TITLE:

Selection on flowering phenology mediated by seed predators and dependent on the community context

Butterfly seed predators and ants as selective agents for flowering phenology

Butterfly seed predators and their second hosts as selective agents for flowering phenology

Selection on flowering phenology: the role of (butterfly) seed predators and their second hosts

Selection on flowering phenology mediated by (butterfly) seed predators and their second hosts

Shifts in selection on flowering phenology mediated by (butterfly) seed predators and their second hosts

Context-dependent seed predation shifts direction of selection on flowering phenology

Predator-mediated and context-dependent (shifts in) selection on flowering phenology

Or: Seed predator-mediated shifts in selection on host flowering phenology depend on a second host.

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INTRODUCTION

Timing of reproduction in plants influences interactions both with the physical environment and with other organisms. Both pollinators (mutualists) and predispersal seed predators (antagonists) can exert selection on flowering phenology (Elzinga *et al.*, 2007) and this leads to different and sometimes opposing selective forces (Brody, 1997; Strauss & Irwin, 2004). Phenotypic selection on floral traits by pollinators is positively related to the degree of pollen limitation (Totland, 2001), and pollinator preference for certain floral traits does not always translate into higher fitness. However, preference of a phenotype by seed predators most likely decreases the fitness of that particular phenotype (Parachnowitsch & Caruso, 2008). Nowadays, there is weak empirical support for pollinator-mediated selection on flowering time (Ehrlén, 2015), while evidence of phenotypic selection mediated by predispersal seed predators on plant traits, such as flowering time, is accumulating (Leimu *et al.*, 2002, Kolb *et al.*, 2007, Ehrlén & Münzbergová, 2009, Kolb & Ehrlén, 2010). … Direct vs. indirect selection .. For example, earlier flowering plants usually have more available resources and thus are able to grow higher and produce more flowers than later-flowering plants (Ison & Wagenius, 2014).

Spatiotemporal variation in species interactions may result in selection mosaics, and lead to different coevolutionary trajectories of plants and their animal interactors in different populations (Thompson, 2005). Differences in selection can be the result of differences both in the intensity of interactions and in the trait preferences of one of the interacting partners. For example, the relationship between plant reproductive traits and predispersal seed predation may vary among populations and years (Kolb *et al.*, 2007), and contribute to variation in selection (Benkman, 1999; Rey *et al.*, 2006). Interaction intensities and preferences of seed predators might, in turn, be related to the environmental context. Both the physical environment where the interaction occurs (Kolb & Ehrlén, 2010; König *et al.*, 2014, 2015; von Euler *et al.*, 2014) and other community members (i.e. the community context, Brandt & Foitzik, 2004; Strauss & Irwin, 2004; Siepielski & Benkman, 2007; Chamberlain et al. 2014) might influence interactions. A simple example of effects of community context is that the outcome of an interaction between a plant and its seed predator depends on the interaction with a third species that is an alternative host species or a natural enemy of the predator.

Large Blue butterflies (*Maculinea* spp.) are predispersal seed predators of specific host plants during their first larval instars. have been shown to bedevelopmentalas well as *Maculinea* larvae need also a second host to complete their development, and they are parasites of ant nests (*Myrmica* spp.) during later instars (Als *et al.*, 2004). The community context in terms of the abundance of the second host might thus indirectly affect phenotypic selection on host plant traits through effects on butterfly abundance and predation intensity. In this study, we examined how phenotypic selection on flowering phenology in the perennial herb *Gentiana pneumonanthe* is mediated by the interaction with its specialist predispersal seed predator, the butterfly *Maculinea alcon*, and how this interaction depends on the community context in terms of the abundance of the second host, *Myrmica* ants . We addressed two main questions: 1) Is the direction and strength of phenotypic selection on flowering phenology in *G. pneumonanthe* influenced by interactions with the seed predator?, and 2) Is butterfly presence and seed predation intensity related to the community context, in terms of the abundance of their second host ()?

