

## Reprotoxic effects of the systemic insecticide fipronil on the butterfly *Pieris brassicae*

Rieta Gols, Michiel F. WallisDeVries and Joop J. A. van Loon

### Article citation details

*Proc. R. Soc. B* **287**: 20192665.

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### Review timeline

Original submission: 15 November 2019

1st revised submission: 16 January 2020

2nd revised submission: 12 February 2020

Final acceptance: 12 February 2020

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

## Review History

### RSPB-2019-2665.R0 (Original submission)

#### Review form: Reviewer 1

##### 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?**

Acceptable

**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**

Review for Manuscript RSPB-2019-2665 'Reprotoxic effects of the systemic insecticide fipronil on the butterfly *Pieris brassicae* occur at concentrations around the limit of detection'

This paper investigates the effects of larval exposure to fipronil on the butterfly *Pieris brassicae*. The exposure occurs either through plants grown from fipronil-treated seeds or from fipronil topically applied to leaf discs. Very little research has been carried out on the effects of systemic insecticides on butterflies so this is an important piece of research and it has been thoroughly carried out. I do have some comments that I would like to see addressed before I recommend the manuscript for publication.

The concentrations at which an effect is seen differs greatly between the two methods of exposure and I think that this is important to discuss more fully and mention in the abstract. It is currently quite difficult to follow all the exposure concentrations and which concentration caused what effect - some additional clarification would be helpful, particularly as this is such a controversial topic and published studies are subject to high levels of scrutiny from all interested parties. Specifically, which concentrations of topically applied fipronil actually had significant impacts compared to the control treatment (from figure 3 it looks like 15ppb but some definite results here are needed). On L365-367 you state 'the extrapolated concentration of fipronil in the leaf-disc experiment causing similar effects as in the whole-plant assay in terms of life-time egg production was approximately 18ppb..' - the calculations here need to be more exact and conclusive, it is important not to only extrapolate and estimate.

The discussion needs some improving - L412-421 repeats the introduction and I suggest moving L347 - 359 from the discussion to the introduction as it would also be good to have more about fipronil in the introduction (including how fipronil is specifically different to neonicotinoids?). Also, L26-28 in the abstract and L436-438 in the discussion are the same - elaboration as to what exactly is meant is needed in the discussion.

Other comments:

L20: Less should be 'fewer' (also on L342).

L26: State what this limit of detection is so we know what sort of concentration you are referring to.

L27: 'reached a level' sounds like the degree of neurotoxicity has been steadily increasing - better to say 'at a level'.

L42: Do you have a reference for the inappropriate management of potential habitat including the abandonment of marginal lands?

L43: Important to specify the influence of pesticides on non-target insect survival.

L51: 'other non-target organisms' – it would be better to give an example of another beneficial insect here. Perhaps predatory insects (natural pest control).

L53: This sentence is not very clear – how are insects exposed 'via drainage of contaminated water'. It would be good to clarify that there is uptake by field margin plants.

L63: Missing the word 'by' before EFSA.

L72: *Pieris brassicae* is not always a 'non-target species' – you need to acknowledge here that it is sometimes considered a pest.

L84: I would find it helpful to have a table summarizing the treatments with all the fipronil doses and samples sizes – it is currently quite difficult to follow how many larvae were exposed to which treatment.

L120: Were the caterpillars contained at this stage? Did they ever leave their food plant?

L169: This conversion to dry mass equivalent is currently not very clear. How are these numbers estimated? – how accurate are they? And are these the numbers you then use in the analysis?

L202: Why specifically 81.7  $\mu$ l?

L228: It would be clearer to give these concentrations in  $\mu$ g/kg so everything is then in parts per billion and the numbers are easier to compare.

L252: What do you mean by 'dishes served as experimental units'? – is it a random effect in the model?

L265: The subtitles are different throughout – it would be clearer if they were consistent.

L285: Compared to how many eggs in the control treatment?

L304: You should say here that treatments 1 and 2ppb were not significantly different from the control (as shown in fig 2).

L344: You say exposure to fipronil only affected the adult stage but in L273 you show that development time was slower in the treated groups - it would be good to discuss this result here. This effect was also shown by Whitehorn et al. (2018) (for reference see below).

L393: Do you measure adult size? – it is not mentioned in the methods or results (only pupal mass).

L427: Additional references needed:

Forister, M.L., Cousens, B., Harrison, J.G., Anderson, K., Thorne, J.H., Waetjen, D., Nice, C.C., De Parsia, M., Hladik, M.L., Meese, R. and van Vliet, H., 2016. Increasing neonicotinoid use and the declining butterfly fauna of lowland California. *Biology letters*, 12(8), p.20160475.

