Response Letter

"Exploring the effect of scent emission and exposition to diurnal versus nocturnal pollinators on selection patterns on floral traits"

New title:

"Investigating the effects of diurnal and nocturnal pollinators on male and female reproductive success and on floral trait selection in *Silene dioica*"

Dear Professors Juan Arroyo and Violeta Simón-Porcar,

Thank you for considering our manuscript entitled "Exploring the effect of scent emission and exposition to diurnal versus nocturnal pollinators on selection patterns on floral traits" (ID #798) for publication in PCIEvolBiol. We appreciate the thorough evaluation and feedback, which helped us clarify the interpretations of our results and streamline the manuscript.

While the reviewers recognized the value of our work, they raised concerns about result synthesis and interpretation, which we have addressed in this revised version. Below is a point-by-point response to each comment, along with the relevant line numbers. Due to compatibility issues between the different computer systems used by the authors, tracking changes consistently proved technically challenging. Consequently, we were unable to produce a fully coherent version with tracked modifications. To address this, we have prepared a PDF document that outlines all modifications made in response to each of the reviewers' comments. This document clearly indicates the specific sections and text revisions in the manuscript, ensuring transparency and ease of review.

We hope these revisions adequately address all concerns.

On behalf of the authors,

Estelle Barbot

Recommenders' comments

Dear E Barbot et al.

After careful examination and review by four reviewers of your preprint "Exploring the effect of scent emission and exposition to diurnal versus nocturnal pollinators on selection patterns on floral traits" submitted to PCI in Evolutionary Biology for its possible recommendation, we consider that the preprint has enough merits as to be considered for recommendation. However, reviewers expressed some concerns that should be addressed properly before a final decision. These concerns are variable in strength and depth, but we believe all of them can be rightly addressed as they do not affect the design and there are no major contradictory concerns among reviewers. In fact, they affect mostly to writing details and interpretations. We hope you consider they will contribute to a significant improvement of your preprint and thus will wish to submit it again to PCI Evol Biol. In such a case, please when resubmitting a new version explain in detail how you addressed the points raised, or why you did not follow that, if this is the case.

Looking forward to receiving your new version

Best regards

Juan Arroyo and Violeta Simón-Porcar

Luis Gimenez-Benavides's comments

This is an interesting experimental study on the pollinator-mediated selection patterns exerted by diurnal and nocturnal pollinators in a dioecious plant with a mixed pollination system, Silene dioica. In the experimental setup, male and female plants were exposed to diurnal vs. nocturnal pollinators, and the flower scent of some plants was altered in a fully crossed design (sex x exclusion time x scent).

The manuscript is an interesting contribution because it explores at the same time the selection on floral traits mediated by diurnal versus nocturnal pollinators on female versus male plants. The manipulation of the flower phenotype through artificial increase of emission of one key compound (phenylacetylaldehyde) adds a further point of complexity to the study. The manuscript is very well written and is valuable, so I suggest its publication after taking into account the following comments.

Comment 1 - My main concern is related with the way in which the authors estimate the female reproductive success to calculate selection gradients on floral traits (multiplying the mean seed number per fruit, total number of non-predated fruits and germination rate). Authors use non-predated fruits because one of the nocturnal pollinators, Hadena bicruris, is a nursery pollinator which oviposits on some of the flowers it pollinates, and its larvae prey on the fruits. Each Hadena larvae normally consume 5-10 fruits before hatching, so authors removed the fruits containing young larvae (primary fruits) to avoid excess of damage to the experimental plants. However, by doing this, authors underestimate the effect of predation on plant fitness. To evaluate the net selection of Hadena bicruris on the floral traits of the plant, it would be necessary to analyze its contribution as a pollinator in adult stage and its contribution as a predator in larval stage. If the authors want to focus only on the study of pollinator-mediated selection (including Hadena), I think they should use total fruit production to estimate female reproductive success, without excluding primary predated fruits. This could significantly change some results.

> **Response:** You are right, we acknowledge that a part of the net selection exerted by *H. bicruris* has been overlooked in our study, as we diminished the effects of predation by removing emerging larvae. An alternative way to do it is to estimate female reproductive success by multiplying seed set and seed germination rate by total fruit production, rather than considering only non-predated fruits. We have reanalyzed the data using this alternative estimator, and it did not change our conclusions: all selection gradients on female traits remained qualitatively unchanged, meaning that traits that significantly influenced fitness estimated based on non-predated fruits only had the same effect when considering overall fruit production, regardless of the impact of predation. These additional results are now included in the supplementary material, and we have updated the main text accordingly.
>
> *L251: The number of viable seeds was also computed using total fruit production instead of just non-predated fruits. Therefore, we have two estimators of female fitness, one that takes predation into account and one that does not.*

L473: All these results were identical using total fruit production instead of non-predated fruits in the estimation of female reproductive success (Figure S4, Table S4).

Comment 2 - L16: Please describe pollination efficiency when it is first mentioned (now in L28)

Response: It was indeed an oversight, we added the definition of pollination efficiency earlier in the paper, at its first mention.

L16: This role is likely to depend on several parameters of the plant-pollinator interaction, in particular (i) the relative abundance of that pollinator in the local community, (ii) its visitation rate and pollination efficiency (defined as the amount of pollen transported and deposited on the stigmas of flowers visited later in the sequence; Wu et al., 2018: Caruso et al., 2019), as well as (iii) the effect that floral traits have on its visitation rate and pollination efficiency.

Comment 3 - L52-53: H. bicruris may act as nursery pollinator of many Caryophyllaceae. There is also evidence that S. dioica interacts with other Hadena species (up to 8 according to the last reviews: Kephart et al. 2006 NPhyt, Prieto-Benitez et al. 2017 Flora). In general, I think that brood pollination by Hadena should be described in more detail, may be in the study system (L96-105).

Response: Thank you for these references. We updated the section about the brood pollination system in Hadena-Caryophyllaceae in the Introduction. We also added further information about the duality between pollination and predation of this system in the study system description of the Material & Methods.

L62: S. dioica (i) is visited by diurnal pollinators but also nocturnal moths (Jürgens et al., 1996), (ii) can be predated by larvae of Hadena species, a moth genera involved in a nursery pollination interaction with many Caryophyllaceae species (Kephart et al., 2006; Prieto-Benítez et al., 2017) including the sister species S. latifolia (Dufaÿ & Anstett, 2003; Bopp & Gottsberger, 2004), and (iii) emits comparable amounts of scent during night and day (Waelti et al., 2008).

