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Drosophila glue protects from predation

Flora Borne, Stéphane R. Prigent, Mathieu Molet and Virginie Courtier-Orgogozo

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Original submission: 13 January 2021
Revised submission: 19 February 2021
Final acceptance: 22 February 2021

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

Review History

RSPB-2021-0088.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?
Marginal

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

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.
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
Pupal stage is important part of the insects' lifecycle, but we know relatively little about the selective environment during that stage. This study focuses on this 'less known' life-stage and aims to understand the mechanism and function of pupa adhesion in Drosophila species. Authors show that strength of adhesion varies among Drosophila species and pupae attached on the substrate are more likely to survive than non-attached pupae. However, between species variation in the strength of adhesion in D. suzukii and D. simulans does not have a strong effect on the survival benefit against ant predators.

In general, I find this study interesting. If we want to understand how organisms defend against predators and how those adaptations evolve throughout the life-stages, we also need to understand the physical and physiological mechanisms behind them. Furthermore, authors were very open about the potential limitation of their experimental designs (non-natural substrate where individuals attached during the pupal stage) but since these species have different preferences for the substrates you need to make compromises to what to control. Also, methods were reported in detailed enough to repeat the experiment and sample sizes were good. Please find my more detailed comments below.

1) Methods: I appreciate the very detailed reporting of methods. However, perhaps authors could give some estimates of how distributed the buckets in the field predation experiment were. Based on the figure, they were sometimes quite close to each other which means that same predators are likely to visit many of them and they are not necessarily independent samples of each other. For example were they all distributed on the same location or were there more than one location?
2) Statistical analyses and experimental design. Benefit of the pairwise/simultaneous choice designs used here is that it is less sensitive for the spatial and temporal variation in e.g. predator abundances of different species among prey locations. However, the problem in simultaneous choice assays is that predator always makes its choice in a certain context which makes generalisation of the results difficult outside of this context (when predator don't have both attached and non-attached pupae available to choose from simultaneously). For example, I think in this case the pairwise design in a field predation experiment may undermine the difference in survival benefit for attached pupae. As the amount of detached pupae decreases towards the end of the experiment, predators are more likely to find and use energy for preying upon attached pupae. Fortunately, this is very conservative way to test the benefits for pupa adhesion. I was also wondering why authors did not use survival analysis to test survival during the experiment? That would have enabled them to include the location (i.e. bucket ID) of the prey as a random factor into the model. Perhaps this was not possible due to pairwise-set up which in this case could be clearly explained in statistical methods.
3) Results (minor comment): very small p-values could be reported as $p<0.001$, but this of

course depends on the journal's instructions.

4) Overall, authors could have discussed their results in a bit broader context in the discussion which was now quite Drosophila-centered. Authors go through some alternative explanations for the pupa adhesion in the introduction, but this kind of comparison was lacking from the discussion. Also the paragraph in the end of the discussion about other defensive mechanisms of pupae were not very tightly linked with the results of the paper. For example, I was missing some alternative explanations that would make it beneficial to stick in a certain environment for these study species. I agree that predation risk is likely to be very important but how about other natural enemies or abiotic conditions for example?

5) It was also unclear for me what was authors' working hypothesis for the maintenance of variation in adhesion strength among species. I now got the impression that since the backgrounds where the pupa is glued can vary a lot among species, the artificial surfaces used in this experiment may undermine the benefit of 'weaker glue' for some species? Perhaps this could be clarified.

6) Often in studies with insects, the focus is on one life-stage at the time, but the fitness of an insect individual is a sum of all the life-stages it needs to go through until reproduction. Also in this manuscript I was missing more discussion about what is known how this pupa attachment relates to behaviour and ecology during the larval stage and adult stage among studied Drosophila species.

Review form: Reviewer 2

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

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Do you have any ethical concerns with this paper?
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I really enjoyed reading this manuscript, which is the first to demonstrate that the glue that adhere Drosophila pupae to the substrate deters predators. The experiments are simple and clear, and neatly address the question posed by the authors. I only have a few comments that I think might improve the manuscript.

