade-offs between growth and reproduction, and quantified plasticity in response to host species. We used common garden experiments to evaluate trait differences between 11 *Euphrasia* taxa grown on a common host, document phenotypic plasticity when a single *Euphrasia* species is grown on eight different hosts, and relate observations to trait differences recorded in the wild. *Euphrasia* exhibited variation in life history strategies; some individuals transitioned rapidly to flowering at the expense of early season growth, while others invested in vegetative growth and delayed flowering. Life history differences were present between some species, though many related taxa lacked clear trait differences. Species differences were further blurred by phenotypic plasticity — many traits were plastic and changed with host type or between environments. Trait differences present between some species and populations demonstrate the rapid evolution of distinct life history strategies in response to local ecological conditions.

## Introduction

Parasitism is a ubiquitous feature of the natural world, with parasitic organisms found living in every ecosystem and exploiting all free-living organisms (Price 1980; Windsor 1998). Parasitic plants are a group of ca. 4500 species of 12 separate evolutionary origins that have evolved a modified feeding organ, the haustorium, which allows them to attach to a host plant and extract nutrients and other compounds (Westwood et al. 2010; Ownley and Trigiano, n.d.; Twyford 2018). Parasitic plants are morphologically diverse and present a broad range of life history strategies and host interactions (Schneeweiss 2007; Tesitel et al. 2010). Hemiparasitic plants, i.e., those that are parasitic but also photosynthesise, can often attach to a broad range of hosts; the well-studied grassland parasite *Rhinanthus* has been found to attach to over 50 co-occurring grass and herbaceous species (Cameron, Coats, and Seel 2007). All hemiparasitic plants are exoparasites; leaves, stems, roots, and flowers grow outside the host, and only the haustorium invades and grows within the host (Twyford 2017).

Research to date has largely focused on three aspects of life history variation in parasitic plants. First, a body of work has looked to understand variation for specific traits between populations and related species. For example, work on natural populations of the hemiparasite *Pedicularis* has shown how investment in male reproductive organs depends on extrinsic environmental conditions (Guo, Mazer, and Du 2010a), while seed mass is primarily determined by intrinsic factors such as plant size rather than extrinsic factors such as elevation (Guo, Mazer, and Du 2010b). Second, researchers have investigated how parasite life history traits are affected by interactions with their host. In the widespread and weedy obligate holoparasite *Phelipanche*

*ramosa*, the duration of the life cycle differs depending on the host (Gibot-Leclerc et al. 2013), with a more rapid life cycle on local rather than non-local hosts. In hemiparasitic *Rhinanthus minor*, biomass depends on the host species and the number of haustorial connections (Rowntree et al. 2014). Finally, a number of studies have looked at life history variation between species studied in a phylogenetic context (Schneeweiss 2007; Tesitel, Plavcova, and Cameron 2010). For example, broad-scale analyses of the Rhinanthaeae clade in the Orobanchaceae has shown a shift from a perennial ancestor to annuality, with correlated shifts to a reduced seed size (Tesitel et al. 2010). Despite the diversity of this research, there are still considerable gaps in our knowledge as to how life history trait variation is maintained (e.g., how common are trade-offs between life history traits), how much of this variation is genetic and how much is plastic, and which traits are the targets of natural selection.

In this study, we explore trait variation in generalist hemiparasitic eyebrights (*Euphrasia*, Orobanchaceae). *Euphrasia* is one of the largest genera of parasitic plants and is characterized by recent transoceanic dispersal and rapid species radiations (Gussarova et al. 2008). In the United Kingdom, there are 21 *Euphrasia* species, which are mostly indistinguishable at DNA barcoding loci (Wang et al. 2018), show complex morphological variation (Yeo 1968; Metherell and Rumsey 2018), and readily hybridise (Liebst 2008; Stace, Preston, and Pearman 2015). Despite shallow species differences due to postglacial divergence, *Euphrasia* species demonstrate substantial ecological divergence, with many taxa restricted to specific habitats such as coastal turf, mountain scree, heathland, or open grassland. Habitat differences would be expected to exert strong selection on life history traits, and this may include selection on growth to match seasonal water availability and to exploit local hosts, or selection on flowering time in response to local competition from surrounding plants, or in response to mowing or grazing (Hellstrom et al. 2004).

Our research builds on a large body of experimental work, with *Euphrasia* used in common garden studies for over 125 years (Koch 1959). The first experimental work on *Euphrasia* revealed that phenotypic differences between two related species, *E. rostkoviana* and *E. montana*, are maintained in a common garden environment (Wettstein 1895). Experimental work in the 1960s showed the growth of various *Euphrasia* species differs depending on the host species (Wilkins 1963; Yeo 1964). More recent experiments using large sample sizes in common gardens (Matthies 1998; Zopfi 1998; Lammi, Siikamaki, and Salonen 1999: @RN947) or in experimental field sites (Seel and Press 1994; Hellstrom et al. 2004) have shown the effect of commonly encountered hosts such as grasses and legumes on hemiparasite biomass, mineral accumulation, plant architecture and reproductive output. Despite this extensive experimental work, studies in *Euphrasia* have yet to compare life history strategies of different species and the extent of phenotypic plasticity in life history traits. This work is critical for improving our knowledge of hemiparasite evolution and for understanding the nature of species differences in a taxonomically complex group. It is also unclear whether *Euphrasia* are restricted to growing on hosts such as grasses and herbaceous species or can parasitize a broad range of taxa including novel hosts rarely encountered in the wild. To address these questions requires simultaneously investigating the growth of multiple *Euphrasia* species and multiple host species with sufficient replication to enable suitable statistical comparisons.