MATERIALS AND METHODS

**Study system**

The marsh gentian (*Gentiana pneumonanthe* L.) is a rare, long-lived perennial herb that reaches 45 cm height. It overwinters as a rosette of short shoots with small leaves (Simmonds, 1946). Plants can have one to many shoots and produce deep blue flowers that are pollinated by bumblebees. The species is entirely self-compatible and flowers in July and August in SW Sweden. Fruits are capsules containing a high number of minute, wind-dispersed seeds. The species occurs in open habitats, such as wet heathlands and grasslands. *Gentiana pneumonanthe* is the primary host of the Alcon Blue butterfly (*Maculinea alcon*), a specialist predispersal seed predator which lays its eggs on young buds during its flight period (July and August, Appelqvist *et al.*, 2007). The caterpillars consume a large fraction of developing seeds in some populations. Fourth-instar caterpillars drop to the ground and are picked up by their second host, *Myrmica* ants. In our study area, probably *M. ruginodis* is the commonly used ant host species (Appelqvist *et al.*, 2007). Caterpillars mimic the surface chemistry of the ant brood (Nash *et al.*, 2008), and this makes ants carry them to their nest, where they spend the rest of their larval period as parasites (Mouquet *et al.*, 2005). Contrary to the majority of *Maculinea* species, which prey on ant brood, *M. alcon* is a “cuckoo” species (Als *et al.*, 2004), and larvae are fed primarily on regurgitations from ant workers, trophic eggs and prey items.

**Data collection**

We collected data on reproductive traits, plant fitness and interaction intensity in 100 marked individuals in each of the study populations during 2010 and 2011. For each individual, we selected one focal shoot as the one having median length. All measures were carried out on this focal shoot. We measured shoot height, phenological state, number of flowers and number of *M. alcon* eggs in late July – early August. Phenology of each individual, in terms of the developmental stages of flowers at a given date, was estimated by counting the number of buds and flowers in each of six developmental stages: a) small bud (the sepals covering the bud completely), b) medium-sized bud (from the time the bud begins to protrude outside the sepals until it grows over the sepals) c) large bud (the bud becomes elongated and rather high), d) not fully blooming flower (from the time the bud starts changing into blue until it becomes a solid blue, tightly twisted bud) e) blooming flower (from the time the bud starts to open, when the petals start to spin up, to a fully opened flower) and f) wilted flower (merged and started to wither). Using this data, we derived two different measures of the developmental stage of each individual: (1) the mean developmental stage of all flowers and buds within a shoot, and (2) the state of the most advanced bud in each shoot.

Populations were visited until all fruits had matured in mid-October. The maximum number of eggs observed during these visits was used as an estimate of interaction intensity. The number of intact (i.e. not damaged by the butterfly) mature fruits was used as an estimate plant fitness. In 2011, no information on fruit production was available for 4 of the populations.

Ant abundance was estimated in each of the 20 study populations, using ant counts along transects with a sugar cube was placed every meter. after that the sugar cube was presented to antsIn 2010, 20-m transects were established , and in 2011 we used a 40-m transect in each population. We used the highest yearly value of the average number of ants per sugar cube in each population as a measure of ant abundance. This was to partly decrease errors associated with variation in ant abundance due to varying weather conditions.

**Statistical analyses**

To assess phenotypic selection on flowering phenology, we performed selection gradient analyses in each of the two years by regressing relative fitness on standardized estimates of phenology, flower number and shoot height (Lande & Arnold, 1983). Fitness, in terms of the number of intact fruits, was relativized within populations by diving individual values by population mean values. Traits were standardized by subtracting the population mean and dividing by the population standard deviation. To test for differences in phenotypic selection gradients among populations, we included also interactions between standardized reproductive traits and population. The main effect of population was not included because fitness was relativized within populations prior to analysis. We fitted a linear model with Type II sums of squares. In addition to tests of directional selection, we also tested for correlational selection by examining the effects of interaction terms in a model also including the linear terms, and for non-linear selection by examining the effect of quadratic terms in a model also including the linear terms. Results for models using mean and most advanced flower developmental states within shoots were very similar in all cases. Below, we present only results for the state of the most advanced bud, hereafter referred to as “phenology”. Higher values indicate earlier flowering.

To test if linear selection on reproductive traits differed among populations with vs. without *M. alcon*, we created a variable “Predation”, coded as 0 in populations without *M. alcon* and as 1 in populations with *M. alcon*, and constructed a linear hierarchical mixed model (Type II sums of squares) for each year. Predictors included standardized reproductive traits and their interactions with population as random effects, and predation as fixed effects. Effects of trait × population and trait x predation interactions were only included in these models if the trait x population interaction was significant. We did not include an individual intercept by population because fitness was relativized within populations prior to analysis.