In the laboratory assays for ant predation, there seems to be a discrepancy with regards to the experimental set up. In the methods section, it says there six pupae per slide: attached, loosely attached, and detached. While in the results, you mention these three categories (lines 307-308), differences between the three categories aren't described. I think this is because the "loosely attached" pupae are actually D suzukii, but the way this is described in the methods and results isn't clear.

Minor comments:

- I'm not sure what you mean by aerial environments in the context of Drosophila pupae, especially since they are most likely to pupate on vegetable matter or in the soil.
- Line 148: Consider revising to "15 larvae were PLACED IN EACH dish."
- Line 184: Consider revising to "…wet cotton and LEFT to pupate for…".
- Line 379-381: Consider revising to "Because pupae WERE CONTAINED within the lid of petri dishes, we can infer that pupae were not blown away by LIGHT wind, but we cannot BE SURE that THE PUPAE THAT HAD disappeared were IN FACT predated.".

Decision letter (RSPB-2021-0088.R0)

12-Feb-2021

Dear Dr Courtier-Orgogozo,

Thank you for submitting this really interesting manuscript to Proceedings B - I found it fascinating. The 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. We are all very positive about the general interest value of the manuscript, but as you will see, the reviewers 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:

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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.

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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.

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It is a condition of publication that you make available the data and research materials supporting the results in the article. Please see our Data Sharing Policies (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.

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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,
Professor Loeske Kruuk
mailto: proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
RSPB-2020-088

Borne et al., Drosophila glue protects from predation

Associate Editor, Board Member Recommendation - Comments to Authors:

This study seeks to examine whether and how pupal adhesion / glue protects Drosophila flies from predation, as has been previously hypothesized for insects. Specifically, in addition to maintaining the animal in a place where it might not be detectable by predators, pupal adhesion might also prevent predators from being able to detach the pupa. In their study, the authors test the latter hypothesis by measuring the adhesion of Drosophila species sampled from the same area and observed that pupa adhesion is variable among species – this variability might potentially be explained by different species using different glue production strategies. The authors then compared attached and manually detached pupae and found that attached pupae remain on site 30 % more than detached pupae in the field after three days, potentially indicating reduced predation risk. In support of this notion, the authors found – using laboratory assays – that attached pupae are less efficiently predated by ants. In summary, I feel that this study provides robust evidence that pupal adhesion / glue reduces predation risk in Drosophila flies.

I enjoyed reading this interesting natural history paper. It is clearly and well-written and examines the function of an interesting, evolutionarily conserved, behavioural and physiological phenomenon (pupal attachment via glue) in Drosophila flies that should be of broad interest. The study uses technically simple but elegant and clever field and lab observations and behavioural experiments to examine variability in pupal adhesion 'strategies' among several Drosophila species and the potential role of adhesion in protection against ant predators. The results reported here should be of broad interests to evolutionary biologists, behavioural ecologists, entomologists, and insect physiologists, and so forth. The paper seems to be first to demonstrate that pupal adhesion (1) varies among Drosophila species and (2) can protect flies from predation. To my mind, this study opens up important opportunities for improving our understanding of the potential survival function of pupal adhesion / glue, its ecology, physiology and genetics.

I have now obtained two external reviews of this manuscript. Both reviewers agree, and I concur, that this manuscript is interesting and may be suitable for publication in Proceedings B. Neither of the reviewers nor I have major concerns about the design of the study or the results. Both

referees make a number of valuable suggestions for improvements. For the detailed reviewer comments please see below; here I just briefly summarize a few key points. Reviewer 1 has a few questions regarding the statistical analyses that require clarification, e.g. whether it would not have been possible to use survival analysis to examine survival during the experiment and whether one could not have included location (bucket ID) as a random factor in a linear mixed model. (A very minor quibble from my side is that the authors sometimes write "chi-squared" and sometimes "Chi2" – it would be good to be consistent here and maybe to use the Greek symbol for Chi.) Another point made by Reviewer 1 is that both the introduction and discussion would benefit from some more general context in terms of our understanding of the ecology and evolution of antipredator defenses during the pupal stage and beyond. I agree that this would improve the manuscript. Reviewer 2 also makes a number of helpful suggestions for improvements; this reviewer has also noticed a small discrepancy with respect to the experimental setup of the laboratory assays for ant predation which needs clarification.

