13-Jan-2016  
  
Dear Editor,

Following the recommendation made by you and the reviewers on 10-Dic-2015, we are sending you a revised version of the manuscript now entitled: ‘Butterfly seed predators mediate shifts in selection on flowering phenology in their host plant’ (ID ELE-01135-2015). This version is a thorough review that takes into account all suggestions made by the three reviewers and the editor to correct the manuscript. We provide an answer for all the comments and have tried to argue about the points where our opinion differs from that of the reviewer, providing a justification to our position. We have particularly worked on reducing the emphasis given to the effects of the community and focusing more on the effects of seed predators in phenotypic selection in their host plant.

Below you will find our response to the review, specifying all the changes we have made in the manuscript (original comments by reviewers and editor are in italics).

We thank you again in advance for your consideration of our manuscript.

Yours sincerely,

Alicia Valdés (corresponding author)

*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 referee is right when saying that the main focus of the paper is in comparing selection between populations with and without the butterfly, as our most important finding is that the butterfly mediates a shift in selection from early to late flowering. However, we agree with the referee in that there is considerable remaining variation in selection when butterflies are present. This variation might indeed be related to variation in butterfly abundance, and we would expect that selection for later flowering was stronger in populations with higher butterfly abundances. We had in fact considered this hypothesis, although we did not include it in the paper because we are lacking detailed data on abundance of butterfly individuals. We can, however, use the mean number of eggs per population and year as a proxy for butterfly abundance and relate it to the selection gradients. We have now included the results of such analyses for both study years in the results section (P11L294-300), but we do not find any significant relationship (although the trend goes on the expected direction: selection gradients are more negative, i.e. selecting more strongly for later flowering, when mean number of eggs is higher). However, there could be other factors causing variation in selection where butterflies are present, for example the degree of overlap between the presence of the preferred developmental stages for oviposition and the oviposition period of the butterflies, or differences in butterfly individual abundances among populations. Nevertheless, we do not think that lacking an explanation for the remaining variation in phenotypic selection in populations with the butterfly invalidates our study, as it is focused on demonstrating how the butterfly is able to shift the direction of selection. We have however tried to acknowledge more the existence of this remaining variation in the discussion on the revised version of the manuscript (P14L395-396, 409-414).

*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?*

Our study focuses on assessing variation in selection and its causes, but we acknowledge that evaluating to what extent this variation could have an evolutionary impact is of major importance. We are not able to give specific conclusions about evolutionary consequences in the current study, as we only have measured plant traits in local populations that differ in phenotypic selection but probably also in many other variables. The variability in phenological responses that we have observed may have both an environmental and a genetic component, and we are aware that only the genetically-determined part of this variability will contribute to evolutionary changes in flowering time driven by the selection exerted by seed predators. With our current observational data, we are not able to say if variation in phenology among populations is due to a genotypic response to selection, or to the response to the different environments experienced by different plant populations. According to our results, we would expect populations where the predator is present to evolve towards later flowering and populations where it is absent to evolve towards earlier flowering. Our data do not show significant differences in flowering phenology among field populations where the predator is present and absent in any of the two study years (data not shown). This would suggest that the observed variation in flowering time is mostly due to phenotypic plasticity in response to the different environment of each population. However, ongoing common garden experiments (A. Valdés and J. Ehrlén, unpublished data) show that there are differences among populations in flowering phenology when environmental variation is removed, although we are still lacking data to confirm if these differences are related to the presence of the predator in the population of origin. However, these differences suggest that at least some of the phenotypic variability observed in flowering time can be attributed to genetic variance, supporting the existence of an evolutionary effect of phenotypic selection. Further experiments including controlled crosses of individuals with different phenotypes will allow to estimate heritability of flowering time, and to assess the ultimate evolutionary consequences of the observed variation in selection mediated by the butterfly seed predator. We now mention in the discussion (P15L432-436) the fact that genetic variance in flowering time is needed for an evolutionary effect of phenotypic selection.

In any case, we believe that studies of phenotypic selection are interesting *per se*, and in our case, we have found a pattern of divergent selection between populations that differ in the presence of an antagonistic interactor, meaning that we have detected spatial variation in selection, but, more importantly, we have also identified a factor causing this variation. This is a major advancement, as most studies until date do not identify the causes of variation in selection. Moreover, we have identified the community context as a factor related to this variation in selection. We are confident that these results are a major first step in assessing the potential of interactions (and the context where they occur) to drive evolutionary changes.

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

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

*P8, line 7: “placed at one-meter…”*

We have changed the text now according to the three comments above.