Whitehorn, P.R., Norville, G., Gilburn, A. and Goulson, D., 2018. Larval exposure to the neonicotinoid imidacloprid impacts adult size in the farmland butterfly *Pieris brassicae*. *PeerJ*, 6, p.e4772.

L602: Graph legend states lifetime egg production (mean $\pm$ se) – but the figure gives no standard errors. These would be a useful addition to the graph. Also, the results to the control group are directly on the y-axis, which is not clear. It would be useful to know which treatments are significantly different from the control group.

## Review form: Reviewer 2

### Recommendation

Major revision is needed (please make suggestions in comments)

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

Good

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

Excellent

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

Good

**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.**

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?**

N/A

**Is it clear?**

N/A

**Is it adequate?**

N/A

**Do you have any ethical concerns with this paper?**

No

### **Comments to the Author**

This manuscript reports the results of experimental dietary exposures of lepidopteran larvae to fipronil. Adults reared on seed-treated plants showed lower fecundity than controls. A dose-response relationship was obtained from adults reared on surface-treated leaf discs, which revealed both mortality and fecundity effects. Based on residue analysis of the seed-treated plants, the authors conclude that almost imperceptible doses of fipronil can effect non-target insects.

The study appears to be technically sound and the paper is largely well-written. The study is perhaps the first to examine effects on non-target lepidoptera and the results will have a high profile among ecotoxicologists, regulators and environmentalists.

Before it can reach its full impact, some improvements should be made, however. I describe these below along with some minor line-by-line edits.

#### 1. Dose-response relationship.

The relationship fitted in Fig. 3 is not right. I suggest that there is no detectable effect below about 7 ppb and then a quasi-exponential increase. So, something like a sigmoidal dose-response is better - for convenience, you could fit to a dose- vs-%-reduction in performance.

This will strengthen your position that the concentrations in the adult plants were higher during early larval development (perhaps you could also estimate them).

#### 2. Undetectable exposures

Your claim that exposures can be both harmful and undetectable depends on the analysis being conducted on the plant tissues sufficiently late. Probably, residue levels were higher when the

harm was inflicted (see above). It's an unnecessary and inflammatory claim, which I suggest that you delete.

### 3. Methodology and statistics

It's tricky to follow your protocol in lines 130-135 because 'groups' could refer to the treatment groups or the plant groups. Best to call caterpillars from a single plant a different term (set?). Then at some point (line 183-185) you report pooling pupae from treatment levels (across discs) - that decision has reduced your level of replication per treatment to  $n = 1$ ; make sure the statistical testing reflects this throughout (any draws from this pool are now pseudoreplicates).

### 4. Analytical procedures

I suggest adding more details about standing curves etc. Did spike the residues with a labelled standard? If not, how are the recovery rates determined? When percentage recovery is as low as 70%, how variable is it? Definitely, this section needs to be clear and convincing.

Minor edits by line number

100 seeds were purchased?

157 solvent?

109 2.5 L

114 tea spoon?

274 don't interpret non-significant patterns (it isn't 'slower' if the comparisons are not significant)

328 Variation in ng/g by dose?

347 full name of chemical should appear first in introduction

365 Explain the extrapolation

367 stick with ng/g or ppb throughout

405 Label speculation.

432 Omit 'drastically' - this is a sentiment, not a conclusion.

## Decision letter (RSPB-2019-2665.R0)

17-Dec-2019

Dear Dr Gols:

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. 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=\(Document not available\)](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 John Hutchinson, Editor  
mailto:proceedingsb@royalsociety.org

Associate Editor  
Board Member: 1  
Comments to Author:

Both reviewers were enthusiastic about this submission on the effects of a systemic pesticide on a non-target butterfly, but raise some important concerns about the analysis and resulting conclusions which need to be addressed. I strongly encourage the authors to pay very careful attention to these concerns and to modify analyses (e.g. for Figure 3) and text (e.g. interpretations of some material in the Discussion, including regarding undetectable harmful exposures) according to the referees' recommendations.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)

Review for Manuscript RSPB-2019-2665 'Reprotoxic effects of the systemic insecticide fipronil on the butterfly *Pieris brassicae* occur at concentrations around the limit of detection'

This paper investigates the effects of larval exposure to fipronil on the butterfly *Pieris brassicae*. The exposure occurs either through plants grown from fipronil-treated seeds or from fipronil topically applied to leaf discs. Very little research has been carried out on the effects of systemic insecticides on butterflies so this is an important piece of research and it has been thoroughly carried out. I do have some comments that I would like to see addressed before I recommend the manuscript for publication.