Board Member: 2
Comments to Author(s):
RSPB-2020-088

Borne et al., Drosophila glue protects from predation

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This study seeks to examine whether and how pupal adhesion / glue protects Drosophila flies from predation, as has been previously hypothesized for insects. Specifically, in addition to maintaining the animal in a place where it might not be detectable by predators, pupal adhesion might also prevent predators from being able to detach the pupa. In their study, the authors test the latter hypothesis by measuring the adhesion of Drosophila species sampled from the same area and observed that pupa adhesion is variable among species – this variability might potentially be explained by different species using different glue production strategies. The authors then compared attached and manually detached pupae and found that attached pupae remain on site 30 % more than detached pupae in the field after three days, potentially indicating reduced predation risk. In support of this notion, the authors found – using laboratory assays – that attached pupae are less efficiently predated by ants. In summary, this study provides robust evidence that pupal adhesion / glue reduces predation risk in Drosophila flies.

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The subject matter of the paper and the reported findings are potentially well suited for publication in Proceedings B, pending in-depth peer review.

Reviewer(s)' Comments to Author:
Referee: 1
Comments to the Author(s)
Pupal stage is important part of the insects' lifecycle, but we know relatively little about the selective environment during that stage. This study focuses on this 'less known' life-stage and

aims to understand the mechanism and function of pupa adhesion in Drosophila species. Authors show that strength of adhesion varies among Drosophila species and pupae attached on the substrate are more likely to survive than non-attached pupae. However, between species variation in the strength of adhesion in D. suzukii and D. simulans does not have a strong effect on the survival benefit against ant predators.

In general, I find this study interesting. If we want to understand how organisms defend against predators and how those adaptations evolve throughout the life-stages, we also need to understand the physical and physiological mechanisms behind them. Furthermore, authors were very open about the potential limitation of their experimental designs (non-natural substrate where individuals attached during the pupal stage) but since these species have different preferences for the substrates you need to make compromises to what to control. Also, methods were reported in detailed enough to repeat the experiment and sample sizes were good. Please find my more detailed comments below.

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3) Results (minor comment): very small p-values could be reported as $p<0.001$, but this of course depends on the journal's instructions.
4) Overall, authors could have discussed their results in a bit broader context in the discussion which was now quite Drosophila-centered. Authors go through some alternative explanations for the pupa adhesion in the introduction, but this kind of comparison was lacking from the discussion. Also the paragraph in the end of the discussion about other defensive mechanisms of pupae were not very tightly linked with the results of the paper. For example, I was missing some alternative explanations that would make it beneficial to stick in a certain environment for these study species. I agree that predation risk is likely to be very important but how about other natural enemies or abiotic conditions for example?
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Referee: 2

Comments to the Author(s)
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Minor comments:

• I'm not sure what you mean by aerial environments in the context of Drosophila pupae, especially since they are most likely to pupate on vegetable matter or in the soil.
• Line 148: Consider revising to "15 larvae were PLACED IN EACH dish."
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Author's Response to Decision Letter for (RSPB-2021-0088.R0)

See Appendix A.

Decision letter (RSPB-2021-0088.R1)

22-Feb-2021

Dear Dr Courtier-Orgogozo

I am pleased to inform you that your manuscript entitled "Drosophila glue protects from predation" 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,
Professor Loeske Kruuk
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor:
Comments to Author:
The authors have done a thorough job with their minor revisions; I have nothing further.

