



Insects and mycorrhizal fungi influence maternal seed provisioning in *Senecio vulgaris*

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Abstract

The performance of plants in any one generation can be influenced not just by the prevailing biotic and abiotic factors, but also by those factors experienced by the parental generation. These maternal effects have been recorded in an array of plant species, but most studies tend to focus on abiotic factors over two generations. Here we show that maternal effects in the annual forb *Senecio vulgaris* may be influenced by beneficial arbuscular mycorrhizal fungi and insect herbivory over four successive generations. These effects were very much determined by seed provisioning, wherein C:N:P ratios were altered by both fungi and aphids. There was little evidence of epigenetic changes induced by the fungi or insects, instead the driving forces seemed to be allocation of N and P to the seeds. However, changes in seed chemistry were not cumulative over generations, often decreases in seed nutrient content were followed by recovery in subsequent generations. The changes in seed stoichiometry can have important consequences for viability, germination and subsequent seedling growth rates. We conclude that studies of maternal effects need to be conducted over multiple generations, and also need to be multifactorial, involving variation in abiotic factors such as water and nutrients, combined with biotic factors.

Introduction

The performance of plants in any one generation can be affected by the environmental conditions experienced by their parents, through maternal effects (Roach and Wulff, 1987; Latzel et al., 2023). Such effects can influence the growth and survival of seedlings (Elwell et al., 2011), through effects on seed size, dormancy mechanisms and viability (Fernandez-Pascual et al., 2019). These effects are often seen in annual plants, in both changing and stable environments (Yin et al., 2019) and are thought to be adaptive if the offspring experience similar environmental conditions to those of the parents (Herman and Sultan, 2011).

Clearly, seeds are the vehicle by which maternal effects can be transmitted from one generation to another. To ensure the survival of the next generation, maternal plants can increase the provisioning of the seeds to give them a better chance of survival (Haig and Westoby, 1988). The local environment plays a role in how such provisioning is given to the seeds. Maternal plants experiencing crowding were found to produce smaller seeds, likely due to intraspecific competition for nutrients. However, smaller seeds were dispersed further from the crowded environment (Larios and Venable, 2015). Meanwhile, high levels of soil nutrients can result in larger seeds, leading to higher rates of germination and seedling growth (Sills and Nienhuis, 1995). While absolute levels of resources such as carbon, nitrogen and phosphorus in seeds are important, it is the stoichiometric ratios between them that are of most ecological relevance (Maskova and Herben, 2021).

As plant growth rates are controlled by protein synthesis, it is mainly the ratio of C:P and N:P within seeds that is important, originally formulated as the growth rate hypothesis (Elser et al., 1996). Low C:N:P ratios support this hypothesis, suggesting that a high growth rate is associated with higher P content, because fast growth requires more P-rich ribosomal RNA leading to greater protein synthesis (Elser et al., 2000; Matzek and Vitousek, 2009). This is because germinating seeds show high metabolic activity, requiring high levels of nutrients for growth until autotrophy is established (Jia et al., 2022). However, correlations between growth rate and stoichiometric ratios involving P depend on the identity of the plant species (Peng et al., 2011). Furthermore, there is some evidence that C:N ratios are also linearly correlated with growth rate (Agren, 2004; Chen et al., 2022) thereby showing the importance of considering C:N:P ratios in studies of seed provisioning (Sardans et al., 2021). However, such ratios are rarely considered in studies of maternal effects.

The limited evidence suggests that abiotic conditions such as water or nutrient availability experienced by parent plants can alter seed stoichiometry and thus the growth rate of the seedling generation, however, abiotic conditions experienced by the progeny may override these effects (Steinger et al., 2000; Jiang et al., 2021). In addition, biotic factors such as beneficial arbuscular mycorrhizal (AM) fungi can also influence maternal effects on seedling

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performance (Varga et al., 2013). These fungi are instrumental in facilitating phosphate and nitrate uptake in many plants (Bolan, 1991; Bucking and Kafle, 2015), thereby potentially altering C:N:P stoichiometry in plant tissues. By increasing nutrient uptake, AM fungi may reduce C:N and N:P ratios in seeds, leading to enhanced germination and seedling growth rate in the progeny generation (Guo et al., 2013). However, experiments that study how AM influences maternal seed provisioning have yet to be conducted over multiple plant generations (Tian et al., 2020).

In natural (i.e., non-crop) conditions, virtually every living plant that is colonized by AM fungi is also simultaneously attacked by insect herbivores. Insect herbivory can also influence maternal effects in plants, though virtually all of these experiments focus on induced resistance and have been conducted over just two (i.e., parent and progeny) generations (Rasman et al., 2012; Singh et al., 2017; Kafle and Wurst, 2019). Meanwhile, other studies have failed to find an effect of herbivores on maternal effects (Bustos-Segura et al., 2021). When multiple generations are considered, very different patterns of maternal effects on plant performance are revealed, with some effects being cumulative, while others show initial decreases and then recovery (Neylan et al., 2018; Chitty and Gange, 2022a). Herbivory may also override abiotic factors, for example, seeds of *Verbascum thapsus* showed higher viability when parental plants were grown in warm conditions, compared to those in colder conditions. However, if the plants were also attacked by insects, this difference disappeared (Alba et al., 2016). Furthermore, whether or not herbivory influences maternal effects through seed provisioning is unclear, with both increases and decreases being reported in C:N ratios in two different *Brassica* species (Soufbaf et al., 2017). In addition, it has long been known that the presence of AM fungi may affect insect performance and vice versa (Koricheva et al., 2009; Barto and Rillig, 2010), yet how such interactions affect seed provisioning in maternal effects is unknown.

In addition to maternal effects being transmitted through resource provisioning, epigenetic changes to the DNA structure in seeds can also be important in determining the performance of the next generation (Michalak et al., 2013; Adrian-Kalchhauser et al., 2020). An epigenetic change to the DNA structure is the addition or removal of methyl groups on cytosine residuals, which can pass stably between one generation of plants to the next (Herman and Sultan, 2016). This addition or removal of methyl groups can change transcription on specific loci, with the transcriptional changes potentially altering environmental parental effects (Herman and Sultan, 2016). Plants generally leave their epigenome intact, whereas animals wipe out the majority of methyl markers when reproduction is taking place (Akst, 2017). By leaving the epigenome intact, plants can inherit alleles that are altered by methyl groups which can change phenotypes, such as drought tolerance (Herman and Sultan, 2016). Experimental manipulation of DNA methylation can be achieved with the chemical zebularine, allowing for the examination of relations between phenotypic and epigenetic variation in plants (Alonso et al., 2017). For example, Herman and Sultan (2016) used zebularine on *Polygonum persicaria* to explore what influence DNA methylation had on drought tolerance between generations. Eliminating methyl groups removed the epigenetic effects of drought tolerance but did not significantly change the phenotypic expression in control plants. To date, there are very few studies of DNA methylation in studies of maternal effects, but there is some evidence that this process can mediate transgenerational environmental effects (Baker et al., 2018). Furthermore,

DNA methylation in seeds may also be influenced by the mycorrhizal status of the parent plants (Varga and Soulsbury, 2017).