Here, we used a series of common garden experiments, in conjunction with field observations, to understand life history trait evolution, species differences, and phenotypic plasticity in hemiparasitic *Euphrasia*. Our first experiment assessed the morphological distinctiveness among several *Euphrasia* species and their hybrids when grown on a single host species in standardised common garden conditions. This experiment also addressed whether there is life history trait divergence among recently diverged hemiparasite species and whether these trait differences correspond to life history trade-offs. We then inspected the plasticity of a single focal *Euphrasia* population grown on many different hosts. This experiment quantified the magnitude of trait change when *Euphrasia* are grown on different hosts. It also tested whether they are truly generalist parasites by observing their growth on a wide range of hosts and without a host. Finally, we related our trait observations in a common garden to records of herbarium specimens collected in the wild. This comparison will help us understand whether life history traits and species' morphological differences are consistent between the

common garden and the wild. Overall, our joint observations of phenotypic variation between closely related taxa and the extent of host-induced plasticity within a species, in an experiment and in the wild, provide new insights into variation in life history strategies in these hemiparasitic plants.

## Materials and Methods

### Experimental design and plant cultivation

We performed two common garden experiments to investigate phenotypic variation in *Euphrasia*. Both common garden experiments took place in parallel in 2016. The experiments used wild-collected, open-pollinated *Euphrasia* seeds that were pooled across individuals in a population. Seeds were contributed by plant recorders as part of the Eye for Eyebrights (E4E) public engagement project and as such included a scattered geographic sample across Great Britain (Appendix S1). All *Euphrasia* species were identified from the herbarium specimens of field collections, and from living material grown in the glasshouse, by *Euphrasia* referee Chris Metherell. Host seeds were sourced from commercial suppliers and from field collections (Appendix S2).

### Species differences experiment

We observed trait differences of 24 populations from five *Euphrasia* species and six natural *Euphrasia* hybrids when grown on clover (*Trifolium repens*). This experiment included sampling multiple populations of three widespread and closely related grassland species, *E. arctica*, *E. confusa*, and *E. nemorosa*, and sparse population sampling of the moorland specialist *E. micrantha* (one population) and calcareous grassland specialist *E. pseudokernerii* (two populations). We chose clover as a host because it usually supports vigorous hemiparasitic growth and confers high survival (Zopfi 1998).

### Phenotypic plasticity experiment

We measured traits of a focal *Euphrasia* taxon, *E. arctica*, when grown with eight potential hosts (*Arabidopsis thaliana*, *Equisetum arvense*, *Festuca rubra*, *Holcus lanatus*, *Marchantia polymorpha*, *Pinus sylvestris*, *Plantago lanceolata*, and *Trifolium repens*) and without a host. These hosts were chosen to include a broad representation of functional groups and phylogenetic diversity, with species encountered in the wild and with novel hosts (full details in Appendix S2). The novel hosts were included to see the limits to which parasitic *Euphrasia* can associate, namely with a tree (*Pinus*), a pteridophyte that produces adventitious roots (*Equisetum*), and a liverwort that produces rhizoids (*Marchantia*).

### Cultivation protocol

Reliable cultivation of *Euphrasia* can be challenging due to low seed germination, variation in time to establishment, the requirement of seed stratification, and high seedling mortality when transplanted (Yeo 1961; Zopfi 1998). We developed cultivation protocols that combine winter germination cues that improve germination and mimic nature, but also used highly standardised and replicated pot conditions that avoid transplanting *Euphrasia* and thus maximise survivorship. We filled 9-cm plastic pots with Melcourt Sylvamix Special growing medium (Tetbury, Gloucestershire, UK) in December, placed one *Euphrasia* seed per pot, and left pots outside over winter at the Royal Botanic Garden Edinburgh (RBGE) for seeds to experience natural seed stratification. Hosts were planted in seed trays in April. *Euphrasia* plants were moved to an unheated and well-ventilated greenhouse in the spring once the cotyledons were fully expanded, and a single

seedling from each host (or a 1-cm<sup>2</sup> clump of *Marchantia*) was transplanted into the pot containing *Euphrasia*. Hosts that died within 10 days of planting were replaced. Twenty or more replicates were grown for each host–parasite combination. Plants were subsequently grown to flowering with regular watering, the locations of pots randomised at weekly intervals, and foreign weed seedlings removed.

