20-Jan-2016  
  
Dear Editor,

Thank you for the invitation to submit a revised version of our manuscript ELE-01135-2015, now entitled ‘Butterfly seed predators mediate shifts in selection on flowering phenology in their host plant’. We found the comments by you and the three reviewers very helpful and think that thoroughly revising the manuscript according to these suggestions has substantially improved it. We now discuss to what extent our results can provide insight into evolutionary patterns and we have added results from additional analyses examining variation in selection among populations where the butterflies are present. In the revision, we also acknowledge that our patterns are based on observational data. We agree with Reviewer 2 and 3 that this fact limits our ability to make inferences regarding the effects of ant abundance on butterfly-mediated selection in host plant populations. Although we have not completely eliminated the part about ants in this version, we have downplayed the role of community context considerably in the current version.

Below, we provide a point-by-point list with answers to all questions and detailing the changes made in response to each of the comments.

Thanks again for your consideration of our manuscript.

Yours sincerely,

Alicia Valdés, on behalf of all authors

*Response to review*

*Referees' comments to the author(s):  
  
Referee: 1  
  
Comments for the Authors  
This is a nice study that uses an array of appropriate analyses to demonstrate that seed predation by larvae of a butterfly causes selection against early flowering in a species of gentian, and that this selection contrasts with opposing selection experienced by the gentian where the butterfly is absent. These results are consistent with some previous studies showing the importance of selection exerted by antagonists on flower phenology. Because the causes of variation in selection are not documented often, this manuscript goes beyond many previous studies by demonstrating that the occurrence of ants, a second host of the butterfly, is necessary (but not sufficient) for the occurrence of the butterfly and thus influences selection. The analyses are mostly limited to comparing selection in the presence and absence of the butterfly, yet there remains considerable variation in selection when butterflies are present. This variation is not addressed. To what extent is this variation accounted for by variation in the abundance of butterflies?*

The reviewer is right in that the main focus of the study is comparing the direction of selection between populations with and without the butterfly. We find that populations without the butterfly always experience selection for earlier flowering while populations with the butterfly experience selection for later flowering in most of the populations, see Appendix S4). Still, we agree with the referee in that there is considerable variation in selection also in populations where the butterflies are present. We also agree that it is reasonable to assume that this variation is related to variation in butterfly abundance, selection for later flowering being stronger in populations with higher butterfly abundances. We had in fact considered to include a test of this hypothesis. The reason why we did not include this aspect in the previous version was that we were lacking detailed independent data on the abundance of butterfly individuals within populations. We can, however, use the mean number of eggs per recorded plant individual within each population as a proxy for butterfly abundance and examine how this measure of abundance is related to differences in the selection gradient for flowering time among populations with the butterfly. We have now included the results of such analyses for both study years in the results section (P10-11, L229-232). We do not find any significant relationships between mean egg number and selection gradients for flowering time, although the trend is in the expected direction (selection gradients are more negative, i.e. selecting more strongly for later flowering, when mean number of eggs is higher). In addition to reporting these results, we now also clearly acknowledge the existence of variation in selection in populations with the butterfly in the discussion (P14, L314-319).

*My main remaining question concerns the impact of this variation in selection on the evolution of flowering phenology in this species of gentian. Is this variation in selection important evolutionarily, and for example can it account for local variation in flowering phenology, or for larger geographic scales of variation? As the authors point out there are increasing numbers of studies showing spatial variation in selection, and I realize their emphasis is on the cause of the variation, but to what extent does this variation have detectable and consequential evolutionary impacts? Is the overall result stabilizing selection because of the fine-scale nature of the variation, or is selection divergent enough to cause spatial or geographic variation and evolutionary divergence?*

We certainly agree with the reviewer that the ultimate aim of any evolutionary ecological study is to link environmental variation to spatial or temporal variation in selection. At the same time, however, we think that examinations the two major components of this relationship – the causes of variation in selection and the responses to differences in selection – are major undertakings in themselves, and keys to our understanding of evolutionary processes. As pointed out by the reviewer, spatial variation in selection has been shown many times. Yet, the causes of differences in selection among a large set of populations have rarely been examined. We thus believe that studies of phenotypic selection are interesting *per se*, and that our study, demonstrating variation in the direction of selection among 20 plant populations during two years, identifying the selective agent causing this variation, and finding a correlation between the presence of this selective agent and a second host, is a major advancement of our understanding of how biotic interactions might lead to evolutionary divergence.