Senecio vulgaris L. (Asteraceae) is a fast-growing annual forb that shows little seed dormancy and which can cycle through three or four generations a year within the same area (Grime et al., 1988). In the non-radiate flower form, selfing occurs with no outcrossing, producing large amounts of viable seed (Aarssen and Burton, 1990), making *S. vulgaris* an ideal 'model plant' for studies of maternal effects (Walter et al., 2020). In natural communities, most plants form an association with AM fungi (Gange et al., 1999), while most are also attacked by insects, particularly the aphid *Myzus persicae* (Sulzer) (Dunne, 1971). We have used this plant to investigate how aphid attack and AM fungi influence maternal effects over four successive generations (hereafter termed 'inductions'), previously reporting effects on the aphid and fungi (Chitty and Gange, 2022b) and plant growth parameters (Chitty and Gange, 2022a). Amongst the latter, seed size was reduced and germination time increased after one induction (i.e., in the second generation); effects which were enhanced by aphids. However, a gradual recovery in these parameters was seen over subsequent inductions. Aphids reduced seed N content, but this depended on the number of inductions and the presence of AM fungi. Here, we report on seed provisioning and viability in these plants, in order to understand the mechanisms behind the maternal effects observed. We hypothesized that AM fungi would reduce seed C:N and N:P ratios due to the enhancement of N and P uptake, respectively, by these fungi, while aphids would increase the ratios, due to negative effects of herbivory on plant N and P. We further hypothesized that any effects of AM fungi would be mediated by the presence of aphids, due to the interactions between insects and fungi (Hartley and Gange, 2009). Alteration of these ratios may have consequences for seed viability and so we hypothesized that AM fungi would increase viability (through lowering of the C:N and N:P ratios). The effect of insects would be far less clear, depending on whether herbivory altered seed N or P content or both. In addition, we studied whether epigenetic changes could also account for apparent maternal effects through the use of zebularine to manipulate DNA methylation. Here, we hypothesized that some of the effects caused by AM fungi would be lessened by the application of zebularine.

Materials and methods

Plant growth conditions

The experimental design is described fully in Chitty and Gange (2022a, 2022b) and shown in Supplementary Fig. S1. In brief, seeds of the non-radiate form of *S. vulgaris* were collected from a wild population and grown for one generation in a controlled environment room (CER) (20°C, 78% RH and 16 h of daylight) to minimize any influence of the parental environment from which the seeds were collected (Latzel, 2015). Seeds were taken from these plants and thereafter plants were grown for four generations. Hereafter, these are referred to as 'induction events' (0, 1, 2 and 3), for comparison with other similar studies (Neylan et al., 2018). Therefore, there were three induction events over the four generations (Supplementary Fig. S1). For each induction, there were four treatments: control (no mycorrhizal fungi or insects), addition of a mixed inoculum of AM fungi ('Rootgrow' (PlantWorks Ltd., Sittingbourne, Kent, UK), containing *Claroideoglossum claroideum*, *Funneliformis geosporus*, *Funneliformis mosseae*, *Glomus*

microaggregatum and *Rhizophagus irregularis*), infestation by aphids (*Myzus persicae*) and both AM fungi and aphids. Non-mycorrhizal plants received autoclaved inoculum, together with a microbial wash (inoculum filtered through a 38 μ membrane) to correct for the non-AM fungal community. Three apterous adult *M. persicae* were taken from an aphid colony maintained on Chinese cabbage (*Brassica rapa* subsp. *chinensis* L.). When a total of 10 nymphs had been produced, the adults were removed with the remaining nymphs forming the aphid treatment. Seeds from any one treatment were used to start that same treatment in the next generation and all generations were grown in identical conditions in the CER. In addition, seeds from aphid only, mycorrhiza only and aphid + mycorrhizal plants were taken from generations one, two and three and plants grown from these without insects or fungi. These are depicted in Supplementary Fig. S1 as 2A, 2B, 2C, 3A, 3B, 3C and 4A, 4B and 4C plants. In all cases, there were 20 replicates of each treatment. Each plant was grown singly in a pot containing 165 g of John Innes grade 3 compost (Westland Horticulture, Huntingdon, UK) (55% sterilized loam, 25% peat, 17% grit, 3% ground limestone) and grown in the CER (Chitty and Gange, 2022a, 2022b).

Seed measurements

Seed nitrogen content (%) was reported in Chitty and Gange (2022b), while seed weight and germination time were reported in Chitty and Gange (2022a). Here, we report on seed carbon and phosphorus content and the associated stoichiometric ratios (C:N and N:P) and seed viability (percentage germination).

It was logistically impossible to measure the carbon content of seeds produced by every plant over all of the generations. Therefore, over 200 seeds from each treatment in each generation were pooled, and six random sub-samples were taken for analysis, as done for nitrogen measurements (Chitty and Gange, 2022b). To measure the percentage of carbon, seeds were oven dried at 60°C for 48 h, ground to a fine powder and 10 mg weighed out into tin capsules (CE instruments, Wigan, UK) and sealed. Carbon content was measured with combustion-gas chromatography using an NC soil analyser flash EA 1112 series with a CHNS configuration. There were quality controls (Sulphanilamide STD) (CE instruments, Wigan, UK) with known carbon concentrations added to the autosampler throughout the sample run. The carbon concentrations of the quality controls were checked against the standards to ensure the results were not drifting through the sample run.

Phosphorus content was determined using six random sub-samples from the same 200 seeds that were used to measure C and N contents. The method used for *S. vulgaris* was adapted from West (1995). Samples of 0.2 g of ground material were weighed into a digestion vessel with 6 ml of concentrated (68%) nitric acid. In a Mars Xpress microwave (CEM technologies, Buckingham, UK), the temperature was ramped to 140°C over 10 min and held for 20 min. The digestion vessels were left to cool for one hour until they were roughly 55°C. Once the digestion was complete, the contents were filtered into a 50 ml volumetric flask.