## Common garden trait measurements and statistical analyses

We measured seven morphological traits at first flowering related to life history variation, indicators of plant vigor, or characters used in taxonomy. In addition to date of first flowering, we recorded corolla length, the ratio of cauline leaf length to internode length below the measured leaf (“internode ratio”), number of leaf teeth on the lower floral leaf (bract), number of nodes to flower, number of branches, and plant height. All lengths were measured to the nearest millimeter as done by Metherell and Rumsey (2018). For the phenotypic plasticity experiment, we also recorded early season growth (height 6 weeks after transplantation of potential host) and height at the end of season after senescence. We did not directly observe host attachment, as preliminary investigations revealed a fine root structure where haustoria were difficult to observe. Instead, we inferred that attachment is likely to have taken place based on observations of height according to Yeo (1964). In his study, *Euphrasia* that attached to a “good” host tended to grow tall with elongated internodes, while *Euphrasia* that did not attach or attached to a “bad” host were much smaller (see discussion for more details).

We used a combination of fixed effect and mixed models to gain insights into the differences in means and the magnitude of variability in our data. In all models, response variables were analysed as either Gaussian (and log-transformed if necessary) or Poisson. If the response variable was analyzed as Poisson, the model was checked for overdispersion and if it was overdispersed, an observation-level random effect was fitted. All correlations between variables were Pearson’s correlations. Multiple correlation comparisons were corrected using Holm’s correction method. Phenotypic clustering was inspected using principal component analysis (PCA). All analyses were done in R version 3.4.3, with the packages lme4 (Bates et al. 2015) and MCMCglmm (Hadfield 2010) for generalised linear mixed effects models, base R for linear models, RcmdrMisc for correlations (Fox 2020) and ggplot2 for data visualisation (Wickham 2016). MCMCglmm models were run for a minimum of 70,000 iterations using either inverse Wishart or parameter-expanded priors with a minimum burn-in period of 30,000 iterations. Model convergence was assessed visually by plotting the posterior distributions and Markov chains.

In the species differences experiment, species of *Euphrasia* was fitted as a fixed effect, and population of *Euphrasia* was treated as a random effect. We excluded hybrids from these analyses because we were interested in testing differences between species. In the case of height and cauline to internode ratio, the traits were log transformed. Likelihood ratio tests calculated the overall significance of species, where this was not possible, deviance information criteria were used to test better model fit. We calculated proportion variance explained by population of *Euphrasia* (after accounting for fixed effects) by dividing the population random effect variance by the total variance in the model. Tukey post hoc tests were performed on each pairwise comparison of *Euphrasia* species and adjusted p-values calculated, using Tukey honestly significant difference (HSD) test and correcting for family-wise error rate in the emmeans R package (Lenth 2020). For the phenotypic plasticity experiment, host species was fitted as a fixed effect. The models were re-levelled so that “no host” was the baseline. Analysis of variance was used to determine overall significance of host species. Tukey post hoc tests were then performed on each pairwise comparison of host species, with adjusted p-values calculated in base R and the multcomp package (Hothorn, Bretz, and Westfall 2008).

## Trait variation in the wild

We tested how phenotypes in the experiments related to those in nature by comparing results from the species differences experiment to phenotypic measurements of herbarium specimens of the same population sampled in the wild. Three individuals were measured from each collection sheet for a given population for each trait. Pressed plants submitted by collectors varied in quality, and therefore, we were unable to measure the height of these plants, nor was it possible to infer date of first flowering. We analysed the data using generalised linear mixed effect models with where individuals were grown (i.e., common garden or wild-collected) as a fixed effect, with each of five traits as the response variable. We treated species and population of *Euphrasia* as random effects to understand the relative contributions of each to the overall variability in a given trait. Response variables that were considered count data were analysed with a Poisson distribution, in all other cases a Gaussian distribution was used. R-values were calculated using Pearson’s correlations of the population level means between the common garden and the wild samples.

## Results

### Species differences

Our species differences experiment revealed extensive morphological trait variation across *Euphrasia* species when compared at first flowering. From the 222 *Euphrasia* individuals that survived to flower on their clover host, the greatest variation was seen in number of branches (9-fold difference between species), internode ratio (2.7-fold) and height (2.5-fold), while traits such as node to flower (1.6-fold) and corolla length (1.6-fold) proved less variable (Figure 1A–D; Appendix S3). A large degree of this variation was separated by species and by population (Table 1). The species with the most distinct life history strategy was *E. micrantha*, which flowered from a low node on the plant ( $8.3 \pm 0.2$  nodes) while it was short ( $70 \pm 8$  mm; Appendix S3). It also formed a partly distinct cluster in the PCA (Appendix S4). *Euphrasia pseudokerneri* was relatively distinct, flowered once it had grown tall ( $176 \pm 16$  mm) and from a high node on the plant ( $13.2 \pm 0.4$  nodes), but showed little separation in the PCA. The morphologically similar *E. arctica*, *E. confusa*, and *E. nemorosa* differed for some traits, with *E. nemorosa* initiating flowering 14 days later and from 3.3 nodes higher than *E. arctica*, but overlapped in many other traits and in overall multi-trait phenotype (Appendices S4, S5). Despite species being a significant factor in the models, and some notable differences in specific traits, there were few significant pairwise Tukey comparisons due to substantial within-species variation (Appendix S6). Of the seven significant pairwise trait differences, three were for node to flower and three for number of leaf teeth, with four of the seven significant comparisons involving *E. micrantha*. In most cases, hybrids combined morphological characters of their parental progenitors. For example, hybrids involving *E. nemorosa* flowered later in the season and initiated flowering from a higher node than *E. arctica* 6 hybrids (Figure 1A–D).

