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Pollen grain size associated with pollinator feeding strategy

Kai Hao, Zhi-Xi Tian, Zi-Chen Wang and Shuang-Quan Huang

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Review timeline

Original submission:	11 March 2020
1st revised submission:	24 May 2020
2nd revised submission:	15 July 2020
Final acceptance:	24 July 2020

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History

RSPB-2020-0548.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Major revision is needed (please make suggestions in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Acceptable

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
Marginal

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
Yes

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
Yes

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
Yes

Is it clear?
No

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
Hao et al investigate variation in pollen grain size among flowering plants. They introduce the two main hypotheses that have been put forward to explain this variation: one related to pre-pollination pollinator behaviour, and another one related to post-pollination pollen-stigma/style interactions. Using a dataset derived from a community of flowering plants in China, for which they quantified pollen grain size, style length and overall flower size, and for which they characterized pollinator type and behaviour, as well as phylogenetic relationships, they perform a range of tests to evaluate support for either of the two hypotheses.

They find differences in pollen grain size related to pollinator type and behaviour, they find support for correlation between grain size and style dimensions, but they ultimately argue that a model-based approach identified pollinator foraging behaviour as the most important determinant of pollen grain size in their community. They further present pollen grain size data for over 1000 angiosperm species and plot these on a family level phylogenetic tree.

The topic is worthy of investigation, as no conclusive assessment appears to have been done (although there is no reason why both hypotheses may be an appropriate explanation). I have a range of minor comments, listed below and in a marked-up file which I have attached.

However, my main issue with the study by Hao et al is that it tries to explain an angiosperm wide phenomenon (as they show nicely with their angiosperm-wide data set), but in terms of their statistical analyses it relies exclusively on a data set collected in a single community of 80 plant species. Although the data may have been collected with great accuracy, it remains unclear to what extent the results can be generalized. Specific local habitat conditions (temperate alpine network, with a specific pollen-collecting fauna), the small sample size of plant species in relation to all angiosperms, and the specific community context (pollen collecting behaviour may be plastic, dependent on whatever other species co-flower, and may therefore vary across communities), may all decrease the possibility to generalize from the results presented. Indeed, the study by Harder (1998) clearly showed for instance that pollen grain size variation is inconsistent across lineages; similarly, these patterns may depend on the factors mentioned above and not only on pollinator behavior. It would be helpful if the relationships presented here can be shown to be more widely applicable, and it appears that the authors already have some data (their angiosperm-wide dataset presented in Table S3) to perform a meta-analysis in a similar way to which they analysed their own community data.

Minor comments:

-The authors perform a large number of tests, and refer to several hypotheses, but I found the link between the hypotheses, methods, and results not clear. I suggest to include a paragraph at the end of the introduction in which a number of hypotheses with associated predictions is put forward, and use this framework to structure the materials and methods, and results section. I found the phrasing of some of the hypotheses a bit unclear, I think these should be unambiguous and result in testable predictions.

-I found the purpose of the two angiosperm-wide datasets (Tables S1 and S3) unclear. They are used in a descriptive way, but it seems to me that more can be done with them. It was also unclear how the raw data from Table S1 were summarized into Table S3.

-The language is generally understandable, but is not up to scratch for publication.

-I found the discussion somewhat disjointed; given that the two hypotheses have received ample attention in the literature, these need not be discussed in detail. I rather expected that the discussion would mostly deal with differences in findings between the current study and similar previous studies, and that the discussion should formulate an argument why the results drawn from the community study could apply angiosperm-wide.

-Based on my own experience with using ITS sequences at the genus and tribal level, I do not think it is feasible to accurately align this across angiosperms. I therefore argue against its use. It might be useful to consult the literature for published trees that can be used for meta-analyses, e.g. consider Janssens et al. Biodiversity Data Journal 8: e39677

-For the phylogenetic analysis, rather than using a substitution model partitioned among genes, it may be more useful to partition among codon positions, as third codon positions are likely to evolve according to a different substitution model than first and second codon positions. It was also not clear whether convergence in model parameter estimation was reached?

-The purpose and methods underlying the phylogenetic mapping are not clear. What algorithm was used to reconstruct ancestral character states, and what were these used for?

-What was the purpose of calculating a frequency distribution of pollen grains? This is not clearly articulated.

-In the phylogenetic analysis, the use of congeners in case a sequence for the study species was unavailable on genbank is fine, as long as this is done for a single representative. This approach becomes more problematic if 2 or more genus members for which genbank accessions are lacking are being included in the analyses.

-Although I am not an expert in statistical methods involving model selection, my understanding is that this approach should not be used to exhaustively assess support for any possible model, but that this approach needs to be based on careful a priori selection of several competing models, in the context of some theoretical debate. Given the importance of the results derived from this analysis, a better justification for the selected approach is desirable.

-In the results it is clarified that the 80 micron threshold is arbitrary. I wonder what the use is of this threshold in that case. What is the basis for the 80 micrometer division? This is not justified anywhere in the introduction.

Review form: Reviewer 2 (David Inouye)

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Excellent

General interest: Is the paper of sufficient general interest?
Excellent

Quality of the paper: Is the overall quality of the paper suitable?
Excellent

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

> **Is it accessible?**
> Yes
>
> **Is it clear?**
> Yes
>
> **Is it adequate?**
> Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
Manuscript describes an interesting study that will be of interest to a broad audience. I think the relevant literature is cited, and the tables and figures are clear. The writing is good (I've made some minor editorial suggestions on the PDF). This topic hasn't received much attention in the existing literature, and this analysis is a good contribution.

In supplementary files, change
Table S1. Data of mean pollen grain diameter in 1271 species from 208 angiosperm families (APG IV), which species with aggregated pollen (dyads, tetrads, polyads or pollinia) are marked in yellow.
to
….families (APG IV). Species with ….

Decision letter (RSPB-2020-0548.R0)

15-Apr-2020

Dear Dr Huang:

I am writing to inform you that your manuscript RSPB-2020-0548 entitled "The evolution of pollen size in animal- pollinated plants is largely driven by pollinator foraging behaviour" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of referees, who have recommended that substantial revisions are necessary. With this in mind we would be happy to consider a resubmission, provided the comments of the referees are fully addressed. However please note that this is not a provisional acceptance.

The resubmission will be treated as a new manuscript. However, we will approach the same reviewers if they are available and it is deemed appropriate to do so by the Editor. Please note that resubmissions must be submitted within six months of the date of this email. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office. Manuscripts submitted after this date will be automatically rejected.

Please find below the comments made by the referees, not including confidential reports to the Editor, which I hope you will find useful. If you do choose to resubmit your manuscript, please upload the following:

1) A ‘response to referees’ document including details of how you have responded to the comments, and the adjustments you have made.
2) A clean copy of the manuscript and one with 'tracked changes' indicating your 'response to referees' comments document.
3) Line numbers in your main document.

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Sincerely,
Dr Daniel Costa
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
Pollen grain size varies astonishingly between plant species and while several hypotheses have been suggested, few have been well-tested. This manuscript tests two of these hypotheses: 1) pollen grain size is driven by post-pollination interactions with style length and 2) pollen grain size is driven by pollen feeding insects. The manuscript finds correlative evidence that pollen grain size and style lengths are associated and also that large pollen grains are associated with animal foragers that do not collect pollen. In the end, the authors utilize model selection to reject the hypothesis that pollen size is driven by post-pollination stylar interactions. I found this to be an extremely interesting manuscript and I really enjoyed reading it. The manuscript was reviewed by two excellent reviewers who were somewhat conflicting in their comments. While both were quite positive about the manuscript, one of the reviewers picked out several potential statistical issues and suggested that we send the manuscript to a specialist statistician. This

reviewer also questions the community study part of the manuscript is used to generalize for all angiosperms.

While I found this study interesting, it does require some work and justification to get it up to Proc Roy Soc standard. Below I outline some of the things that the authors need to concentrate on to increase the chances of acceptance. The points reiterate some of the main points made by reviewers as well as some of my own.

1) I agree with Reviewer 1 that when trying to explain angiosperm-wide phenomena it would be useful to use data from across the angiosperm phylogeny and not just a simple community analysis. Since the authors appear to have these data at their disposal, I think that such an analysis would go a long way to nullifying these concerns.

2) Reviewer 1 was not convinced that post-pollination stylar interactions could be excluded as a driver of pollen size from the model selection process employed by these authors. I was also a little sceptical that so much faith was placed in a process of model selection to support one hypothesis over another. The authors really need to make sure that this approach is sound, or interpret their results in a more egalitarian manner.

3) In this respect, I was also a little concerned about the general tone of the manuscript. The general tone is that pollen grain size has been driven by pollinator foraging behaviour (see title). However, in reality, all the authors have done is show some correlations and collect field data to show that pollen foraging takes place mostly on plants that have small pollen grains. While this is all very interesting it is far from being definitive evidence that pollen foraging has actually driven the variation in pollen grain size. Even the field data collected does not show that pollen foraging has driven the evolution of small pollen – pollen consuming insects may simply prefer to collect pollen from flowers with small pollen grains – perhaps it packs better in corbiculae. Here I advocate a more cautious approach to the interpretation of these data.

4) I suspect that grain size is also negatively correlated on with the number of grains produced. This makes me question whether selection is directly on grain size or whether selection is actually on grain number. At the very least, this needs to be addressed in the manuscript. Do the authors have data on the number of pollen grains produced and how this correlates with foraging insects?

5) I noticed that the regression in Fig 3B may be driven by a single outlying data point. I suggest the authors re-run this correlation after removing the data point to see if it still holds. Obviously, if the regression does not stand up to the removal of a single data point, it would lend support to the line taken by this manuscript (rejecting the style length hypothesis).

6) Reviewer 1 suggested some restructuring which I support. While the introduction is presently very short (around 600 words), I do not think it requires lengthening for the sake of lengthening. At the moment, the manuscript is short and punchy and fits nicely with the journal style. But the point is that there is plenty of word-space left to explain things better where necessary.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
Hao et al investigate variation in pollen grain size among flowering plants. They introduce the two main hypotheses that have been put forward to explain this variation: one related to pre-pollination pollinator behaviour, and another one related to post-pollination pollen-stigma/style interactions. Using a dataset derived from a community of flowering plants in China, for which they quantified pollen grain size, style length and overall flower size, and for which they

characterized pollinator type and behaviour, as well as phylogenetic relationships, they perform a range of tests to evaluate support for either of the two hypotheses.