The samples were run through a Skalar segmented flow analyser comprised of SA 1050 random access autosampler, chemistry unit SA 4000, SA853 SFA interface with a digital photometer head and Flowaccess software package. The ammonium hepta molybdate and potassium antimony (III) oxide tartrate react in an acidic medium with diluted solutions of phosphate to form an antimony-phospho-molybdate complex. This complex was

reduced by 0.1 M ascorbic acid to an intensely blue-coloured complex which was measured spectrophotometrically at 880 nm. A standard was used throughout the sample run, so that any drift was corrected for. The standard was made by dissolving 4.39 g (0.03 M) of potassium orthophosphate (KH₂PO₄) in 800 ml of deionized water. The standard was diluted down from 1000 ppm to 8, 6, 4, 2, 1 and 0 to be used throughout the sample run (West, 1995).

To measure seed germination, 10 90 mm diameter petri dishes per plant (i.e., 20 replicates per treatment) each filled with 5 mm of damp sand (with 30 ml added water) were used. Five seeds were placed at random onto the sand in each dish. The dishes were sealed and placed in complete darkness at 15°C and checked daily for 4 weeks. The percentage of seeds that had germinated after this time was recorded. Any ungerminated seeds were subjected to the tetrazolium test (França-Neto and Krzyzanowski, 2019) and all were found to be dead.

DNA methylation

To explore whether DNA methylation was causing any of the differences in parameters between generations, an experimental method was adapted from Herman and Sultan (2016). The parental generation was grown from seeds collected from generation one of the main experiments. Potential DNA methylation was then examined after one induction event (i.e., generation two). The progeny generation was grown in the same conditions as the parental generation and 20 replicates of the same four treatment groups were used; however, each treatment was split into 2 with 10 being treated with zebularine (demethylating agent) and 10 not (Supplementary Fig. S2). Two days after seedling emergence, the addition of the demethylating drug zebularine (Sigma-Aldrich, Gillingham, UK) began. 60 ml of 45 μ M zebularine solution was watered into each treated pot. This concentration has previously been shown not to disrupt plant development (Herman and Sultan, 2016). When plants were mature, seeds were collected and weight, germination time and viability were measured.

Statistical analysis

All analyses were performed in R 4.0.5. All seed chemistry data were expressed on a dry weight basis. Normality tests were performed on whole datasets and data were transformed when necessary using lambda calculated by the Box-Cox transformation. Percentage data were subjected to the logit transformation (Warton and Hui, 2011). Differences in seed chemistry and germination over treatment groups in each generation were tested using a three-way ANOVA, employing aphid presence/absence, AM fungal presence/absence and induction event as the main effects. In each analysis, the minimal adequate model was considered, following step-wise deletion of non-significant interaction terms (Chitty and Gange, 2022a, 2022b). A one-factor ANOVA was used to examine whether cessation of any treatment caused differences in plant parameters compared with successive inductions. Thus, for example, for each plant parameter, AM fungi-only plants grown in generation two (i.e., after one induction) were compared with those from the same parents, but lacking the fungi (2A plants in Supplementary Fig. S1). Meanwhile, aphid-only plants in generation two were compared with those from the same parents but without aphids (2B plants in Supplementary Fig. S1), etc.

For the demethylation experiments, within-generation effects were examined using linear models, employing aphid presence/absence and mycorrhiza presence/absence as the main effects for comparing within the first generation of seeds. Effects within generation two were examined using a linear model, employing zebularine presence/absence, aphid presence/absence and mycorrhiza presence/absence. Another linear model was deployed to examine between generation effects, employing generation, zebularine presence/absence, aphid presence/absence and mycorrhiza presence/absence as the main effects.

Results

Overall, seed carbon percentage differed between induction events ($F_{3,42} = 18.1$, $P < 0.001$), where seeds following one induction (generation two) had the lowest C content (Fig. 1A). Aphid attack increased seed carbon overall ($F_{3,42} = 4.087$, $P < 0.05$) with this elevation being most apparent after zero, one and three inductions (i.e., generations one, two and four). Colonization by mycorrhizal fungi tended to increase seed C, but this was not significant across all inductions. However, there was a significant interaction term between all three variables ($F_{3,42} = 2.9$, $P < 0.05$), as AM fungal colonization elevated seed C when aphids were absent, this being most clearly seen after zero, one and three inductions (Fig. 1A). When progeny plants in generations 2, 3 and 4 (Supplementary Fig. S1) were grown without aphids, the increase

in seed C persisted (comparing treatments 2B v. 3B v. 4B and 2C v. 3C v. 4C, Fig. 1B).

With seed P content, insufficient seed material was available in the no aphid (with or without AM fungi) treatments, following one induction. Seed P was measured for one replicate, but as a result, data for seed P in generation two (induction one) were excluded from the statistical analysis. However, the values for seed P in the no aphid treatments were broadly in line with those where aphids were present following one induction (generation two) (Fig. 2A).

Seed P content varied across inductions ($F_{2,32} = 79.2$, $P < 0.001$), with an increase seen after 1 or 2 inductions followed by a reduction to previous levels after 3 inductions (i.e., generation 4) (Fig. 2A). Over all inductions, aphid attack decreased seed P content ($F_{1,32} = 7.5$, $P < 0.01$), but there was an interaction term found between inductions and aphids, ($F_{2,32} = 3.8$, $P = 0.05$), because aphid herbivory decreased seed P after zero and two induction events, but increased it after three inductions. Mycorrhizal fungal colonization had no effect on seed P overall, as effects varied depending upon inductions. These fungi tended to increase seed P after three inductions, but did not do so after zero, one or two inductions (Fig. 2A). When plants were grown without aphids or AM fungi, the reduction in seed P resulting from herbivory persisted after one or two inductions, but then recovered after three inductions (Fig. 2B).