In populations where the butterfly was present, we investigated predator preferences, by regressing the frequency (0 or 1) and intensity (number of eggs) of attack by *M. alcon* on reproductive traits, population and their interactions in each of the two years. We also examined the direct effects of reproductive traits on fitness, and the indirect effects mediated by the probability of predator attack or interaction intensity, using path analyses (Grace, 2006). To test for differences among populations, we used multigroup analysis (Grace, 2006). Because populations differed significantly, we fitted separate models for each population and year. We excluded population D in 2010 from these analyses because only one plant individual was attcked. Details and results of this additional analysis are given in Appendix S2.

Finally, we examined if the abundance of the second host of *M. alcon* influenced phenotypic selection by increasing the probability of predator presence or the intensity of the interaction. First, we performed a logistic regression of butterfly presence on log-transformed ant abundance, using the 20 study populations. Second, performed linear regressions of the mean number of eggs per plant and the proportion of plants with *M. alcon* eggs in each of the two years on log-transformed ant-abundance, using the 11 populations where the butterfly was present.

Path analyses were carried out in Amos 16.0 (Arbukle, 2007). All other analyses were conducted in R 3.1.2 (R Core Team, 2014).

RESULTS

We found evidence for directional selection on flowering phenology, flower number and shoot height in both study years (Table 1A). Linear selection on flowering phenology and flower number differed among populations in both study years and selection on shoot height differed in 2011. We also found evidence of correlational selection, although effects differed among populations (Table 1B). In both study years, we found a significant quadratic effect of flower number on fitness, but also this effect differed among populations (Table 1C).

Phenotypic selection on flowering phenology differed between populations where the predator was absent and populations where it was present (Table 2, Appendix S3). In populations where the predator was absent, there was selection for earlier flowering (mean ± 95% CI of selection gradients, 2010: 0.22 ± 0.15, 2011: 0.30 ± 0.17), while in populations where the predator was present there was selection for later flowering (mean ± 95% CI of selection gradients, 2010: -0.19 ± 0.15, 2011: -0.10 ± 0.11, Fig. 1).

In both study years, early flowering increased the probability of being attacked by the predator, and this effect did not differ significantly among populations (Table 3, Appendix S4). In 2010, there were differences between populations in the relationship between probability of attack and flower number, while in 2011 the predator consistently preferred plants with higher number of flowers in all populations. In 2011, there were differences between populations in the relationship between probability of attack and shoot height. There were also differences between populations in the effects of phenology and flower number on predation intensity (number of eggs, Table 3, Appendix S4) in both years studied.

Multigroup analyses revealed significant among-population differences in trait-fitness relationships (Tables S2.1-2). Neverhteless, models fitted for each population and year consistently identified effects of phenology on fitness mediated by seed predator preference for early-flowering plants, while direct effects of traits on fitness were less consistent (Tables S2.3-5).

The probability of *M. alcon* presence increased with increasing abundance of *Myrmica* ants in the population (Fig. 2A). Although there were populations with high ant abundance where *M. alcon* was absent, ant abundance was seldom low in populations where it was present. In populations where the butterfly was present, neither the mean number of eggs per plant (Fig. 2B) nor the proportion of plants with *M. alcon* eggs (Fig. 2C) was related to ant abundance.

DISCUSSION

In this study, we have shown that the butterfly seed predator *M. alcon* mediates phenotypic selection on flowering phenology *G. pneumonanthe*. In the absence of the seed predator, phenotypic selection favors earlier flowering. Because *M. alcon* preferentially attacks earlier-flowering individuals, selection favors later flowering in populations where the predator is present. Butterfly-mediated selection on flowering phenology, in turn, depends on community context, i.e., the incidence of the predator in host plant populations increases with ant abundance.

**xxx**Our finding that there is phenotypic selection for later flowering phenology mediated by the interaction with a butterfly seed predator agrees with previous studies demonstrating predator-mediated selection on phenology (Pilson, 2000; Parachnowitsch & Caruso, 2008). However, other studies (see review by Kolb *et al.*, 2007) have reported predators selecting for both early and late flowering. . Therefore, plants flowering late have a higher fitness in presence of the predator because they are less prone to be attacked.