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Minor comments:

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Referee: 2

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In supplementary files, change
Table S1. Data of mean pollen grain diameter in 1271 species from 208 angiosperm
families (APG IV), which species with aggregated pollen (dyads, tetrads, polyads or
pollinia) are marked in yellow.
to
….families (APG IV). Species with ….

Author's Response to Decision Letter for (RSPB-2020-0548.R0)

See Appendix A.

RSPB-2020-1191.R0

Review form: Reviewer 2 (David Inouye)

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Excellent

General interest: Is the paper of sufficient general interest?
Excellent

Quality of the paper: Is the overall quality of the paper suitable?
Excellent

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

> **Is it accessible?**
> Yes
>
> **Is it clear?**
> Yes
>
> **Is it adequate?**
> Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
This revision addresses the concerns I had with an earlier version. I have made some minor editorial suggestions/corrections on the PDF, but they should be easy to change. I have no other significant concerns with the manuscript. I think it will be a good contribution to the literature about pollen size.
David Inouye

Decision letter (RSPB-2020-1191.R0)

14-Jul-2020

Dear Dr Huang:

Your manuscript has now been peer reviewed and the reviews have been assessed by an Associate Editor. The reviewers' comments (not including confidential comments to the Editor) and the comments from the Associate Editor are included at the end of this email for your reference. As you will see, the reviewers and the Editors have raised some concerns with your manuscript and we would like to invite you to revise your manuscript to address them.

We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Associate Editor, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available we may invite new reviewers. Please note that we cannot guarantee eventual acceptance of your manuscript at this stage.

To submit your revision please log into http://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions", click on "Create a Revision". Your manuscript number has been appended to denote a revision.

When submitting your revision please upload a file under "Response to Referees" in the "File Upload" section. This should document, point by point, how you have responded to the reviewers' and Editors' comments, and the adjustments you have made to the manuscript. We require a copy of the manuscript with revisions made since the previous version marked as 'tracked changes' to be included in the 'response to referees' document.

Your main manuscript should be submitted as a text file (doc, txt, rtf or tex), not a PDF. Your figures should be submitted as separate files and not included within the main manuscript file.

When revising your manuscript you should also ensure that it adheres to our editorial policies (https://royalsociety.org/journals/ethics-policies/). You should pay particular attention to the following:

Research ethics:
If your study contains research on humans please ensure that you detail in the methods section whether you obtained ethical approval from your local research ethics committee and gained informed consent to participate from each of the participants.

Use of animals and field studies:
If your study uses animals please include details in the methods section of any approval and licences given to carry out the study and include full details of how animal welfare standards were ensured. Field studies should be conducted in accordance with local legislation; please include details of the appropriate permission and licences that you obtained to carry out the field work.

Data accessibility and data citation:
It is a condition of publication that you make available the data and research materials supporting the results in the article (https://royalsociety.org/journals/authors/author-guidelines/#data). Datasets should be deposited in an appropriate publicly available repository and details of the associated accession number, link or DOI to the datasets must be included in the Data Accessibility section of the article (https://royalsociety.org/journals/ethics-

policies/data-sharing-mining/). Reference(s) to datasets should also be included in the reference list of the article with DOIs (where available).

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should also be fully cited and listed in the references.

If you wish to submit your data to Dryad (http://datadryad.org/) and have not already done so you can submit your data via this link http://datadryad.org/submit?journalID=RSPB&manu=(Document not available), which will take you to your unique entry in the Dryad repository.

If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link.

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All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI. Please try to submit all supplementary material as a single file.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rspb.[paper ID in form xxxx.xxxx e.g. 10.1098/rspb.2016.0049].

Please submit a copy of your revised paper within three weeks. If we do not hear from you within this time your manuscript will be rejected. If you are unable to meet this deadline please let us know as soon as possible, as we may be able to grant a short extension.

Thank you for submitting your manuscript to Proceedings B; we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Best wishes,
Dr Daniel Costa
mailto: proceedingsb@royalsociety.org

Associate Editor Board Member
Comments to Author:
see attached doc

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s).
This revision addresses the concerns I had with an earlier version. I have made some minor editorial suggestions/corrections on the PDF, but they should be easy to change. I have no other significant concerns with the manuscript. I think it will be a good contribution to the literature about pollen size.
David Inouye

Author's Response to Decision Letter for (RSPB-2020-1191.R0)

See Appendix B.

Decision letter (RSPB-2020-1191.R1)

24-Jul-2020

Dear Dr Huang

I am pleased to inform you that your manuscript entitled "Pollen grain size associated with pollinator feeding strategy" has been accepted for publication in Proceedings B.

You can expect to receive a proof of your article from our Production office in due course, please check your spam filter if you do not receive it. PLEASE NOTE: you will be given the exact page length of your paper which may be different from the estimation from Editorial and you may be asked to reduce your paper if it goes over the 10 page limit.

If you are likely to be away from e-mail contact please let us know. Due to rapid publication and an extremely tight schedule, if comments are not received, we may publish the paper as it stands.

If you have any queries regarding the production of your final article or the publication date please contact procb_proofs@royalsociety.org

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Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

Thank you for your fine contribution. On behalf of the Editors of the Proceedings B, we look forward to your continued contributions to the Journal.

Sincerely,
Dr Daniel Costa
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor:
Board Member
Comments to Author:
I have enjoyed reading this manuscript and have suggested some more minor revisions (see below)

Minor edits
L17: consider simplifying this to: Here we propose that visits by pollen foraging pollinators have selected against large pollen grains

L18: Delete the bit about the hypothetical role and write: An association between pollinator behaviour and pollen grain size was confirmed by field studies of 80 flowering species…

L38: delete the short sentence as replace with something like: For example, harvested pollen is often packed on the corbiculae of bees where it is not available for stigmatic deposition or ovule fertilization (REF)

L42: You may want to add another possible evolutionary response to pollen harvesting at the end of this paragraph: It is also thought that dispensing pollen very slowly may reduce grooming behaviour in bees – I think there are some nice refs by James Thompson

L43, comma after species

L47: delete have

L58: The null hypothesis of allometric scaling between sexual organs and flower size…

L60: Early workers proposed that pre-pollination foraging economics could also select on

L62: grain >>>grains

L62: envisioned to have

L66: per >>>pre

L67: …on bee limbs would be more efficient at grooming large pollen grains than small ones

L69: may >>>would

L84: Delete: although available evidence for the pollinator foraging hypothesis is unviable to date.

L87: …whereas in species visited by pollen-collecting foragers, large numbers of small pollen grains may enhance reproductive success by increasing the chances that some pollen grains are not groomed.

L104: Delete pollinator

L204: delete: The Pearson's correlation analysis indicated that. So start the sentence with: Pollen size….

L271: I have suggested some changes to this paragraph. Please check that it still says what you wanted it to say:

We observed that the two most abundant bumblebee species did not collect pollen from species in Cucurbitaceae, Malvaceae, Geraniaceae and Liliaceae, despite the fact that these pollen were relatively large (diameter > 80 m). They did however collect pollen from other species with relatively small grains (Fig. 1, Figs. S2 & S3; Tong & Huang 2018). Pollen depletion by bees accounts for a high proportion of pollen loss during pollen transfer (Harder & Routley 2006), however many plants appear to have evolved adaptive strategies to avoid pollen overexploitation by collectors (Hargreaves et al. 2009). In a few plant groups, for example cotton and pumpkin flowers (Thorp 2000), anecdotal observations showed that honeybees did not groom and pack pollen into the corbiculae. Instead, pollen was cleaned from their bodies and discarded. It is thought that spines on the pollen grains of cotton (Gossypium) make pollen packing physically difficult (Lunau et al. 2015) and act as a defence against exploitation. Alternatively large pollen grains may be unfavourable to bumblebees (Vaudo et al. 2016) if they are starch-rich but protein-poor (Baker & Baker 1979). To our knowledge, physical and chemical defences protecting pollen from bee collection have been little-studied, but they could account for the lack of harvesting from some of the large-grained species in this study.

L286: Delete: Bees rely on pollen for a protein source. Also delete the next sentence.

L286: Replace with: The conflict of interest between pollen consumers and plants also appears to have influenced the evolution of other floral rewards

Appendix A

Dear Prof. Daniel Costa,
Editor, Proceeding B

We are resubmitting a manuscript (ID RSPB-2020-0548) on the evolution of pollen grain size.

We sincerely thank the associated editor and two experts providing positive comments and helpful suggestions to improve the study!

Considering their suggestions and the data that we currently have, we have re-organized the manuscript. We omit the large-scale comparative analysis of pollen size evolution across angiosperm families that we did in an early version, given that this part of analyses on foraging pollen behaviours of bees that were unclear in the literature, usually supposed to be pollen collectors. Our field observations in bee-pollinated plant species showed that these bumblebees foraged nectar only, but did not positively forage and collect pollen in around 50% bee-pollinated plant species. As suggested, we input data of pollen number per flower and focus on possible pollen-related traits and potential factors affecting variation in pollen size. In the revision, we are trying to figure out the driving forces on evolution of pollen size by an empirical study in the natural communities. To address the large variation in pollen size (diameter from 10 to over 100 microns) in the 80 flowering species which major pollinators and feeding pollen behaviour were studied, we are able to test both the pre- and post-pollination hypotheses for evolution of pollen grain size.

As previous studies did not find that pollinator types relate to variation in pollen size across species, an early hypothesis of pollinator mediated selection on pollen size evolution (pre-pollination hypothesis) has been abandoned. We observed that the same bumblebee species rarely collected large pollen grains into their corbiculae but collected small pollen from other co-flowering species, suggesting that the size matters pollen collection by bees. These results seemed surprising, as bees rely on pollen as sole protein source. Our comparison of pollen size and number in these bee-pollinated plant species showed that pollen grains in those species pollinated by pollen collecting bees were significantly smaller than species pollinated by non-pollen feeding bees. The bee rejection collecting pollen allows pollen grains to enlarge in size, as little pollen loss from pollen collectors.

Please see our response to each comment from the AE and referees below. We are submitting a tracking-changes version to show where we modified the manuscript, and a clean text file of the revision. The clean file is easier for the editor and reviewers to read, as we changed a lot on the early version.

We greatly appreciated colleagues to review the revision in this particularly difficult time as the field work of every evolutionary biologist

is interrupted!

Stay safe and keep healthy!

Sincerely yours,

Shuang-Quan Huang
on behalf of coauthors (three postgraduate students).