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

They are actually not “errors” and we have now changed this sentence.

*P11-12: “in turn, depended on…”*

This is changed now.

*P12, line 28: delete “of”*

We have now rephrased this sentence in order 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.*

Our aim was not to infer causality, but to show the effects of the interaction with the seed predator on selection, as well as the effect of the community context on the presence of the seed predator. It is definitely true that, with our study design and due to the observational nature of the data, we cannot depict true causality. However, we would like to point out that we are using multiple regression models, where we express a dependent variable as a function of several independent variables that have been chosen on the basis of previous scientific evidence. We have shown that neither the proportion of plants with eggs nor the mean number of eggs per plant were related to ant abundance, i.e. there is no direct link between the community context and interaction intensity at the population level. However, we found that the probability of butterfly presence in a population increased significantly with increasing ant abundance. We acknowledge that this result is based on a correlation and we have no means to confirm that the presence of a certain amount of ants is what determines the presence of the butterfly. For example, we cannot rule out other explanations, as the presence of a confounding factor that influences ant and butterflies in the same way. Another of the referees also suggested this as a critical issue, so we have carefully tried to moderate our wording in this part of the manuscript and to give less overall importance to the effect of the community context. Still, we think that it is interesting to keep this result in the manuscript, as the increased incidence of butterflies in populations with more ants agrees with what is known about the biology of the butterfly, which needs ants as a second host to survive. Therefore, we have now changed our wording in order to present ant abundance not as a cause of changes in selection, but as a factor related to the presence of the interactor, which is a prerequisite for selection to occur (as there could be no butterflies without ants). On the other hand, although the influence of the seed predators on selection is also assessed from observational data, we have proved that predators prefer attacking early-flowering plants (see Table 3, Appendix S4 and S5), which supports the hypothesis that predators actually drive selection for later flowering in their host plant.

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

Number of shoots per individual ranged from 1 to 44 (mean=3). This is included in the text now.  
 *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 changed 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 *Myrmica* ants, which were easy to differentiate visually on the sugar cubes. *Myrmica* is the only host for the larvae of *Phengaris alcon* (Mouquet et al., 2005). *P.8, l.45: Correlational selection should be estimated in models including both linear and quadratic terms.*

We have revised the bibliography on the topic and acknowledge that the referee is right. We are very thankful for this suggestion. We have now run models including both linear and quadratic terms to estimate non-linear (i.e. quadratic and correlational) selection. Table 1 as well as the text (P9L231-238, P11L304-306) are modified accordingly. *P.9, l.22: “...if the trait × pop interaction was significant.” Should this be trait × pop × predation interaction?*

The writing is right here. 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. No three-way interactions were included.  *P.10, l.25 and 43: “..., but selection differed...”. Change but to and and differed to varied.*

This is changed now. *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 earlier flowering. Therefore, selection gradients are positive when the predator is absent (selection for early flowering) and negative when the predator is present (selection for later flowering).

*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 is included in the text now.  *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 about the second part of the study, and we acknowledge that we might have given too much emphasis to this part. We reckon that with our data we cannot confirm that ant abundance has an effect on seed-predator mediated selection. However, our results prove that the probability of presence of the predator in a population is related to ant abundance on that population. Although this cannot prove ant abundance as a cause of selection, it suggests that the presence of a certain amount of ants is needed for the predator to be present. This is in agreement with what is known about the biology of the butterfly, which needs its second host to complete its life cycle. We have tried to reduce this part of the manuscript and to carefully modify our wording to show that we do not intend to present the community context as the ultimate cause of predator-mediated selection, but to suggest it as a factor related to predator presence, which can (possibly) influence selection (although this cannot be assessed with our data). As we think that the effects of the community context on interactions are still poorly understood, and as the other two referees though that this was an interesting point, we would still like to keep this part on the manuscript. However, we can remove it if the editor or the referees thinks it is strictly necessary.

*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.*

The title is changed now according to the referee’s suggestions, and the new title is “Butterfly seed predators mediate shifts in selection on flowering phenology in their host plant”. We have tried to highlight the role of the butterfly as a seed predator and removed the focus on 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 prefer to only include this information on the Methods section (see sub-section entitled “Study system”).

*Introduction  
Emphasis in Community context should be reduced.*

We have now tried to focus less in the community context throughout the introduction and the whole 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 seek to prove that ant abundance influences seed-predator mediated selection. Instead, we try to relate ant abundance to the presence of the predator and the intensity of predation. If any of these relations are significant, this might be an indication that butterfly-mediated selection is influenced by the community context, but this is only a suggestion, as we cannot truly prove this with our data. In this sense, we have changed now hypothesis 2 to “Community context, in terms of host ant abundance, is related to the probability of butterfly presence and to predation intensity within plant populations”. Later on, we show that the first part of this hypothesis is confirmed (host ant abundance is related to the probability of butterfly presence) but the second is rejected (host ant abundance is not related to predation intensity).