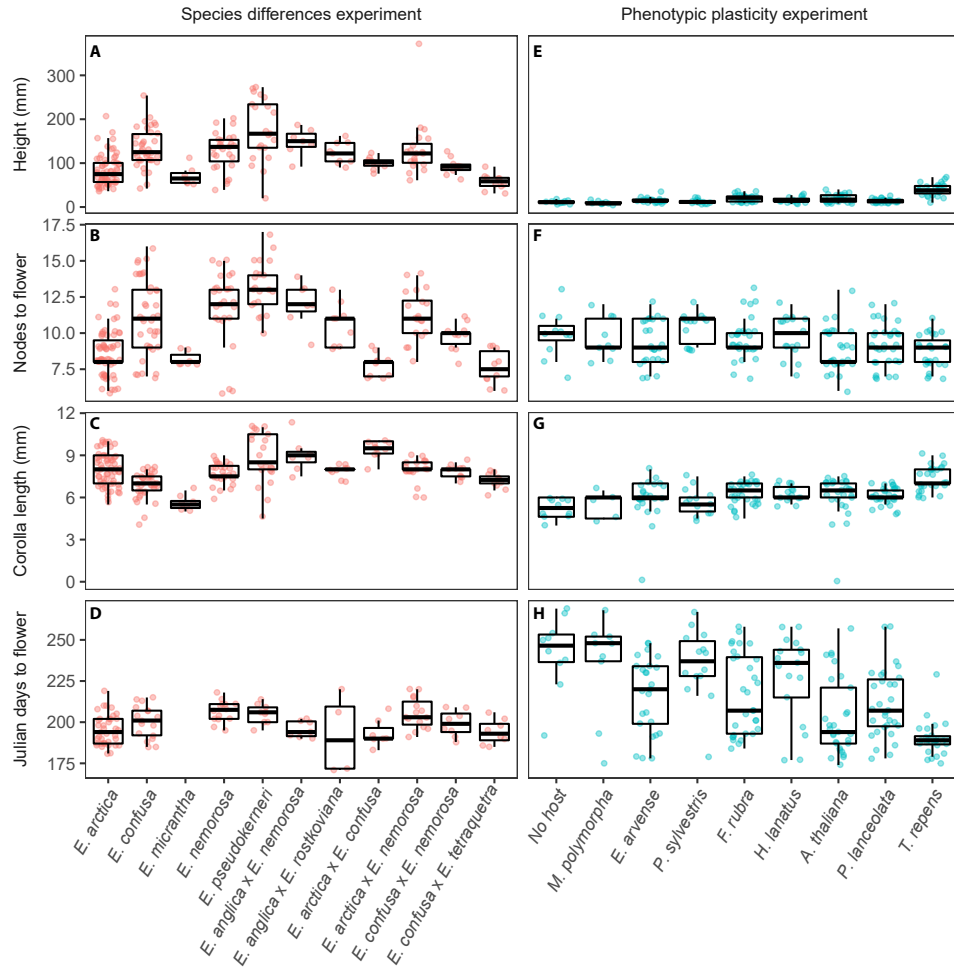


Figure 1: Trait variation in a common garden experiment of diverse *Euphrasia* species and hybrids grown on clover (A–D); *Euphrasia arctica* grown on many different hosts (E–H). The edges of the box plots show the first and third quartiles, the solid lines the median, the whiskers the highest and lowest values within 1.5-fold of the inter-quartile range, and the jittered dots each individual measurement. Length measurements were recorded in millimeters.

Table 1: Summary of generalized linear mixed effects models for *Euphrasia* trait values measured in a common garden environment. Model outputs are summarized for five *Euphrasia* species grown with clover in the species differences experiment and for *E. arctica* grown with eight hosts and without a host in the phenotypic plasticity experiment. For the phenotypic plasticity experiment, we report model outputs with all potential hosts, as well as models excluding *Pinus* and *Marchantia* when there was no evidence of attachment or interactions (reported in square brackets). The percentage variance explained by random effects are reported in parentheses along with the 95% credibility interval. \*\*\*p < 0.001, \*\*p < 0.01, \*p < 0.05.