Proceedings B - Decision on Manuscript ID RSPB-2020-0548
From: "Proceedings B" onbehalfof@manuscriptcentral.com
To: sqhuang@whu.edu.cn

15-Apr-2020

Dear Dr Huang:

I am writing to inform you that your manuscript RSPB-2020-0548 entitled "The evolution of pollen size in animal- pollinated plants is largely driven by pollinator foraging behaviour" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of referees, who have recommended that substantial revisions are necessary. With this in mind we would be happy to consider a resubmission, provided the comments of the referees are fully addressed. However please note that this is not a provisional acceptance.

The resubmission will be treated as a new manuscript. However, we will approach the same reviewers if they are available and it is deemed appropriate to do so by the Editor. Please note that resubmissions must be submitted within six months of the date of this email. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office. Manuscripts submitted after this date will be automatically rejected.

Please find below the comments made by the referees, not including confidential reports to the Editor, which I hope you will find useful. If you do choose to resubmit your manuscript, please upload the following:

1) A ‘response to referees’ document including details of how you have responded to the comments, and the adjustments you have made.
2) A clean copy of the manuscript and one with 'tracked changes' indicating your 'response to referees' comments document.
3) Line numbers in your main document.

To upload a resubmitted manuscript, log into http://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Resubmission." Please be sure to indicate in your cover letter that it is a resubmission, and supply the previous reference number.

Sincerely,

Dr Daniel Costa
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
Pollen grain size varies astonishingly between plant species and while several hypotheses have been suggested, few have been well-tested. This manuscript tests two of these hypotheses: 1) pollen grain size is driven by post-pollination interactions with style length and 2) pollen grain size is driven by pollen feeding insects. The manuscript finds correlative evidence that pollen grain size and style lengths are associated and also that large pollen grains are associated with animal foragers that do not collect pollen. In the end, the authors utilize model selection to reject the hypothesis that pollen size is driven by post-pollination stylar interactions. I found this to be an extremely interesting manuscript and I really enjoyed reading it. The manuscript was reviewed by two excellent reviewers who were somewhat conflicting in their comments. While both were quite positive about the manuscript, one of the reviewers picked out several potential statistical issues and suggested that we send the manuscript to a specialist statistician. This reviewer also questions the community study part of the manuscript is used to generalize for all angiosperms.

While I found this study interesting, it does require some work and justification to get it up to Proc Roy Soc standard. Below I outline some of the things that the authors need to concentrate on to increase the chances of acceptance. The points reiterate some of the main points made by reviewers as well as some of my own.

1) I agree with Reviewer 1 that when trying to explain angiosperm-wide phenomena it would be useful to use data from across the angiosperm phylogeny and not just a simple community analysis. Since the authors appear to have these data at their disposal, I think that such an analysis would go a long way to nullifying these concerns.
Response: Yes, it took us nearly one year to extract data of pollen-related traits, major pollinators and foraging behaviours from over 1000 literature surveys. As we now have relevant data of pollen number and feeding pollen behaviours, particularly in the 64 bee-pollinated plant species, we prefer omitting this part of large-scale analyses across angiosperm families in the revision and re-running the analysis later. The foraging

pollen behaviours of bees were rarely stated clearly in the literature, usually supposed to be pollen collectors. We have planned to collect data of pollinator foraging behaviours from hundreds of plant species in 200 angiosperm families from the field study in our ongoing project. Hope we could accomplish the field survey in two years.

2) Reviewer 1 was not convinced that post-pollination stylar interactions could be excluded as a driver of pollen size from the model selection process employed by these authors. I was also a little sceptical that so much faith was placed in a process of model selection to support one hypothesis over another. The authors really need to make sure that this approach is sound, or interpret their results in a more egalitarian manner.

Response: We agree. As we input another pollen-related trait (pollen number), the capability of model selection to filter the main factor becomes even weaker. In the revision, we give up the analysis of model selection but instead, use the partial correlation analysis to remove the confounding effect of flower size on style length, pollen size and number.

3) In this respect, I was also a little concerned about the general tone of the manuscript. The general tone is that pollen grain size has been driven by pollinator foraging behaviour (see title). However, in reality, all the authors have done is show some correlations and collect field data to show that pollen foraging takes place mostly on plants that have small pollen grains. While this is all very interesting it is far from being definitive evidence that pollen foraging has actually driven the variation in pollen grain size. Even the field data collected does not show that pollen foraging has driven the evolution of small pollen – pollen consuming insects may simply prefer to collect pollen from flowers with small pollen grains – perhaps it packs better in corbiculae. Here I advocate a more cautious approach to the interpretation of these data.

Response: We revised the title to down tone of the pollinator foraging behaviour as one factor.

4) I suspect that grain size is also negatively correlated on with the number of grains produced. This makes me question whether selection is directly on grain size or whether selection is actually on grain number. At the very least, this needs to be addressed in the manuscript. Do the authors have data on the number of pollen grains produced and how this correlates with foraging insects?

Response: Thanks for sharing critical view of pollen size related to number! Fortunately, we had data of pollen number in most of studied species. We now consider trait correlations and possible factors affecting both pollen size and number in the revision.

5) I noticed that the regression in Fig 3B may be driven by a single outlying data point. I suggest the authors re-run this correlation after removing the data point to see if it still

holds. Obviously, if the regression does not stand up to the removal of a single data point, it would lend support to the line taken by this manuscript (rejecting the style length hypothesis).

Response: Following above suggestions, we re-run the pollen-related trait correlation under both simple correlation and the phylogenetic independent contrast (PIC) model, see new Table 1. The results reveal that pollen size, style length and pollen number were strongly correlated with flower size. However, the partial correlation analysis to remove the confounding effect of flower size indicated that the positive correlations between pollen size and style length, pollen number and style length disappeared, but a negative relation between pollen size and number appeared.

6) Reviewer 1 suggested some restructuring which I support. While the introduction is presently very short (around 600 words), I do not think it requires lengthening for the sake of lengthening. At the moment, the manuscript is short and punchy and fits nicely with the journal style. But the point is that there is plenty of word-space left to explain things better where necessary.

Response: To keep the manuscript short as the journal style and follow above suggestions, we expand the Discussion section.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)

Hao et al investigate variation in pollen grain size among flowering plants. They introduce the two main hypotheses that have been put forward to explain this variation: one related to pre-pollination pollinator behaviour, and another one related to post-pollination pollen-stigma/style interactions. Using a dataset derived from a community of flowering plants in China, for which they quantified pollen grain size, style length and overall flower size, and for which they characterized pollinator type and behaviour, as well as phylogenetic relationships, they perform a range of tests to evaluate support for either of the two hypotheses.

They find differences in pollen grain size related to pollinator type and behaviour, they find support for correlation between grain size and style dimensions, but they ultimately argue that a model-based approach identified pollinator foraging behaviour as the most important determinant of pollen grain size in their community. They further present pollen grain size data for over 1000 angiosperm species and plot these on a family level phylogenetic tree.

The topic is worthy of investigation, as no conclusive assessment appears to have been done (although there is no reason why both hypotheses may be an appropriate explanation). I have a range of minor comments, listed below and in a marked-up file which I have attached.

However, my main issue with the study by Hao et al is that it tries to explain an angiosperm wide phenomenon (as they show nicely with their angiosperm-wide data set), but in terms of their statistical analyses it relies exclusively on a data set collected in a single community of 80 plant species. Although the data may have been collected with great accuracy, it remains unclear to what extent the results can be generalized. Specific local habitat conditions (temperate alpine network, with a specific pollen-collecting fauna), the small sample size of plant species in relation to all angiosperms, and the specific community context (pollen collecting behaviour may be plastic, dependent on whatever other species co-flower, and may therefore vary across communities), may all decrease the possibility to generalize from the results presented. Indeed, the study by Harder (1998) clearly showed for instance that pollen grain size variation is inconsistent across lineages; similarly, these patterns may depend on the factors mentioned above and not only on pollinator behavior. It would be helpful if the relationships presented here can be shown to be more widely applicable, and it appears that the authors already have some data (their angiosperm-wide dataset presented in Table S3) to perform a meta-analysis in a similar way to which they analysed their own community data.

Response: Thanks for pointing out the plasticity of pollen collecting behaviours! To minimize the effect of pollinator species and their foraging behaviours, we collected data of the 80 flowering species of 25 angiosperm families from the field station of our university where pollen grains of these co-flowering species are interviewed by the same pollinator assemblages (local insect fauna). For example, around 4-6 bumblebee species serve as major pollinators for over 50 flowering plant species in our samplings, but bumblebees consistently rejected pollen collection from plant species of several families. Different from previous studies where bees were usually proposed to be pollen collectors, our field observations showed that bumblebees actually chose to collect pollen in species whose grains were small in general but to reject large pollen grains in other species. The variation in pollen size could be responsive to selection of pollen feeding behaviour.

Our preliminary results showed that large pollen grains had thicker pollen wall, perhaps due to relatively poor nutrient (less protein) in large grains rejecting bee collection (unpublished data).

Minor comments:

-The authors perform a large number of tests, and refer to several hypotheses, but I found the link between the hypotheses, methods, and results not clear. I suggest to include a paragraph at the end of the introduction in which a number of hypotheses with associated predictions is put forward, and use this framework to structure the materials

and methods, and results section. I found the phrasing of some of the hypotheses a bit unclear, I think these should be unambiguous and result in testable predictions.
Response: The Introduction section was revised following above and the AE and Reviewer 2's suggestions.

-I found the purpose of the two angiosperm-wide datasets (Tables S1 and S3) unclear. They are used in a descriptive way, but it seems to me that more can be done with them. It was also unclear how the raw data from Table S1 were summarized into Table S3.

-The language is generally understandable, but is not up to scratch for publication.

-I found the discussion somewhat disjointed; given that the two hypotheses have received ample attention in the literature, these need not be discussed in detail. I rather expected that the discussion would mostly deal with differences in findings between the current study and similar previous studies, and that the discussion should formulate an argument why the results drawn from the community study could apply angiosperm-wide.

-Based on my own experience with using ITS sequences at the genus and tribal level, I do not think it is feasible to accurately align this across angiosperms. I therefore argue against its use. It might be useful to consult the literature for published trees that can be used for meta-analyses, e.g. consider Janssens et al. Biodiversity Data Journal 8: e39677

-For the phylogenetic analysis, rather than using a substitution model partitioned among genes, it may be more useful to partition among codon positions, as third codon positions are likely to evolve according to a different substitution model than first and second codon positions. It was also not clear whether convergence in model parameter estimation was reached?

-The purpose and methods underlying the phylogenetic mapping are not clear. What algorithm was used to reconstruct ancestral character states, and what were these used for?
Response: The above comments on phylogenetic methods disappeared as the large-scale meta-analysis was omitted in the revision.