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The discussion needs some improving - L412-421 repeats the introduction and I suggest moving L347 - 359 from the discussion to the introduction as it would also be good to have more about

fipronil in the introduction (including how fipronil is specifically different to neonicotinoids?). Also, L26-28 in the abstract and L436-438 in the discussion are the same – elaboration as to what exactly is meant is needed in the discussion.

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L602: Graph legend states lifetime egg production (mean $\pm$ se) – but the figure gives no standard errors. These would be a useful addition to the graph. Also, the results to the control group are directly on the y-axis, which is not clear. It would be useful to know which treatments are significantly different from the control group.



Referee: 2

#### Comments to the Author(s)

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Your claim that exposures can be both harmful and undetectable depends on the analysis being conducted on the plant tissues sufficiently late. Probably, residue levels were higher when the harm was inflicted (see above). It's an unnecessary and inflammatory claim, which I suggest that you delete.

#### 3. Methodology and statistics

It's tricky to follow your protocol in lines 130-135 because 'groups' could refer to the treatment groups or the plant groups. Best to call caterpillars from a single plant a different term (set?).

Then at some point (line 183-185) you report pooling pupae from treatment levels (across discs) – that decision has reduced your level of replication per treatment to  $n = 1$ ; make sure the statistical testing reflects this throughout (any draws from this pool are now pseudoreplicates).

#### 4. Analytical procedures

I suggest adding more details about standing curves etc. Did spike the residues with a labelled standard? If not, how are the recovery rates determined? When percentage recovery is as low as 70%, how variable is it? Definitely, this section needs to be clear and convincing.

#### Minor edits by line number

100 seeds were purchased?

157 solvent?

109 2.5 L

114 tea spoon?

274 don't interpret non-significant patterns (it isn't 'slower' if the comparisons are not significant)

328 Variation in ng/g by dose?

347 full name of chemical should appear first in introduction

365 Explain the extrapolation  
 367 stick with ng/g or ppb throughout  
 405 Label speculation.  
 432 Omit 'drastically' - this is a sentiment, not a conclusion.

## Author's Response to Decision Letter for (RSPB-2019-2665.R0)

See Appendix A.

## RSPB-2019-2665.R1 (Revision)

### Review form: Reviewer 1

#### **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?**

Good

#### **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?**

No

#### **Is it clear?**

N/A

#### **Is it adequate?**

N/A

**Do you have any ethical concerns with this paper?**

No

**Comments to the Author**

Review for Manuscript RSPB-2019-2665 'Reprotoxic effects of the systemic insecticide fipronil on the butterfly *Pieris brassicae* occur at concentrations around the limit of detection'

This manuscript has been substantially improved after addressing the comments of the two reviewers. I have a few minor comments, detailed below:

L59-63: This sentence has become a bit long and unclear – the additions are all great but I think it needs a bit of re-wording to make sense.

L63-65: This additional sentence is not relevant to the context of this study.

L79: Here you state that in developed countries the most common application of fipronil is seed coating but on L369 you write that fipronil-coated seeds are no longer available – is this just for Brussel sprouts? Is there any reason for this (for example a suspension of use due to suspected harmful side effects)? It would be useful to know.

L195: How much ethanol?

L241: Here you write tissues were frozen but on L161 you write samples were stored at 20°C – both these sections refer to the plants grown for the whole plant experiment so please clarify which is correct.

L236-260: What is the reason that the leaf tissue samples were dried by two different methods? Is it known if these different methods will impact the concentrations of fipronil detected?

L278: 'recovery of pupae' – does this mean survival?

L402 & 448: Your finding that exposure to fipronil during larval development primarily affected the adult stage was also found by Whitehorn et al. (2018), who looked at the effects of the neonic imidacloprid on the same species of butterfly:

Whitehorn, P.R., Norville, G., Gilburn, A. and Goulson, D., 2018. Larval exposure to the neonicotinoid imidacloprid impacts adult size in the farmland butterfly *Pieris brassicae*. *PeerJ*, 6, p.e4772.

Very minor corrections:

L32: probability to pinpoint → probability of pinpointing

L70: Perhaps better to start a new sentence after [20]. In contrast, neonicotinoids....

L345: 'been' should be 'between'

L467: check formatting of name of ref [2]

**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?**

Good

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

Excellent

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

Good

**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.**

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?**

Yes

**Is it adequate?**

Yes

**Do you have any ethical concerns with this paper?**

No

**Comments to the Author**

Dear Authors,

Good work in modifying your manuscript.

I have one concern - the dose-response curve in Fig 3. The shape is now much better. Two things need fixing:

(a) The 'zero reproduction' points at 32 ppb need to display on the figure otherwise the tail looks like a complete guess, when it isn't.