Across generations, seed C:N ratio was reduced after one or two inductions ($F_{3,42} = 11.4$, $P < 0.001$), while aphid presence

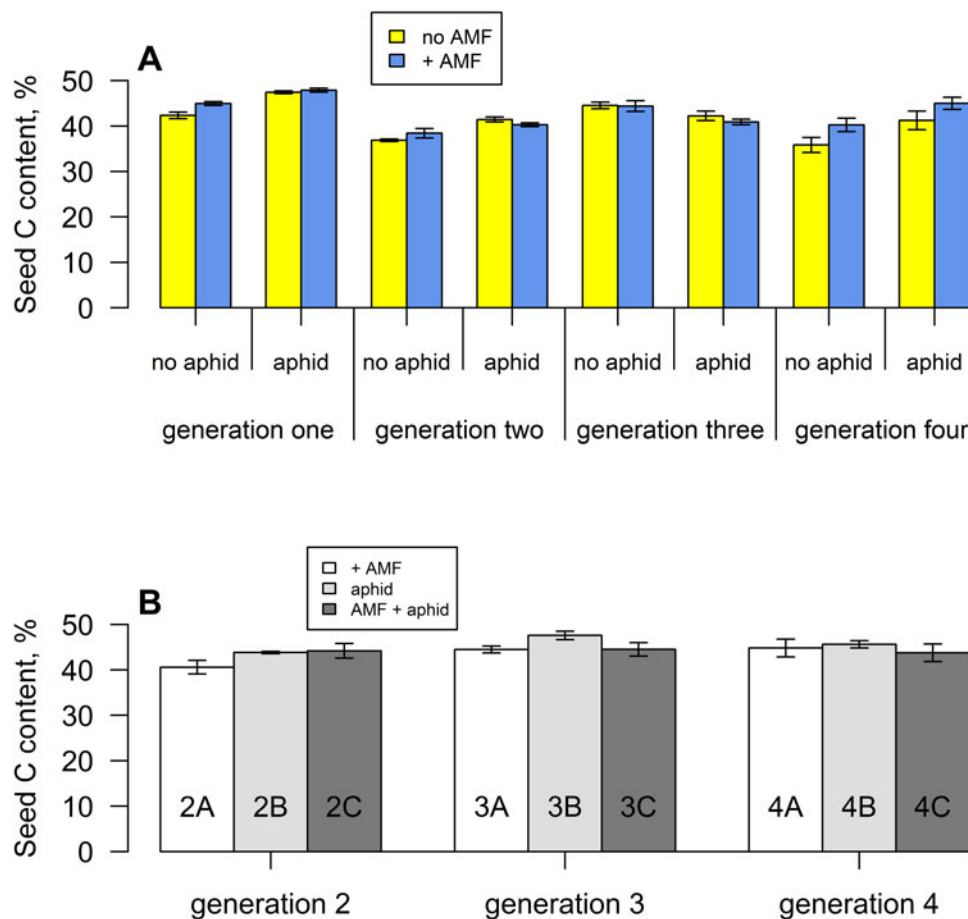


Figure 1. (A) Mean seed carbon content (%) of *Senecio vulgaris* grown with or without aphids and with (blue bars) or without (yellow bars) arbuscular mycorrhizal fungi for four successive generations. Vertical lines represent \pm one standard error, $n = 6$. (B) Mean seed carbon content of *S. vulgaris* grown without aphids, AM fungi or both, from parents that experienced these treatments (2A, B & C; 3A, B & C and 4A, B & C plants in Supplementary Fig. S1).

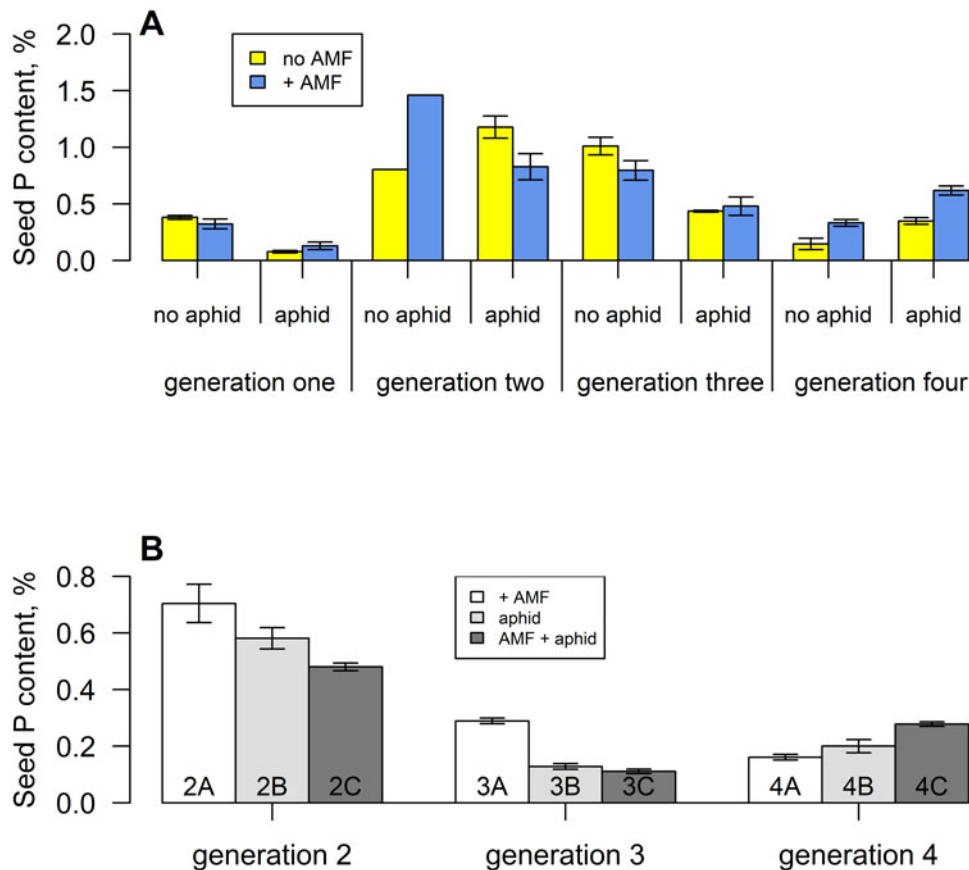


Figure 2. (A) Mean seed phosphorus content (%) of *Senecio vulgaris* grown with or without aphids and with (blue bars) or without (yellow bars) arbuscular mycorrhizal fungi for four successive generations. Vertical lines represent \pm one standard error, $n = 6$. (B) Mean seed phosphorus content of *S. vulgaris* grown without aphids, AM fungi or both, from parents that experienced these treatments (2A, B & C; 3A, B & C and 4A, B & C plants in Supplementary Fig. S1).

increased this ratio ($F_{1,42} = 4.9$, $P < 0.05$) (Fig. 3A). However, the reduction in the ratio after inductions one and two was only seen in plants that were not attacked by aphids, leading to a significant interaction term ($F_{3,42} = 11.8$, $P < 0.001$). Mycorrhizal inoculation had no effect on the seed C:N ratio, but reductions were seen in the combined treatment after zero and three inductions, leading to a significant three-way interaction term ($F_{3,42} = 3.1$, $P < 0.05$). When plants were grown without aphids or AM fungi, C:N ratios recovered more in seeds from plants arising from the combined treatment (Fig. 3B).