L129: Beyond their role as pollinators, nocturnal pollinators of the genus Hadena are also recognized as predators of Silene dioica (Prieto-Benítez et al., 2017). This interaction between Hadena moths and Silene dioica flowers occurs within a brood pollination system, where adult moths not only pollinate the flowers but also deposit their eggs inside them. The emerging larvae then feed on the reproductive tissues of the host plant. (Kephart et al., 2006).

Comment 4 - L65: should read Benzenoid

Response: This was corrected.

Comment 5 - L126: how many days did the experiment last? Please provide dates

Response: We relocated this information to the beginning of the Materials and Methods section and additionally included the specific dates of the experiment.

L139: The experiment spanned seven days, from July 6th to July 12th, 2019, and was set up in a common garden on the campus of Lille University in France (50°36'27.9''N 3°08'36.3''E), several kilometers away from the nearest wild populations of Silene dioica.

Comment 6 - L133: Why do you choose to double the amount of PAA? Is that a reasonable amount compared to other species in the wild?

Response: This aspect of our experiment aimed at detecting whether increased PAA emission modified selection on other traits. We had no clue about any possible effect of this sort, and we decided to strongly exaggerate PAA emission to have a chance to detect any

effect. Our current results suggest that PAA may modify some aspects of plant/pollinator interactions and modulate selective pressures on some traits, but as pointed by the referee this has been done through an exaggerated phenotypic manipulation, which only allows us to suggest the PAA effect. This prevents any attempt of quantification of these effects and we are now more cautious with our interpretation.

To accurately assess the quantitative impact of PAA on reproductive success, one would need to measure scent emission and analyze the selection gradients for each compound. In light of this, we have revised our discussion to nuance our conclusions and emphasize that future studies should focus on natural scent emission to better understand PAA's role in reproductive success and its effect on selection on other floral traits.

L563: One aim of this study was to assess whether variation in one type of signal (volatile compound) could influence patterns of selection on other floral characteristics (visual signals).

L659: Nonetheless, given that the strong artificial increase of PAA is unlikely to reflect natural variation in PAA among individuals, future studies are now needed to verify and quantify these effects, through estimates of selection gradients for individual compounds involved in scent emission. This would allow for a more accurate assessment of the selection gradient on PAA emission and its interaction with the selection on other floral traits.

Comment 7 - L336: should read "Results of the ANCOVAs analyzing female (left)…"

Comment 8 - L379: should read " * : P < 0.05, ** : P < 0.01 and *** : P < 0.001"

Comment 9 - L419: should read "a trend that has…"

Response to comments 7-9: Corrected, thank you..

Comment 10 - L443: please describe the term fertility selection

Response: We indeed forgot to define fertility selection, this was corrected.

L589: The positive selection on flower number in all treatments suggests that the functional category of pollinators does not significantly influence this pattern. In other terms, selection on flower number could only be ascribed to fertility selection: females that produce many flowers have a better fitness because they produce more gametes and not because they attract more pollinators.

Comment 11 - L453-454: I consider this data not shown very relevant to the discussion of this study, why don't you present it?

Response: We have decided to remove this statement from the discussion. The result was not statistically significant, and the hypothesis was only an attempt to explain the observed positive selection on calyx height during the night. Furthermore, this finding contradicts previous studies (Kula et al. 2013; Miyake et al. 2018). We have now reworded the section to simply state that we were unable to provide a clear explanation for this unexpected result.

L598: A notable exception was observed regarding calyx height, which was found to be under positive selection but only in plants exposed during the night and with an artificial increase of PAA. This result could be associated with the observed rise in fruit predation within this particular group of plants. Nonetheless, calyx height has been reported to be positively associated with likelihood of oviposition by Hadena sp in other Caryophyllaceae species (Kula et al., 2013; Miyake et al., 2018), so the proximal cause of this pattern remains undetermined.

Comment 12 - L484-491: This result may be related with the flower part responsible for PAA scent emission. In S. latifolia, most benzenoids like PAA are emitted by the petals (Dotterl & Jurgens 2005). Selection on corolla width by nocturnal pollinators under natural conditions of scent emission could disappear with the addition of PAA since the pollinator would not olfactorily perceive differences in corolla size between plants.

> **Response:** Indeed, this is a highly relevant hypothesis. Thank you for bringing it to our attention; we have included this in the discussion as a possible explanation for the fact that selection on corolla width diminishes when PAA emission is intensified.
>
> *L645: If, as in the sister species Silene latifolia (Dötterl et al., 2005), the majority of benzenoids, including PAA, are released by the petals in S. dioica, then variation in corolla diameter among individuals may result in differences in natural scent emission levels. The selection pressure exerted by nocturnal pollinators on corolla width could thus diminish with the exacerbation of PAA emission, as pollinators may fail to detect olfactory differences in corolla size among plants.*

ANDREA COCUCCI'S COMMENTS

Title and abstract

- Does the title clearly reflect the content of the article? [x] Yes, [ ] No (please explain), [ ] I don't know
- Does the abstract present the main findings of the study? [x] Yes, [ ] No (please explain), [ ] I don't know

Introduction

- Are the research questions/hypotheses/predictions clearly presented? [x] Yes, [ ] No (please explain), [ ] I don't know
- Does the introduction build on relevant research in the field? [x] Yes, [ ] No (please explain), [ ] I don't know

Materials and methods

- Are the methods and analyses sufficiently detailed to allow replication by other researchers? [x] Yes, [ ] No (please explain), [ ] I don't know
- Are the methods and statistical analyses appropriate and well described? [ ] Yes, [ ] No (please explain), [x] I don't know. I have see an issue in lines 188. See my comments below

Results

- In the case of negative results, is there a statistical power analysis (or an adequate Bayesian analysis or equivalence testing)? [x] Yes, [ ] No (please explain), [ ] I don't know
- Are the results described and interpreted correctly? [x] Yes, [ ] No (please explain), [ ] I don't know

Discussion

- Have the authors appropriately emphasized the strengths and limitations of their study/theory/methods/argument? [x] Yes, [ ] No (please explain), [ ] I don't know.
- Are the conclusions adequately supported by the results (without overstating the implications of the findings)? [x] Yes, [ ] No (please explain), [ ] I don't know. However,

one part of discussion must be reviewed in the light of the results obtained (lines 563-565). See my comments below.