At the same time, we agree with the reviewer that adding information about the potential for evolutionary divergence is relevant. Based on our current knowledge of the study system, we are not yet able to assess to what extent plant populations have genetically differentiated in flowering time in response to selection mediated by the butterfly. What we do know, is that common garden experiments (A. Valdés and J. Ehrlén, unpublished data) show that there are significant, genetically based differences among populations in flowering phenology. However, we still lack data from a sufficient number of populations to confirm that these differences are related to the presence of the predator in the population of origin. We have now added a discussion of the necessary pre-requisites for the observed differences in selection to translate into genetic divergence, as well as what we now about these factors in our study system (P16, L352-365).

*P4, second line from bottom: “for particular developmental stages”?*

Changed

*P6, L34: “and prey items brought to the nest by ants”*

Changed

*P8, line 7: “placed at one-meter…”*

Changed

*P8, line 14: are they “errors” or “variation”?*

It is true that they are not “errors” and we have now changed this sentence.

*P11-12: “in turn, depended on…”*

Changed

*P12, line 28: delete “of”*

We have rephrased this sentence to make it easier to understand.

*Referee: 2  
  
Comments for the Authors  
This manuscript examines phenotypic selection on flowering phenology and plant size in 20 populations of Gentiana pneumonanthe. In half of the populations, the predispersal seed predator Phengaris alcon is present, and interaction intensity with this antagonist is measured as the number of eggs recorded on the plant. Abundance of a third species, a Myrmica ant that hosts the later life-cycle stage of the seed predator, is also estimated in all populations. The results show that there is always selection for earlier flowering in populations with no seed predators, and selection for later flowering in populations with the seed predator present. In addition, path analyses consistently indicate seed predator-mediated selection for late flowering (i.e. seed predators prefer early-flowering plants). Finally, the probability of seed predator presence is found to increase with ant abundance. The authors conclude that ant abundance drives seed predator-mediated shift in selection on phenology.  
  
I find the topic of this paper very interesting, with focus on the role of community context (ant abundance) in determining selection mediated in pairwise interaction. The system is well suited to ask this kind of questions, and the sample size of the study is good, with a high number of populations (20) as well as individuals per population (100), across two years. The results are clearly presented and the manuscript is well-written.  
  
My only concern with this study is that it relies on purely correlative data, and thus cannot claim to show causality in any relationships. Strictly, a manipulation of butterfly interaction intensity in populations with the butterfly present would be needed to demonstrate butterfly-mediated selection. And similarly, a manipulation of ant abundance would be necessary to conclusively identify this component of the community as the driving factor of changes in selection. As it is, it cannot be ruled out that there is some associated environmental factor that underlies the observed patterns. Populations with seed predation may experience selection for later flowering due to a favourable local environment allowing later seed maturation, or due to higher competition, leading to reduced plant growth rate. Or due to a higher abundance of pollinators later in the season. And ant abundance may be correlated with density of butterfly food plants, or with the abundance of another butterfly species that competes with Phengaris alcon. In my view, the authors need to acknowledge this in the manuscript, and moderate some of their wording accordingly. In particular, I think the link between ant abundance and butterfly interaction intensity is weak.*

We agree with the reviewer that in a strict sense experiments are needed to infer causality. We thus also agree with the reviewer’s recommendation to acknowledge that our study is based on correlative data and to moderate some of our wording. For the relationship between butterfly presence and ant abundance, it is indeed true that some other environmental factor may influence both ant abundance and butterfly presence (although it is known that some minimum abundance of ants is necessary to enable the butterfly to complete its’ life cycle). For the relationship between selection on flowering time and butterfly presence, it is also true that we have not proved causation in a strict sense. In this case, however, we know from direct observations that: (1) butterfly attack reduces fitness by larval feeding on seeds, and (2) that butterflies preferentially attack early-flowering individuals (Table 3 and Appendices S5 and S7-S9 in the ms). In the revision, we now acknowledge that our results are based on correlative data. We have also moderated our wording, in particular with regards to the relationship between ant abundance and butterfly presence. Also based on comments by another reviewer, we now overall give less weight to the results regarding the effects of community context in the manuscript.