Our results also show that in the absence of the predator, phenotypic selection favored early flowering, being consistent with the direction of the general trend shown by Munguía-Rosas *et al.* (2011). This could be due to early flowering directly increasing fitness (e.g. by favoring outcrossing, Munguía-Rosas *et al.*, 2011), but also to indirect selection on other correlated reproductive traits (e.g. early-flowering plants might have more resources and produce in general higher shoots and more flowers), or to environmental covariance (i.e. phenology and fitness being both influenced by the environment, Ehrlén, 2015). In our study, the effect of phenology on fitness remained significant also after including two traits, that are likely to be correlated with plant resource state, as covariates in our models. This suggests that early flowering may have positive effects on fitness in the absence of the predator. Xxx. Our results demonstrate that the direction of selection on phenology differs between populations with vs. without the seed predator. This strongly suggests that the predator mediates shifts in selection from favoring early to favoring late flowering. Xxx. In a recent meta-analysis (Siepielski *et al.*, 2013), selection was reported to vary mainly in strength, but less in direction among populations. We have found evidence of variation both in direction (i.e. favoring early or late flowering depending on absence or presence of the predator) and in strength (cf. linear selection gradients in Appendix S3). Xxx. If predator distribution is constant in time, and there is sufficient genetic variance in flowering time to allow a genotypic selection response (Putterill *et al.*, 2004), this divergent selection could also lead to local adaptation (Thompson, 2005) and result in genetic differences in flowering phenology among populations. Besides, the differences in selection strength could lead to populations experiencing stronger selection adapting more quickly than populations experiencing weak selection (Siepielski *et al.*, 2013). Our data do not show significant differences in flowering phenology among populations where the predator is present and absent (results not shown). However, common garden experiments are ongoing in order to assess if these populations differ in flowering phenology when environmental variation is removed.

We also found evidence for phenotypic selection on other plant traits. Butterflies might prefer to lay eggs on tall shoots, as this indicates better plant quality and hence more resources available for larval development (Czekes *et al.*, 2014; Wynhoff *et al.*, 2014). Phenotypic selection on flower number varied among populations, but these differences were not mediated by predation, and could be explained by variation in the strength of the relationship between flower and fruit production (due to e.g. variation in pollination success or in resources available for fruit set, Zimmerman & Aide, 1989). consistently The visibility of food plants for butterflies may be determined by both its height and the size of its floral display (Nowicki *et al.*, 2005). However, in our study system, the first factor is not very important, and the importance of the second varies between years.

Selection on flowering phenology in *G. pneumonanthe* depends on the presence of the predator, and thus is also indirectly determined by the factors that determine predator presence. We have shown that the community context in terms of the abundance of the second host is a key factor for predator presence, and thus it will indirectly condition phenotypic selection on flowering phenology. Xxx. Previous studies have shown that the community context can affect the likelihood or intensity of plant-animal interactions (Strauss & Irwin, 2004), as selection on plant traits by either mutualists or antagonists can be influenced by other community members. For example, nectar robbers can affect selection by pollinators (Irwin, 2006), and plant neighborhood may alter selection by insect herbivores (Agrawal *et al.*, 2006). In our study system, the presence of *M. alcon* is more probable in sites with a high abundance of *Myrmica* ants, as the butterfly needs them to complete its life cycle. Therefore, the plant-seed predator interaction can only take place and mediate phenotypic selection in these sites. However, in populations where the butterfly is present, the intensity of seed predation on *G. pneumonanthe* is not related to ant abundance. This suggests that the second host is crucial for antagonist presence, but does not determine its abundance. Further studies relating predation to ant abundance near individual plants would clarify if community context determines interaction intensity at an individual scale, as the evidence for ant-related oviposition patterns is nowadays controversial (van Dyck *et al.*, 2000; Nowicki *et al.*, 2005; Fürst & Nash, 2010; Wynhoff *et al.*, 2014).

Our results illustrate how biotic interactions can shift the direction of phenotypic selection, and how the community context of these interactions can explain differences in selection among populations. In our study system, variation in selection on flowering phenology is determined by a seed predator, whose presence is conditioned by the abundance of its second and host. Thus, both antagonist interactors and the community context may act respectively as direct and indirect selective agents that can determine evolution on flowering time. Longer studies that also investigate the heritability of phenological traits are needed to assess if this shift in selection is maintained in time and could result in genetic differences in flowering phenology among populations.