General comments

This is a very thoroughly planned study on a subject that has been little explored. The characteristics of the system are quite complex since plants are pollinated both during day and nightime, some pollinators may act as seed predators, and an experimental treatment has been made to explore the importance of olfactory flower attractans. All these factors of variation have been accounted for in a carefully designed experiment. Results are sound and discussion, in general, appropiate for the results obtained.

However, I have the following claims which are also included in the ms as comments:

Comment 13 - In line 188, it is not clear to me how the number of gametes that a plant produces can be a target of pollinator mediated selection. How can the number of gametes influence pollinator attraction or pollination efficiency in the way authors are expecting?. In lines 111-112 authors ask whether attraction traits may be targets of selection. Is the number of gametes regarded as an attraction traits or is there another function of gamete number that could influence pollinator attraction and efficiency? If so, this should be better explained. As I see it, the number of gametes could rather be used to estimate reproductive success in terms of the proportion of grains of an individual that sired seeds or the proportion of ovules that set seeds. Consequently, it should be analyzed better as a part of the response variable than as a predictor variable in the phenotypic selection models. In that case, number of gametes could be included in a binomial model where the response variable consists of a two column matrix of successes and failures (see details in the R documentation for function glm).

Response: Pollen is a vital nutritional resource for some pollinators, including in bumblebees, which are among the most frequent visitors of *Silene dioica*.. In *S. dioica*, the stamens are arranged in two whorls, with the upper whorl extending beyond the corolla, potentially acting as a visual signal for insects. The role of pollen as an attractive trait has been highlighted in other systems (Carr et al. 2015), which justifies retaining this trait in the models in our opinion.

Obviously, the same does not apply to ovules: while developing seeds may serve as a resource for *Hadena*, ovule number is not directly observable and therefore does not function as an attractive trait per se. The first reason why we decided to include this trait in the statistical models for females is our desire to keep models for both sexes as similar and comparable as possible.

The second reason is that, even if pollen does not play a role in attracting pollinators in our system, it is highly likely that the number of gametes (in both sexes) affects the reproductive success of individuals. Since the number of gametes—whether pollen grains in males or ovules in females—varies greatly between individuals (Barbot et al. 2023), it is essential to include this trait as a covariate in the models to explicitly account for this source of variation.

We added a sentence to explain that not all traits are linked to attraction :

L196: We measured a set of floral traits on all individuals in the experimental population, including traits that are presumably linked to pollinator attraction (corolla diameter, calyx

height, flower number, pollen production) and traits that are linked to individual fertility (gamete production per flower).

Comment 14 - In lines 477-478 I think that results are not correctly interpreted. In lines 563 to 565, it is stated that, despite a mixed pollination system, nocturnal and not diurnal pollinators are selecting flower phenotype. Consequently, prediction through syndrome of nocturnal visitors as more efficient pollinators was not as bad after all. These is also an interesting, I think the most interesting, resuldata not showt that is not being highlighted because noctural pollinators seem to be moulding flower phenotype despite that there is "no discernible differences in male or female reproductive success between exposure treatments".

Response: We added a sentence in the introduction to clarify our point: even if there is some indirect evidence of a mixed pollination system, *S. dioica* is generally described in the literature as a diurnal species (and not nocturnal, as the referee suggests here: Jürgens et al. 2002; Jürgens 2004), notably because it keeps its pink flowers open throughout the day, in contrast to its sister species, *S. latifolia* (see lines L68-71). When we set up this experiment, we were convinced that diurnal pollination would be the most effective and predicted that pollen limitation would occur at night. Obviously, this prediction is not supported by our results, and our study is the first to demonstrate that *S. dioica* engages in true mixed pollination.

Furthermore, we are not sure we understand the link the referee makes between pollinator efficiency and selection patterns. For instance, one could imagine a system with an extremely efficient pollinator that does not discriminate between plants based on their floral phenotype. In this case, we would have high-quality pollination but no selection at all on floral traits. Therefore, it is maybe not that surprising that, despite an equally efficient pollination between night and day, selection patterns can vary between these treatments.

Minor comments

Comment 15 - line 202. replace "reported" with "recorded"

Response: This was corrected.

Comment 16 - line 211. specification of the lamp used is needed as perception depends on light quality

Response: We indeed forgot to give this information. We used a mercury vapor lamp, which is known for being effective at attracting moths (Rich and Longcore 2013). We added this information in the Material & Methods.

L226: observations were conducted for one hour the third day of the experiment, at 11pm, and involved (i) hanging a white sheet in the experimental garden and shining a mercury vapor lamp on it to attract, capture and identify nocturnal pollinators and (ii) direct observations on the plants using a flash-light.

Comment 17 - line 211. was the presence of Silene pollen assessed?

Response: It is an excellent suggestion, but we did not perform pollen grain identifications and counts on the pollinators' bodies. Doing so would indeed have provided better insight into which insects are effective pollinators of *Silene dioica*, and this could be explored in future experiments.

Comment 18 - line 221. replace "polinized" with "pollinated"

Comment 19 -line 273. replace "if" with "whether"

Response to comments 18-19: This issues have been addressed, thank you.

Comment 20 - line 286. explain fully which were the terms of this model

Response: We added this information in the statistical analyses section: the proportion of predated fruits was analysed using a generalized linear model with exclusion and odour treatment as explanatory variables to test if predation rate differs between treatments.

L305: To do so, we constructed a generalized linear model with exclusion and PAA treatment as explanatory variables and with a binomial error distribution, and then proceeded to post-hoc Tukey's tests to compare groups.

Comment 21 - lines 349-350. correct negative statement neither … nor…

Response: Corrected, thank you.

L377: Experimental treatments (HP DC, DT, NC and NT) did not affect neither seed set () $\chi^2_{4,609}=2.07, P=.73$ *nor primary fruit-set (*$\chi^2_{4,135}=2.09, P=.72$*), suggesting pollen receipt did not limit female reproductive success in any of the plant groups (Table 1).*

Comment 22 - line 351. replace "pollen" with "pollination"

Response: A word was missing, we corrected the sentence by adding "receipt" (see comment 21).

Comment 23 - line 376. replace "Sphyngidae" with "Sphingidae"

Response: Done.

Comment 24 - lines 414-424. This table could be made more reader friendly by adding subtitles to the parts of the table, in which case the long note would not be needed.

