Peer Community In Evolutionary Biology

More in less: almost everything you wanted to know about sex in flowers is in a single experiment with a single plant species

Juan Arroyo and *Violeta Simón-Porcar* based on peer reviews by *Luis Gimenez-Benavides*, *Andrea Cocucci*, *Giovanni Scopece* and 1 anonymous reviewer

Barbot Estelle, Dufaÿ Mathilde, Godé Cécile, De Cauwer Isabelle (2024) Investigating the effects of diurnal and nocturnal pollinators on male and female reproductive success and on floral trait selection in Silene dioica. Zenodo, ver. 3, peer-reviewed and recommended by Peer Community in Evolutionary Biology.
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Most flowering plants (almost 90% of species) are pollinated by animals (Ollerton et al. 2011). In fact, many plants are completely dependent on pollinator visits for reproductive success, due to the complete inability of selfing if they are self-incompatible or have strong gender differentiation, as in dioecious plants. Others have diminished reproductive output in the absence of pollinators, even being self-compatible, if their flowers present strong herkogamy or dichogamy, making autonomous selfing more difficult. Ultimately, all animal-pollinated plant species rely on pollinators for outcrossing. Depending on the genetic structure of plant populations and the movement patterns of these animals, outcrossing patterns will shape the population genetic variation, which will determine its adaptive fate. Thus, understanding the mechanisms governing the pollination interaction is crucial for unraveling the uncertainties of a huge proportion of biodiversity on Earth. Being mutualistic by definition, the animal side of this interaction is less understood, despite most pollinator groups being likely dependent on it for their persistence and perhaps diversity (Ollerton 2017). The role of pollinators in plant diversification has generated much literature and controversy ever since Darwin and his "abominable mystery" about angiosperm diversification (Friedman 2009). However, the other way around, that of plant's effect on pollinator diversification, is more debatable. A remarkable example of this effect is the

possible case of co-speciation mediated by nursery (brood site) pollination, which also includes antagonistic insect herbivory (Wiens et al. 2015), as in some Silene species and their moth pollinators and herbivores (Hembry and Althoff 2016). A properly functional pollination interaction relies on efficient pollinators being attracted to flowers (by visual and olfactory stimuli), rewarded or deceived by them (in feeding, nesting, basking, mating, etc. sites), fit the flower shape and contact the sex organs to enhance both male and female plant fitness. Whereas flower rewards, visually attractive stimuli, and flower architecture and shape greatly dominate pollination studies; there are much fewer studies of olfactory attractive stimuli through flower volatile organic compounds (VOC), due to inherent methodological difficulties (Raguso 2008). Most of the studies dealing with flower volatiles are correlative by nature, whereas manipulative experimental approaches are far less common. Albeit still plant-centered, the manuscript by Barbot et al. (2024) on *Silene dioica* and its varied pollinator arrays has great merit in including many of the issues mentioned above to solve long-standing questions in plant reproduction. It elegantly fills a gap with well-designed and performed experiments in a particular pollination mode, nursery pollination, which is now considered more frequent and diverse than formerly thought (Nunes et al. 2018, Haran et al. 2023, Suetsugu 2023). The authors demonstrate that *Silene dioica* has a truly mixed pollination system, including not only generalist diurnal pollinators as it was formerly considered but also nocturnal pollinators of similar proven efficiency. Although the specialized nursery pollination system of *Silene-Hadena* is widely reported and described in the literature (Kephart et al. 2006; Prieto-Benítez et al. 2017), it was not formerly considered important for Silene dioica, based on its floral syndrome. However, the experiments designed by Barbot et al. (2024) explore in detail the mechanisms of attraction of nocturnal pollinators and their consequences for plant reproductive success, fully confirming the proper functioning of nursery pollination in this plant species. All these prospects are robustly performed through classical sound phenotypic selection analyses and manipulative experiments, together with other approaches less frequent due to technical difficulties but critical when testing authors' hypotheses. In particular, male fitness estimates using suitable microsatellite markers are especially appropriate in this dioecious species, although they are also very useful in hermaphroditic species when different pollinators and flower variants are interacting (Kulbaba and Worley 2013, Simón-Porcar et al. 2015). Finally, the manipulation of flower fragrance (in fact, of a single VOC) has proved also critical to getting insight into its effect on night pollinators and their joint role as ovipositors and thus predators. All these questions are addressed with a fully crossed experimental design that allows unveiling interactions between experimental factors, a challenge in experimental biology, especially under natural conditions. In evolutionary biology, most experiments are carried out in laboratories, where factors under scrutiny are carefully controlled and hence their effects are easy to reproduce. The cost of this approach is that the variables of interest are oversimplified and difficult to extrapolate to real ecological conditions. In evolutionary ecology, experiments under field conditions greatly solve this shortcoming, but the cost arises in the difficulties of dealing with several interacting and uncontrolled factors. The study by Barbot et al. (2024) nicely addresses real-world questions in a specialized pollination plus herbivory interaction. The results are robust and pave the road for further overarching pursuits, as mentioned by the reviewers. Thus, it would be interesting to assess actual, absolute values of pollen dispersal distance in natural populations, the selection exerted through the complete reproductive period of male and female plants, provided that they are different, or the effect of using more natural flower bouquets, in the next steps. However, as it stands, this outstanding study will be of high interest to scholars on any of the many topics dealt with there, but also to students willing to start research in the fascinating field of experimental pollination biology, given the wide array of questions addressed and the modern methodological approaches provided. **Acknowledgments**