*Details  
P.7, l.3: How many shoots did plants have in total?*

The number of shoots per individual ranged from 1 to 44 (mean=3). This is now included in the text.  
 *P.7, l.7: “From the end of July to the beginning of August” – unclear. Once per pop during that time?*

Traits were measured once during this period. This is now clarified in the text.  *P.8, l.10: Were there any other species of ants than Myrmica? Could there be alternative hosts for the larvae?*

Yes, there were other species of ants (e.g. belonging to the genus *Formica* or *Lasius*), but we only counted the number of *Myrmica* ants. *Myrmica* is the only known host genus for the larvae of *Phengaris alcon* (Mouquet et al., 2005). This is now clarified in the text.  *P.8, l.45: Correlational selection should be estimated in models including both linear and quadratic terms.*

We agree, and we are very thankful to the reviewer for pointing this out. We have now run models including both linear and quadratic terms to estimate correlational selection. Table 1 as well as the text (P8-9, L176-183, P11, L235-237) have been modified accordingly. *P.9, l.22: “...if the trait × pop interaction was significant.” Should this be trait × pop × predation interaction?*

No, the text is correct. We first tested the significance of the trait x population interaction, and only in cases where this interaction was significant, we included the trait x predation interaction in this model. Three-way interactions trait × pop × predation are not possible as predation is a population-level factor.  *P.10, l.25 and 43: “..., but selection differed...”. Change but to and and differed to varied.*

Changed *P.10, l.33-38: Are given selection gradients with CI swapped? They are positive when the predator was absent and negative when the predator was present – should be the other way around?*

The selection gradients given are right. Higher values of the “phenology” variable indicate later stages of flower development, and thus earlier flowering. Therefore, selection gradients are positive when the predator is absent (selection for early flowering) and mostly negative when the predator is present (selection for later flowering). This is now clarified in the text.

*Should also include information on the number of populations with statistically significant selection on phenology, 5 out of 20 in 2010 and 3 out of 16 in 2011 (A S2)?*

This information has now been included in the text.  *Referee: 3  
  
Comments for the Authors  
Revision of the manuscript “Butterfly-mediated shifts in selection on flowering phenology depend on host ant abundance” by Alicia Valdés and Johan Ehrlén. In this manuscript the Authors assessed phenotypic selection on flowering phenology in 20 populations of  Gentiana pneumonanthe, a perennial herb, and how selection is affected by a seed predator (Phengaris alcon) and a second host (Myrmica ants). The approach was observational and comparative. Selection gradient were calculated in 11 G. Pneumonanthe populations with the predator and 9 populations without the predator, then, the authors compared the selection gradients between these two groups. Additionally, the Authors measured ant abundance (at population level) and correlated it with predator incidence. According to the results, early flowering phenotypes are favoured when the seed predator was absent while late flowering phenotypes were selected when seed predator was present. Also, ant abundance was a good predictor of seed predator incidence.  The authors concluded that phenotypic selection is being mediated by the seed predator and that seed predator mediated selection also depends on the abundance of the second host  (Myrmica ants). The authors highlight the role of community context when assessing phenotypic selection mediated by an antagonistic interaction.  
  
The manuscript is well written, concise and methods are, in general, sound. I think the Authors successfully show that phenotypic selection is mediated by seed predators; this is an interesting result which is also supported by previous studies (Pilson 2000, Kolb et al 2007, Paracnowitsh and Caruso 2008 among others, see references). My major concern, however, is that the second part of the story: influence of ant abundance on seed-predator mediated selection (see title), is not supported by data. Phenotypic selection works at plant level, but ant abundance was assessed at population level. The authors clearly showed that ant abundance predicts seed predator presence at population level, but there is no link between ant abundance, flowering phenology and intensity of seed predation at plant level. This is a critical issue as this aspect is an important component of the manuscript. I would suggest eliminate this part and only present the results related to seed predation.*

We understand the concerns of the referee regarding the second part of the study and we agree that our conclusions in this regard might have been too far-reaching. Based on our data, we are not able to establish that ant abundance has an effect on seed-predator mediated selection, only that the probability of predator presence in a population is correlated with ant abundance. In the revision, we have considerably reduced the parts of the manuscript dealing with community context and ant abundance. We have also carefully moderated our wording to clarify that we do not claim to have demonstrated that ant abundance influences selection on flowering time. Instead, we argue that once we have identified a biotic interaction, such as seed predation, as an important predictor of the direction of selection, then the next step in linking environmental variation to variation in selection becomes to identify the factors that determine whether a certain biotic interaction is present or not. In our study system, where we know that the butterfly needs both a plant and an ant host to complete its life cycle, it is reasonable to predict that ant abundance is one factor that influences the likelihood of seed predator presence. Given that our results were consistent with such a prediction, and that the other two referees found this part interesting, we have therefore downplayed, but not yet completely removed, the parts about ant abundance in this version of the manuscript.