(b) You should delete the statistical testing for Fig 3's sigmoidal regression; you do not appear to have replication at each dose because you pooled the subjects. It doesn't matter - your other experiment shows that fipronil reduces reproduction and nobody is going to contest that fipronil is dose-dependent reprotoxic - the only thing that matters is the fit. The fit looks great, but I can't find the r-squared in text or figure legend (please add). You can test the decrease in reproduction with dose using the group means if you want but you don't have many doses; but you really, really mustn't use pseudoreplicates as independent data in stats testing as their inter-relationships invalidates the p-value. But I'm perfectly happy that you use the pseudoreps to fit the sigmoidal, as the only assumption is least-squares. You'll need to justify this in methods somewhere.

**Decision letter (RSPB-2019-2665.R1)**

05-Feb-2020

Dear Dr Gols

I am pleased to inform you that your manuscript RSPB-2019-2665.R1 entitled "Reprotoxic effects

of the systemic insecticide fipronil on the butterfly *Pieris brassicae* " has been accepted for publication in Proceedings B. Congratulations!!

The referee(s) have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, I invite you to respond to the referee(s)' comments and revise your manuscript. Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript within 7 days. If you do not think you will be able to meet this date please let us know.

To revise your manuscript, log into <https://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. You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you will be able to respond to the comments made by the referee(s) and upload a file "Response to Referees". You can use this to document any changes you make to the original 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.

Before uploading your revised files please make sure that you have:

- 1) A text file of the manuscript (doc, txt, rtf or tex), including the references, tables (including captions) and figure captions. Please remove any tracked changes from the text before submission. PDF files are not an accepted format for the "Main Document".
- 2) A separate electronic file of each figure (tiff, EPS or print-quality PDF preferred). The format should be produced directly from original creation package, or original software format. PowerPoint files are not accepted.
- 3) Electronic supplementary material: this should be contained in a separate file and where possible, all ESM should be combined into a single file. 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.

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].

- 4) A media summary: a short non-technical summary (up to 100 words) of the key findings/importance of your manuscript.

- 5) Data accessibility section and data citation

It is a condition of publication that data supporting your paper are made available either in the electronic supplementary material or through an appropriate repository.

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should be fully cited. To ensure archived data are available to readers, authors should include a 'data accessibility' section immediately after the acknowledgements section.

This should list the database and accession number for all data from the article that has been made publicly available, for instance:

- DNA sequences: Genbank accessions F234391-F234402
- Phylogenetic data: TreeBASE accession number S9123
- Final DNA sequence assembly uploaded as online supplemental material
- Climate data and MaxEnt input files: Dryad doi:10.5521/dryad.12311

NB. From April 1 2013, peer reviewed articles based on research funded wholly or partly by RCUK must include, if applicable, a statement on how the underlying research materials – such as data, samples or models – can be accessed. This statement should be included in the data accessibility section.

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=\(Document not available\)](http://datadryad.org/submit?journalID=RSPB&manu=(Document%20not%20available)) 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. Please see <https://royalsociety.org/journals/ethics-policies/data-sharing-mining/> for more details.

6) For more information on our Licence to Publish, Open Access, Cover images and Media summaries, please visit <https://royalsociety.org/journals/authors/author-guidelines/>.

Once again, thank you for submitting your manuscript to Proceedings B and I look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Sincerely,

Professor John Hutchinson  
Editor, Proceedings B  
<mailto:proceedingsb@royalsociety.org>

Associate Editor:

Comments to Author:

Both reviewers were very positive about the revised version of this manuscript, but had a few remaining requests for the authors. Please pay careful attention to referee 2's request for clarification / modification of the presentation and analysis for figure 3: please ensure that datapoints appear clearly on the graph for all treatments, and that your statistical test in the legend or around line 363 is appropriate (make sure it is clear where data were pooled, and whether therefore the datapoints on the figure are independent). Please address the comments also of referee 1, including for example simplifying lines 59-63, and please check that data are available to accompany the submission.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)

Review for Manuscript RSPB-2019-2665 'Reprotoxic effects of the systemic insecticide fipronil on the butterfly *Pieris brassicae* occur at concentrations around the limit of detection'

This manuscript has been substantially improved after addressing the comments of the two reviewers. I have a few minor comments, detailed below:

L59-63: This sentence has become a bit long and unclear – the additions are all great but I think it needs a bit of re-wording to make sense.

L63-65: This additional sentence is not relevant to the context of this study.

L79: Here you state that in developed countries the most common application of fipronil is seed coating but on L369 you write that fipronil-coated seeds are no longer available – is this just for Brussel sprouts? Is there any reason for this (for example a suspension of use due to suspected harmful side effects)? It would be useful to know.

