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.

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ABSTRACT

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INTRODUCTION

Timing of reproduction in plants influences interactions both with the physical environment and with other organisms. For plants in temperate regions several abiotic and biotic factors may mediate selection for earlier flowering. …. have been shown to be under selection for earlier flowering due to abiotic conditions (e.g. climate) and pollinator-mediated selection (Munguía-Rosas *et al.*, 2011). On balance, several factors may act to favor later flowering. …. abiotic factors … species interactions … For example, growing evidence shows that predispersal seed predators can mediate selection on flowering phenology (Pilson, 2000; Leimu *et al.*, 2002; Ehrlén & Münzbergová, 2009; Kolb & Ehrlén, 2010), and that this sometimes favors later flowering (REFS…). 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. The physical environment where the interaction occurs ….. (Kolb & Ehrlén, 2010; König *et al.*, 2014, 2015; von Euler *et al.*, 2014). Also other community members, being natural enemies, competitors or alternative hosts, i.e. the community context, might influence interactions (Brandt & Foitzik, 2004; Strauss & Irwin, 2004; Siepielski & Benkman, 2007; Chamberlain *et al.*, 2014). For example, nectar robbers can affect selection by pollinators (Irwin, 2006), and plant neighborhood may alter selection by insect herbivores (Agrawal *et al.*, 2006). One interesting way in which community context may influence selection mediated by a focal interaction is that the outcome of a plant-consumer interaction depend on the abundance of a second resource for the consumer. For example, ….. Analyses of selection mediated by species interactions have mostly focused on pair-wise interactions, and analyses of more complex food web structures rarely have assessed effects on selection. Unravelling the ways in which community context influences species interactions and the resulting selection, is therefore a key step to link analyses of selection to community ecology.

Large Blue butterflies (*Maculinea* spp.) are specialist predispersal seed predators during their first larval instars and oviposition often occurs only on specific plant developmental states (Thomas & Elmes, 2001; Van Dyck & Regniers, 2010; 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). Selection on plant traits might thus be influenced by the community context, in terms of the abundance of the ant host through effects on butterfly abundance and seed 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) Do butterfly seed predators influence the direction and strength of phenotypic selection on flowering phenology in *G. pneumonanthe*?, and 2) Does community context, in terms of the abundance of their second host, influence butterfly occurrence and seed predation intensity?

MATERIALS AND METHODS

**Study system**

The marsh gentian (*Gentiana pneumonanthe* L.) is a rare, long-lived perennial herb, occuring in open habitats, such as wet heathlands and grasslands (Simmonds, 1946). Plants can have one to many up to 45 cm high 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 per capsule, Appelqvist *et al.*, 2007). *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 inside the capsule until they reach the fourth-instar, when then they drop to the ground to be picked up by *Myrmica* ants (Mouquet *et al.*, 2005). 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. In our study area, *M. ruginodis* is thought to be the commonly used ant host species (Appelqvist *et al.*, 2007).

**Data collection**

The 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 20 study populations during 2010 and 2011. For each individual, we selected one shoot of median length. All measurements were carried out on this focal shoot. We measured shoot height, reproductive phenological state, number of flowers, and number of *M. alcon* eggs during late July – early August. Phenology of each individual, in terms of the developmental stages of flowers on the focal shoot at a given date, was estimated by counting the number of buds and flowers in each of six developmental stages: a) the sepals covering the bud completely, b) from the time the bud begins to be visible between the sepals until it grows over the sepals, c) from the time the bud grows over the sepals until it starts to turn blue, d) from the time the bud starts to turn blue until it becomes a solid blue, tightly twisted bud. e) from the time the bud starts to open, until the flower is fully opened and f) from the time the flower shows signs of wilting. Using this data, we derived two different measures of the reproductive 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, h

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 depending on the time needed for 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, information on fruit production was not 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. Assessments were only carried out under dry (no precipitation) and warm conditions. The number of *Myrmica* sp. ants at each of the sugar cubes was counted 30 minutes after presenting them 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 remaining 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.

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 also from these analyses.

Finally, we examined if the abundance of the ant host of *M. alcon* influenced phenotypic selection in the plant host 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).

The direction of 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 within all populations where the predator was present (Table 3, Appendix S4). The probability of being attacked was higher also in plants with higher number of flowers in 2011, while the effect differed among populations in 2010. The relationship between probability of attack and shoot height wasnot significant in 2010 and differed among populations in 2011. 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 (Tables S2.3-5). Direct effects of traits on fitness were less consistent.

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 pre-dispersal seed predator *M. alcon* shifts the direction of phenotypic selection on flowering phenology in its host plant *G. pneumonanthe*. In the absence of the seed predator, phenotypic selection favored earlier flowering. Because *M. alcon* preferentially attacked earlier-flowering individuals within populations, selection favored later flowering in populations where the predator was present. Butterfly-mediated selection on host plant flowering phenology, in turn, did depend on community context, i.e., the incidence of the predator in host plant populations increased with the abundance of the butterfly ant host.