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TABLES

Table 1: Effects of three standardized traits, flowering phenology, flower number, and shoot height, population and their interactions on relative fitness (number of intact fruits) of *G. pneumonanthe* in 2010 (N = 2000 plants in N =20 populations) and 2011 (N = 1598 plants in N = 16 populations). Results are from linear models with Type II sums of squares, including: A) only linear effects, B) linear effects and interactions and C) linear and quadratic effects. Estimates are given for significant main effects where the Population x trait interaction is not significant.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Source of variation | |  | 2010 |  |  |  | 2011 |
| df | F | Estim |  | df | F |
| A) Linear terms | |  |  |  |  |  |  |
|  | Phenology (early flowering) | 1 | 0.02 |  |  | 1 | 0.50 |
|  | Flower number | 1 | 183.73\*\*\* |  |  | 1 | 50.81\*\*\* |
|  | Shoot height | 1 | 7.18 \*\* | 0.128 |  | 1 | 0.47 |
|  | Population x Phenology | 19 | 3.61\*\*\* |  |  | 15 | 1.98\* |
|  | Population x Flower number | 19 | 3.88\*\*\* |  |  | 15 | 2.90\*\*\* |
|  | Population x Shoot height | 19 | 1.19 |  |  | 15 | 2.35\*\* |
|  |  |  |  |  |  |  |  |
| B) Interaction terms | |  |  |  |  |  |  |
|  | Phenology x Flower number | 1 | 1.78 |  |  | 1 | 0.08 |
|  | Phenology x Shoot height | 1 | 3.21 |  |  | 1 | 2.16 |
|  | Flower number x Shoot height | 1 | 9.59\*\* |  |  | 1 | 4.57\* |
|  | Population x Phenology x Flower number | 19 | 2.66\*\*\* |  |  | 15 | 1.61 |
|  | Population x Phenology x Shoot height | 19 | 1.02 |  |  | 15 | 1.31 |
|  | Population x Flower number x Shoot height | 19 | 1.82\* |  |  | 15 | 2.37\*\* |
|  |  |  |  |  |  |  |  |
| C) Quadratic terms | |  |  |  |  |  |  |
|  | Phenology 2 | 1 | 2.47 |  |  | 1 | 0.13 |
|  | Flower number 2 | 1 | 4.38\* |  |  | 1 | 2.02 |
|  | Shoot height 2 | 1 | 0.03 |  |  | 1 | 1.50 |
|  | Population x Phenology 2 | 19 | 1.40 |  |  | 15 | 0.94 |
|  | Population x Flower number 2 | 19 | 2.40\*\*\* |  |  | 15 | 3.38\*\*\* |
|  | Population x Shoot height 2 | 19 | 1.22 |  |  | 15 | 1.80\* |

\* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001

Table 2: Effects of three standardized traits, flowering phenology, flower number, and shoot height, predation and their interactions on relative fitness (number of intact fruits) of *G. pneumonanthe* in 2010 (N = 2000 plants in N =20 populations) and 2011 (N = 1598 plants in N = 16 populations). Results are from linear hierarchical mixed models with Type II sums of squares (Wald 2 values are shown). Only significant trait x population interactions are included in the model.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Source of variation | 2010 | |  | 2011 | |
| df | 2 |  | df | 2 |
| Phenology (early flowering) | 1 | 0.00 |  | 1 | 0.86 |
| Flower number | 1 | 53.85\*\*\* |  | 1 | 27.82\*\*\* |
| Shoot height | 1 | 7.82\*\* |  | 1 | 0.21 |
| Predation | 1 | 0.11 |  | 1 | 0.00 |
| Predation x Phenology | 1 | 14.72\*\*\* |  | 1 | 14.95\*\*\* |
| Predation x Flower number | 1 | 0.01 |  | 1 | 3.69 |
| Predation x Shoot height | 1 | - |  | 1 | 5.69\* |

\* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001

Table 3: Effects of population, phenology, flower number and shoot height of *G. pneumonanthe* on probability (0 or 1) and intensity (number of eggs) attack by *M. alcon* in 10 populations in 2010 (N = 1000 plants) and 11 populations in 2011 (N = 1099 plants) where the predator was present. 2 values are shown for logistic regressions, and F values for linear regressions. Estimates (from a model without interaction terms) are given for significant main effects where the interaction with population is not significant

