TITLE:

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

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

Valdés, Alicia\* and Ehrlén, Johan

Department of Ecology, Environment and Plant Sciences, Stockholm University, SE-106 91 Stockholm, Sweden

\* Corresponding author. E-mail: [alicia.valdes@su.se](mailto:alicia.valdes@su.se)

INTRODUCTION

Timing of reproduction in plants influences interactions both with the physical environment and with other organisms. Plants in temperate regions have been shown to be under a strong selective pressure for flowering earlier due to abiotic conditions (e.g. climate) and pollinator-mediated selection (Munguía-Rosas *et al.*, 2011). On the other hand, growing evidence shows that predispersal seed predators can also mediate phenotypic selection on phenology and other plant traits (Pilson, 2000; Leimu *et al.*, 2002; Ehrlén & Münzbergová, 2009; Kolb & Ehrlén, 2010). A phenotype which is preferentially attacked by seed predators will most likely decrease in fitness (Parachnowitsch & Caruso, 2008). Predispersal seed predators might attack preferentially early-flowering plants if the weather conditions early in the season are more favorable for female fecundity and larval development. In this case, seed predators could shift the direction of selection towards late flowering if selection to escape from predation prevails over other selective pressures. Seed predators might also mediate indirect selection if they have a preference for other traits which are correlated with plant phenology (Lande & Arnold, 1983). 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). Seed predators might prefer attacking plants with higher inflorescences (Sletvold & Grindeland, 2008), or with many flowers (Leimu *et al.*, 2002), which could indirectly select for late flowering.

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. For example, nectar robbers can affect selection by pollinators (Irwin, 2006), and plant neighborhood may alter selection by insect herbivores (Agrawal *et al.*, 2006). A simple example of effects of community context is that the outcome of an interaction between a plant and its seed predator may depend on the interaction with a third species that is an alternative host 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. Oviposition patterns of *Maculinea* butterflies have been shown to be closely related to plant developmental state (Thomas & Elmes, 2001; Van Dyck & Regniers, 2010) as well as to other reproductive traits Czekes *et al.*, 2014). *Maculinea* larvae need also a second host to complete their development, and most species 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 (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 (mean seed weight = 0.044 mg, Simmonds, 1946), wind-dispersed seeds (usually 300-700, Appelqvist *et al.*, 2007). 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 feed and live inside the seedpods until they reach the fourth-instar, when then they drop to the ground and are picked up by their second host, *Myrmica* ants (Mouquet *et al.*, 2005). 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 (i.e. nutritious, infertile eggs which are fed to the queens and larvae) and prey items.

**Data collection**

This study was carried out in 20 populations of *G. pneumonanthe* located in the county of Västra Götaland in SW Sweden (see Appendix S1 for details). The populations are mainly located in moist heathlands. *M. alcon* was present in 11 of the study populations and absent from 9.

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:. 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 stage of the most advanced bud in each shoot. in both cases

Populations were visited once at the end of July-beginning of August and from 1 to 5 times from the end of August until all fruits had matured in mid-October (the number of visits depended on the duration of fruit maturation). 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 where a sugar cube was placed every meter. The number of *Myrmica* sp. ants in each of the cubes was counted 30 minutes after presenting the sugar cube to ants. In 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 done 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 stages within shoots were very similar in all cases. Below, we present only results for the stage of the most advanced bud, hereafter referred to as “phenology”.

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 × predation interactions were only included in these models if the trait × 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 probability (0 or 1) and intensity (number of eggs in all individuals) of attack by *M. alcon* on reproductive traits, population and their interactions in each of the two years. We excluded population D in 2010 from these analyses because only one plant individual was attacked. As an additional analysis, 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 also excluded population D in 2010 from these analyses. 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, we 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 this effect also 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). The probability of being attacked was higher in plants with higher number of flowers in all populations in 2011, but this relation differed among populations in 2010. In 2011, the relationship between probability of attack and shoot height also differed among populations. The effects of phenology and flower number on predation intensity (number of eggs, Table 3, Appendix S4) differed between populations in both years studied.

Multigroup analyses revealed significant among-population differences in trait-fitness relationships (Tables S2.1-2). Nevertheless, 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* shifts the direction of phenotypic selection on flowering phenology in *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.