*I also have a few minor suggestions  
  
Title page  
I would suggest change the title. Butterfly-mediated selection is not informative, I would emphasize the role of the butterfly i.e. “seed-predator mediated selection”. Dependence of phenotypic selection on host ant abundance is not clear, I would eliminate this part from the title.*

We agree with both of these points. The title is now changed to “Butterfly seed predators mediate shifts in selection on flowering phenology in their host plant”, highlighting the role of the butterfly as a seed predator and removing the reference to the community context from the title.  *Abstract.  
Please explain a bit more about natural history of butterfly-ant interaction.*

Due to the 150-word limit for the abstract, we found it very difficult to include much more information about the natural history here.

*Introduction  
Emphasis in Community context should be reduced.*

We now put less emphasis in community context in the introduction, as well as in rest of the manuscript.

*There is no prediction regarding the influence of Ants on seed-predator mediated phenotypic selection.*

We have now changed the title and some parts of the introduction in order to make clear that we do not aim to directly demonstrate that ant abundance influences seed-predator mediated selection. Instead, we try to relate ant abundance to the presence of the seed predator and the intensity of predation. If any of these relations would be significant, it would be consistent with (but not evidence of) that butterfly presence, and as a consequence, seed predator-mediated selection, is influenced by the community context. To clarify this, we have now changed hypothesis 2 to “Community context, in terms of host ant abundance, is related to the probability of butterfly presence within plant populations”.

*Study system  
P 4 L 54-56. “...is a rare, long-lived perennial herb...”  how long is life time in this plant species? I mean, how many years. Does this species reproduce since the first year? Please clarify.*

This information is now included in the text.

*Data Collection  
P 6 L 49-51. “P alcon was present in 11 of the study population”  Do the authors have any data about predator abundance or intensity of seed predation?   To show P alcon as a two-levels categorical variable may mask lots of variance.*

We do not have data on abundance of adult butterflies, but information about the proportion of plants with eggs and the mean number of eggs per plant individual for the 100 marked plants in each population in each year is now provided in Appendix S1.

*P 7 L 1-26. For me it was a bit difficult to follow how flowering time was assessed. It must be describe better and the redaction improved. In what scale flowering phenology was measured (e.g. ordinal, interval)?  Which are the units? What is the range? What was the distribution (e.g. mean, mode)?*

There are two different approaches to estimate differences in flowering phenology: (1) record the date at which individuals reach a pre-defined developmental stage (e.g. first day of flowering), and (2) record the developmental stage of all individuals at a given date. In this study we used the second approach because we considered it to have two major advantages. First, it makes it possible to record a large number of individuals at a single visit to a population. Second, differences in flower development stage at a given date are, in contrast to differences e.g. in date of first flower, independent of factors such as temperature that may cause differences rate of development over the flowering season. We have now modified the text to clarify how the phenology estimates were calculated. The developmental stage of individual flowers was recorded on an ordinal scale, ranging from 1 to 6. The intervals corresponded to pre-defined developmental stages possible to identify unambiguously in the field. Higher values indicate a more advanced floral development and thus correspond to an earlier flowering. At the level of plant individuals, we calculated two values: (1) the mean development stage of all flowers and buds (a continuous variable), and (2) the stage of the most advanced bud (an ordinal variable). We now also provide ranges for these two measures of phenology.

*P 7 L 45-60 P 8 L 1-19. How many times phenology, fruit production and ant abundance were assessed each year? If phenology was the subject, time dimension is very important.*

As stated in the response to the previous comment, reproductive traits were measured once per plant in each of the two years (at the end of July-beginning of August). This has now been clarified in the text. We have also modified the text to clarify that plant fitness, i.e. fruit production, was estimated by the maximum number of intact mature fruits on the focal shoot. This maximum number was assessed by counting fruits during one to five visits to each population (the number of visits depending on the time needed for maturation of all fruits). Ant abundance was estimated once per study year in each population. This is now also mentioned in the text.

*P 8 L 47 “...Correlational selection” what is meant with “correlational selection”? Please include a reference.*

This part has been modified according to comments by another reviewer, and we now talk about “non-linear selection” which includes both quadratic and correlational selection. Definitions and references are now included for both terms.

*P 8 L 54-56 “Results for models using mean and most advances flower developmental stages within the shoots were very similar in all cases” please show data and statistics.*

We now show the results for the selection gradient analyses (i.e. analogous to Table 1 in the main text) using the mean flower developmental stage, rather than the stage of the most advanced bud/flower, in Appendix S2. It should now be evident to the reader that the results for the two different measures are indeed very similar.