L195: How much ethanol?

L241: Here you write tissues were frozen but on L161 you write samples were stored at 20°C – both these sections refer to the plants grown for the whole plant experiment so please clarify which is correct.

L236-260: What is the reason that the leaf tissue samples were dried by two different methods? Is it known if these different methods will impact the concentrations of fipronil detected?

L278: 'recovery of pupae' – does this mean survival?

L402 & 448: Your finding that exposure to fipronil during larval development primarily affected the adult stage was also found by Whitehorn et al. (2018), who looked at the effects of the neonic imidacloprid on the same species of butterfly:

Whitehorn, P.R., Norville, G., Gilburn, A. and Goulson, D., 2018. Larval exposure to the neonicotinoid imidacloprid impacts adult size in the farmland butterfly *Pieris brassicae*. *PeerJ*, 6, p.e4772.

Very minor corrections:

L32: probability to pinpoint ? probability of pinpointing

L70: Perhaps better to start a new sentence after [20]. In contrast, neonicotinoids....

L345: 'been' should be 'between'

L467: check formatting of name of ref [2]

Referee: 2

Comments to the Author(s)

Dear Authors,

Good work in modifying your manuscript.

I have one concern - the dose-response curve in Fig 3. The shape is now much better. Two things need fixing:

(a) The 'zero reproduction' points at 32 ppb need to display on the figure otherwise the tail looks like a complete guess, when it isn't.

(b) You should delete the statistical testing for Fig 3's sigmoidal regression; you do not appear to have replication at each dose because you pooled the subjects. It doesn't matter - your other experiment shows that fipronil reduces reproduction and nobody is going to contest that fipronil is dose-dependent reprotoxic - the only thing that matters is the fit. The fit looks great, but I can't find the r-squared in text or figure legend (please add). You can test the decrease in reproduction with dose using the group means if you want but you don't have many doses; but you really, really mustn't use pseudoreplicates as independent data in stats testing as their inter-relationships invalidates the p-value. But I'm perfectly happy that you use the pseudoreps to fit the sigmoidal, as the only assumption is least-squares. You'll need to justify this in methods somewhere.

**Author's Response to Decision Letter for (RSPB-2019-2665.R1)**

See Appendix B.

## Decision letter (RSPB-2019-2665.R2)

12-Feb-2020

Dear Dr Gols

I am pleased to inform you that your manuscript entitled "Reprotoxic effects of the systemic insecticide fipronil on the butterfly *Pieris brassicae* " 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](mailto:procb_proofs@royalsociety.org)

Your article has been estimated as being 10 pages long. Our Production Office will be able to confirm the exact length at proof stage.

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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,

Proceedings B

<mailto:proceedingsb@royalsociety.org>



# Appendix A

## Response Letter

Associate Editor

Board Member: 1

Comments to Author:

Both reviewers were enthusiastic about this submission on the effects of a systemic pesticide on a non-target butterfly, but raise some important concerns about the analysis and resulting conclusions which need to be addressed. I strongly encourage the authors to pay very careful attention to these concerns and to modify analyses (e.g. for Figure 3) and text (e.g. interpretations of some material in the Discussion, including regarding undetectable harmful exposures) according to the referees' recommendations.

*RESPONSE: We are glad about the enthusiastic reactions of the reviewers and have tried to carefully address each of their comments in our revision.*

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)

Review for Manuscript RSPB-2019-2665 'Reprotoxic effects of the systemic insecticide fipronil on the butterfly *Pieris brassicae* occur at concentrations around the limit of detection'

This paper investigates the effects of larval exposure to fipronil on the butterfly *Pieris brassicae*. The exposure occurs either through plants grown from fipronil-treated seeds or from fipronil topically applied to leaf discs. Very little research has been carried out on the effects of systemic insecticides on butterflies so this is an important piece of research and it has been thoroughly carried out. I do have some comments that I would like to see addressed before I recommend the manuscript for publication.

The concentrations at which an effect is seen differs greatly between the two methods of exposure and I think that this is important to discuss more fully and mention in the abstract. It is currently quite difficult to follow all the exposure concentrations and which concentration caused what effect - some additional clarification would be helpful, particularly as this is such a controversial topic and published studies are subject to high levels of scrutiny from all interested parties. Specifically, which concentrations of topically applied fipronil actually had significant impacts compared to the control treatment (from figure 3 it looks like 15ppb but some definite results here are needed). On L365-367 you state 'the extrapolated concentration of fipronil in the leaf-disc experiment causing similar effects as in the whole-plant assay in terms of life-time egg production was approximately 18ppb..' – the calculations here need to be more exact and conclusive, it is important not to only extrapolate and estimate.