There was a large decrease in seed N:P ratios from generation one (before any inductions took place) to subsequent generations ($F_{2,32} = 59.5$, $P < 0.001$) (Fig. 4A). Both AM fungi ($F_{2,32} = 21.2$, $P < 0.001$) and aphid attack ($F_{2,32} = 13.5$, $P < 0.001$) increased the ratio overall, but in both cases significant interactions were found between inductions and AM fungal and aphid presence. This was because the increase caused by AM fungi and aphids was only seen clearly in generation one (Fig. 4A). Indeed, after three inductions (generation four), AM fungi reduced seed N:P ratios. When AM fungi and aphids were removed, the effects were not consistent, an increase was seen in plants grown following two inductions, but a decrease occurred in those grown following three inductions (Fig. 4B).

Seed viability, as measured by percent germination, also varied greatly across the generations ($F_{3,127} = 14.7$, $P < 0.001$), with a large decrease seen in generation two, after one induction (Fig. 5A). AM fungal colonization increased seed viability across generations

($F_{1,127} = 6.9$, $P < 0.01$), but this was most apparent when plants had experienced zero or three inductions, leading to a significant interaction term. Meanwhile, seed viability was increased by aphid herbivory in plants experiencing no or one induction, but decreased after two or three inductions, producing another significant interaction term ($F_{3,127} = 6.6$, $P < 0.001$) (Fig. 5A). When plants were grown without aphids or AM fungi, the decreases seen after one induction disappeared (Fig. 5B).

There was no overall effect of either AM fungi or aphids on seed weight after one induction ('gen 2' in Fig. 6A). AM fungal colonization appeared to reduce seed weight, and while this was cancelled out by aphid presence, there was no significant interaction term. The addition of zebularine had no effect on seed weight in the presence of AM fungi or aphids.

As in the main experiment, there was a large reduction in seed viability after one induction (Fig. 6B), with decreases seen in all treatments ($F_{1,71} = 53.6$, $P < 0.001$). The addition of zebularine had a weak effect ($F_{1,71} = 2.85$, $P < 0.05$) in that viability was not reduced to such an extent when AM fungi were absent.

Germination time was similarly reduced in all treatments after one induction (Fig. 6C) ($F_{1,71} = 84.3$, $P < 0.001$) but as with seed weight, no effects of zebularine addition were found.

Discussion

We found clear evidence of maternal effects on seed provisioning in *S. vulgaris*. All of the parameters measured (seed C, P, C:N

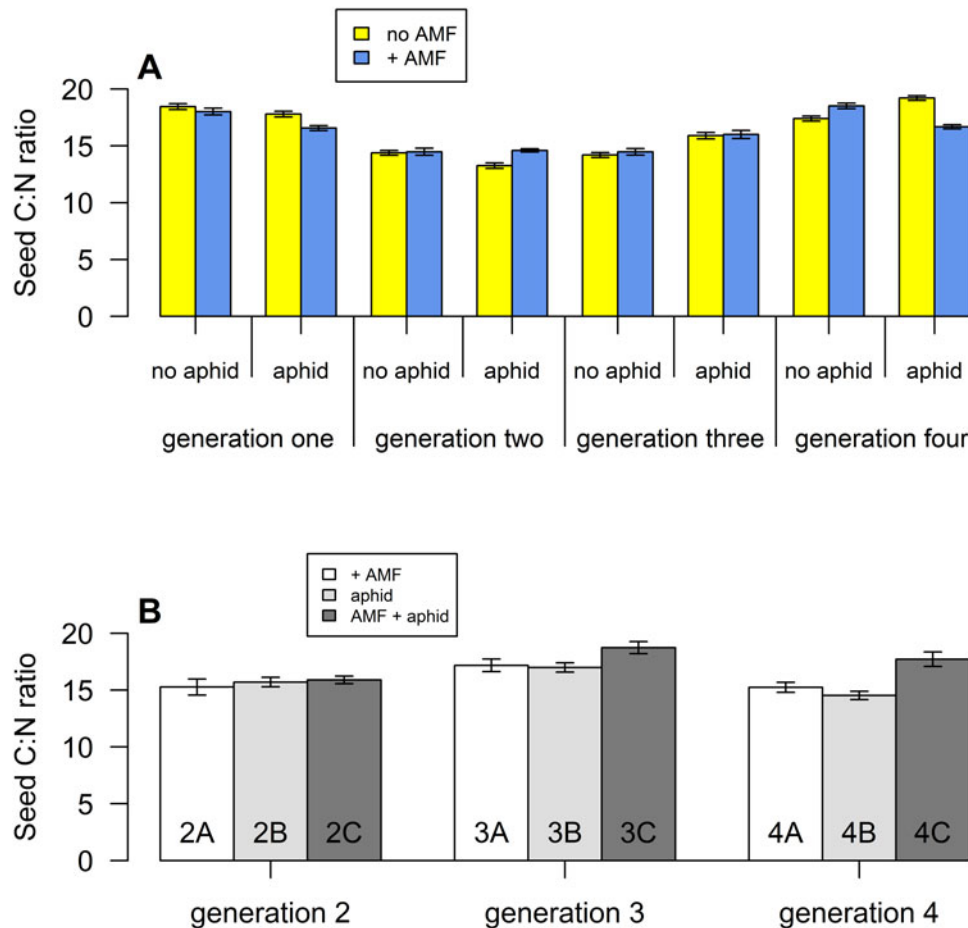


Figure 3. (A) Mean seed C:N ratio of *Senecio vulgaris* grown with or without aphids and with (blue bars) or without (yellow bars) arbuscular mycorrhizal fungi for four successive generations. Vertical lines represent \pm one standard error, $n = 6$. (B) Mean seed C:N ratio of *S. vulgaris* grown without aphids, AM fungi or both, from parents that experienced these treatments (2A, B & C; 3A, B & C and 4A, B & C plants in Supplementary Fig. S1).

ratio, N:P ratio and viability) changed over inductions; however, in all cases, these changes were not consistent; often decreases in the parameter were followed by recovery after further inductions. AM fungal colonization decreased C:N and N:P ratios, while aphids increased them, but the effects were not consistent, providing partial support for our first hypothesis. There were some interactions found between the presence of these fungi and aphids, providing support for our second hypothesis. Meanwhile, our third hypothesis found strong support; AM colonization increased seed viability, while aphid herbivory decreased it, though again this depended on previous inductions. The transient nature of the maternal effects was often exemplified by those plants that no longer experienced the treatments and were grown without fungi or aphids. Frequently, the parameters measured recovered in these plants to be more similar to the controls (no insects or fungi) in the main experiment. Furthermore, plant parameters in the zebularine treatments (albeit over one induction (two generations) showed few differences to those in untreated plants, suggesting that epigenetic changes played a minor role in the maternal effects seen.