| Trait                 | Species differences  |   | Phenotypic plasticity  |
|-----------------------|--|---|--|
| Branches              | DIC <sub>full</sub> = 676.08;<br>DIC <sub>spp</sub> = 679.48 | DIC <sub>full</sub> = 676.08;<br>DIC <sub>pop</sub> = 714.87<br>(25.9%, 13.4–57.4%) | NA   |
| Corolla length        | $\chi^2(4) = 11.91^*$  | $\chi^2(1) = 41.38^{***}$<br>(54.1%, 24.4–69.2%)                                    | (F <sub>8,173</sub> = 9.85)***<br>[(F <sub>6,157</sub> = 11.38)***]  |
| Height                | $\chi^2(4) = 11.67^*$  | $\chi^2(1) = 57.13^{***}$<br>(61.2%, 35.0–79.7%)                                    | (F <sub>8,185</sub> = 23.14)***<br>[(F <sub>6,164</sub> = 24.39)***] |
| Internode ratio       | $\chi^2(4) = 13.00^*$  | $\chi^2(1) = 34.38^{***}$   | (F <sub>8,184</sub> = 3.36)**<br>[(F <sub>6,163</sub> = 4.11)***]    |
| Julian days to flower | $\chi^2(3) = 2.26$   | $\chi^2(1) = 1.42$ E-14<br>(58.7%, 28.6–80.9%)                                      | $\chi^2(8) = 192.39^{***}$<br>[ $\chi^2(6) = 141.67^{***}$ ]         |
| Node to flower        | $\chi^2(4) = 15.42^{**}$                                     | $\chi^2(1) = 2.87$ (14.1%,<br>1.0–33.5%)  | $\chi^2(8) = 5.02$ [ $\chi^2(6) =$<br>3.04]                          |
| Number of leaf teeth  | $\chi^2(3) = 12.45^{**}$                                     | $\chi^2(1) = 0.0059$ (0.12%,<br>2.8%–23%)   | $\chi^2(8) = 26.79^{***}$ [ $\chi^2(6)$<br>= 17.04**]                |

Correlation analyses across species revealed clear suites of traits that are related. Significant correlations were found between 12 of the 21 pairwise comparisons after correcting for multiple tests, with five of these correlations with  $R > 0.6$  (Table 2A). Plants flowering at a late node are more likely to be tall, more highly branched, and have many teeth on the lower floral leaf. The relationship of traits is also supported in the PCA, with many traits contributing to multiple principal components (Appendix S5). Traits related to height and flowering node were largely uncorrelated with internode ratio and corolla length.

Table 2: Pearson’s correlation coefficients for seven phenotypic traits measured in a common garden experiment for (a) five *Euphrasia* species and six hybrids, (b) *Euphrasia arctica* grown with eight hosts and without a host. \*\*\*p < 0.001, \*\*p < 0.01, \*p < 0.05. Asymptotic p-values values are reported from the Hmisc package in R using the rcorr() function.

| (A) Species differences experiment   |                     |             |                 |                       |            |                 |
|--------------------------------------|---------------------|-------------|-----------------|-----------------------|------------|-----------------|
| Trait                                | Corolla length (mm) | Height (mm) | Internode ratio | Julian days to flower | Leaf teeth | Nodes to flower |
| Branches                             | 0.260**             | 0.609***    | -0.116          | 0.057                 | 0.658***   | 0.775***        |
| Corolla length (mm)                  |                     | 0.319***    | -0.161          | -0.127                | 0.197*     | 0.049           |
| Height (mm)                          |                     |             | 0.246**         | 0.292*                | 0.563***   | 0.628***        |
| Internode ratio                      |                     |             |                 | 0.204                 | -0.120     | 0.076           |
| Julian days to flower                |                     |             |                 |                       | 0.053      | 0.249*          |
| Leaf teeth                           |                     |             |                 |                       |            | 0.651***        |
| (B) Phenotypic plasticity experiment |                     |             |                 |                       |            |                 |
| Trait                                | Corolla length (mm) | Height (mm) | Internode ratio | Julian days to flower | Leaf teeth | Nodes to flower |
| Branches                             | 0.524***            | 0.834***    | -0.299***       | -0.572***             | 0.694***   | -0.572**        |
| Corolla length (mm)                  |                     | 0.503***    | 0.098           | -0.406***             | 0.536***   | -0.166          |
| Height (mm)                          |                     |             | 0.477***        | -0.481***             | 0.692***   | -0.186          |
| Internode ratio                      |                     |             |                 | -0.034                | 0.168      | -0.009          |
| Julian days to flower                |                     |             |                 |                       | -0.691***  | 0.530***        |
| Leaf teeth                           |                     |             |                 |                       |            | -0.239**        |

## Phenotypic plasticity

Our phenotypic plasticity experiment showed substantial morphological variation across 194 *E. arctica* plants grown with eight different potential host species and the 22 plants grown without a host. Plants growing on clover transitioned to flower quickly ( $189.8 \pm 2.0$  Julian days), grew tall by the time of first flowering ( $39 \pm 3$  mm), and produced large flowers ( $7.4 \pm 0.2$  mm; Figure 1E–H, Appendix S7). These results contrast with *Euphrasia* with no host, which flowered on average 52 days later ( $241.3 \pm 7.9$  Julian days), were extremely short at first flowering ( $11 \pm 1$  mm), and produced small flowers ( $5.3 \pm 0.2$  mm). *Euphrasia arctica* grown



on *Arabidopsis*, *Equisetum*, *Festuca*, *Holcus*, or *Plantago* were all statistically significantly different from no host for at least one trait (Tukey comparisons,  $p < 0.05$ ), while *E. arctica* on *Marchantia* or *Pinus* was not significantly different from no host for any trait ( $p > 0.05$ ; Appendix S8). While the overall effect of host was significant for all traits except nodes to flower (Appendices S9–S11), three traits showed relatively little plasticity, with few statistically significant pairwise Tukey comparisons for nodes to flower (0 significant comparisons), number of leaf teeth (3), and internode length (4), while the other three traits showed many pairwise differences (days to flower, 21 significant comparisons; height, 16; corolla length, 12; Appendix S8). Our comparison of growth traits across host treatments measured through the year showed that height at the end of the season was weakly predicted from height 6 weeks after introducing a host ( $R = 0.47$ ), but strongly correlated with height at first flowering ( $R = 0.82$ ; Appendix S12). Plants that flowered early were more likely to grow larger by the end of season ( $R = -0.55$ ) and become more highly branched ( $R = -0.57$ ; Appendix S12).