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Source of variation | Response: attack | | | | | | |  | Response: number of eggs | | | | |
| 2010 | | |  | 2011 | | |  | 2010 | |  | 2011 | |
| df | 2 | Estim |  | df | 2 | Estim |  | df | F |  | df | F |
| Phenology (early flowering) | 1 | 39.32\*\*\* | 0.420 |  | 1 | 39.00\*\*\* | 0.545 |  | 1 | 79.57\*\*\* |  | 1 | 46.87\*\*\* |
| Flower number | 1 | 27.69\*\*\* |  |  | 1 | 81.15\*\*\* | 0.794 |  | 1 | 75.43\*\*\* |  | 1 | 240.98\*\*\* |
| Shoot height | 1 | 1.93 |  |  | 1 | 0.04 |  |  | 1 | 0.01 |  | 1 | 0.03 |
| Population | 9 | 181.74\*\*\* |  |  | 10 | 138.77\*\*\* |  |  | 9 | 29.09\*\*\* |  | 10 | 46.00\*\*\* |
| Population x Phenology | 9 | 8.62 |  |  | 10 | 16.65 |  |  | 9 | 9.91\*\*\* |  | 10 | 5.55\*\*\* |
| Population x Flower number | 9 | 29.67\*\*\* |  |  | 10 | 14.11 |  |  | 9 | 8.77\*\*\* |  | 10 | 33.61\*\*\* |
| Population x Shoot height | 9 | 9.98 |  |  | 10 | 18.38\* |  |  | 9 | 0.52 |  | 10 | 0.72 |

\* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001

FIGURE LEGENDS

Figure 1: Differences in linear selection gradients for phenology between populations where the predator was present (*M. alcon* =1, N = 11) and absent (*M. alcon* = 0, N = 9) in 2010 and 2011. P-values from one-way ANOVAs are shown.

Figure 2: Effects of abundance of the second host (log-transformed maximum *Myrmica* abundance on A) *M. alcon* presence (p from logistic regression fit is shown, N = 20 populations), B) mean number of eggs per plant, and C) proportion of plants with *M. alcon* eggs. In B) and C), N = 11 populations where the predator was present, Black symbols in B and C are estimates for 2010 and grey are for 2011.

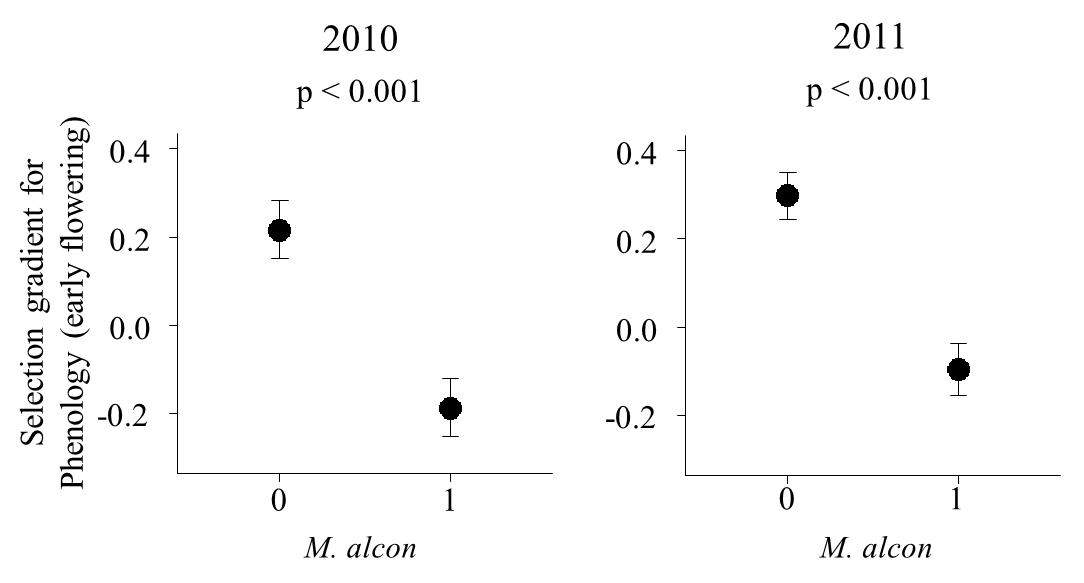
Figure 1

Figure 2