Previously, from this experiment, we have shown that maternal effects result in altered seedling performance and plant growth rates in the progeny generations (Chitty and Gange, 2022a). The majority of studies involving maternal effects concentrate on parental stresses, such as lack of water or nutrients (e.g.

Walter et al., 2016) wherein progeny performance is often altered depending upon the environmental conditions experienced by the parent. In this respect, *S. vulgaris* is no exception; Aarssen and Burton (1990) found that maternal plants that experienced nutrient stress produced smaller seeds which resulted in smaller seedlings, but which were able to survive for longer if the stressful conditions persisted. In these experiments, environmental conditions were kept constant through all generations, but the obvious next step is to conduct larger, multi-factorial studies in which biotic factors such as fungi and herbivores are combined with abiotic factors such as water and nutrient availability (Latzel et al., 2023). The critical point is that we have shown maternal effects on seed provisioning to be strong, and so likely to affect plant performance in natural communities. However, whether these effects are influenced to any extent by variation in temperature and/or rainfall from year to year is unknown.

AM fungi represent a significant sink for photosynthetically fixed carbon, with estimates of up to 30% of annual production consumed (Olsson et al., 2010). However, these fungi can also elevate photosynthetic rates, largely compensating for the cost of the sink (Zhu et al., 2022). Meanwhile, insect herbivores also have little overall effect on the carbon contents of plant foliage (Aguilar-Chama and Guevara, 2016; Lucas-Barbosa et al., 2017). We found some increases in seed carbon resulting from herbivory, but these depended on the number of inductions that parental

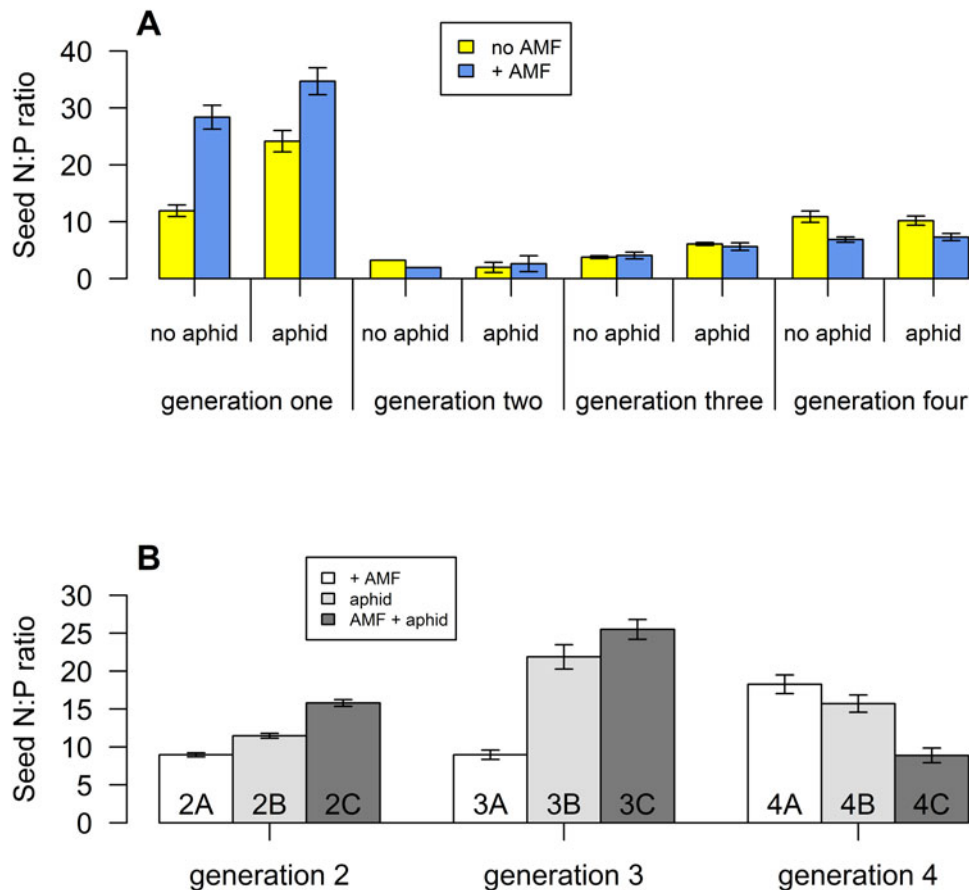


Figure 4. (A) Mean seed N:P ratio of *Senecio vulgaris* grown with or without aphids and with (blue bars) or without (yellow bars) arbuscular mycorrhizal fungi for four successive generations. Vertical lines represent \pm one standard error, $n=6$. (B) Mean N:P ratio of *S. vulgaris* grown without aphids, AM fungi or both, from parents that experienced these treatments (2A, B & C; 3A, B & C and 4A, B & C plants in Supplementary Fig. S1).

plants had experienced. Furthermore, AM fungal colonization also tended to elevate seed C, but this was only when aphids were absent, and also depended on induction number. Aphid attack may reduce AM colonization and functioning (Babikova et al., 2014), which may explain the mycorrhizal effect when they were absent (upholding our second hypothesis), but it may also have no influence (Charters et al., 2022). Such contrasting effects may be a result of their having been no control for parental effects in those experiments (Latzel, 2015).

We expected seed P to be increased by the presence of AM fungi, as it has long been known that these fungi play an important role in P uptake (Bolan, 1991), leading to higher rates of P provisioning and improved seedling performance (Poulton et al., 2002; Varga et al., 2013; Wang and Tang, 2022). However, in this study, seed P depended upon induction number, being increased after three inductions, but showing no effect before then. Mycorrhizal colonization was around 10% in generation one and four plants, and around 5% in generations two and three (Chitty and Gange, 2022a). It is intriguing that seed P was highest in the generations where AM fungal colonization was lowest and this may be a result of *S. vulgaris* being a weakly mycorrhizal plant (West, 1995) or even that it is antagonized by the fungi to some degree (Jin et al., 2017). Meanwhile, aphid herbivory tended to decrease seed P, but this was also dependent on the induction number, with the effect disappearing after three inductions. It may also be no coincidence that seed P was lowest in the

generations when aphid growth rate was highest (Chitty and Gange, 2022b), suggesting that the insect attack negatively affected P provisioning of seeds in these generations. To our knowledge, there are no previous reports of how aphid herbivory affects seed P content, but Charters et al. (2020) found decreases in shoot P content in wheat cultivars when attacked by aphids.