-What was the purpose of calculating a frequency distribution of pollen grains? This is not clearly articulated.
Response: Omitted.

-In the phylogenetic analysis, the use of congeners in case a sequence for the study species was unavailable on genbank is fine, as long as this is done for a single representative. This approach becomes more problematic if 2 or more genus members for which genbank accessions are lacking are being included in the analyses.
Response: This problem disappeared in the analysis of the 80 flowering

species.

-Although I am not an expert in statistical methods involving model selection, my understanding is that this approach should not be used to exhaustively assess support for any possible model, but that this approach needs to be based on careful a priori selection of several competing models, in the context of some theoretical debate. Given the importance of the results derived from this analysis, a better justification for the selected approach is desirable.
Response: The model selection was omitted in the revision.

-In the results it is clarified that the 80 micron threshold is arbitrary. I wonder what the use is of this threshold in that case. What is the basis for the 80 micrometer division? This is not justified anywhere in the introduction.
Response: The part of an arbitrary categorization the 80 micron in pollen diameter as large pollen was omitted, as the large-scale analysis omitted.

Referee: 2

Comments to the Author(s)
Manuscript describes an interesting study that will be of interest to a broad audience. I think the relevant literature is cited, and the tables and figures are clear. The writing is good (I've made some minor editorial suggestions on the PDF). This topic hasn't received much attention in the existing literature, and this analysis is a good contribution.

In supplementary files, change
Table S1. Data of mean pollen grain diameter in 1271 species from 208 angiosperm families (APG IV), which species with aggregated pollen (dyads, tetrads, polyads or pollinia) are marked in yellow.
to
….families (APG IV). Species with ….
Response: Thanks for kindly improving the writings! Those suggestions on the PDF file are accepted and correspondingly revised in the revision.

In Acknowledgements section, we would like to take this opportunity to thank the associate editor and two anonymous reviewers for helpful suggestions to improve the study.

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Journal Name: Proceedings of the Royal Society B

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Print ISSN: 0962-8452
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MS Reference Number: RSPB-2020-0548
Article Status: REJECTED
MS Dryad ID: RSPB-2020-0548
MS Title: The evolution of pollen size in animal- pollinated plants is largely driven by pollinator foraging behaviour
MS Authors: Hao, Kai; Tian, Zhi-Xi; Wang, Zi-Chen; Huang, Shuang-Quan
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Contact Author ZIP/Postal Code: 430079
Keywords: pollen size evolution, style length, pollinator type, grooming behaviour, size-number tradeoff, phylogeny
Abstract: In flowering plants pollen grain diameter varies greatly from a few to over 100 microns, but the selective forces driving the evolution of pollen size remain unclear. Our phylogeny of 1213 species from 199 angiosperm families indicates multiple origins of large pollen (> 80 μm) in diverse lineages, and large pollen grains are associated with animal foragers that do not collect pollen. The hypothetical role of pollinator behaviour was further confirmed by field studies of natural communities, showing that pollinators positively collected pollen in those species with relatively smaller pollen but rarely did so in species with larger pollen. After excluding the confounding effects of pollinator type and flower size or style length, we suggest that the evolution of pollen grain size in zoophilous lineages could be largely driven by pollen foraging habit. Many plant species whose pollen is collected or consumed by pollinators produce small pollen grains.
EndDryadContent

Appendix B

Dear Prof. Daniel Costa,
Editor, Proceeding B

We are resubmitting a revision of MS ID RSPB-2020-1191 on the evolution of pollen grain size.

We sincerely thank the two experts who reviewed our early version (ID RSPB-2020-0548) and kindly provided the second round views! As I understand, their comments are helpful suggestions to teach us how to clearly set up the testing hypotheses and to improve the scientific writings.

Major concerns from Reviewer 1 are (1) the Introduction is poorly written and (2) need a cautious approach to explain the data associations of pollen-related traits. Following his/her suggestions, we now (1) clearly state the three hypotheses for evolution of pollen size in the Introduction with a short-term for each hypothesis. Meanwhile, we add a Table S1 to present the three hypotheses and evidence as suggested. (2) Different from previous comparative studies of interspecific variation in pollen size, we examined pollinator foraging behaviours across 80 co-flowering species from a field station. The empirical study permits us to examine pollen feeding behaviours of the same pollinator species (major bumblebee) on different flowering species, unveiling a pattern that bumblebees did not collect large pollen grains from some species but small ones in the others. We agree, further studies are needed to clarify whether pollen grain size is directly driven by pollinator foraging habit or indirectly mediated by pollen number trade-offs, as Reviewer 1 pointed out. We now state this unsolved problem at the end of Abstract and add a paragraph to discuss it at the end of Discussion section.

The re-wordings kindly provided by the both reviewers are accepted and have been correspondingly revised in the revision.

Please see our response to each comment from reviewers below. We are submitting a clean text file of the revision. Please see below a tracking-changes version to show where we modified the manuscript behind the responses to the comments.

We greatly appreciated colleagues to review the 2nd revision in this particularly difficult time as the field work of every evolutionary biologist is interrupted!

Hope the third version acceptable!

Stay safe and keep healthy!

Sincerely,

Shuang-Quan Huang
on behalf of coauthors (three postgraduate students).

Proceedings B - Decision on Manuscript ID RSPB-2020-1191
From : "Proceedings B" <onbehalfof@manuscriptcentral.com>
To: sqhuang@whu.edu.cn, sqhuang2001@hotmail.com
Cc: journal-submit@datadryad.org

14-Jul-2020

Dear Dr Huang:

Your manuscript has now been peer reviewed and the reviews have been assessed by an Associate Editor. The reviewers' comments (not including confidential comments to the Editor) and the comments from the Associate Editor are included at the end of this email for your reference. As you will see, the reviewers and the Editors have raised some concerns with your manuscript and we would like to invite you to revise your manuscript to address them.

We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Associate Editor, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available we may invite new reviewers. Please note that we cannot guarantee eventual acceptance of your manuscript at this stage.

To submit your revision please log into http://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions", click on "Create a Revision". Your manuscript number has been appended to denote a revision.

When submitting your revision please upload a file under "Response to Referees" in the "File Upload" section. This should document, point by point, how you have responded to the reviewers' and Editors' comments, and the adjustments you have made to the manuscript. We require a copy of the manuscript with revisions made since the previous version marked as 'tracked changes' to be included in the 'response to referees' document.

Your main manuscript should be submitted as a text file (doc, txt, rtf or tex), not a PDF. Your figures should be submitted as separate files and not included within the main manuscript file.

When revising your manuscript you should also ensure that it adheres to our editorial policies (https://royalsociety.org/journals/ethics-policies/). You should pay particular attention to the following:

Research ethics:
If your study contains research on humans please ensure that you detail in the methods section whether you obtained ethical approval from your local research ethics committee and gained informed consent to participate from each of the participants.

Use of animals and field studies:
If your study uses animals please include details in the methods section of any approval and licences given to carry out the study and include full details of how animal welfare standards were ensured. Field studies should be conducted in accordance with local legislation; please include details of the appropriate permission and licences that you obtained to carry out the field work.

Data accessibility and data citation:
It is a condition of publication that you make available the data and research materials supporting the results in the article (https://royalsociety.org/journals/authors/author-guidelines/#data). Datasets should be deposited in an appropriate publicly available repository and details of the associated accession number, link or DOI to the datasets must be included in the Data Accessibility section of the article (https://royalsociety.org/journals/ethics-policies/data-sharing-mining/). Reference(s) to datasets should also be included in the reference list of the article with DOIs (where available).

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should also be fully cited and listed in the references.

If you wish to submit your data to Dryad (http://datadryad.org/) and have not already done so you can submit your data via this link http://datadryad.org/submit?journalID=RSPB&manu=(Document not available), which will take you to your unique entry in the Dryad repository.

If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link.

For more information please see our open data policy http://royalsocietypublishing.org/data-sharing.

Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI. Please try to submit all supplementary material as a single file.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rspb.[paper ID in form xxxx.xxxx e.g. 10.1098/rspb.2016.0049].

Please submit a copy of your revised paper within three weeks. If we do not hear from you within this time your manuscript will be rejected. If you are unable to meet this deadline please let us know as soon as possible, as we may be able to grant a short extension.

Thank you for submitting your manuscript to Proceedings B; we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Best wishes,

Dr Daniel Costa
mailto: proceedingsb@royalsociety.org

Associate Editor Board Member
Comments to Author:

Using phylogenetically controlled analyses, this manuscript examines associations with pollen grain size across 80 angiosperm species to try and distinguish between a host of hypotheses which attempt to explain the enormous variation found in pollen grain size. From the outset, I have thought that this manuscript holds great potential and in the first round of review, the authors received some good feedback from 2 reviewers as well as myself. Despite a very positive review from one of the first reviewers, I am not particularly happy with the way the authors have handled some of my own comments (and the comments of the other reviewer) on the first submission. While I still think the manuscript has plenty of potential, I do not think that the manuscript, presently stands is up to Proc Roy Soc standards. Below I outline my major concerns and also some general editing.

Major concerns
1. The introduction is still poorly written despite concerns by one of the first reviewers. One of the main problems with the introduction is that it is not clear what hypotheses are going to be tested, and what the predictions of each hypothesis are. This needs to be made crystal clear at the end of the introduction and given the complexity of the hypotheses, I suggest adding a table of hypotheses and the kind of evidence which will/could be used in support or for rejection of each. As it is presently written, the authors appear to write about one or two hypotheses in the introduction, but then start

introducing all sorts of others, and various side-stories in the methods. This really does not work. The introduction needs to be properly structured so that new hypotheses and side-stories are not introduced later.

Response: We sincerely appreciate you providing critical views of the study of pollen size evolution and a framework of testing hypotheses. Following above suggestions, we now add a paragraph at end of Introduction section and a table (Table S1) to present the three hypotheses and collected evidence for pollen size. We refer the allometry hypothesis as a hull model testing the relations between flower size, pollen size and style length. The two functional hypotheses include per-pollination (pollinator foraging) and post-pollination (stylar-interaction).

2. In my last round of comments, I suggested several problems with associative studies which call for a cautious approach to the interpretation of the data. While the authors have attempted to discuss some of these problems, they do a poor job of it. For example, the main finding of this manuscript was that small grains are found in association with pollinators that harvest pollen. The authors conclude that pollinators have selected for small grain size. At my request, the authors analysed and found a strong negative correlation between pollen size and number. This makes it difficult to determine whether pollen foragers are selecting directly on pollen size or whether they are actually selecting on number of grains with size being dragged along pleiotropically or as a trade-off. They attempt to discuss this but what they write is not particularly clear and I do not know what to conclude. The authors probably need to explicitly say in the discussion that it is not clear whether pollen grain size is directly associated with pollinator foraging or indirectly mediated by pollen number trade-offs (i.e. selection is actually on pollen grain number).