Response: The structure of the table and the footnotes were initially chosen to remind the reader that one should first check the significance of three-way interactions before examining lower-order interactions and main effects. However, we agree that this did complicate the message so we now present our results in a more "traditional" manner, starting with main effects, followed by two-way interactions and then three-way interactions. We have removed the grey bar separating the different part of the tables, and drastically reduced the footnote. We hope these changes make the table more conventional and reader-friendly.

L445: Notes: The table represents results from ANCOVAs for the main effect of floral traits and treatments, as well as two-ways and three-ways interactions between each variable. Statistics (F-values) and their associated P-values are indicated for each variable effect on female or male relative reproductive success.

Comment 25 - line 457. what are these error bars, SE, SD, CI?

Response: We indeed forgot to specify that these bars are standard-errors (SE) of the selection gradient estimates, we added this information in the legend of the figure.

L482: Figure 1 - Estimates of selection gradients (± SE) on floral traits in each sex, and for the four treatment combinations.

Comment 26 - line 462. replace "significance values" with "asterisks"

Comment 27 - line 469. repalce "shapes" with "shape"

Response to comments 26 and 27: Agreed.

ANONYMOUS REVIEWER #1'S COMMENTS

Title and abstract

- Does the title clearly reflect the content of the article? [ ] Yes, [X] No (please explain), [ ] I don't know

  No. This study is entitled "Exploring the effect of scent emission and exposure to diurnal versus nocturnal pollinators on selection patterns on floral traits" and the authors do not study odour emission, but the effect of a single volatile, phenylacetaldehyde , previously isolated from the set of volatiles that make up the floral odour of Silene dioica.

- Does the abstract present the main findings of the study? [X] Yes, [ ] No (please explain), [ ] I don't know

Introduction

- Are the research questions/hypotheses/predictions clearly presented? [X] Yes, [ ] No (please explain), [ ] I don't know
- Does the introduction build on relevant research in the field? [X] Yes, [ ] No (please explain), [ ] I don't know

Materials and methods

- Are the methods and analyses sufficiently detailed to allow replication by other researchers? [X] Yes, [ ] No (please explain), [ ] I don't know
- Are the methods and statistical analyses appropriate and well described? [ ] Yes, [X] No (please explain), [ ] I don't know

  See my comments in the review

Results

- In the case of negative results, is there a statistical power analysis (or an adequate Bayesian analysis or equivalence testing)? [ ] Yes, [ ] No (please explain), [X] I don't know
- Are the results described and interpreted correctly? [ ] Yes, [X] No (please explain), [ ] I don't know

  See my comments in the review

Discussiondata not show

- Have the authors appropriately emphasized the strengths and limitations of their study/theory/methods/argument? [ ] Yes, [X] No (please explain), [ ] I don't know

  See my comments in the review

- Are the conclusions adequately supported by the results (without overstating the implications of the findings)? [ ] Yes, [X] No (please explain), [ ] I don't know

  See my comments in the review

Review

Barbot and co-authors present a continuation of their previously published studies on Silene dioica, a species with a generalized pollination system. This manuscript describes a series of exclusion experiments and the use of a floral volatile aimed at understanding their effects on various measures of female and male fitness, subsequently analysing selection gradients on floral traits. Overall, I think this study is interesting and a good fit for Peer Community in Evolutionary Biology. That said, I believe the paper needs significant revision to clarify the methods. In my opinion, the authors should make some changes to the way they interpret the results of these experiments. I do not think the all conclusions are fully supported by the data. I list my major concerns first, followed by some minor suggestions for improvement.

Major comments

Comment 28 - I have some concerns about the experimental design. A first point is the aim of this study. With only a small population studied during seven days, and some flaws in the methodology (see below), in my opinion, the experimental design does not allow for an explicit test of the intensity of selection on floral traits between treatments. For example, do the authors consider that selection gradients can be assessed in this short period of time? According to this, please indicate the phenological cycle of the species. Did all individuals flower at the same time? Was the number of flowers per individual taken into account? In such a short (time) experimental design, can the authors consider that the experiment and the results are repeatable? In general, the authors should consider toning down the discussion in order to be more realistic.

> **Response:** Our intention in this study was not to measure the overall selection gradient across the entire flowering season—an approach previously taken by Barbot et al. (2023)—but rather to dissect the specific components of selection operating at different times of day. By experimentally isolating the processes occurring during the day and night, we aimed to provide a more nuanced understanding of selection patterns. Sections of the discussion were reworded to clarify that point (see L509, L520, L531).
>
> We are confident in the validity of our conclusions for two reasons.
>
> 1. The results align closely with our previous findings from 2023. For instance, we observed significant selection on gamete number in females, while this selection was absent in males. Additionally, we identified selection for a larger corollsa size in males under certain conditions, but not in females. These consistencies lend strong support to our current findings and suggest that our methodology effectively captures the ongoing selection patterns, and a sentence was added in the discussion to highlight this (see L523).
> 2. Contrary to what the referee suggests, our experiment captures a non-negligible portion of the annual fitness of the individuals. The referee correctly points out that to be convincing, we should include more information about the flowering phenology of *Silene dioica* and about our experimental setup. In *S. dioica*, males flower for a long time (up to 2 months), while females have a shorter flowering period, generally around one month. Female flowering is characterized by slow production at the beginning and end, with a marked peak in the middle. For this experiment, we selected female plants that were at a similar stage in their flowering phenology, with 11.22 (± 9.53 SD) open flowers on average and many flower buds, indicating they were approaching their peak flowering stage. The average number of fruits produced during the experiment was 7.27 (± 4.64 SD), which

represents more than one-fourth of the usual annual fruit production for our collection of plants. Choosing females that were at the same stage obviously precludes us to study the effects of selection on traits such as flowering phenology (this statement was added L522), but this has already been done in previous studies and these traits do not have a direct link with pollinator attraction, which was the central question here.

The details about flowering phenology have been added to the current version of the manuscript (see L146).

Finally, the referee questions whether flower number was included in our statistical models. We agree that flower number is potentially very important for individual fitness, as it likely impacts both pollinator attraction and overall gamete production in both sexes. As indicated in the previous version of the manuscript, flower number was included as a covariate in all relevant analyses: the analysis of pollinator visitation patterns (see L314), selection gradients on floral traits (see L354), and the analysis of pollen dispersal distance (see L362), see also table 3 and Figure 1.