We have previously reported from this experiment that aphid attack dramatically reduced seed N content after one and two inductions (Chitty and Gange, 2022b). Therefore, while aphid feeding increased seed C, the reduction in seed N was much greater, leading to an increase in seed C:N ratios in plants that were attacked. Previous studies have produced similar results; Soufbaï et al. (2017) found that aphids increased foliar C:N ratios in two *Brassica* species, while Zhang et al. (2011) found similar effects with chewing grasshoppers in some (i.e., not all of those studied) plant species. However, our results provided only partial support for our first hypothesis, as the increases were not consistent across generations. Some previous studies of grazing have found no maternal effects on C:N ratios (e.g. Zhang et al., 2011; Ma et al., 2019), but these studies have taken place over two generations (parent and progeny), highlighting the need for longer-term studies.

Changes in seed C:N ratios can have important consequences for seed mass and viability. Both He et al. (2005) and Zhang et al. (2017) found that seed mass increased as the C:N rose, and decreased as the N concentration increased. To an extent, a

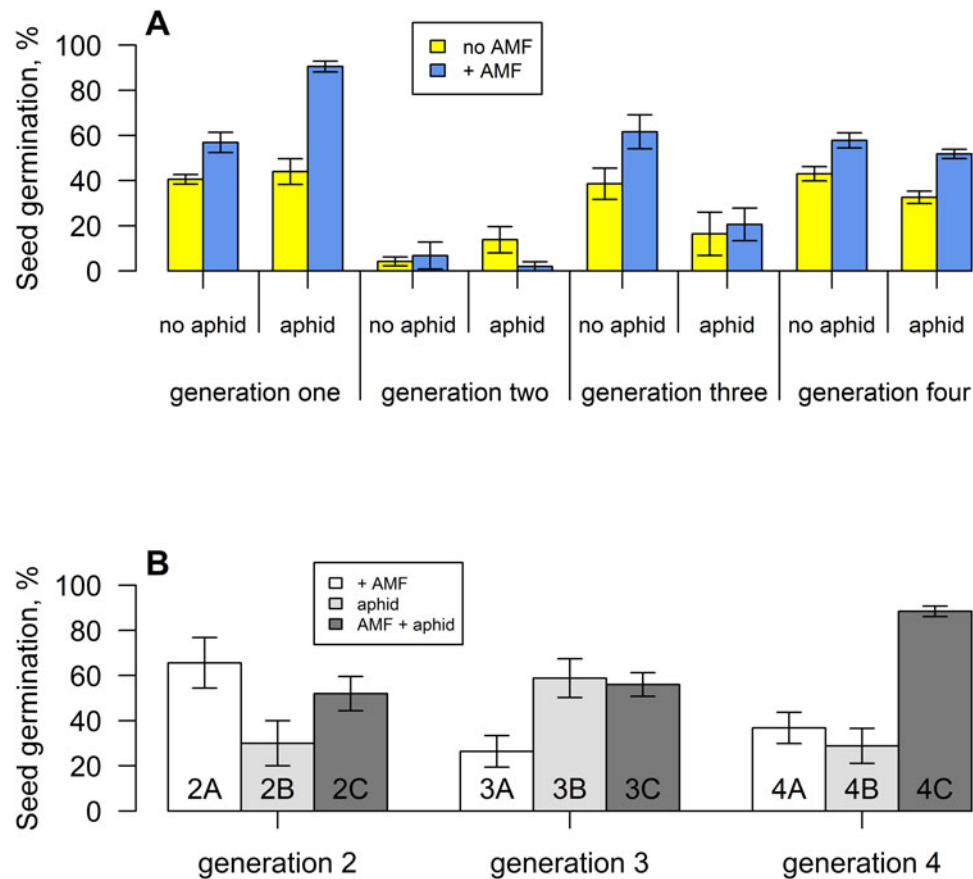


Figure 5. (A) Mean seed viability (percent germination) of *Senecio vulgaris* grown with or without aphids and with (blue bars) or without (yellow bars) arbuscular mycorrhizal fungi for four successive generations. Vertical lines represent \pm one standard error, $n = 6$. (B) Mean seed viability of *S. vulgaris* grown without aphids, AM fungi or both, from parents that experienced these treatments (2A, B & C; 3A, B & C and 4A, B & C plants in Supplementary Fig. S1).

similar pattern was found in the current study; aphid attacks increased seed C:N ratio after one induction, when they also increased seed size (Chitty and Gange, 2022a). However, after two inductions, this effect disappeared. Meanwhile, the lack of a mycorrhizal fungal effect on the C:N ratio was mirrored in the absence of effects on seed size (Chitty and Gange, 2022a). Germination of seeds may also be controlled by variation in the C:N ratio (Osuna et al., 2015), but while the presence of both AM fungi and aphids increased seed viability, this did not tend to correlate with changes in the C:N ratios of those seeds.

In contrast to seed C:N ratios, there were large changes seen across inductions in the N:P ratios. Overall, both AM fungal colonization and aphid attack increased the ratios, the latter fact upholding our first hypothesis. After zero inductions (generation one), seed N:P was greatly increased by the fungi (counter to our hypothesis), but after three inductions, the ratio was decreased, upholding the hypothesis. These effects can largely be explained by the changes seen in seed N rather than P, as AM colonization increased seed N after zero inductions (Chitty and Gange, 2022b), but had no effect on seed P. However, the underlying mechanism was reversed after three inductions; AM fungi increased seed P, while having no effect on N, leading to a decrease in the N:P ratio. Guo et al. (2013) also found that AM fungi could reduce seed N:P ratios in sorghum, though the effect differed between fungal species and was only studied in one plant generation. It is generally agreed that AM fungi play an important role in the uptake of nitrogen from soil, though the contribution of this

relative to P in the nutrient budget of plants is still debated (Buckling and Kafle, 2015). Our results suggest that the outcome of experiments investigating the effect of AM fungi on nutrient uptake may be influenced by the maternal environment of the plants studied (particularly soil nutrient content), which may be a reason for conflicting results seen in the past. Meanwhile, after zero inductions, aphid attack decreased seed P, but elevated seed N (Chitty and Gange, 2022b), leading to increases in the N:P ratio, as hypothesized. However, after one and two inductions, seed N was much reduced by aphids, as was seed P, resulting in little change in the N:P ratios.