Across host treatments, there was a significant negative correlation between Julian days to flower and most other traits (Table 2B). We find that late flowering individuals are likely to be smaller at first flowering and have fewer branches, leaves with fewer teeth, and smaller flowers. While these traits were strongly correlated, there were substantial differences in the magnitude of response. For example, days to flower differed considerably depending on host, with a 3.8-fold greater difference than seen between means for different *Euphrasia* species grown on the same host (Figure 1D,H). In contrast, corolla length and node to flower proved less variable depending on host, with a 1.4-fold and 1.2-fold change between means, respectively.

## Variation in the wild

The comparison between the species differences common garden experiment and wild-collected herbarium specimens revealed population means of a single trait, nodes to flower, are strongly correlated ( $R = 0.79$ ), and trait values are not significantly different ( $p \text{ MCMC} = 0.71$ ) between environments (Figure 2; Appendices S13, S14). All other traits did differ significantly between environments ( $p \text{ MCMC} < 0.05$ ), with *Euphrasia* plants in the common garden having corollas on average 1.4 mm longer, with 0.2 more teeth on the lower floral leaves, an increase in internode ratio of 1.0 mm, and 4 more pairs of branches. Despite these differences, there were correlations between the common garden and the wild-collected specimens for corolla length ( $R = 0.93$ ,  $p \text{ MCMC} < 0.001$ ), internode ratio ( $R = 0.65$ ,  $p \text{ MCMC} < 0.001$ ) and number of branches ( $R = 0.29$ ,  $p \text{ MCMC} < 0.001$ ), but not for number of leaf teeth ( $R = 0.07$ ,  $p \text{ MCMC} = 0.034$ ).

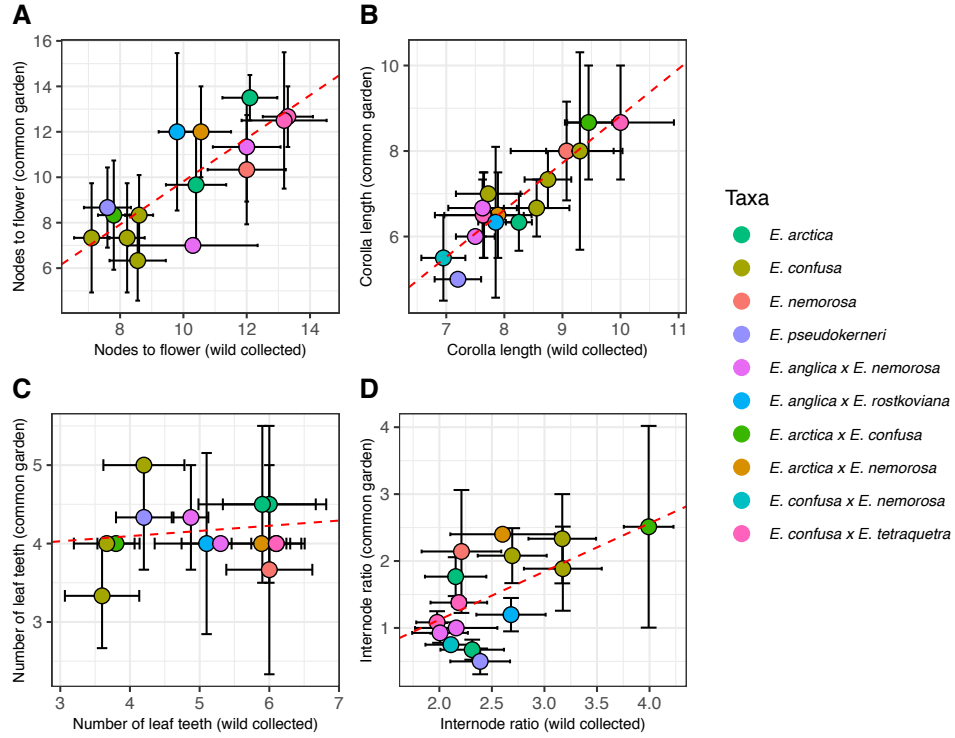


Figure 2: Relationship between morphological trait measurements made in the common garden and on wild-collected herbarium specimens for diverse *Euphrasia* species. Points are *Euphrasia* population means; bars represent the standard error of measurements. The line of best fit was calculated using coefficients from linear regression models on the means of each *Euphrasia* population. Length measurements are reported in millimeters. For an alternative representation of pairwise comparisons, see Appendix S13.