Appendix A

Dr Virginie Courtier-Orgogozo
CNRS Institut Jacques Monod
15 rue Hélène Brion
75013 Paris
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Tél : (+33) 1 57 27 80 43
E-mail: virginie.courtier@ijm.fr
https://virginiecourtier.wordpress.com/

Paris, 13 January 2021

Proceedings B Editor

Dear Editor,

Thank you very much for all these positive reviews. We took all the comments into account in our revised manuscript. Please find below the point-by-point response to the reviewers and to the editor.

With best regards,

Virginie Courtier-Orgogozo and Flora Borne

Associate Editor, Board Member Recommendation - Comments to Authors:

This study seeks to examine whether and how pupal adhesion / glue protects Drosophila flies from predation, as has been previously hypothesized for insects. Specifically, in addition to maintaining the animal in a place where it might not be detectable by predators, pupal adhesion might also prevent predators from being able to detach the pupa. In their study, the authors test the latter hypothesis by measuring the adhesion of Drosophila species sampled from the same area and observed that pupa adhesion is variable among species – this variability might potentially be explained by different species using different glue production strategies. The authors then compared attached and manually detached pupae and found that attached pupae remain on site 30 % more than detached pupae in the field after three days, potentially indicating reduced predation risk. In support of this notion, the authors found – using laboratory assays –

that attached pupae are less efficiently predated by ants. In summary, I feel that this study provides robust evidence that pupal adhesion / glue reduces predation risk in Drosophila flies.

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I have now obtained two external reviews of this manuscript. Both reviewers agree, and I concur, that this manuscript is interesting and may be suitable for publication in Proceedings B. Neither of the reviewers nor I have major concerns about the design of the study or the results. Both referees make a number of valuable suggestions for improvements. For the detailed reviewer comments please see below; here I just briefly summarize a few key points. Reviewer 1 has a few questions regarding the statistical analyses that require clarification, e.g. whether it would not have been possible to use survival analysis to examine survival during the experiment and whether one could not have included location (bucket ID) as a random factor in a linear mixed model.

We performed a survival analysis including buckets as a random effect, as suggested, and we obtained a similar result (see below).

(A very minor quibble from my side is that the authors sometimes write "chi-squared" and sometimes "Chi2" – it would be good to be consistent here and maybe to use the Greek symbol for Chi.)

Thank you.
Line 226, we changed "chi-squared" by "X^2"
Line 235: we changed "chi-squared" by "X^2"
Line 241: we changed "chi-squared" by "X^2"
Line 284: we changed "chi2" by "X^2"
Line 284: we changed "chi2" by "X^2"

Another point made by Reviewer 1 is that both the introduction and discussion would benefit from some more general context in terms of our understanding of the ecology and evolution of antipredator defenses during the pupal stage and beyond. I agree that this would improve the manuscript.

The discussion was modified accordingly (see below).

Reviewer 2 also makes a number of helpful suggestions for improvements; this reviewer has also noticed a small discrepancy with respect to the experimental setup of the laboratory assays for ant predation which needs clarification.

We clarified the text as suggested. Thank you so much for all these useful comments.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
Pupal stage is important part of the insects' lifecycle, but we know relatively little about the selective environment during that stage. This study focuses on this 'less known' life-stage and aims to understand the mechanism and function of pupa adhesion in Drosophila species. Authors show that strength of adhesion varies among Drosophila species and pupae attached on the substrate are more likely to survive than non-attached pupae. However, between species variation in the strength of adhesion in D. suzukii and D. simulans does not have a strong effect on the survival benefit against ant predators.

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1) Methods: I appreciate the very detailed reporting of methods. However, perhaps authors could give some estimates of how distributed the buckets in the field predation experiment were. Based on the figure, they were sometimes quite close to each other which means that same predators are likely to visit many of them and they are not necessarily independent samples of each other. For example were they all distributed on the same location or were there more than one location?

We now provide, as a new supplementary figure (Fig. S1), a more detailed map of the location of the buckets to have a better understanding of the set up.