*RESPONSE; in the abstract we now mention the difference in effect dosage for the two methods and also provide possible explanations for this discrepancy. We now present applied dosages consistently in  $\mu\text{g}$  fipronil/ kg (DW leaf tissue). These dosages are based on estimation of dry weight equivalents of the fresh plant and, therefore, are approximations of the actual dosages. We have re-analyzed the data according to the suggestions of the second referee which indeed reveal that there is no effect of fipronil at concentration of  $\leq 4 \mu\text{g}/\text{kg}$ . The concentration causing similar effects as in the whole plant bioassay is now calculated based on the estimated model parameters in Figure 3 and was found to be  $12.2 \mu\text{g}/\text{kg}$ . This estimation is given in the text.*

The discussion needs some improving - L412-421 repeats the introduction and I suggest moving L347 – 359 from the discussion to the introduction as it would also be good to have more about fipronil in the introduction (including how fipronil is specifically different to neonicotinoids?). Also, L26-28 in the abstract and L436-438 in the discussion are the same – elaboration as to what exactly is meant is needed in the discussion.

*RESPONSE: We have expanded in the introduction the text on fipronil by moving some of the text from the discussion to the introduction as suggested by the reviewer. We also mention in the introduction the difference in mode of activity between fipronil and neonicotinoids as requested. We have rephrased the last sentence of the abstract and discussion to point out our concern that with the current levels of toxicity, it is becoming increasingly difficult to demonstrate harmful effects even when they are present.*

Other comments:

L20: Less should be 'fewer' (also on L342). *RESPONSE: this has been rephrased.*

L26: State what this limit of detection is so we know what sort of concentration you are referring to.

*RESPONSE: we have added the limit of detection for fipronil in the sentence.*

L27: 'reached a level' sounds like the degree of neurotoxicity has been steadily increasing – better to say 'at a level'.

*RESPONSE: we have rephrased the text as suggested.*

L42: Do you have a reference for the inappropriate management of potential habitat including the abandonment of marginal lands?

*RESPONSE: we have added a reference (Bonari et al. 2017, Agriculture Ecosystems & Environment, 246, 243-252.*

L43: Important to specify the influence of pesticides on non-target insect survival.

*RESPONSE: this is a very good point. We have changed this.*

L51: 'other non-target organisms' – it would be better to give an example of another beneficial insect here. Perhaps predatory insects (natural pest control).

*RESPONSE: we have rephrased the text here and have given other examples of beneficial insects.*

L53: This sentence is not very clear – how are insects exposed 'via drainage of contaminated water'. It would be good to clarify that there is uptake by field margin plants.

*RESPONSE: we have clarified this in the text.*

L63: Missing the word 'by' before EFSA.

*RESPONSE: this has been corrected.*

L72: *Pieris brassicae* is not always a 'non-target species' – you need to acknowledge here that it is sometimes considered a pest.

*RESPONSE: we have explained why *Pieris brassicae* is a non-target species in the case of fipronil application and added with references that it has been reported as a pest in other areas of Europe.*

L84: I would find it helpful to have a table summarizing the treatments with all the fipronil doses and samples sizes – it is currently quite difficult to follow how many larvae were exposed to which treatment.

*RESPONSE: we have added a table summarizing the treatments as requested, which we have provided as a supplementary Table.*

L120: Were the caterpillars contained at this stage? Did they ever leave their food plant?

*RESPONSE: when food is not limiting, the caterpillars do not need to be confined during the first four instars. Only when they are in the final fifth instar, caterpillars start to wander. We have mentioned this now in the text.*

L169: This conversion to dry mass equivalent is currently not very clear. How are these numbers estimated? – how accurate are they? And are these the numbers you then use in the analysis?

*RESPONSE: we determined the dry mass of 20 7-cm diameter leaf discs and used the mean dry mass of these discs to estimate the concentration per dry mass equivalent (ca 0.0510 ng applied per cm<sup>2</sup>*

divided by the mean dry mass per  $\text{cm}^2$ ). We clarify in the text how we measured dry mass equivalents and have provided a measure of variation for the dry mass. It is not possible to provide a measure of accuracy for the applied concentrations as this is affected by the accuracy of the pipet and pipetting itself, variation in leaf disc surface area and variation in dry mass of the leaf discs. We used the approximated concentrations of fipronil in the analyses.

L202: Why specifically 81.7  $\mu\text{l}$ ?