Our results 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, but also to indirect selection on other correlated reproductive traits (e.g. flower number and shoot height), or to environmental covariance (i.e. phenology and fitness being both influenced by resource availability, 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 direct positive effects on fitness in the absence of the predator. Flowering early can have several advantages, e.g. avoiding competition for pollinators, favoring outcrossing, and increasing time of seed maturation in suitable weather conditions (Munguía-Rosas *et al.*, 2011).

Nevertheless, the direction of selection on phenology differs between populations with vs. without the seed predator, and this strongly suggests that the predator mediates shifts in selection from favoring early to favoring late flowering. This finding agrees with previous studies demonstrating predator-mediated selection for late flowering (Pilson, 2000; Parachnowitsch & Caruso, 2008), although other studies (see review by Kolb *et al.*, 2007) have reported predators selecting for both early and late flowering. *M. alcon* consistently preferred attacking early-flowering plants in all populations and in both study years. Therefore, plants flowering late have a higher fitness in presence of the predator because they are less prone to be attacked. Previous studies have focused on the relationship between oviposition and bud phenological state, showing that females of *Maculinea* sp. prefer laying eggs on slightly immature buds, increasing time available for brood feeding and development (Thomas & Elmes, 2001; Patricelli *et al.*, 2011). In contrast, we have focused on the response of the predator to the flowering phenology of the plant individual, and demonstrated that plants flowering early in the season are more prone to be attacked by the butterfly than plants flowering late. This may be related to the fact that *M. alcon* butterflies flying early in the season (during the first third of the flight period) deposit higher number of eggs than those flying later, due to the different fecundity of females over time (Arnaldo *et al.*, 2014). Besides, weather conditions early in the season are probably warmer and thus more favorable for larval development.

We also found evidence for phenotypic selection on other plant traits. Butterflies might prefer to oviposit on tall shoots, which indicate more resources for larval development (Czekes *et al.*, 2014; Wynhoff *et al.*, 2015), and might receive more solar radiation, which creates a warmer microclimate suitable for larval growth (Van Dyck & Regniers, 2010). However, we only observed this preference in some populations and years (Fig. S4.3). This could be explained by variation in height of the surrounding vegetation: in sites where gentians are surrounded by tall vegetation, high shoots that protrude out of the vegetation are probably preferred for oviposition (Küer & Fartmann, 2005), but this could not be the case when the surrounding vegetation is short. Phenotypic selection on flower number varied among populations, but it was not mediated by predation. *M. alcon* consistently preferred attacking plants with higher number of flowers in one of the study years, but not in the other. In our study system, plant phenology seems more important than plant height and size of the floral display (Nowicki *et al.*, 2005) for determining butterfly preferences.

In a recent meta-analysis (Siepielski *et al.*, 2013), phenotypic selection was reported to vary mainly in strength, but less in direction among populations. We have found evidence of variation both in direction and in strength of selection on flowering phenology in *G. pneumonanthe*. Phenotypic selection favors early or late flowering depending on if the predator is absent or present. This shift in selection is also indirectly mediated 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 selection on flowering phenology. The presence of *M. alcon* is more probable in sites with a high abundance of *Myrmica* ants. The butterfly is unable to complete its life cycle without ants, and thus the plant-seed predator interaction only occurs in sites where ants are relatively abundant. In these sites, selection on phenology shifts in direction towards favoring late flowering. 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. In our study system, the second host is crucial for antagonist presence, but does not determine its abundance, as the intensity of seed predation on *G .pneumonanthe* is not related to ant abundance.

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 between populations with vs. without the seed predator could 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 current 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. On the other hand, the process of local adaptation could still be ongoing in these populations, and thus it could be too early to see among-populations differences in flowering phenology caused by the predator.

Our results have important implications for understanding the causes of spatial variation in selection and the existence of selection mosaics (Thompson, 2005). We have shown that the occurrence of a biotic interaction can shift the direction of phenotypic selection, and this can lead to opposed selection outcomes in different populations. This mosaic pattern can be related to geographic variation in the community context, as selection can be indirectly determined by the interaction of one of the partners with a third species of the community whose abundance varies spatially.