We indeed performed pairwise choice design where the two conditions (attached and detached pupa) are present within a bucket so that a potential bucket effect would affect the two conditions in the same way and thus would not be an important parameter in our analysis, as Referee 1 mentioned in 2).
We alternated East - West orientation of the conditions inside buckets in the neighbouring buckets.

We added the sentence: “In order to avoid a potential bucket effect on pupa disappearance in the predation assay in the field, we paired both “attached” and “detached” pupae conditions in each bucket and we subsequently used paired tests.” (line 208-210, Quantification and Statistical Analysis, Methods part)

We added reference to Fig. S1 in “The lids of the Petri dishes were put in the center of buckets previously installed in the ornithological reserve of Bois de Vincennes **(Fig S1)**.” line 158.

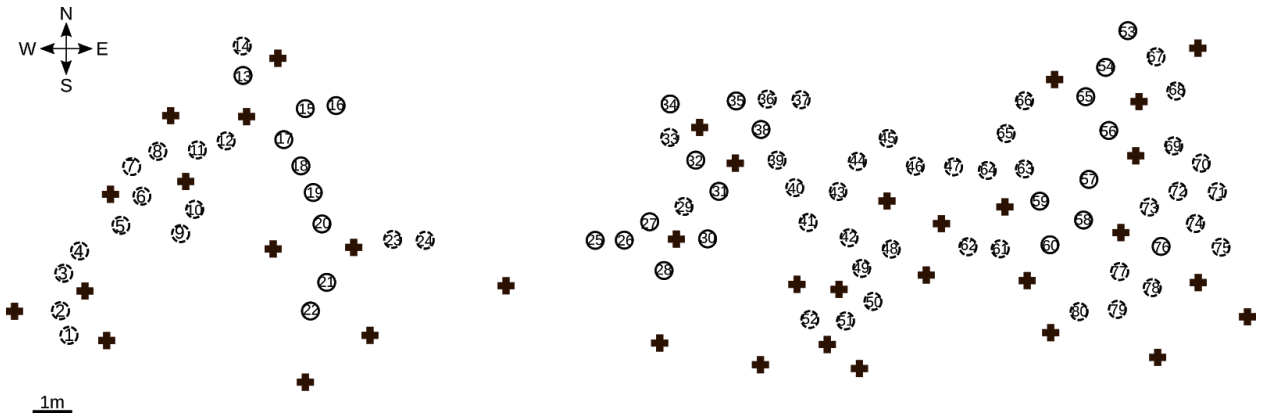
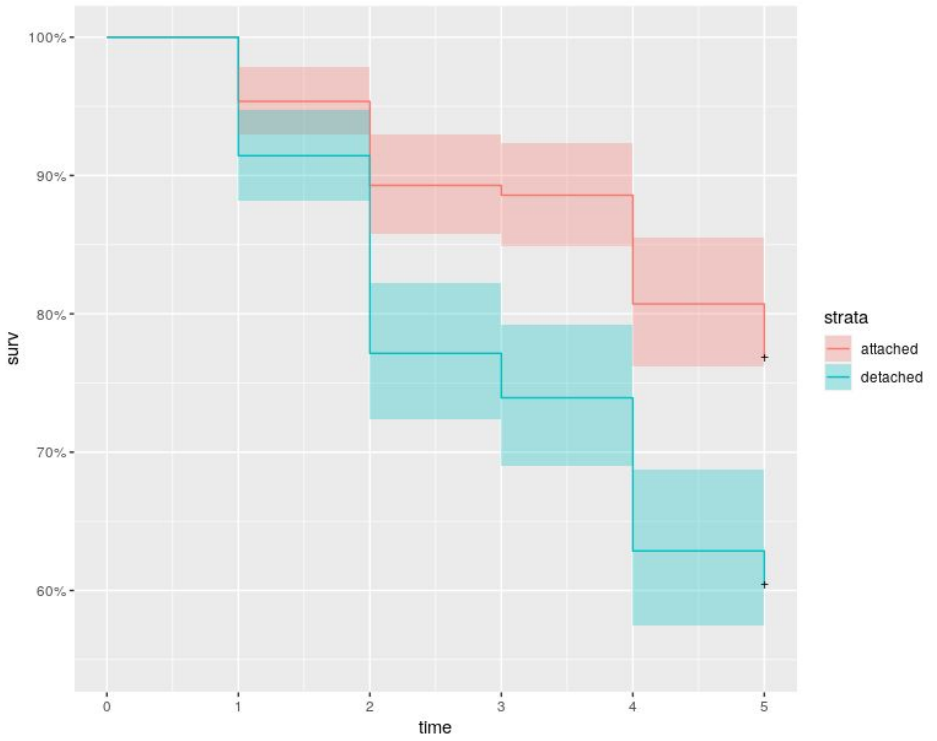