Response: We greatly appreciate the critical view of selection of pollen number which may indirectly affect evolution of pollen size. Our PIC analyses are consistent with the prediction of the pollinator-foraging hypothesis (Table 2), species whose pollen grains are exploited by pollen collectors need produce more grains, leaving for pollination. Only those pollen grains are not consumed could be large in a small amount. Our analysis shows that pollinator foraging behaviours affected pollen size but not pollen number in the bee-pollinated species, see Lines 252-255.

At the moment, we do not know why bumblebees did not positively collect large-sized grains. These large pollen grains are placed on the bumblebees' body but are not gathered into their pollen basket. We saw bees cleaning off and discarding large-sized pollen from their body.

As suggested, we now add one paragraph at the end of Discussion section. See Lines 331-341.

The other problem is that it is not clear whether the association between pollen grain size and pollinator foraging mode is a direct, evolutionary response of plants reducing grain size because of pollinator foraging. OR an ecological association – bees may actually prefer to forage on plants with smaller grains. Again, I think the authors attempt to discuss this but the conclusion of the discussion is not clear. I think that the discussion simply needs a cautionary paragraph about interpreting cause and effect from associative studies. In this para these alternative explanations for the patterns can be entertained.

As suggested, we now add one paragraph at the end of Discussion section. See Lines 331-341.

MINOR COMMENTS

Title: does not read smoothly, partly because of the double "by." Consider: Small pollen gain size associated with pollen-foraging by pollinators. OR. Pollen grain size associated with pollinator feeding strategy

Response: Revised the title as suggested: **Pollen grain size associated with pollinator feeding strategy.**

L 14: replace In flowering plants with Angiosperm

Response: Revised as suggested.

L16+17: Strictly speaking, this paper is a far cry from demonstrating pollinator mediated selection on grain size. You are over-selling your paper

Response: Revised as suggested.

L25 + 26: Last sentences of the abstract should be "discussion type sentences" about the broad significance of the results. But this sentence reads like a result.

Response: Revised as suggested.

L32: this is a bit of a straw man. Biologists have recognized for decades that pollination is complicated and often does not follow the idea of a classic mutualism. Others would argue that as a general rule, mutualisms are complicated and reciprocally exploitative. This starting sentence does not work. Delete this first sentence (and the next) and start with: Pollination mutualisms are often complicated by the fact that the agents of pollen dispersal are usually attracted to flowers by the prospect of nourishment. Floral visitors and plants consequently have conflicting agendas, especially when the nourishment sought by the floral visitors is pollen.

Response: Revised as suggested.

L41: It remains unclear how plants mitigate pollen loss to bee visitors, although in a few species this has been resolved by the evolution of heteranthery, the partitioning in….

Response: Revised as suggested.

L45, 46: mixed measurements (volume and diameter). Stick to one. I suggest diameter.

Response: We just want to remind readers of the magnitude differences between pollen diameter and volume.

L49: Hypotheses have to explain pollen grain size variation can be broadly categorized pre- and post-pollination selection.
Response: Revised as suggested.

L51: Numerous observations support the post-pollination hypothesis, for example, stigma/style length is often positively associated with pollen grain size Cruden & Miller-Ward 1981; Baker & Baker 1982; Plitmann & Levin 1983; Harder 1998; Roulston et al. 2000; Cruden 2009).
Response: Revised as suggested.

L55: Here, larger pollen grains may outperform smaller grains on stigmas because of faster germination or tube growth, resulting in a higher siring success (Cruzan 1990; Williams & Rouse 1990; Endress 1994; Manicacci & Barrett, 1995; McCallum & Chang 2016). I have a problem with this sentence as it does not directly address the previous sentence. The previous sentence is about positive style and grain size associations. But this sentence only addresses why big grains are better than small ones, not why a correlation exists.
Response: Revised.

L58. However correlations between pollen grain size and style length may simply be the result of intrinsic scaling relationships and have nothing to do with variation in fertilization success of different sized grains (Lee 1978; Sarkissian & Harder 2001; Wang et al. 2016).
Response: Revised as suggested.

L60: This sentence does not follow from the last. It feels like a different topic. Try to make the sentences flow and follow better.
Response: Revised.

L63: This sentence also does not follow from the last. It is unclear how the two are connected.
L63 onwards: I have been frustrated with trying to correct this sentence by sentence and have rewritten the remainder of the intro (please see that L63 follows from the previous sentence)

Early workers proposed that foraging economics would select on pollen grain size. In particular, it was suggested that bees would prefer small (lipid-rich, starchless) pollen grains, over large (starchy) grains because larger grains were envisioned as having relatively lower nutritional value (Baker & Baker 1979). However, this pre-pollination hypothesis has not been supported by subsequent studies considering phylogenetic relatedness and pistil characteristics (Harder 1998), or analysis of nutrition components (Roulston et al. 2000). Instead, Harder (1998) proposed another pre-pollination hypothesis, that the comb-like structures on bee limbs would groom large pollen grains

more efficiently than smaller ones. Consequently one may expect that bee pollinated plants may evolve smaller pollen grains to escape grooming. However, Harder (1998) found no evidence for associations between pollen grain size and the effectiveness of grooming. Although this idea appears to have been been abandoned, it was never fully investigated using modern phylogenetic methods coupled with direct examinations of pollinator grooming behaviour and variation in pollen grain size. In this manuscript, we re-visit pre and post pollination hypotheses explaining pollen size variation. We ask whether inter-specific pollen grain size variation is the result of post pollination stigma/stylar interactions or whether variation is associated with pollinator foraging behaviour.
Response: Revised above paragraph as suggested. Great thanks for help of writing!

L107-114: The intro outlines 2 hypotheses for pollen grain variation (stylar interactions and pollinator foraging). But it is unclear what you are trying to propose now and which of the 2 hypotheses you are addressing here. I thought that the main idea was that pollen collectors would more efficiently harvest large pollen, not reject them. And what is this concept of pollen exposure which is now being introduced in the methods?
Response: Revised. As we observed, bees rejected collecting large-sized pollen, perhaps these pollen grains containing more toxic components or poor in protein. As exposed pollen without physical protection from collection, these large-sized grains may have evolved chemical defense against collections.

L133: I am getting a little confused. I thought the two hypotheses were stigma/stylar correlations versus pollinator foraging associations with grain size. In this sentence it sounds like your 2 hypotheses are pollen size evolution and trait correlations which makes no sense to me. You need to ne explicit in the intro about what hypotheses are going to be tested and then use the same terminology throughout when you are referring to the two different hypotheses.
Response: Now, the three hypotheses are stated in the Introduction and Table S1.

L138: The sequences were assembled using Geneious version 11.0 (Biomatters, Auckland, New Zealand), they were aligned using Mafft version 7.3.0 (Katoh & Toh 2010), and were edited using BioEdit version 7.2.5 (Hall 1999).
Revised as suggested.

L151: This is the first time your hypothesis is explicitly stated. This needs to be done properly in the intro and does not need to be fully repeated in the methods. I also found the hypothesis description a little cumbersome, try the following: We propose that pollen grain size should be associated with pollinator behaviour. More specifically, large pollen grains should be associated with species where pollen grains are seldom harvested by pollinators while small pollen grains should be associated with species which are heavily exploited by pollen-collecting foragers. Once you have outlined this hypothesis fully, you can give it a name (e.g. the pollinator foraging hypothesis) and you can refer to it as such from then onwards

Response: Revised as suggested. Now, the three hypotheses are stated in the Introduction and Table S1.

On a side note, how do we know that grain size is actually a selective response to pollinator foraging behaviour. Instead, you may find associations between small grains and pollen foraging because pollen foragers actually prefer foraging small grains.
Response: Revised “affected by” into “associated with” as suggested.

L156: My previous comment underlines the problem with using words like “affected”. This is an associative study, you cannot invoke cause and effect from these associations.
Response: Revised “affected by” into “associated with” as suggested.

L161: So now it looks like you may be getting into testing the idea about stylar interactions and grain size, but also ideas about allometry. Again, I think the intro needs to be more explicit in stating which hypotheses you are testing. State the hypotheses clearly in the intro and give them each a shortened name (e.g. the stylar-interaction hypothesis). While I initially thought you were only testing 2 hypotheses, it seems like you are also testing the idea about allometry and a bunch of others (none of which are particularly clear. It may really help if in the intro, you provide a table of ALL the competing hypotheses that you will test in this paper and then also show which kinds of evidence will reject the hypothesis or support it. Something like what I have done below.

	Evidence		
HYPOTHESIS	Correlation between grain size and flower size	Association between pollen foraging and grain size	Correlation between style length and grain size
Allometry	Support	reject	neither reject nor support
Pollinator foraging	reject	support	reject
Stylar interactions	neither reject nor support	reject	support

Response: Revised as suggested. Now, the three hypotheses are stated in the Introduction and Table S1.

L215: However, pollen grains were significantly larger in species…
Response: Revised as suggested.

L236: Bee-pollinated species had significantly more (Wald $\chi 2 = 11.003$, $P = 0.001$) pollen grains if bees collected their pollen (102754 ± 29441, n = 26) than for species where bees did not collect their pollen (27013 ± 8137, n = 26).
Response: Revised as suggested.

L247: I can’t see that this is a test for whether bees reject large vs small pollen grains. You never did tests on bees preferences or abilities. Again, you are using associations to say something about cause and effect. This is very dangerous territory which needs to be toned down.

Response: Revised as “**confirming that large pollen was usually not exploited by bees”**.

L256-264: This detailed background into the history of pollen size studies belongs in the intro, not in the first paragraph of the discussion.
Response: Omitted.

L284: little-studied
Response: Revised.

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s).
This revision addresses the concerns I had with an earlier version. I have made some minor editorial suggestions/corrections on the PDF, but they should be easy to change. I have no other significant concerns with the manuscript. I think it will be a good contribution to the literature about pollen size.
David Inouye
Response: Thanks again for editing and correcting our writings! All suggested rewordings are accepted. Only for one word “bumblebee”, we note that British journal usually use bumblebee as one word and American Journal as two words “bumble bee”.