RESPONSE: this is the volume of solution applied per  $\text{cm}^2$  (2.12  $\mu\text{l}$ ) times the surface area of a 7 cm disc (38.48  $\text{cm}^2$ ). When spread over the leaf surface this volume will dry in 30 min. In the text we say now that leaf discs were treated with the approximated dosage of 16  $\mu\text{g}$  fipronil per kg dry matter of leaf.

L228: It would be clearer to give these concentrations in  $\mu\text{g}/\text{kg}$  so everything is then in parts per billion and the numbers are easier to compare.

RESPONSE: we use this notation throughout the revised manuscript.

L252: What do you mean by 'dishes served as experimental units'? – is it a random effect in the model?

RESPONSE: it means that each dish generates a single data point that serves as a replicate in the analysis.

L265: The subtitles are different throughout – it would be clearer if they were consistent.

RESPONSE: we have corrected this.

L285: Compared to how many eggs in the control treatment?

RESPONSE: Control females ( $n=19$ ) produced in total 15393 eggs. We added this information in the text.

L304: You should say here that treatments 1 and 2ppb were not significantly different from the control (as shown in fig 2).

RESPONSE: we say now in the text that longevity of butterflies exposed to concentration of 2  $\mu\text{g kg}^{-1}$  or lower did not differ from control butterflies (Fig. 2).

L344: You say exposure to fipronil only affected the adult stage but in L273 you show that development time was slower in the treated groups - it would be good to discuss this result here. This effect was also shown by Whitehorn et al. (2018) (for reference see below).

RESPONSE: actually males developed slower on control plants than on fipronil-treated plants, whereas there was no effect on female development. As this effect was only marginally significant and not significant following a correction for multiple comparisons, we think this is ecologically irrelevant (see also comment by reviewer 2).

L393: Do you measure adult size? – it is not mentioned in the methods or results (only pupal mass).

RESPONSE: we only measured pupal mass which is often considered as an approximation of size. We have replaced 'adult size' by 'pupal mass' in the text.

L427: Additional references needed:

Forister, M.L., Cousens, B., Harrison, J.G., Anderson, K., Thorne, J.H., Waetjen, D., Nice, C.C., De Parsia, M., Hladik, M.L., Meese, R. and van Vliet, H., 2016. Increasing neonicotinoid use and the declining butterfly fauna of lowland California. *Biology Letters*, 12(8), p.20160475.

Whitehorn, P.R., Norville, G., Gilburn, A. and Goulson, D., 2018. Larval exposure to the neonicotinoid imidacloprid impacts adult size in the farmland butterfly *Pieris brassicae*. *PeerJ*, 6, p.e4772.

RESPONSE: we added the suggested Forister et al reference. The Whitehorn et al reference is not so interesting taking into account that the effect of fipronil on development time was not significant.

L602: Graph legend states lifetime egg production (mean $\pm$ se) – but the figure gives no standard errors. These would be a useful addition to the graph. Also, the results to the control group are directly on the y-axis, which is not clear. It would be useful to know which treatments are significantly different from the control group.

RESPONSE: a new Figure 3 has replaced the previous one and the legend has been rephrased.

Referee: 2

#### Comments to the Author(s)

This manuscript reports the results of experimental dietary exposures of lepidopteran larvae to fipronil. Adults reared on seed-treated plants showed lower fecundity than controls. A dose-response relationship was obtained from adults reared on surface-treated leaf discs, which revealed both mortality and fecundity effects. Based on residue analysis of the seed-treated plants, the authors conclude that almost imperceptible doses of fipronil can effect non-target insects.

The study appears to be technically sound and the paper is largely well-written.

The study is perhaps the first to examine effects on non-target lepidoptera and the results will have a high profile among ecotoxicologists, regulators and environmentalists.

Before it can reach its full impact, some improvements should be made, however. I describe these below along with some minor line-by-line edits.

#### 1. Dose-response relationship.

The relationship fitted in Fig. 3 is not right. I suggest that there is no detectable effect below about 7 ppb and then a quasi-exponential increase. So, something like a sigmoidal dose-response is better – for convenience, you could fit to a dose- vs-%-reduction in performance.

This will strengthen your position that the concentrations in the adult plants were higher during early larval development (perhaps you could also estimate them).

*RESPONSE: we agree with the referee that dose-response curves tend to be sigmoidal. However, a linear model analysis resulted in a reasonable fit ( $R^2 = 0.84$ ). We have analyzed the data using logistic regression to generate a sigmoidal relationship and have replaced the original analysis with this one. We were not able to estimate the concentration in younger plants.*

#### 2. Undetectable exposures

Your claim that exposures can be both harmful and undetectable depends on the analysis being conducted on the plant tissues sufficiently late. Probably, residue levels were higher when the harm was inflicted (see above). It's an unnecessary and inflammatory claim, which I suggest that you delete.