Fig S1. Distribution of the buckets in Bois de Vincennes. Each circle represents one bucket. Dashed lined circles indicate unused buckets and solid lined circles buckets used for the experiment. The number in the circle indicates the ID of the bucket. Crosses indicate the location of the trees. Orientation of the location is indicated in the top left corner.

2) Statistical analyses and experimental design. Benefit of the pairwise/simultaneous choice designs used here is that it is less sensitive for the spatial and temporal variation in e.g. predator abundances of different species among prey locations. However, the problem in simultaneous choice assays is that predator always makes its choice in a certain context which makes

generalisation of the results difficult outside of this context (when predator don't have both attached and non-attached pupae available to choose from simultaneously). For example, I think in this case the pairwise design in a field predation experiment may undermine the difference in survival benefit for attached pupae. As the amount of detached pupae decreases towards the end of the experiment, predators are more likely to find and use energy for preying upon attached pupae. Fortunately, this is very conservative way to test the benefits for pupa adhesion. I was also wondering why authors did not use survival analysis to test survival during the experiment? That would have enabled them to include the location (i.e. bucket ID) of the prey as a random factor into the model. Perhaps this was not possible due to pairwise-set up which in this case could be clearly explained in statistical methods.

The pairwise choice design was indeed motivated by the interest to not lose statistical power by adding bucket location in the model as we had a limited number of replicates. As Referee 1 suggested, we tested survival using Cox's proportional hazards model to compare conditions “attached” and “detached” and using buckets ID as covariates. All pupae which were not gone before the end of the experiments were censored. We found similar results with a difference in survival between attached and detached pupae (p=8.59e-06) and a significant effect of buckets (p=1.34e-08).



However, we decided not to use survival analysis to test survival because, as Referee 1 mentioned, the samples present in the same bucket are probably not independent and could

interact differently overtime: we can assume that over time, as the number of pupae in one dish is decreasing, predators would then go in the neighbouring dish. This would lead to a change in the ratio of the hazard functions between the individuals in the same bucket overtime. However, one assumption to use this test is that the ratio of the hazards for any two individuals is constant over time.

We added: "As the number of detached pupae decreases towards the end of the experiment, predators might be more likely to prey upon attached pupae at later time points. Therefore we did not use a Cox proportional hazards model for survival analysis as the ratio of the hazard functions for individuals within the same bucket may change overtime." (line 210-214, Quantification and Statistical Analysis, Methods part).

3) Results (minor comment): very small p-values could be reported as $p<0.001$, but this of course depends on the journal's instructions.

We prefer to keep it as it is, as small p-values are more precise than $p<0.001$, but we will modify it if the editor finds it necessary.

4) Overall, authors could have discussed their results in a bit broader context in the discussion which was now quite Drosophila-centered. Authors go through some alternative explanations for the pupa adhesion in the introduction, but this kind of comparison was lacking from the discussion. Also the paragraph in the end of the discussion about other defensive mechanisms of pupae were not very tightly linked with the results of the paper. For example, I was missing some alternative explanations that would make it beneficial to stick in a certain environment for these study species. I agree that predation risk is likely to be very important but how about other natural enemies or abiotic conditions for example?