The authors of this recommendation benefitted from grants provided by grants PID2021-122715NB-I00 and TED2021-131037B-I00 funded by MCIN/AEI/ 10.13039/501100011033 and by the "European Union NextGeneration EU/PRTR", and by MSCA-IF-2019-89789. **References**

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Reviews

Evaluation round #1

DOI or URL of the preprint: https://doi.org/10.5281/zenodo.11488687
Version of the preprint: 2

Authors' reply, 01 November 2024

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Decision by Juan Arroyo and Violeta Simón-Porcar, posted 03 August 2024, validated 05 August 2024

Decision on the first version of Bardot et al preprint "Exploring the effect of scent emission and exposition to diurnal versus nocturnal pollinators on selection patterns on floral traits"

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

Reviewed by Luis Gimenez-Benavides, 23 July 2024

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

Barbot E., Dufaÿ M., Godé C. and De Cauwer I.

Review for PCI Evol Biol

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.

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.

Other comments:

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

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).

L65: should read Benzenoid

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

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

L336: should read "Results of the ANCOVAs analyzing female (left)..."

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

L419: should read "a trend that has..."

L443: please describe the term fertility selection

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

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.

Reviewed by Andrea Cocucci, 02 July 2024

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:

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).

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, result 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".

Minor comments

line 202. replace "reported" with "recorded"

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

line 211. was the presence of Silene pollen assessed?

line 221. replace "polinized" with "pollinated"

line 273. replace "if" with "whether"

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

lines 349-350. correct negative statement neither ... nor...

line 351. replace "pollen" with "pollination"

line 376. replace "Sphyngidae" with "Sphingidae"

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.

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

line 462. replace "significance values" with "asterisks"

line 469. repalce "shapes" with "shape"

Download the review

Reviewed by anonymous reviewer 1, 02 July 2024

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

Discussion

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

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.

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?

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?

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.

Minor comments

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

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.

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

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.

Line 142. Why not use ‘pollen supplementation rather than ‘hand pollinations?

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

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

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

Line 197. mother’s? Do you mean father’s?

Line 275. Weights?

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

Line 290. P = 0.093 is not marginally significant.

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.

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

Did you find relatedness between the multiple estimates of fitness?

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.

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

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

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

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

Line 448. A notable exception? And gamete number?

Line 458. Replace This by These.

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.

Line 469. Correct italicised references.

Line 472. Replace 'wile' by 'while'.

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

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

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

Tables and Figures.

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

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

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

Reviewed by Giovanni Scopece, 16 July 2024

· 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.

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).

Abstract:

Line 2: of selection ON floral traits

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.

Main text:

Through the main text, I recommend the authors to be more consistent.

Discussion: In this study, we investigated how pollinator communities shapes pollen dispersal distances, access to sexual partners and reproductive success in Silene dioica.

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"

Line 31: remove italics from the parenthesis

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

Line 119: correct "individuals.m-²"

Line 157: add a dot after "Barbot et al"

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

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

Line 419: Has (not as)

Line 444: insert a dot after "pattern"

Line 452: caryophyllaceae with capital C

Line 458: These (not This)

Line 468: Italicize "bicruris"

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

Line 472: While (not wile)

Line 482: nocturnal pollinatorS

Line 500: insert a dot after "selection"