## Discussion

Our study sheds light on species differences, life history evolution and phenotypic plasticity of the generalist parasitic plant *Euphrasia*. We found different life history strategies between recently diverged species, with some species rapidly transitioning to flower at the expense of growth-related traits, while others delay flowering and invest in early-season vegetative growth. However, many traits are phenotypically plastic and change in response to the host. While plants in benign common garden conditions grew vigorously, the correlation between life history traits in a common garden and in the wild suggests our experimental observations are indicative of patterns observed in nature. Morphological differences between species in the common garden also suggest that the currently delimited *Euphrasia* species are, at least in part, distinct. Overall, our study highlights the value of integrating trait data from multiple common garden experiments and field collections to study life history strategies in parasitic plants and demonstrates the rapid evolution of life history differences in a postglacial radiation of hemiparasites.

## Life history variation in a generalist hemiparasitic plant

We found evidence for different life history strategies in British *Euphrasia*. *Euphrasia arctica*, *E. micrantha*, and hybrids such as *E. arctica*  $\times$  *E. confusa*, transition rapidly to flower, flower while they are short, and produce their first flower from a low node on the plant. These rapid flowering species contrast with *E. pseudokerneri*, *E. nemorosa* and hybrids involving *E. nemorosa* that delay flowering until later in the season, grow tall before flowering, and produce their first flower from a late node on the main axis. These different life history strategies correspond to the known ecology of these species, with *E. nemorosa* flowering late in tall mixed grassland, while *E. micrantha* flowers early in patchy heathland (Metherell and Rumsey 2018). While species show some general differences in life history strategies, there is also significant variation between populations within species. A relationship between internode number and habitat has previously been observed within *Euphrasia* species, with populations of *E. rostkoviana* in Sweden flowering at a lower node in a common garden if they have been collected from intensely grazed pasture (Zopfi 1998). Overall, these observations within and between species are consistent with the classic life history trade-off between growth and reproduction (Stearns 1992; Roff 2002). For *Euphrasia* growing in the wild, early reproduction allows the plants to reliably complete their life cycle before summer competition, herbivory, mowing, summer drought, and other seasonal abiotic and biotic stresses. However, early flowering involves reproducing at the expense of early-season growth and at a time when the resource budget may be constrained by relatively few haustorial connections. These trait trade-offs pose an interesting comparison to the well-studied *Mimulus guttatus* (syn. *Erythranthe guttata*), a nonparasitic relative in the Lamiales that shares the same basic plant architecture. In *M. guttatus*, multiple traits related to growth and reproduction are correlated, both within and between populations, due to genetic trade-offs between time to flower and fecundity (Mojica et al. 2012; Friedman et al. 2015). In *Euphrasia*, the genetics underpinning this life history trade-off have yet to be characterized and may be a consequence of multiple independent loci or trade-offs at individual loci (Hall, Lowry, and Willis 2010).

While much life history variation is captured by differences in time to flower and growth-related traits, we also see evidence for flower size representing a separate axis of variation across *Euphrasia* species. In our common garden, *E. micrantha* has small corollas, while *E. arctica* and *E. nemorosa* have larger corollas, and corolla size is not strongly correlated with other traits. *Euphrasia* species are well known to have flower size variation, with a continuum between small-flowered species that are highly selfing (e.g., *E. micrantha*, corolla size = 4.5–6.5 mm, inbreeding coefficient  $F_{IS} > 0.88$ ; Stone (2013)) and large-flowered species that are highly outcrossing (e.g., *E. rostkoviana*, flower size 8–12 mm,  $F_{IS} = 0.17$ –0.25; French et al. (2005)). Such wide variation in outcrossing rate has been documented in species of *Datura* (Motten and Stone 2000), *Mimulus* (Karron et al. 1997), and *Nicotiana* (Breese 1959). Small flowers have shorter anther–stigma separation and thus increased potential for autogamous selfing (Karron et al. 1997), while also having reduced attractiveness to pollinators and thus receiving less outcross pollen (Mitchell et al. 2004). In addition to differences in corolla size between *Euphrasia* species, corolla size also shows a change of up to 2 mm in response to host species. This change in flower size is of a magnitude that may potentially affect the mating system (Luo and Widmer 2013) and suggests host species represents a previously unaccounted factor affecting the mating system of parasitic plants.

Our comparisons of *Euphrasia* species in a common garden also shed light on the distinctiveness of these recently diverged species and can be used to refine the suite of traits that are reliable in telling *Euphrasia* species apart. *Euphrasia* is a taxonomically complex plant genus, with the 21 currently described British species presenting complex and often overlapping morphological variation (French et al. 2008; Metherell and Rumsey 2018; Wang et al. 2018). Our study suggests varying degrees of morphological distinctiveness of *Euphrasia* species. We see *E. micrantha* is morphologically distinct in the common garden and *E. pseudokerneri* somewhat distinct, while the closely related species *E. arctica*, *E. confusa*, and *E. nemorosa* differ in life history traits such as nodes to flower, but overlap in many other traits and are not clearly separated in the PCA. The morphological trait differences between species observed under standardised conditions are correlated with