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Keywords: pollen size evolution, style length, pollinator type, grooming behaviour, size-number tradeoff, phylogeny

Abstract: In flowering plants pollen grain diameter varies greatly from a few microns to over 100, but the selective forces driving the evolution of pollen size remain unclear. Although both pre- and post-pollination hypotheses have been proposed, empirical evidence for pollinator-mediated selection is absent. Here we propose that relaxed selection by non-pollen-foraging visitors would allow plants to evolve large pollen grains. The hypothetical role of pollinator behaviour was confirmed by field studies of 80 flowering species in natural communities, showing that pollinators positively collected pollen in those species with relatively smaller pollen grains but rarely did so in species with larger ones. Allowing for the confounding effects of pollinator type, flower size or style length and pollen grain number, we found a significant effect of pollen foraging behaviour on variation in pollen grain size, particularly in bee-pollinated plants. Many plant species whose pollen is collected or consumed by pollinators produce small pollen grains.

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Pollen grain size associated with pollinator feeding strategy~~Are large pollen grains favoured by relaxed selection by pollen-feeding flower visitors?~~

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Short title: ~~How big should~~Evolution of pollen grain~~s be?~~ size

Study area: Ecology and Evolution of flowers, Plant-pollinator interactions

The manuscript has two figures and two tables, supplementary materials including four figures, and three~~wo~~ tables. Table S1 presents three testing hypotheses and collected evidence, and Table S2 and S3 present~~ing~~ collected data.

Abstract

Angiosperm~~In flowering plants~~ pollen grain diameter varies greatly from a few microns to over 100, but the selective forces driving the ~~evolution of~~inter-specific variation in pollen size remain unclear. Although both pre- and post-pollination hypotheses have been proposed, empirical evidence ~~for pollinator-mediated selection i~~remains ~~absent~~scarce. Here we propose that relaxed selection by non-pollen-foraging visitors would allow plants to evolve large pollen grains. The hypothetical role of pollinator behaviour was confirmed by field studies of 80 flowering species in natural communities, showing that pollinators positively collected pollen in those species with relatively smaller pollen grains but rarely did so in species with larger ones. Allowing for the confounding effects of pollinator type, flower size or style length and pollen grain number, we found a significant effect of pollen foraging behaviour on variation in pollen grain size, particularly in bee-pollinated plants. While these results ~~our analyses of pollen-related trait correlations~~ suggest that ~~M~~many plant species whose pollen is collected or consumed by pollinators produce small pollen grains, it remains unclear whether pollen grain size is directly affected by pollinator foraging habit or indirectly mediated by pollen number trade-offs.

Key words: pollen grain size evolution, style length, pollinator type, grooming behaviour, size-number tradeoff, phylogeny

Introduction

Pollination mutualisms are often complicated by the fact that the agents of pollen dispersal are usually attracted to flowers by the prospect of nourishment. Floral visitors and plants consequently have conflicting agendas, especially when the nourishment sought by the floral visitors is pollen~~Plant-pollinator interactions have long been recognized as classic mutualisms: pollinators transfer pollen that contains the male gametes of flowering plants, while flowers reward pollinators, usually with nectar and/or pollen (Willmer 2011). The interaction becomes complicated in that diverse flower visitors consume pollen, which is therefore lost for plant sexual reproduction. This conflict of interest is particularly common in bee-pollinated flowers~~ (Westerkamp 1997; Parker et al. 2016; Tong & Huang 2018). Bees have evolved various structural and behavioural adaptations to promote pollen collection, as the development of their larvae relies on the pollen ~~is the sole~~ protein ~~source for the development of their larvae~~ (Thorp 2000). Pollen taken from anthers by foragers is not available for fertilization. It remains unclear how plants ~~reduce~~ mitigate ~~this competition for~~ pollen loss to bee visitors (Minnaar et al. 2019), although in a few species this ~~can~~ has been resolved by the evolution of heteranthery, a partitioning in function in anthers between pollinating and feeding (Vallejo-Marín et al. 2010).

Among angiosperm species pollen grain volume ranges over almost five orders of magnitude, the diameter ranging from less than 10 µm (e.g., in forget-me-not, *Myosotis*) to over 100 µm (in cotton or cucumber) (Wodehouse 1935; Muller 1979; Wang et al. 2005; Willmer 2011). Why are pollen grains so large in some species but relatively smaller in most species (ca. 30-50 µm)? ~~Several h~~Hypotheses have ~~proposed~~ to explain ~~the variation in~~ pollen grain size variation can be broadly categorized ~~into two:~~ pre- or post-pollination selection. ~~To date, n~~Numerous observations support the post-pollination hypothesis, for example, stigma depth/style length is often ~~have shown a~~ positive~~ relation between~~ly associated with pollen grain size ~~and pistil characteristics (stigma depth and/or style length), in support of the post-pollination hypothesis~~ (Cruden & Miller-Ward 1981; Baker & Baker 1982; Plitmann & Levin 1983; Harder 1998; Roulston et al. 2000; Cruden 2009~~);~~). Here, larger pollen grains may outperform smaller grains on stigmas in a long race because of faster germination or tube growth, resulting in a higher siring success (Cruzan 1990; Williams & Rouse 1990; Endress 1994; Manicacci & Barrett, 1995; McCallum & Chang 2016). ~~Such~~However, ~~a~~ correlations between pollen grain size and style length~~, however, could~~ may ~~reflect~~ simply be the result of ~~an~~ intrinsic scaling relationships and have nothing to do with variation in fertilization success of ~~with flower~~different sized grains (Lee 1978; Sarkissian & Harder 2001; Wang et al. 2016). The null model of allometric growth of sexual organs with flower size is referred as the allometry hypothesis here (Table S1).~~ Given that there is a size-number tradeoff (Vonhof & Harder 1995; Yang & Guo 2004), the evolution of pollen grain size relates to the numbers of grains per flower, reflecting an allocation strategy for male investment (Geber & Charnov 1986).~~

Early workers proposed that foraging economics would select on pollen grain size. In particular, it was suggested that bees would ~~result in bee~~ prefer~~ence for~~ small (lipid-rich, starchless) pollen grain~~s, given that~~ over large (starchy) grains because larger grains were envisioned as having relatively lower~~are likely to provide less~~ nutritional value (Baker & Baker 1979). However, this pre-pollination hypothesis ~~of pollinator-mediated selection on pollen grain size~~ has not been supported by ~~later~~ subsequent studies considering phylogenetic relatedness and pistil characteristics (Harder 1998), or analysis of nutrition components (Roulston et al. 2000). Instead, Harder (1998) proposed another per-pollination hypothesis, that the comb-like structures on bee limbs would groom large pollen grains ~~should be collected~~ more ~~easily~~ efficiently than small ones~~ as bee grooming involves comb-like structures,~~. ~~so that relatively small grains~~Consequently, one may expect ~~would be favoured~~that~~ in~~ bee-pollinated plants ~~because~~ may evolve smaller pollen grains to ~~their~~ escape from grooming~~ would facilitate pollen transfer between flowers~~. However, Harder (1998) found no evidence for ~~The predicted~~ associations between pollen grain size and ~~strength~~ the effectiveness of grooming~~ behaviour, however, was not supported by the data (Harder 1998)~~. Although the idea appears ~~pre-pollination hypothesis that the evolution of pollen size could be driven by pollinator behaviour~~to h~~ has~~ave been abandoned, ~~there has~~it was ~~not yet been an~~never fully ~~investigation~~ investigated ~~of pollen grain size evolution based on~~using modern phylogenetic methods coupled with direct examinations of ~~the relation between~~ pollinator grooming behaviour and variation in pollen grain size.

Here, we re-visit pre- and post-pollination hypotheses explaining pollen size variation (Table S1). The evolution of pollen grain size may be constrained by the numbers of grains per flower given that there is a size-number tradeoff (Vonhof & Harder 1995; Yang & Guo 2004), reflecting an allocation strategy for male investment (Geber & Charnov 1986). To disentangle confounding effects of the pollen size evolution, we ask whether inter-specific pollen size variation is the result of allometric growth of flower size, or post-pollination stigma/stylar interactions (i.e., the stylar interaction hypothesis) or whether variation is associated with pollinator foraging behaviour (the pollinator foraging hypothesis). We propose that pollen grain size should be associated with pollinator behaviour, although available evidence for the pollinator foraging hypothesis is unviable to date. More specifically, large pollen would be favoured in the species whose pollen is little exploited by pollinators, whereas in species visited by pollen-collecting foragers smaller pollen grains, generally in larger numbers, would be beneficial for reproductive success given that a proportion of the pollen grains could escape collection by pollen consumers and be left for plant sexual reproduction.

Materials and methods

(a) Measurement of pollen grain size and number

We collected pollen grains from open flowers of 80 native species from 25 families in a field station of Central China Normal University, Shangri-La Alpine Botanical Garden (SABG, 27°54′N, 99°38′E, 3300-3350 m above sea level) in Yunnan Province, southwest China. These pollen grains were made into temporary slides with gelatin. To estimate pollen grain size, equatorial and/or polar diameters of 5-20 grains per species were measured under a light microscope based on pollen shape (Fig. S1; Table ~~S1~~S2). As the sampled pollen was basically spherical or ellipsoidal, the value of the long (polar) axis was used as pollen diameter in the comparisons across species. Pollen grain numbers per flower were collected from our previous studies in SABG sampling 10 flowers that were nearly opening (Gong & Huang 2014) or 20 flowers per species (Gao et al. 2015).

(b) Pollinator pollen-foraging behaviour

To examine the pollinator foraging hypothesis ~~that pollen grain size is driven by pollinator pollen-feeding habits~~, we investigated pollinator groups and pollinator foraging pollen behaviour on flowers in natural communities in SABG. Our previous studies over years there indicated that diverse insects acted as effective pollinators including bumblebees, solitary bees, hoverflies, other flies, butterflies, hawkmoths and other moths (see Gong & Huang~~,~~ 2009; Fang & Huang~~,~~ 2012, 2013; Xiong et al. 2019). To identify whether pollinators collect pollen, we spent hundreds of hours on clear days observing pollinator foraging activities on 80 flowering species from 25 families (Table ~~S1~~S2). These species were native, flowering in the wild and open to natural visitors. We observed for at least 20 foraging bouts of each floral visitor or for more than four hours to record whether the insect bodies contacted anthers/pollen and conspecific stigmas during foraging, and whether the visitors consumed or groomed pollen, particularly into the bees' corbiculae or scopae (Figs. S2 & S3). As large pollen grains commonly appear in Cucurbitaceae, Geraniaceae, Malvaceae and Liliaceae whose pollen grains are usually exposed to visitors (Xiong et al. 2019) without physical protection from pollen collectors, ~~we hypothesize that if pollen collectors reject large pollen grains, selection for concealing pollen from collectors will be weak. To test the prediction that large pollen grains are associated with pollen exposure,~~ we examined pollen visibility in these 80 species to test whether large pollen grains are associated with pollen exposure and pollinator feeding behaviour. Pollen in each species was categorized as exposed (anthers and pollen are visible to visitors) or concealed (anthers and pollen are hidden in the corolla tube) (see Xiong et al. 2019).