Our study shows that in the absence of the predator, phenotypic selection favored early flowering. This is consistent with the general trend shown by Munguía-Rosas *et al.* (2011). Such patterns could potentially be the result both of that early flowering directly increases fitness, and of selection on other plant traits that are correlated with flowering phenology. Moreover, positive correlations between early flowering and high fitness may also be the result of that early flowering and fitness are correlated with favorable microsite conditions and a high resource availability, i.e., they are the result of environmental covariance (Ehrlén, 2015). In our study, we tried to alleviate these problems by incorporating traits that we considered likely to be correlated with flowering phenology and traits likely to be correlated with plant resource state as covariates in our models. This suggests that the positive effects of early flowering in the absence of the predator may indeed be direct and causal in our system. Flowering early in *Gentiana pneumonanthe* 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).

Our results also show that such selection for earlier flowering in the absence of antagonists may shift to selection for later flowering if antagonists are present. In our study system, the direction of selection on phenology differed markedly between Gentian populations with vs. without the butterfly pre-dispersal seed predator. This strongly suggests that the predator mediates shifts in selection from favoring early to favoring late flowering. Moreover, within populations *M. alcon* consistently preferred attacking early-flowering plants, thus increasing the relative fitness of late-flowering plants. Previous studies with this study system have focused on the relationship between oviposition and bud phenological state and showed that females of *Maculinea* sp. prefer laying eggs on not fully developed buds, thereby increasing time available for brood feeding and development (Thomas & Elmes, 2001; Patricelli *et al.*, 2011). In contrast, our study focused on the response of the predator to the timing of bud development of individuals, and showed that given the butterfly preference for a given phonological stage, plants starting bud development early in the season are more prone to be attacked by the butterfly than plants flowering late. A possible explanation for this pattern is that *M. alcon* butterflies flying early in the season deposit higher number of eggs than those flying later, due to the different fecundity of females over time (Arnaldo *et al.*, 2014). It is also true that weather conditions are probably more favorable for larval development early in the season. Ours with *G. pneumonanthe* … SUMMARIZING SENTENCE …

We also found evidence for phenotypic selection on other plant traits. Butterflies preferred to oviposit on taller shoots. This may be an effect of that taller shoots provide more resources for larval development (Czekes *et al.*, 2014; Wynhoff *et al.*, 2015). It is also possible that taller shoots receive more solar radiation, createing a more suitable microclimate for larval growth (Van Dyck & Regniers, 2010). The preference for taller shoots was only observed in some populations and years (Fig. S4.3). This could possibly be explained by variation in height of the surrounding vegetation. High shoots may protrude out of the vegetation and being preferred for oviposition (Küer & Fartmann, 2005). This effect is likely to vary with the height of the surrounding vegetation. *M. alcon* attacked plants with higher numbers of flowers in one of the study years, but not in the other.

In a recent meta-analysis (Siepielski *et al.*, 2013), phenotypic selection was reported to vary mainly in strength, but less in direction among populations. Our study not only provided evidence of that variation in the direction of selection on flowering phenology among populations was mediated by the presence of a butterfly seed predator, but also suggests that seed-predator presence depends on the abundance of its second host. *Maculinea alcon* was more probable to be present in host plant populations with a high abundance of *Myrmica* ants. This finding agrees with what is known about the biology of the butterfly. The butterfly is unable to complete its life cycle without ants … , Previous studies with other systems 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. …

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

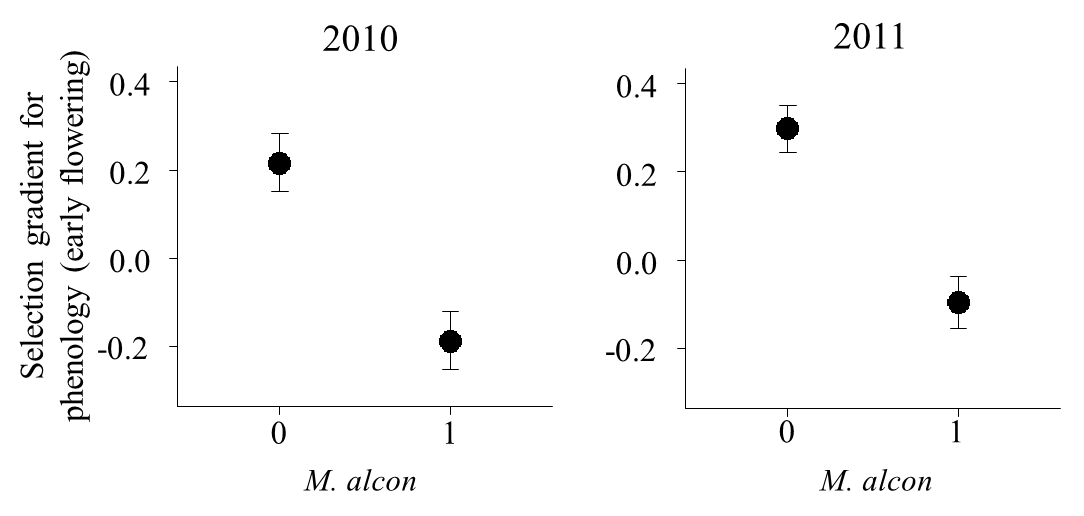


Figure 2