Comment 29 - The second point focuses on the use of the benzenoid phenylacetaldehyde for odour manipulation The flower scent composition of Silene dioica is quite complex. For example, monoterpenoids are quite abundant in Silene dioica, and may be also involved in pollinator attraction. I think some reframing of the discussion (when dealing with the effect of floral scent) should be considered, and mention that in natural populations the results could be very different. Furthermore, in my opinion the amounts of odour compounds (ng/ h) used in the experiment are higher than suggested. Do such high amounts, not existing in nature, allow drawing clear conclusions on the effect of odour on pollinator-mediated selection? Furthermore, it is not clear whether the PAA-containing tubes were put in at the beginning of the experiment and left there for the whole week, as this would greatly affect the emission rate from day 1 to day 7. Finally, the plants with the "increased odour" treatment that had such a high amount of PAA were placed too close to the control plants (c. 4 plants per m2). Do the authors think that they were too close and that the odour could spread to adjacent plants? If not, why were the HP plants separated?

Response: We agree with the referee that, as in any experimental study, our observations may not reflect what would occur in a natural population. Any experiment is necessarily a simplification, but a fine understanding of the effect of scent on individual fitness was not our aim here (see also how the pros and cons of such approach were already presented in the previous version of the ms : L72); instead, we sought to assess whether (admittedly strong) variation in one type of signal (scent), could affect patterns of selection on other floral characteristics (visual signals). This was done by exaggerating the phenotype: we chose to add PAA in a quantity corresponding to a doubling of the total amount of scent emitted on average by the plants. In doing so, we indeed reached PAA emission rates that would not be observed in nature. However, as the referee rightly points out, the floral bouquet of *S. dioica* is complex, consisting of nearly thirty different compounds (Waelti et al. 2008), and it would have been very difficult if not impossible to experimentally recreate such a complex floral bouquet. A more detailed understanding of selection on VOC emissions — including PAA — and the interaction between selection on scent signals and visual signals will require measuring natural scent emission and quantifying the strength and direction of selection acting on these traits. This presents an exciting avenue for future research, and a sentence has been added to the discussion to reflect this idea (see L563-575).

We are unsure why the referee believes that the amounts of odor compounds used in the experiment were higher than suggested. We verified this by placing identical experimental devices (glass tubes containing the scented solution wrapped in aluminum foil) in the same experimental garden and weighing them daily before the experiment took place to check the emission rates. Obviously, there was some level of fluctuation due to natural variations in temperature, but these were moderate, and contrary to what the referee implies, emission was still occurring at the end of the experiment.

Finally, the referee also states that the plants were too close to one another, suggesting that both PAA-exaggerated and control plants probably displayed the same phenotype, and he seems to imply that we may have "hidden" HP plants in another corner of the experimental garden. In response, we would like to clarify the following points: 1. The densities were chosen to fall within the range typically observed in nature; spacing them too far apart could significantly modify insect behavior. 2. While we acknowledge that scent can diffuse around plants, it is not accurate to claim that exaggerated and control plants displayed the same phenotype; otherwise, the PAA-treatment would never have yielded significant results in the statistical analysis. 3. HP plants were positioned very close to the other plants, but not in the same spot due to logistical constraints: D plants needed to be moved indoors at night, N plants during the day, while HP plants remained in their position throughout. The position of HP plants was therefore slightly offset to avoid obstructing the movement of carts between the greenhouse and the experimental garden.

Comment 30 - Third, the artificial population could be too small to detect pollen movement distances of pollinators that are known to be able to disperse pollen over long distances. I understand that the authors cite papers where low pollen dispersal distances are also observed, although many studies suggest, on the contrary, that Bombus and moths can move pollen over long distances. Additionally, a question arises: did all plants have the same number of flowers, and was this variable taken into account in the analyses?

Response: Bumblebees and moths can indeed travel long distances, but that does not mean they frequently carry viable pollen over those distances. The method we used here (Oddou-Muratorio et al. 2018) directly estimates the parameters of the dispersal kernel, i.e. the mathematical function that describes the probability distribution of distances over which successful pollen grains move from a source male. These parameters are independent of the surface area of the population or the exact configuration of plants within the population. It is worth noting that we tested several dispersal kernels and that the best fit was obtained with an exponential probability distribution, meaning that the likelihood of long-distance dispersal events is relatively low (confirming what is often seen in insect-pollinated plants, e.g Van Rossum et al. 2011). Additionally, please note that the average pollen dispersal distances that we estimated here (well under two meters, both during day and night) are well below the average distance between plants. The restricted dispersal distances we report here are thus not artifacts of the experimental setup; rather, they reflect the fact that a majority of successful pollination events occur between neighboring plants. Long-distance pollen dispersal is the exception rather than the rule.

Regarding flower number, obviously not all plants did carry the same number of flowers otherwise we could not have been able to measure any selection gradient on this trait.

Flower number was nonetheless taken into account, as a covariate, for other analyses: please refer to our answer to comment 28.

Comment 31 - Finally, although the amount of lab work on mate number was remarkable, microsatellite genetic data are significantly underutilised. I believe that additional valuable information could be provided. The authors have genotyped almost 2000 seeds and all adult plants, and the results are reduced to just a few lines.

Response: Progeny genotyping was used not only to estimate mating success (number of reproductive partners) but also to quantify selection gradients on floral traits in males, with paternity analysis serving as our method for assessing male reproductive success. We believe that genotypic data was explored to the fullest extent possible without deviating from the objectives of the study, unless there is something we are overlooking.

Minor comments

Comment 32 - Line 50. Correct the italics at the beginning of the line. The same for line 51 in (Jürgens….)

Response: this may have been due to incompatibility issues between our respective computer systems. We have deposited a pdf version of our manuscript for this second round, just in case.

Comment 33 - Lines 77-83. Two hypotheses are put forward focusing on how a single benzenoid compound can weaken or strengthen selection on several floral traits by affecting pollinator behaviour. In my opinion, these hypotheses should be more clearly stated, as they represent a large part of the objective of the study.

Response: The section to which the referee is referring here is as follows:

"Finally, PAA emission could interact with selection on the other attractive floral traits, if PAA enhances diurnal or nocturnal pollinator attraction. Indeed, this could weaken selection on other traits because scent emission would be a more important feature to pollinators (thus effectively removing the selective advantage of other attractive floral traits). On the contrary, PAA emission could strengthen selection on other traits by enhancing visits by specific pollinators, which also use floral traits as visual cues when visiting the plants."