We agreed with Referee 1 about the lack of alternative explanations of the role of adhesion for Drosophila pupa in the discussion and we added more details in the discussion.

We changed the title of the last paragraph in the discussion, from "Other strategies can protect pupae from predation" to "Alternative functions of Drosophila glue and alternative strategies to protect pupae from predation" (line 375-376)

In the discussion, we added "Here, we demonstrated that pupal adhesion protects from predation by preventing predators like ants from taking the pupa away. Pupa adhesion may have alternative roles, such as maintaining the individual in a favorable environment (3, 9, 14) so that the pupa would be hidden from predators, protected from microorganism contaminants or/and have optimal conditions for pupal development. If not attached, the pupa could be moved away by abiotic factors such as wind or rain or biotic factors such as competitors. Pupa attachment could

also help the adult to emerge from the pupal case, as suggested in butterflies (11) or facilitate pupal aggregation and thus dilution of predation risk as in freshwater caddisflies (12). Pupal congregation has been observed in Drosophila species (39,42) but its contribution to protection from predators has not been tested." (line 377-386)

And we removed: "Additionally, pupae have evolved different behaviors such as interacting with conspecifics during larval stage particularly to form aggregation. Aggregated pupae may be more visible to visual predators but predation risk is diluted in group living (12). This last strategy is found in Drosophila species (39,42) but its contribution to protection from predators has not been tested."

5) It was also unclear for me what was authors' working hypothesis for the maintenance of variation in adhesion strength among species. I now got the impression that since the backgrounds where the pupa is glued can vary a lot among species, the artificial surfaces used in this experiment may undermine the benefit of 'weaker glue' for some species? Perhaps this could be clarified.

We indeed did not explain clearly our hypothesis to explain variation of adhesion strength observed between species.

We added the following sentences in the discussion: "The differences we observed in adhesion strength among species may reflect differences in their ecology. For example, an adhesive substance might not be required for animals pupating on sticky substrates (rotten fruits and mushrooms, sap, ...). Furthermore, species exhibiting distinct adhesion strengths in our laboratory conditions may nevertheless stick with similar forces in their respective natural habitats." (Discussion, line 343-347)

We removed: "As many parameters seem to influence pupation behavior, it is hard to know how differences in pupa ecology lead to differences in pupa adhesion between species."

We changed the sentence "We note that our experiment might not reflect natural conditions as we have not tested adhesion on natural substrates and in natural conditions." to "We note that our experiment might not reflect natural conditions as we have not tested adhesion on natural substrates and in natural **habitats**." and moved it from line to line 324-326.

6) Often in studies with insects, the focus is on one life-stage at the time, but the fitness of an insect individual is a sum of all the life-stages it needs to go through until reproduction. Also in this manuscript I was missing more discussion about what is known how this pupa attachment relates to behaviour and ecology during the larval stage and adult stage among studied Drosophila species.

We agreed with Referee 1 about the importance of relationships between the different stages of Drosophila. Larval behavior and ecology will really impact on pupal fitness as the pupation site choice will be crucial for the survival and the good development of the pupa. And correct development of the pupa will be necessary to form healthy adults. Pupa attachment is conditioned by larvae finding a suitable place to pupate. Nothing is known about the effect of pupa attachment on adult fitness but it has been suggested that it could help the adults to emerge from the pupal case.

We added: “At the end of the larval stage, larvae stop feeding and start to search for a site to pupate. Pupation site choice during the larval stage is important for pupal survival.” (line 331-332)

We changed: “Pupation behavior and pupation sites choice have been thoroughly investigated in the lab” by “ **Pupation site choice has** been thoroughly investigated in the lab” (line 333)

We changed: “**This choice** depends on abiotic factors such as temperature (37), darkness (40)...” (line 334)

We changed: “Additionally, **pupation site preference** depends on biotic factors and particularly on the presence of conspecifics and alien species.” (line 339-340)

Referee: 2

Comments to the Author(s)
I really enjoyed reading this manuscript, which is the first to demonstrate that the glue that adhere Drosophila pupae to the substrate deters predators. The experiments are simple and clear, and neatly address the question posed by the authors. I only have a few comments that I think might improve the manuscript.