Variation in seed C:N:P ratios, in particular N:P, have been used to evaluate potential nutrient limitations in plants (Reich and Oleksyn, 2004). For example, decreases in the N:P ratio suggested that N is more limiting to growth than P in rice (Li et al., 2019). In particular, stoichiometric ratios are linked to growth rates (Elser et al., 2000), with high P content (i.e., lower N:P ratio) associated with faster growth rates (Peng et al., 2011). To an extent, this was seen in the current study; after three inductions colonization by AM fungi reduced the N:P ratio, which was correlated with greater seed viability and faster germination, upholding our third hypothesis (Chitty and Gange, 2022a). However, after zero inductions, the fungi increased the N:P ratio but this also resulted in greater viability. *S. vulgaris* displays rapid growth rates in its native and introduced ranges (Robinson et al., 2003) and it may be that the plant is sensitive to both nitrogen and phosphorus amounts in soil.

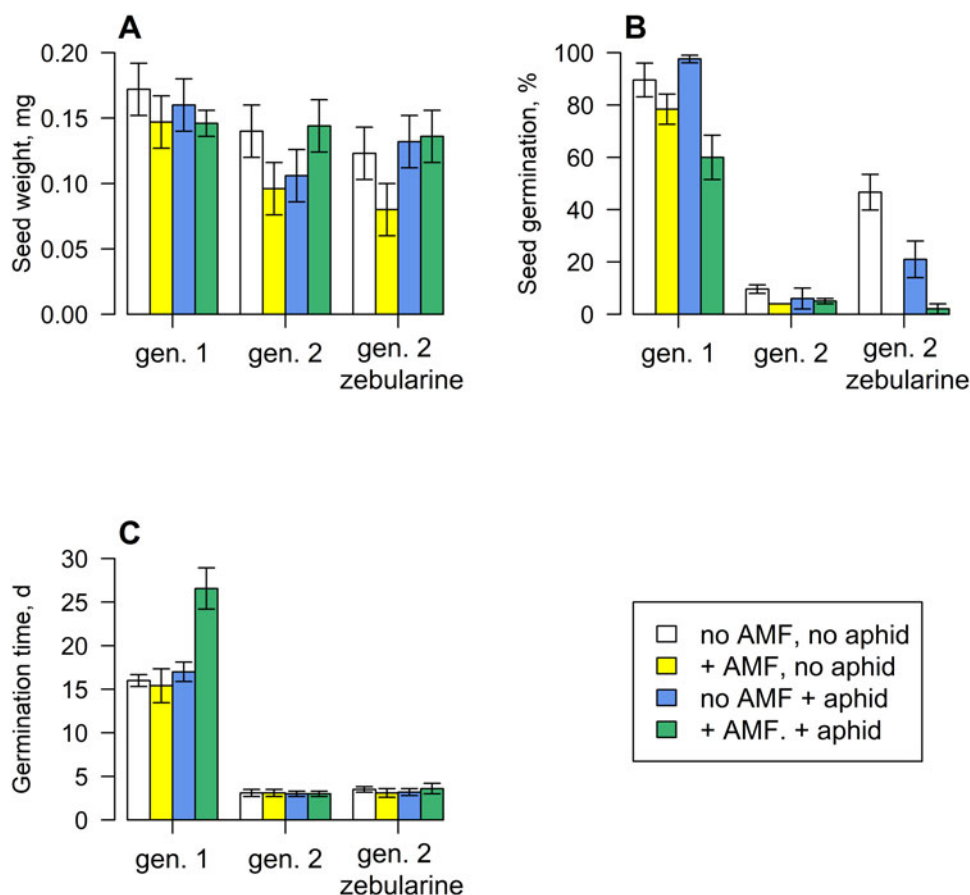


Figure 6. Mean weight of seeds (A), viability (B) and germination time (C) of *Senecio vulgaris* in plants ($n = 10$ in each case) grown from parents experiencing no inductions (generation one) or one induction (generation two) and grown with or without aphids and with or without AM fungi. Plants following one induction were also grown with the demethylating agent zebularine. Vertical lines represent \pm one standard error.

Increases in plant nitrogen uptake facilitated by AM fungi are thought to be one way by which aphid growth is increased on mycorrhizal plants (Wilkinson et al., 2019), as the fungi change plant physiology, enabling phloem location to be more successful (Simon et al., 2017). However, over the course of these experiments, we found little evidence for improved aphid growth on mycorrhizal plants (Chitty and Gange, 2022b). Therefore, our second hypothesis, that interactions would be seen between the fungi and the aphids in their effects on stoichiometry, was not upheld. It is important to note that any such interactions are two-way; while AM fungi may increase aphid growth, aphids can reduce AM colonization (Babikova et al., 2014), which was also seen in this study (Chitty and Gange, 2022b). It may be that these interactions cancel each other out, resulting in an overriding effect of inductions in the analyses of the different parameters.

Throughout these studies, the maternal effects appear to be via a mechanism of seed provisioning and not through epigenetic changes, which may frequently enable plants to cope with changing environments (Ashapkin et al., 2020). In most cases, when seeds were taken from plants and grown without the treatments (2A, 2B, 2C, 3A, etc. plants in Supplementary Fig. S2), the resulting parameters were different from those in the main experiment, often showing immediate recovery when there had been a reduction. This is further supported by the results of the zebularine experiment. Here, the application of the demethylation agent had little or no effect on seed weight, viability or germination

time, suggesting that the alterations in seed mass and viability were not inherited thereby providing no support for our fourth and final hypothesis.

In conclusion, we have shown that both AM fungi and insect attacks can shape the maternal effects of seed provisioning in *S. vulgaris*. This is likely to influence plant performance in the field, where the species often grows in the same situation from season to season and from year to year. The fact that these maternal effects may be influenced by environmental parameters of water and temperature means that the maternal effects may vary in their intensity in different seasons, as the species may have three or even four generations per year (Grime et al., 1988). While being ecologically important for the performance of the plant and its associated organisms, a more general point is that when researchers collect seeds from the wild to begin their experiments, the precise history of the plant and its parents are always unknown. This reinforces the plea of Latzel (2015) to grow at least one generation before any experiments begin, to remove this form of environmental variation from their work.

Supplementary material. The supplementary material for this article can be found at <https://doi.org/10.1017/S0960258524000266>.

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