We thus describe the two ways in which our treatment (PAA addition) might impact selection on other floral traits, either impeding it or strengthening it, and why this could happen. We are uncertain about how we could clarify this but any further suggestions would be welcome.

Comment 34 - Line 130. It would be interesting if you could add some results (you put here only personal observations).

Response: We do not have quantitative data on the shift between nocturnal and diurnal pollinators, only qualitative observations conducted prior to the experiment.

Comment 35 - Line 133. You are not using the mean absolute amounts of PAA emitted by Silene doica described in previous studies, but the mean absolute amount of all odour compounds.

Response: You are right, the sentence needed clarification. We now explain that we doubled PAA emission compared to the total emission of all odour compounds reported in a previous study.

L176: Preliminary experiments were conducted to design a protocol allowing PAA emission in the T group to be twice the total average emission of all VOCs described for S. dioica in the literature (110 ng.h^{-1} per flower, with 20 flowers on average per plant, thus 2200 ng. h^{-1} per plant, Waelti et al. 2008).

Comment 36 - Line 142. Why not use 'pollen supplementation rather than 'hand pollinations?

Response: The title was changed accordingly (L190).

Comment 37 - Lines 158-162. Does this mean that on average one male flower per individual plant was analysed?

Response: Indeed pollen production was estimated using a particle counter on one mature bud per male plant at the beginning of the experiment. We clarified the sentence.

L209: For males, total pollen production per flower was estimated. To do so, one nearly opened flower bud per male was collected just before the experiment and dissected.

Comment 38 - Lines 164-166. Please, indicate how many hours were spent observing diurnal flower visitors.

Response: We proceeded to a total of three sessions of pollinator observations over the course of the experiment, each one lasting 20 minutes. We modified the sentence to announce the observation time per plant.

L217: Pollinator observations were conducted for each individual of the D group (i.e. diurnal pollination exposure treatment), using three 20 minutes sessions in the afternoon spread over the course of the experiment (for a total of pollination observations of 60 minutes per plant).

Comment 39 - Lines 170-171. With only 1 h of observation for nocturnal pollinators, I consider that it is too little to draw any conclusions.

Response: We are actually not drawing any quantitative conclusions based on the observations of nocturnal pollinators. As stated above, *S. dioica* is typically described as diurnal in the literature (Jürgens et al. 2002; Jürgens 2004). With our observations, we simply wanted to determine whether pollinating insects visited the common garden and the plants in the artificial population at night. If reproductive success had been zero at night and if we did not have these qualitative observations, it would have been impossible to know if pollinators were simply absent or present but ineffective at pollinating *S. dioica*. Our observations only serve to confirm pollinators occurrence during the night. Our conclusions are based on other data, such as the measurements of pollen limitation and reproductive success.

Comment 40 - Line 197. mother's? Do you mean father's?

Response: No, we mean "mother". When deciding which seedlings to genotype, the number of seedlings per female was weighted by her seed production to ensure that the genotyped sample was representative of population-level reproduction. And after this, we could use our data to determine who the father was.

Comment 41 - Line 275. Weights?

Response: We now added an example when introducing this term.

L332: For males, we accounted for uncertainties in the estimation of male reproductive success by modeling prior weights of a posteriori distributions of the MEMM model (i.e., individuals

with narrower confidence intervals in their estimation of reproductive success have less influence on the model).

Comment 42 - Line 285. Plant group? Replace by treatment and exclusion, but please follow the same terminology across the manuscript.

Response: You are right; this was the only instance in the manuscript where we used this terminology. We introduced the term 'experimental treatments' to refer to all experimental groups (HP, NC, DC, NT, and DT) at the beginning of the Materials & Methods section (under 'Experimental treatments,' L155). For consistency, we have replaced the term 'plant group' with 'experimental treatments.

L377: Experimental treatments (HP DC, DT, NC and NT) did not affect neither seed set ($\chi^2_{4,609}=2.07, P=.73$ *) nor primary fruit-set (*$\chi^2_{4,135}=2.09, P=.72$*), suggesting pollen receipt did not limit female reproductive success in any of the experimental treatments (Table 1). Effective fruit-set (i.e. only non-predated fruits) significantly differed among experimental treatments (* $\chi^2_{4,135}=19.23, P<.001$*).*

Comment 43 - Line 290. P = 0.093 is not marginally significant.

Response: Since the threshold for labeling a p-value as 'marginally significant' varies and is a subject of debate (including or not values < .10), we have revised the sentence accordingly.

L381: Tukey's test revealed that it was significantly lower for NT females compared to DC females ($P<.01$*) or DT females (*$P=.015$*, Table 1), and lower but not significantly so compared to NC females (*$P=.093$*) and HP females (*$P=.069$*).*

Comment 44 - Lines 316-318. Could you please explain this better? Above, you indicated that PAA treatment did not significantly modify independent visits or total visits, while here indicate that PAA addition increased the total number of visited flowers. Explain why the data are analysed differently.

Response: This is because the dataset is different: in this particular analysis, we focused on plants that received at least one pollinator's visit. This allows to capture pollinators behavior once they have arrived on the plant. This model was mentioned in the statistics part in Material and Methods but we added a sentence to catch the reader's attention on this particular analysis, and we now explain how this could be interpreted, compared to the other analyses on pollinators observations both in Material and Methods (L327) and in the results (L418). We also now explicitly use this result in the discussion (L656.

Comment 45 - Line 369. Please, indicate the meaning of DM and NM.

Response: We corrected this and no longer use abbreviations (DM for diurnal males; NM for nocturnal males).

L477: Mean pollen dispersal distance was higher during the night (diurnal males: 1.62m ± 0.032, nocturnal males: 1.98m ± 0.074; $F_{1,115}=4.62, P<.001$*; Figure S3) but was not affected by PAA treatment (*$F_{1,115}=0.42, P=.16$*).*

Comment 46 - Did you find relatedness between the multiple estimates of fitness?

Response: For females, overall fitness was estimated by calculating the number of viable seeds produced during the experiment. This was done by multiplying the mean seed number per fruit by the total number of non-predated fruits and the germination rate. We are not

entirely sure to understand what the referee has in mind here, but examining potential correlations between overall fitness and its components (average number of seeds per fruit, number of fruits, and germination rate) would be meaningless, as overall fitness was derived from these components.