(c) Measurements of flower size and style length

To test the post-pollination stylar-interaction hypothesis for the interspecific ~~evolution of~~ pollen ~~grain~~ size variation, we examined the relationship between pollen grain diameter and style length. Previous analysis of pollen grain size and number suggested that the size of sexual organs could be related to flower size (Vonhof and Harder 1995, Wang et al. 2016). To test the allometry hypothesis, we measured flower size. To estimate style length, we measured the distance from the corolla base to the top of the pistil with a digital caliper on 3-30 fresh flowers (34 species) or on photos of herbarium specimen from Chinese Virtual Herbarium (http://www.cvh.ac.cn/) (46 species) using Digimizer software (version 4.6.0). Meanwhile, the surface area of the corolla of each of the 80 species was measured to estimate flower size with herbarium specimens using Digimizer software (Salvarzi et al. 2018). For bowl-shaped flowers, we measured the total area of the corolla. For tubular and bilaterally symmetrical flowers, flower size was calculated as the lateral area multiplied by two. If species had special corolla shapes such as the beak-like upper lips in *Pedicularis* species, areas of these parts were then added to the total area (Gong & Huang 2009).

(d) Data analysis

To test the ~~two~~ three hypotheses ~~of~~ for pollen size evolution (Table S1) ~~and trait correlations~~, we built a phylogenetic tree of the 80 species from SABG with one outgroup based on Internal Transcribed Spacer (*nr*ITS) and two chloroplast markers (*mat*K, *rbc*L regions). All gene sequences were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/). GenBank accession numbers are shown in Table S1. The sequences were assembled using Geneious version 11.0 (Biomatters, Auckland, New Zealand), ~~and~~ they were aligned using Mafft version 7.3.0 (Katoh & Toh 2010), ~~then~~ and were edited using BioEdit version 7.2.5 (Hall 1999). Aligned matrices of three DNA regions were combined using SequenceMatrix version 1.8 (Vaidya et al. 2011). Bayesian Inference (BI) methods were used for phylogenetic reconstruction. Partitioned BI analyses were performed using MrBayes version 3.2.6 (Ronquist & Huelsenbeck 2003), with DNA substitution models selected for each gene partition by the Bayesian information criterion (BIC) using jModeltest version 2.0 (Darriba et al. 2012; Guindon and Gascuel 2003). Markov Chain Monte Carlo (MCMC) analyses were run in MrBayes for 10 million generations for each dataset with each run comprising four incrementally heated chains. The first 25% of the trees were discarded as burn-in. The remaining trees were used to generate a majority-rule consensus tree. Both BI analyses and jModeltest were performed at the CIPRES Science Gateway (http://www.phylo.org).

~~We propose that large pollen would be favoured in the species whose pollen is little exploited by pollinators, whereas in species visited by pollen-collecting foragers smaller pollen grains, generally in larger numbers, would be beneficial for reproductive success given that a proportion of the pollen grains could escape collection by pollen consumers and be left for plant sexual reproduction.~~ To see whether variation in pollen size is a~~ffected by~~ssociated with pollen consumption by flower visitors (Fig. S1, S2 & S3), we mapped pollen diameter, pollinator foraging habits (whether or not pollinators consume/collect pollen), pollen visibility (whether or not pollen is concealed or physically protected from consumption) and pollen number on the phylogenetic tree at the Interactive Tree Of Life (https://itol.embl.de/) (Fig. 1).

To examine the association between flower size (visual area of corolla), style length, and pollen number and diameter, we conducted bivariate correlation in SPSS 22.0 (IBM Inc., New York, NY, USA). As pollen-related traits usually correlate with flower size, partial correlation analysis with flower size as the control variable was performed to account for the effect of flower size. The phylogenetically independent contrast (PIC) analyses and calculation of Felsenstein's contrasts correlation (Felsenstein 1985) between flower size, pollen grain size, pollen number and style length were performed in MESQUITE v.2.75 (Maddison & Maddison 2011) with the phenotypic diversity analysis program (PDAP) package (Midford et al. 2005).

To examine the effects of pollinator type, grooming behaviour and pollen visibility on pollen grain size and pollen number, we logarithmically transformed data of pollen grain size and number and then conducted GLM analysis (normal distribution and an identity function) with pollen size or pollen number as the dependent variable, and pollinator type, grooming behaviour and pollen visibility as the fixed factors. Also, we conducted the same analysis using a phylogenetic linear model by maximum likelihood using Pagel's lambda model (Pagel 1999). This analysis was performed with the function phylolm of the package phylolm (Ho & Ane 2014) in R 3.5.0 (R Core Team

2018) separately. The outgroup from the BI tree was pruned before analyses.

To remove the confounding effects of flower size and style length on pollen grain size and number in bee-pollinated species, we further calculated the ratio of pollen diameter to style length, the ratio of pollen diameter to flower size and the ratio of pollen number to flower size (see Stroo 2000). Then we conducted GLM analysis (normal distribution and an identity function) with these ratios as the dependent variable and grooming behaviour as fixed factors. To examine whether exposed species' pollen is less likely to be depleted by pollen collectors than the concealed pollen in bee-pollinated species, GLM analysis (normal distribution and an identity function) was performed with the proportion of species with exposed pollen as dependent variable and pollen grooming behaviour as the fixed factor.

Results

(a) Correlations of pollen-related traits

Pollen-related traits including pollen grain size and number, flower size and style length varied greatly among the 80 species for which pollinator foraging behaviour was observed in natural communities (Figure 1; Table S1). For example, pollen diameter (mean ± SE = 42.0 ± 2.4 μm, n = 80; Fig. S4) varied around 10-fold from the smallest (11.8 μm in *Onosma confertum* (Boraginaceae)) to the largest (106.1 μm in *Herpetospermum pedunculosum* (Cucurbitaceae)). Pollen number per flower (mean ± SE = 62991 ± 14621, n = 64) varied from ~~less~~ fewer than 700 in *Geranium sibiricum* to over 700,000 grains in *O. confertum* (Figure 1). The Pearson's correlation analysis indicated that pollen size was correlated positively with flower size and style length, and negatively with pollen number. These correlations between flower size and pollen-related traits were confirmed based on phylogenetically independent contrasts except for pollen size and number (Table 1). However, the partial correlation analysis with flower size as the control variable indicated that only pollen size and pollen number were correlated (r = -0.653, P < 0.001), while there was no significant correlation between pollen size and style length (P = 0.563), or pollen number and style length (P = 0.218). These results suggest an intra-sexual trade-off between pollen size and number that was strongly correlated with flower size, an intrinsic factor, while interspecific variation in allocation to pollen size and number could be driven by extrinsic factors.

(b) Factors affecting pollen size and number

~~Bumblebees, solitary bees, syrphid flies, other flies, butterflies and moths were abundant pollinators in the 80 flowering species (Table S1).~~ Under a generalized linear model, pollen size or number in species mainly pollinated by bees did not differ significantly from that of species pollinated by other insects (Table 2A). However, ~~P~~pollen grains were significantly larger in species with exposed pollen than in those with concealed pollen (Table 2A), but the relationship between pollen visibility and pollen size disappeared under phylogenetic analysis (Table 2B), perhaps because pollen exposure is a conservative trait within plant families. Under the phylogenetic linear model, effects of either pollinator type or pollen visibility on both pollen grain size and number were not significant, but the presence or absence of pollen-foraging behaviour by pollinators significantly affected both pollen size and number (Fig. 2; Table 2B).

Our field observations of pollinator foraging behaviours showed that bees did not collect pollen in 33 (51.6%) of the 64 bee-pollinated plant species; bees collected nectar but rarely or never positively gathered pollen into their corbiculae (Figs. 1, S2 & S3). Pollen grains of plant species pollinated by pollen collectors were significantly smaller (Fig. 2C) and more numerous (Fig. 2F) than those of species pollinated by insects ~~which~~ that did not positively collect pollen from that species.

To isolate the confounding effect of pollinator type on pollen grain size, we analysed the 64 species whose major pollinators were bees, showing that pollen grain diameter was significantly larger (Wald $\chi^2 = 32.981$, $P < 0.001$) in species ~~on~~ from which bees did not collect pollen (48.8 ± 3.7 µm, n = 33) than in species ~~on~~ from which bees did collect pollen (30.5 ± 1.9 µm, n = 31). Correspondingly, the proportion of species with exposed pollen was significantly higher (Wald $\chi^2 = 12.470$, $P = 0.001$) in plants ~~on~~ from which bees did not collect pollen (66.7 ± 8.3%) than in those ~~on~~ from which they did positively collect it (25.8 ± 8.0%). ~~In b~~Bee-pollinated species~~, the pollen number of species with pollen collection (102754 ± 29441, n = 26) was~~ had significantly ~~larger~~

more (Wald χ2 = 11.003, $P = 0.001$) pollen grains if bees collected their pollen (102754 ± 29441, n = 26) than ~~that of~~for species ~~whose~~ where ~~pollen grains were~~bees did not ~~collected~~ collect their pollen (27013 ± 8137, n = 26). However, pollen number did not significantly differ (Wald χ2 = 1.268, $P = 0.260$) between species with concealed (78716 ± 31421, n = 24) and exposed pollen (53026 ± 12988, n = 28).

A positive relationship between pollen grain size and style length ($r = 0.313$, $P = 0.012$) among the 64 bee-pollinated species was also observed under the PIC analysis. ~~To further remove the confounding effect of flower size, we calculated t~~The ratio of pollen grain diameter to style length in these bee-pollinated species~~. It~~ was significantly higher (Wald χ2 = 4.795, $P = 0.029$) in species in which bees did not positively collect pollen (6.8 ± 1.0) than in those they did collect it (4.1 ± 0.7), confirming that large pollen was usually ~~rejected~~ not exploited by bees. Similarly, the ratio of pollen diameter to flower size was significantly higher (Wald χ2 = 14.546, $P < 0.001$) in species without pollen collection (0.98 ± 0.06) than in species with pollen collected by bees (0.73 ± 0.03). However, the ratio of pollen number to flower size did not differ significantly (Wald χ2 = 0.035, $P = 0.851$) between species with (2.28 ± 0.08) and without (2.26 ± 0.11) pollen collection, indicating that pollen size rather than number was likely to be affected by pollen collection by pollinators.