values from field-collected herbarium specimens where plants have associated with diverse hosts, been exposed to different ecological conditions, and were collected at different life-stages. These correlations suggest that our common garden results generalize to observations in nature. However, our study is likely to overestimate the distinctiveness of taxa by only including a subset of UK species and by choosing populations that could be identified to species level in the field. We suspect adaptive divergence between closely related *E. arctica*, *E. confusa*, and *E. nemorosa* is a consequence of differential natural selection for local ecological conditions such as soil water availability or mowing. Selection appears to be operating at a fine spatial scale, with significant life history trait differences evident between populations within species. *Euphrasia* taxa may be genetically cohesive, either showing genome-wide divergence or divergence in genomic regions underlying life history differences (Twyford and Friedman 2015), or alternatively these taxa may be polytopic and not genetically cohesive (Blackmore and Oldfield 2017). Genomic sequencing of natural populations will help resolve the nature of species differences in *Euphrasia*.

### Phenotypic plasticity in response to host

Our phenotypic plasticity experiment shows *Euphrasia* are affected by growing with a range of different hosts. Specifically, *E. arctica* with a host such as clover rapidly transitions to flowering. At the other extreme, *Euphrasia* grown without a host are small and flower late. These differences in growth are established early in the season, and early-flowering plants go on to grow the tallest and are more highly branched. Most other hosts result in a continuum of *Euphrasia* phenotypes between these extremes. Two surprising results were that *E. arctica* parasitizing *Arabidopsis* grew relatively tall despite the host senescing early in the growth season and that growth of *Euphrasia* with *Equisetum* was similar to growth on the commonly encountered grass *Holcus lanatus*. This result suggests that it attached to *Equisetum*, which would need to be confirmed by excavating root systems and observing haustoria, or it indirectly benefits without attachment through association with *Equisetum* fungal symbionts (Bouwmeester et al. 2007). Less surprising was the poor growth of *E. arctica* with *Pinus*. However, an association between *Melampyrum pratense* and *Pinus sylvestris* suggests at least some hemiparasitic Orobanchaceae benefit from attachment to woody host species or from interactions with their associated ectomycorrhizal fungi (Salonen, Setälä, and Puustinen 2000).

The diverse effects of host on parasite growth are complex, but the variation we saw in our experiments may be attributed to host root architecture, germination time, and resource availability, as well as the presence of mechanisms to defend against parasite attack, such as cell wall thickening, localized host dieback, and chemical defence (Cameron, Coats, and Seel 2007; Twyford 2018). While *Euphrasia* is generally thought to have low reliance on host resources, deriving only ~30% of carbon heterotrophically (Tsitel, Plavcova, and Cameron 2010), at least under our experimental conditions *Euphrasia* only produced multiple flowers on certain hosts. Overall, our results point to *E. arctica* being a true generalist hemiparasite, but one where vigorous growth is only observed with a subset of potential hosts.

In terms of specific traits, only three pairs of trait correlations show consistent correlation coefficients in both *Euphrasia* common garden experiments (between height, number of branches, and leaf teeth), with other correlations between species breaking down when *Euphrasia* are grown on different hosts. The most notable plasticity is seen in flowering time, with plants on clover rapidly transitioning to flower within ~100 days of germination, while plants with a more typical host (e.g., *Holcus lanatus*) flower a month later. Phenotypic plasticity in flowering time in response to resource availability is well documented in many plant groups, particularly *Arabidopsis* (e.g. Zhang and Lechowicz (1994)), but has received less attention in studies of parasitic plants, which are more likely to look at growth-related traits such as biomass (Ahonen, Puustinen, and Mutikainen 2006; Matthies 2017). However, date of first flowering has been shown to differ by up to 10 weeks in populations of *Rhinanthus glacialis* across Switzerland (Zopfi 1995). Overall, we expect date of first flowering to be critical for the lifetime reproductive success of parasitic plants in the wild.

In contrast to seeing traits with extensive plasticity, we also saw evidence of developmental constraint in number of nodes to flower. For *E. arctica*, this trait showed the least plasticity with different hosts, is consistent between populations within species, and between the common garden and the field. Thus, the developmental event of transitioning to flower may be genetically determined, with changes in flowering time altered by plasticity in internode length and not nodes to flower. This developmental constraint may explain why nodes to flower is such an important diagnostic trait for species identification in *Euphrasia* and related species in the Rhinanthaeae (Jonstrup, Hedren, and Andersson 2016). Despite nodes to flower changing little in response to host species, our overall impression is that *Euphrasia* show considerable plasticity and little developmental constraint in many aspects of growth. In particular, differences between individuals on a given host also suggests other sources of variation, such as genetic background in host and parasite, as well as the timing of attachment, may be crucial in determining performance.

## Conclusions

Despite over a century of experimental studies in parasitic plants, our understanding of the evolution of life history strategies in these diverse organisms is extremely limited. Our results with *Euphrasia* provide strong support for the rapid evolution of distinct life history strategies in response to local ecological conditions, with phenotypic plasticity further altering plant growth in response to host availability. We anticipate that future studies that test lifetime reproductive success of many parasitic plant species grown on many different host species will give further insight into the complex nature of host–parasite interactions.

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