*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 and the corresponding references are included now 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 butterfly individuals, but we do have measures of intensity of seed predation from the 100 marked plants in each population. Two measures of predation intensity (mean number of eggs per plant and proportion of plants attacked) are now provided for each of the populations with the predator and study years 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)?*

We would like to clarify that we have not measured flowering time, but instead we have used estimates of phenology based on the reproductive development stage of individuals at a given date. This is, we have measured phenology once per year in each individual. Measurements were carried out in approximately the same dates in all populations and in both years (within the same week). This allows this static measure of phenology to be comparable among populations and years. We have now tried to improve the writing on this part of the manuscript, in order to clarify how the phenology estimates were calculated. We state now in the text the ranges for the two measures of phenology (both range from 1 to 6, with the mean development stage of all flowers and buds being a continuous variable, and the stage of the most advanced bud being an ordinal variable). There are no units for these variables, as they represent developmental stages, but, as stated in the text, higher values indicate in both cases an earlier phenology (i.e. plants flowering earlier in the season). We hope that these modifications make it easier to understand out methods.

*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 above, and now also written in the main text, the three reproductive traits were measured once per plant in each of the two years (at the end of July-beginning of August). We have now modified the text to clearly state 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 from 1 to 5 counts of fruits on different visits each population (the number of visits depending on the time needed for fruit maturation). 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 referee, and we now talk about “non-linear selection” which includes both quadratic and correlational selection. Definitions and references are included now 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 show now the results for the selection gradient analyses (i.e. analogous to Table 1 in the main text) using the mean flower developmental stage in Appendix S2. If compared to the results on Table 1, the reader can see that they are indeed very similar and the same effects are significant in both tables.

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

This is corrected now.

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

We would like to clarify that in Table 1, as stated in the table legend, the parameter estimate (from a model without interaction terms) is given for the only significant main effect where the population × trait interaction is not significant (i.e. shoot height in 2010). As in the other cases the population × trait interaction is significant, we have decided to include the linear selection gradients for each population in Appendix S3. These linear selection gradients were calculated from multiple regressions of relative fitness on standardized traits performed within each population. We have preferred to include these values in an appendix for the sake of brevity.

*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 they do not represent the major results of the study and besides, showing the results of all the models for each population does not seem feasible in terms of space. However, we have included now in the results section (P12L331-336) some details on the percentage of models showing direct and indirect effects of phenology on fitness, which we believe is one of the most interesting results that we can extract from these analyses.

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

We have now tried to moderate our wording in this part of the discussion. Our results about the effects of the community context are limited to a positive relationship between the abundance of the second host and the presence of the butterfly. We acknowledge that this is not enough to point out the community context as a driver of differences in selection, but we still think that this could be a plausible hypothesis to be tested with further data. Thus, we now present the community context only as a potential driver of this among-population variation in selection.

*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 have now tried to include more discussion about the study limitations. However, we do not think that evaluating only female fitness represents a problem, because the average fitness of males and females should be the same…. On the other hand, we totally agree with the reviewer in that assessing fitness using population growth rate would be a better approach, which would allow to assess phenotypic selection considering all the fitness components and their effects on lifetime fitness. However, unfortunately we only have demographic data in one of the populations and gathering this kind of data in the rest of populations would have been a massive effort that we could not tackle. We have now included a paragraph in the discussion where we present this limitation of our study (P14-15L415-424).

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

As stated before, in these tables 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. The linear selection gradients for each population are included in Appendix S3, in order to make the manuscript shorter.

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

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

Both figures are changed now according to the referee’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 worked in addressing all the comments from the referees and prepared a revised version of the manuscript including all their suggestions. We agree on the importance of the evolutionary impact of phenotypic selection and discuss this now on the manuscript, as well as the remaining variation in selection when the predator is present, and its possible causes. We have also worked on reducing the importance given to the community context in the manuscript, as some of the reviewers expressed concerns about this part. We now focus more on phenotypic selection mediated by the predator, and we acknowledge that we cannot demonstrate effects of the community context on phenotypic selection with our current data. We have also removed the reference to the second host in the title of the manuscript. However, we still think that the relation between the presence of the predator and the abundance of its second host is an interesting result, and would like to keep this on the manuscript if the editor and referees agree.

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.