Comment 47 - The discussion does not follow the same order as the results and is sometimes difficult to follow. In my opinion the flow and impact would be improved from restructuring the paragraphs.

Response: We respectfully disagree with the referee on this point. A complete interpretation requires us to integrate the results rather than interpret them in isolation. We prefer to structure the discussion around biological questions instead of following a series of statistical models.

Comment 48 - Line 384. Pollinator communities? Or exclusion experiments and the addition or a single volatile?

Response: In the discussion, for a sake of clarity, we discuss separately the effects of exclusion experiments and PAA addition. In the sentence which the referee refers to here, we discuss the overall effect of exclusion experiments (i.e. different pollinators groups). We added a sentence in the beginning of this paragraph to make this clear (L500).

Comment 49 - Lines 406-426. Why is the addition of phenylacetaldehyde treatment not mentioned in this section?

Response: As indicated in the subtitle, this section of the discussion specifically focuses on the effect of the exposure to different groups of pollinators on pollen dispersal distances and number of mates (as mentioned in our previous response: we aimed to structure the discussion around biological questions). Therefore, the PAA treatment is not addressed here, but a bit later in the discussion.

Comment 50 - Line 429. Phenylacetaldehyde is also implicated in diurnal pollination. Please, indicate.

Response: Yes, the referee is correct, and we have modified the sentence accordingly (L569).

Comment 51 - Line 443. Functional category? Do you mean diurnal vs nocturnal? This separation is not usually considered 'functional group'.

Response: We remove this term and now use “pollinator group” instead. This was done throughout the whole manuscript.

L589: The positive selection on flower number in all treatments suggests that the pollinator group (diurnal versus nocturnal) does not significantly influence this pattern.

Comment 52 - Line 448. A notable exception? And gamete number?

Response: We are not sure what the referee means here. Here, we discuss the fact that interaction between trait and treatment was found non-significant for most traits in females (thus implying that pollinators identity or behavior are not involved in these selective pressures). The notable exception in this sentence is calyx height, which is indeed the only trait for which gradients were found different among treatments (as indicated by the significant interaction presented in the results). Regarding gamete number, as mentioned

L467, the interaction was not significant, suggesting that the treatment had no effect on selective pressures on this particular trait.

Comment 53 - Line 458. Replace This by These.

Response: This was corrected, thank you.

Comment 54 - Lines 456-457. I do not agree with this statement. According to table S3, only the number of flowers (as in females) and corolla width in NC are subject to selection. The difference between males and females in my opinion is not so significant and should be treated with more caution.

Response: We are not sure to understand the referee's comment. We cannot quantitatively compare selection gradients between males and females, since different methods are used for each sex. Throughout the whole text, we thus compare qualitatively our results to investigate whether selective processes were potentially different between sexes. Whereas in females, number of flowers is under positive selection in all plants group (with no interaction between treatment and trait), we found a completely different result in males: the three-way interaction is significant in males, as illustrated by a significant gradient only all plant groups except DC plants.

Comment 55 - Line 469. Correct italicised references.

Comment 56 - Line 472. Replace 'wile' by 'while'.

Response to comments 55-56: This was corrected, thank you.

Comment 57 - Line 489: Not the emission of phenylacetaldehyde, but a significant artificial increase of the natural emission of this VOC.

Response: The sentence was modified (L660).

Comment 58 - Line 494: No pollinator communities. Indicate groups of pollinators (nocturnal vs. nocturnal).

Response: The appropriate changes were made throughout the whole manuscript (see our answer to comment 51)

Comment 59 - As indicated above, conclusions are not fully supported by the data presented. The authors could tone down certain statements.

Response: as explained in both the introduction (L40) and the discussion (L512), because the only difference that stands between plants pollinated during day *vs.* night is the identity of pollinators, when differences were found in selection gradients, it could be attributable only to pollinators. When focusing on control plants, differences in selection gradients imputable to pollinators (differences between day and night) occurred only for males. It thus seems that pollinators play a greater role in selection in males compared to females, which is our main conclusion.

Comment 60 - Table 1. Please standardized to 3 decimals for all results. For Effective fruit-set, the NC and HP the superscrips should be 'ab'.

Response: Done, thank you.

Comment 61 - Table 2. Could you include significant differences in pairwise comparisons?

Response: Yes, thank you for the suggestion, this was done in this new version

Comment 62 - It would be interesting to add a table listing diurnal and nocturnal insect pollinators and visitation patterns.

Response: As noted in the manuscript, we did not conduct pollinator observations at night, so we do not have visitation patterns for these. However, the raw visitation data, including insect identity and visit sequences, are available on Dryad, and the link will be provided with the article.

GIOVANNI SCOPECE'S COMMENTS

Title and abstract

- Does the title clearly reflect the content of the article? Yes, but it can be improved, see below.
- Does the abstract present the main findings of the study? Yes, but it can be improved, see below.

Introduction

- Are the research questions/hypotheses/predictions clearly presented? Yes
- Does the introduction build on relevant research in the field? Yes

Materials and methods

- Are the methods and analyses sufficiently detailed to allow replication by other researchers? Yes
- Are the methods and statistical analyses appropriate and well described? Yes

Results

- In the case of negative results, is there a statistical power analysis (or an adequate Bayesian analysis or equivalence testing)? Yes
- Are the results described and interpreted correctly? Yes

Discussion

- Have the authors appropriately emphasized the strengths and limitations of their study/theory/methods/argument? Yes
- Are the conclusions adequately supported by the results (without overstating the implications of the findings)? Yes

In this study the authors explored the effect of diel cycle and of increasing emission of PAA (i.e., a compound known to be attractive to Hadena nursery pollinators) on male and female reproductive success in Silene dioica. Overall, I liked the manuscript and I think it represents a nice case study that push forward our understanding of pollination mechanisms in an interesting plant group as the Silene genus. In particular, I have highly appreciated the manipulative approach, the full-crossing design and the paternity analyses of the seedlings as a way to estimate male function. I also think that the analyses were formally correct, and the results well presented. Therefore, my comments are mainly focused on presentation, and I hope that can help improving the manuscript.

In my opinion, title and abstract can be improved.