Discussion

~~An early comparative study of 990 angiosperm species showed that species pollinated by pollen-collecting insects generally had smaller, starchless pollen but species pollinated by Lepidoptera or birds had larger, starchy pollen (Baker & Baker 1979). Comparisons of pollen grain diameter between nectared and nectarless bumblebee-pollinated *Pedicularis* species as well as bee- and hummingbird-pollinated congeners did not reveal variation in pollen size related to pollinator types (Harder 1998). It is clear that flowers pollinated by large pollinators such as Lepidoptera, bats or birds usually have large pollen and a long style (Baker & Baker 1979; Stroo 2000), given that these species have relatively larger flowers.~~ The PIC analysis suggested a positive relation between pollen grain size and style length across species, not inconsistent with the post-pollination hypothesis for the evolution of pollen grain size. These pollen-related trait correlations disappeared in the partial analysis as the control of flower size, however, a trade-off between pollen size and number appeared. To reduce the confounding effects of pollinator type and flower size, our comparison of pollen size/style length ratios in 64 bee-pollinated species showed that pollen size was strongly affected by pollen feeding habits.

In contrast to a basic assumption in previous analyses that bees are generalized pollen collectors, our direct observations in the field showed that bumblebees foraged for nectar only and avoided collecting pollen on 52% of bee-pollinated species (Fig. 2B); pollen grains of those species were lodged on the bee bodies but were rarely groomed into the corbiculae. We observed that the two most abundant bumblebee species consistently rejected pollen from species in Cucurbitaceae, Malvaceae, Geraniaceae and Liliaceae whose pollen grains were relatively large (diameter > 80 μm) and usually exposed to visitors, but they collected pollen from other species whose grains were relatively small (Fig. 1, Figs. S2 & S3; Tong & Huang 2018). Pollen depletion by bees accounts for a high proportion of pollen loss during pollen transfer (Harder & Routley 2006). Plants may have evolved adaptive strategies to avoid this pollen overexploitation by collectors (Hargreaves et al. 2009). To our knowledge, however, physical and chemical defenses protecting pollen from bee collection have been ~~little ~~little-studied. In a few plant groups, for example cotton and pumpkin flowers (Thorp 2000), anecdotal observations showed that honeybees did not groom pollen into the corbiculae; pollen deposited on their bodies was cleaned off and discarded. The pollen of cotton (*Gossypium*) is rejected by bees, perhaps ~~in that~~because spines on pollen grains make pollen packing physically difficult (Lunau et al. 2015), or ~~in that~~because large pollen grains are starch-rich (Baker & Baker 1979) but protein-poor, a type of pollen unfavourable to bumble~~-~~bees (Vaudo et al. 2016).

Bees rely on pollen for a protein source. The evolution of pollen-related traits could be under selection by the conflict of interest between pollen consumers and plants. Unpalatable and toxic floral nectar may filter ineffective pollinators and protect nectar from robbers (Johnson et al. 2006; Barlow et al. 2017). Recent studies have found that pollen usually contained greater quantities of toxic components than nectar (Palmer-Young et al. 2019), while a chemical defense protecting pollen from collection was confirmed in two bumblebee-pollinated *Dipsacus* species with exposed pollen on unconcealed anthers (Wang et al. 2019). Compared to species in which pollen grains were heavily incorporated into the bees' diet, grains that were rejected were observed to be effectively delivered to stigmas, facilitating pollen transfer (Wang et al. 2019).

Comparative analyses showed positive relations between pollen size and style length and tradeoffs between pollen size and number in some but not in other plant lineages (Table S3), but the confounding effect of flower size ~~was~~ has rarely been considered ~~(Table S2)~~. Flowers pollinated by large pollinators such as Lepidoptera, bats or birds usually have large pollen and a long style (Baker & Baker 1979; Stroo 2000), these plants likely to have relatively larger flowers than bee-pollinated plants. The PIC analysis showed that pollen size and style length ~~were~~ are strongly correlated with flower size across the 80 species (Table 1), supporting the allometry hypothesis, but the partial correlation analysis excluding the effect of flower size showed pollen size was only correlated with pollen number. If a number of pollen grains are exploited by pollinators as rewards, a partition in allocation to feeding and pollinating would balance the size of pollen grains, as indicated by pollen size and number tradeoffs. Our survey of pollen grain size and pollinator feeding habits indicated that pollen grains ~~were~~ are significantly smaller in species whose pollen ~~was~~ is collected or consumed. The coefficient of variation (CV) in pollen-concealed species ~~was~~ is smaller than that in pollen-exposed ones (Table 2). While pollen size (and number) ~~was~~ is consistently smaller (and higher) across species in concealed species, exposed species ~~had~~ have either large or small (or few or many) pollen grains. This difference in CV may explain why we found no significant variation in pollen size or number between pollen-exposed and pollen-concealed species (see Fig. 2).

Our analyses removing the confounding effects of pollinator types and flower size showed that the effect of pollen-feeding behaviour on variation in pollen grain size (but not pollen number) remained significant in bee-pollinated species, supporting the pollinator foraging ~~ging~~ hypothesis~~ of pollinator-mediated selection on the evolution of pollen size~~. Pollen grains in species pollinated by non-pollen-collecting Lepidoptera, bats or birds are relatively large, perhaps as a result of the same relaxed selection by pollen loss to consumers. Interspecific variation in pollen number per flower can be affected by intrinsic factors such as flower size, pollen size and nutritional content, and extrinsic factors including pollen vector, pollen collection intensity and visitation frequency (Cruden 2000; Roulston and Cane 2000; Harder and Routley 2006; Muchhala et al. 2010; Song et al. 2019). For example, bat-pollinated flowers usually produce more and larger pollen grains than hummingbird-pollinated species in a cloud forest in Ecuador (Muchhala and Thomson 2010). An increase of pollen production would be favoured if larger amounts were efficiently transferred, resulting in a more linear male fitness gain curve under a scarcity of pollinator visits and non-discarding-pollen behaviour (Muchhala and Thomson 2010; Song et al. 2019), which could explain some species (i.e., Lepidoptera-pollinated Liliaceae species) producing a large number of relatively large pollen grains.

Our study of pollinator foraging behaviours showed that large pollen grains were associated with species where pollen grains were seldom harvested by pollinators while small pollen grains associated with species which were heavily exploited by pollen-collecting foragers. Further studies are needed to clarify whether pollen grain size is directly driven by pollinator foraging habit or indirectly mediated by trade-offs between pollen size and number (i.e. selection is actually on pollen grain number). As predicted, our results indicated that large pollen grains would be favoured where pollen collection is weak or absent. However, it remains unclear why the major pollinators (i.e., bumblebees here) reject collecting large pollen grains. A perspective of pollen-pollen consumer competition could open a new avenue for understanding the evolution of flower-pollinator interactions and male reproductive success in flowering plants.

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Table 1. ~~Hypothesis~~	~~Evidence~~		
	~~Correlation between flower size and grain size~~	~~Association between pollen foraging and grain size~~	~~Correlation between style length and grain size~~
~~Allometry~~	~~Support~~	~~Reject~~	~~Support~~
~~Pollinator foraging~~	~~Neither reject nor support~~	~~Support~~	~~Neither reject nor support~~
~~Stylar interactions~~	~~Neither reject nor support~~	~~Reject~~	~~Support~~

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Table 1. The Pearson's correlation and Felsenstein's contrast (left/right) values (upper right) and P-values (lower left) between flower size, style length, pollen number and pollen grain diameter based on bivariate correlation analysis and the phylogenetically independent contrast (PIC) analysis of the 80 wild species in Shangri-La, southwest China. Significant R values are in bold.

	Flower size	Style length	Pollen number	Pollen size
Flower size		**0.583/0.649**	**0.620/0.609**	**0.267/0.302**
Style length	<0.001/<0.001		0.229/**0.358**	**0.267/0.382**
Pollen number	<0.001/<0.001	0.068/0.004		**-0.384**/-0.082
Pollen size	0.016/0.007	0.017/<0.001	0.002/0.517	

Table 2. Comparison of pollen size and number per flower between different pollinator types, different pollinator foraging behaviour (whether or not visitors positively collect pollen) and pollen visibility under (A) Generalized linear model analysis, with the coefficient of variation (CV) in pollen grain diameter and number, and (B) Phylogenetic linear model analysis. Bold values indicate significant differences at $P < 0.05$.

(A) GLM analysis

Factors		Pollen grain diameter (μm)			Pollen number		
		Wald χ^2	***P***	CV	Wald χ^2	***P***	CV
Pollinator type	Bee pollinators	2.531	0.112	0.49	0.057	0.812	1.78
	Other pollinators			0.60			2.33
Grooming behaviour	Grooming	31.928	**< 0.001**	0.35	10.729	**0.001**	1.46
	No grooming			0.48			2.20
Pollen visibility	Exposed pollen	9.418	**0.002**	0.51	1.625	0.202	1.64
	Concealed pollen			0.39			2.00

(B) PIC analysis

Dependent variable	Factors	Estimate	SE	t	***P***
Pollen size	Pollinator type	7.686	5.921	1.298	0.198
	Grooming behaviour	-19.357	5.092	-3.802	**<0.001**
	Pollen visibility	7.042	5.011	1.405	0.164
Pollen number	Pollinator type	-0.214	0.203	-1.054	0.296
	Grooming behaviour	0.485	0.186	2.601	**0.012**
	Pollen visibility	-0.076	0.178	-0.424	0.673

Fig~~. 1.~~ure legends

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Figure 1. Reconstruction of the phylogeny of 80 flowering species from 25 families which were studied in Shangri-La, southwest China with pollinator types (bee pollinators, other pollinators), pollen grooming/collecting behaviour (positively collecting, not or rarely grooming/collecting), pollen visibility (exposed, concealed pollen) indicated by closed or open symbols respectively, pollen grain diameter, style length and pollen number (related to bar lengths) mapped onto it.

Figure 2. Comparison of pollen grain size (mean ± SE, n = 80 species) and number (mean ± SE, n = 64 species) between pollinator types (**A, D**), pollen visibility (**B, E**) and pollen-foraging behaviour whether pollinators positively collect pollen or not (**C, F**), all estimated by phylogenetic linear model analysis (*, $P < 0.05$; ns, no significant difference). The number of species is shown under each bar.