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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 × 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 × Phenology | 19 | 3.61\*\*\* |  |  | 15 | 1.98\* |
|  | Population × Flower number | 19 | 3.88\*\*\* |  |  | 15 | 2.90\*\*\* |
|  | Population × Shoot height | 19 | 1.19 |  |  | 15 | 2.35\*\* |
|  |  |  |  |  |  |  |  |
| B) Interaction terms | |  |  |  |  |  |  |
|  | Phenology × Flower number | 1 | 1.78 |  |  | 1 | 0.08 |
|  | Phenology × Shoot height | 1 | 3.21 |  |  | 1 | 2.16 |
|  | Flower number × Shoot height | 1 | 9.59\*\* |  |  | 1 | 4.57\* |
|  | Population × Phenology × Flower number | 19 | 2.66\*\*\* |  |  | 15 | 1.61 |
|  | Population × Phenology × Shoot height | 19 | 1.02 |  |  | 15 | 1.31 |
|  | Population × Flower number × 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 × Phenology 2 | 19 | 1.40 |  |  | 15 | 0.94 |
|  | Population × Flower number 2 | 19 | 2.40\*\*\* |  |  | 15 | 3.38\*\*\* |
|  | Population × 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). Effects of trait × population (random effects, not shown) and trait × predation interactions were only included in the models if the trait × population interaction was significant.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| 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 × Phenology | 1 | 14.72\*\*\* |  | 1 | 14.95\*\*\* |
| Predation × Flower number | 1 | 0.01 |  | 1 | 3.69 |
| Predation × 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) of 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: probability of attack | | | | | | |  | Response: intensity of attack | | | | |
| 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 × Phenology | 9 | 8.62 |  |  | 10 | 16.65 |  |  | 9 | 9.91\*\*\* |  | 10 | 5.55\*\*\* |
| Population × Flower number | 9 | 29.67\*\*\* |  |  | 10 | 14.11 |  |  | 9 | 8.77\*\*\* |  | 10 | 33.61\*\*\* |
| Population × 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. Means±SE are represented (one-way ANOVAs indicated significant differences, with P<0.001 in both years).

Figure 2: Effects of abundance of the second host (log-transformed maximum *Myrmica* abundance) on A) *M. alcon* presence (fitted curve is a logistic regression, P-value 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

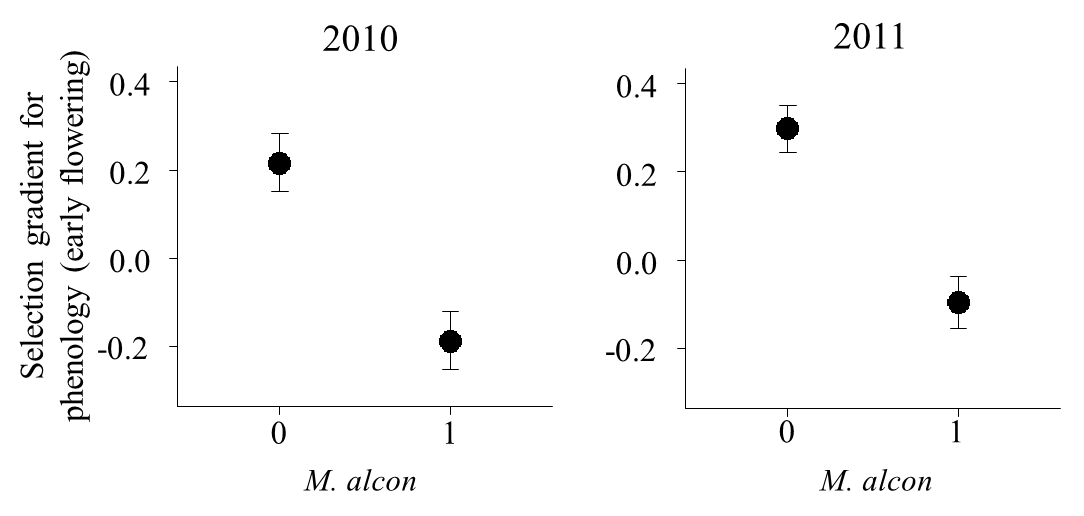


Figure 2