*P 10 L 17 correct reference is “R Development Core Team, 2014”*

This is now corrected.

*Results.  
Please show selection gradients.  Only one is shown in table 1 and 2.*

In Table 1, as stated in the legend, the parameter estimate (from a model without interaction terms) is given only for significant main effects where the population × trait interactions were not significant (i.e. shoot height in 2010). In cases where the population × trait interaction was significant, we end up with 20 selection gradient coefficients which are now, for the sake of brevity, presented in Appendix S4.

*P 11 L 14-23. Results of path analyses are only shown in supplementary materials. I suggest presenting some results in the main manuscript as well.*

We prefer to leave the majority of the results of the path analyses in the appendices as we believe that showing the results of path models for all populations is not feasible in terms of space. However, we now include (P11, L248-253) the most important information, in terms of the proportion of models with direct and indirect effects of phenology on fitness, in the results section.

*Discusssion  
P 14 L 35-51 P 15 L 10-14 This is not supported by data (see my major comments above).*

We agree and have moderated our wording in this part of the discussion. We now restrict our inferences to that the positive relationship between the abundance of the second host and the presence of the butterfly that we found is consistent with, but not evidence of, that community context influences the incidence of the seed predator in plant populations. See also response to the corresponding major comment above.

*Please acknowledge study limitations: only female fitness was evaluated. Fruit production may be a NON reliable measure of fitness, germination rate and survival may change the observed trend. Why not a more integrative measure of fitness was selected (.e.g. population growth rate)?*

We could not agree more with the reviewer in that assessing selection based on estimates of lifetime fitness would provide better information about actual differences in net, albeit not seed predator-mediated, selection on flowering time. Unfortunately we only have demographic data from one of the populations and gathering this kind of data in the remaining 19 populations would have been a massive effort. We now acknowledge this limitation and discuss its potential effects on net selection in the discussion (P16, L352-361).

It is also true that we did not measure male fitness. However, we do not think that evaluating only female fitness represents a major problem. This is because in a species with hermaphroditic flowers, the average fitness of males able to fertilize females at a given time should equal the average fitness of females receptive at that time, i.e. if average female fitness increases or decreases with a later development of flowers, then average male fitness should exhibit the same trend with flowering time.

*Table 1, 2 & 3 Please include model coefficients.*

As stated above, we have included the parameter estimates (from a model without interaction terms) for the significant main effects where the population × trait interaction is not significant in these tables. The linear selection gradients for each population are included in Appendix S3.

*Figure 1. I suggest showing years (2010 and 2011) as different series in a single plot.*

Changed according to the reviewer’s suggestion.

*Figure 2. I would eliminate Fig 2 b & c: they do not show statistically significant trends.*

Changed according to the reviewer’s suggestion.

*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*  
Editor  
Editors Comments for the Author(s):  
  
  
I have received the comments from three experts. Overall, they like the study and think that this is solid manuscript. However, all of them have detected some important caveats that need to be solved before making this manuscript suitable for publication. Specifically, Reviewer #1 thinks that it is critical to link your results to some measure of the evolutionary impact. Measures of selection are of limited value if they don’t provide insight into evolutionary patterns. In addition, according to this Reviewer, a large amount of variation in selection occurs when butterflies are present, and it is not addressed. The main concern of Reviewer # 2 is that this study is correlative, and consequently the effect of other factors influencing the results cannot be ruled out.  Reviewer #2 thinks that between ant abundance and butterfly interaction intensity is weak. Reviewer # 3 consider that influence of ant abundance on seed-predator mediated selection is not supported by data. This is an important issue. This reviewer even suggests eliminating this part from the manuscript.  
  
In conclusion, if the authors honestly think that they can address all the issues raised by the Reviewers, I am willing to admit a new and upgraded version of this manuscript. When doing this, please be sure that you indicate in a point-by-point letter how you have responded to all Reviewers' comments*

We have thoroughly re-worked the manuscript to address all the comments by the reviewers, paying special attention to the points highlighted by the editor above. We feel that the comments were very helpful and that the changes resulted in a much improved manuscript. We hope that you find our responses in this letter and the associated changes in the manuscript satisfactory, but would be happy to undertake any further revision that you find necessary.

REFERENCES

Mouquet, N., Belrose, V., Thomas, J.A., Elmes, G.W. & Clarke, R.T. (2005). Conserving community modules: a case study of the endangered lycaenid butterfly *Maculinea alcon*. *Ecology*, 86, 3160–3173.