In the laboratory assays for ant predation, there seems to be a discrepancy with regards to the experimental set up. In the methods section, it says there six pupae per slide: attached, loosely attached, and detached. While in the results, you mention these three categories (lines 307-308), differences between the three categories aren’t described. I think this is because the “loosely attached” pupae are actually D suzukii, but the way this is described in the methods and results isn’t clear.

The reviewer is right, “loosely attached” pupae are *D suzukii*, and this was not clear in the manuscript.

We changed the sentence “To understand further how predators may act when they encounter strongly attached, loosely attached or detached pupae...” by “To further understand how predators may act when they encounter **an attached or a detached pupa**” (line 272-273)

We changed the sentence: “... each ant colony was given on each day one glass slide with two pupae, an attached and a detached one (either two pupae of D. simulans, or two pupae of D. suzukii, we alternated colonies each day).” by “each ant colony was given on each day one glass slide with two pupae, an attached and a detached one. We used either two pupae of D. simulans (strongly attached and detached) or two pupae of D. suzukii (loosely attached and detached) and we alternated colonies each day.” (line 275-278)

Minor comments:

• I’m not sure what you mean by aerial environments in the context of Drosophila pupae, especially since they are most likely to pupate on vegetable matter or in the soil.

Aerial environments were meant as opposed to aquatic environments. This was indeed confusing.
Line 18 and 39, we changed the sentence “aerial environments, …” by “**terrestrial** environments, ...”
Line 76, it is indeed not necessary to precise “aerial pupation sites” as Drosophila pupation sites that we mentioned are all terrestrial.
We changed the sentence “Aerial pupation sites, …” by “Pupation sites” (line 76)

• Line 148: Consider revising to “15 larvae were PLACED IN EACH dish.”

Thank you. We changed the sentence “15 larvae were put per dish” by “15 larvae were **placed in each** dish.” (line 148)

• Line 184: Consider revising to “…wet cotton and LEFT to pupate for…”.
We changed the sentence “...with soft forceps and let to pupate for….” by “with soft forceps and **left** to pupate for…” (line 149)
We changed the sentence “... wet cotton and let to pupate for….” by “wet cotton and left to pupate for…” (line 188)

• Line 379-381: Consider revising to “Because pupae WERE CONTAINED within the lid of petri dishes, we can infer that pupae were not blown away by LIGHT wind, but we cannot BE SURE that THE PUPAE THAT HAD disappeared were IN FACT predated.”.

We changed the sentence “Because pupae **were contained** within the lid of Petri dishes, we **infer** that **they** were not blown away by **light** wind, but we cannot **be sure** that **missing pupae were predated.**” (line 351-353)

Furthermore, when looking again at the raw data, we found 3 errors corresponding to typos when digitizing manually the data.
We corrected the raw data **Table S4. Pupal count in the field experiment**
We changed:

bucket_ ID	Tim e	count_ C	count_N C
16	t4	10	7
17	t3	9	1
31	t3	6	0

to

bucket_ ID	Tim e	count _C	count_N C
16	t4	10	**8**
17	t3	9	**2**
31	t3	**5**	0

We performed the tests again and corrected the figure 2F and the manuscript:
We changed, line 260-262, “day 1 PM: V = 9, p = 0.2; day 2 AM: V = 89.5, p = 0.02; day 2 PM: V = **92.5**, p = 0.01; day 3 AM: V = 91, **p = 0.01**; day 3 PM: V = 110, p = 0.005”
by
“day 1 PM: V = 9, p = 0.2; day 2 AM: V = 89.5, p = 0.02; day 2 PM: V = **92**, p = 0.01; day 3 AM: V = 91, **p = 0.002**; day 3 PM: V = 110, p = 0.005”