Comment 63 - The title in its present form does not include a mention to male and female reproductive functions, that are in my opinion one of the strengths of the study, neither to the investigated species that could be instead attractive to readers as the Silene system is well-known. Also the reference to "scent emission" is too generic, as the experiment is only carried out using one compound (PAA).

> **Response:** We modified the title to follow the referee's suggestion: *"Investigating the effects of diurnal and nocturnal pollinators on male and female reproductive success and on floral trait selection in* Silene dioica*"*. It now mentions the fact that both male and female reproductive functions have been investigated in this study as well as the name of our study species. Also, the anonymous referee asked us to remove the term "scent" from the title, since our results reflect the impact of one particular volatile compound rather than the impact of the whole scent. We tried, as suggested by the anonymous referee, to replace scent by the name of the molecule but it led to a very complex, very long and not so catchy title. Because our results on the effects of PAA are less straightforward and often less easy to interpret, we decided to remove this notion from the title. It will however be visible in the abstract and the keywords.

Abstract:

Comment 64 - Line 2: of selection ON floral traits

> **Response:** Agreed, thank you for pointing this out.

Comment 65 - Line 3-5: This sentence needs rephrasing. In its present form it only focuses on selection gradients, whilst the study also covers male and female reproductive success. Also, the dioecious mating system of the investigated species should be stated at the beginning in order to allow readers to understand why results are then divided into male and female plants.

> **Response:** We agree that this sentence did not fully represent the aim of our experiment and needed more accuracy. We have revised it according to your suggestions.
>
> *L13: In this study, we investigated how female and male reproductive successes of the dioecious species Silene dioica are affected by their floral traits, in relation to (i) the pollinator group the plants were exposed to (diurnal versus nocturnal pollination) and (ii) the level of emission of a volatile organic compound typically linked to pollinator attraction (natural versus enhanced phenylacetylaldehyd (PAA) emission) in a fully crossed design.*

Main text:

Comment 66 - Line 8: Here the logic flow can be improved by specifying that the diversifying role of plant pollinator interactions is thought to be prevalent in specialized plant species, before stating that "It should be noted, however, that only a minority of plant species have highly specialized pollination systems"

> **Response:** The mention of the diversification effect of plant-pollinator interactions in specialized plant species should indeed improve the understanding of our main idea in this paragraph. We have revised the sentence based on your suggestions.
>
> *L4: The increasing number of experimental studies evidencing the occurrence of pollinator-mediated selection on floral traits (Caruso et al., 2019), along with studies linking pollination mode to floral diversification in a phylogenetic framework strongly support this driving role of*

plant-pollinator interactions in shaping floral evolution, particularly in specialized plant species (e.g Graham & Barrett, 2004; Whittall & Hodges, 2007). It should be noted, however, that only a minority of plant species benefit from this highly specialized pollination systems (for instance plants that are engaged in brood-site mutualisms, e.g. Pellmyr 1992, or plants that rely on sexual mimicry to attract their pollinators, e.g. Peakall et al. 2010).

Comment 67 - Line 31: remove italics from the parenthesis

Response: as answered to comment 32, this may have been due to incompatibility issues between our respective computer systems. We have deposited a pdf version of our manuscript for this second round, just in case.

Comment 68 - Line 76: here and through the manuscript, "versus" should be italicized

Comment 69 - Line 119: correct "individuals.m-^{2}"

Comment 70 - Line 157: add a dot after "Barbot et al"

Comment 71 - Line 384: how pollinator communities shape (i.e. not shapes)

Comment 72 - Lines 413-414: I would change as: "or by genotyping seedlings as done in our study and in Barthelmess et al. (2006)"

Comment 73 - Line 419: Has (not as)

Comment 74 - Line 444: insert a dot after "pattern"

Comment 75 - Line 452: caryophyllaceae with capital C

Comment 76 - Line 458: These (not This)

Comment 77 - Line 468: Italicize "bicruris"

Comment 78 - Line 469: Remove italics from (Labouche & Bernasconi, 2009)

Comment 79 - Line 472: While (not wile)

Comment 80 - Line 482: nocturnal pollinatorS

Comment 81 - Line 500: insert a dot after "selection"

Response to comments 68-81: All these issues were addressed.

References

Barbot, E., M. Dufaÿ, and I. De Cauwer. 2023. Sex-specific selection patterns in a dioecious insect-pollinated plant. Evolution; International Journal of Organic Evolution 77:1578–1590.

Carr, D. E., A. I. Haber, K. A. LeCroy, D. E. Lee, and R. I. Link. 2015. Variation in reward quality and pollinator attraction: the consumer does not always get it right. AoB PLANTS 7:plv034.

Jürgens, A. 2004. Flower scent composition in diurnal Silene species (Caryophyllaceae): phylogenetic constraints or adaption to flower visitors? Biochemical Systematics and Ecology 32:841–859.

Jürgens, A., T. Witt, and G. Gottsberger. 2002. Flower scent composition in night-flowering *Silene* species (Caryophyllaceae). Biochemical Systematics and Ecology 30:383–397.

Kula, A. A. R., M. R. Dudash, and C. B. Fenster. 2013. Choices and consequences of oviposition by a pollinating seed predator, *Hadena ectypa* (Noctuidae), on its host plant, *Silene stellata*(Caryophyllaceae). American Journal of Botany 100:1148–1154.

Miyake, T., I. Satake, and K. Miyake. 2018. Sex-biased seed predation in gynodioecious *Dianthus superbus* var. *longicalycinus* (Capryophyllaceae) and differential influence of two seed predator species on the floral traits. Plant Species Biology 33:42–50.

Oddou-Muratorio, S., J. Gauzere, A. Bontemps, J.-F. Rey, and E. K. Klein. 2018. Tree, sex and size: ecological determinants of male vs. female fecundity in three *Fagus* sylvatica stands. Molecular Ecology 27:3131–3145.

Rich, C., and T. Longcore. 2013. Ecological Consequences of Artificial Night Lighting. Island Press.

Van Rossum, F., I. Stiers, A. Van Geert, L. Triest, and O. J. Hardy. 2011. Fluorescent dye particles as pollen analogues for measuring pollen dispersal in an insect-pollinated forest herb. Oecologia 165:663–674.

Waelti, M. O., J. K. Muhlemann, A. Widmer, and F. P. Schiestl. 2008. Floral odour and reproductive isolation in two species of *Silene*. Journal of Evolutionary Biology 21:111–121.