*RESPONSE: we agree with the referee that we should be careful to state that exposure levels of fipronil can be harmful to organisms at or below the detection level based on the results of this study. We indeed measured concentrations 2 to 3 days after the caterpillars stopped feeding and, therefore, cannot exclude that concentrations were higher during caterpillar feeding. Given a half-life of fipronil of 7 days, concentrations in tissues of plants grown from treated seeds would still be near the detection level. It is important here to take into account that ca. 80% of the total caterpillar consumption occurs during the final instar, that is just prior to leaf tissue collection. We have toned down that the effects of fipronil and its degradation products may occur near the level of detection and removed this claim from the title. However, we do think it is important to point out the potential problem that with the current levels of toxicity, that may become even higher in the future, it will become increasingly difficult to demonstrate harmful effects even when they are present. If you do not agree with this opinion, we are prepared to delete the last sentence of the discussion and rephrase the ending of the abstract.*

### 3. Methodology and statistics

It's tricky to follow your protocol in lines 130-135 because 'groups' could refer to the treatment groups or the plant groups. Best to call caterpillars from a single plant a different term (set?).

*RESPONSE: this has been clarified.*

Then at some point (line 183-185) you report pooling pupae from treatment levels (across discs) – that decision has reduced your level of replication per treatment to  $n = 1$ ; make sure the statistical testing reflects this throughout (any draws from this pool are now pseudoreplicates).

*RESPONSE: we agree with the referee that pooling data is an issue here. It would have been better to maintain butterflies in individual cages but this was impossible due to space limitation. We now have not accounted for the variation among the five cages. We consider this as a minor flaw.*

### 4. Analytical procedures

I suggest adding more details about standing curves etc. Did spike the residues with a labelled standard? If not, how are the recovery rates determined? When percentage recovery is as low as 70%, how variable is it? Definitely, this section needs to be clear and convincing.

*RESPONSE: We have provided calibration curves as supplementary data. No stable isotope labels have been used for quantification. Recovery is determined by adding 0.4 and 10 ng/g of the quantified components to blank material and quantification is performed by bracketed calibration upon a reference solution of 5 ng/ml. The resulted concentration of the quantified components is assessed against the theoretical concentration.*

Minor edits by line number

100 seeds were purchased? *RESPONSE: corrected*

157 solvent? *RESPONSE: the solvent was tap water. This has been added in the text.*

109 2.5 L. . *RESPONSE: 'l' is the symbol for litre according to standard formatting of the journal.*

114 tea spoon? *RESPONSE: this was approximately 4 g, we have replaced this in the text.*

274 don't interpret non-significant patterns (it isn't 'slower' if the comparisons are not significant) *RESPONSE: text has been revised.*

328 Variation in ng/g by dose? *RESPONSE: we added the variation in dry mass here. It is impossible to provide variation in ng/g by dose (see response to reviewer 1).*

347 full name of chemical should appear first in introduction. *RESPONSE: has been corrected.*

365 Explain the extrapolation. *RESPONSE: we have redone the results on life-time egg production performance. We used the new Figure 3 to extrapolate the concentration at which the performance would be reduced similarly as in the whole plant bioassay. Females on plants grown from coated-seeds produced 44% of the number of eggs compared to females on control plants.*

367 stick with ng/g or ppb throughout *RESPONSE: we now use  $\mu\text{g}/\text{kg}$  throughout the manuscript*

405 Label speculation. *RESPONSE: ok done.*

432 Omit 'drastically' – this is a sentiment, not a conclusion. *RESPONSE: 'drastically' has been deleted.*

\*\*\*\*\*

Journal Name: Proceedings of the Royal Society B Journal

## Appendix B

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)

Review for Manuscript RSPB-2019-2665 'Reprotoxic effects of the systemic insecticide fipronil on the butterfly *Pieris brassicae* occur at concentrations around the limit of detection'

This manuscript has been substantially improved after addressing the comments of the two reviewers. I have a few minor comments, detailed below:

L59-63: This sentence has become a bit long and unclear – the additions are all great but I think it needs a bit of re-wording to make sense. RESPONSE: the sentence has been spilt into two sentences.

L63-65: This additional sentence is not relevant to the context of this study. RESPONSE: this sentence has been deleted.

L79: Here you state that in developed countries the most common application of fipronil is seed coating but on L369 you write that fipronil-coated seeds are no longer available – is this just for Brussel sprouts? Is there any reason for this (for example a suspension of use due to suspected harmful side effects)? It would be useful to know.

RESPONSE: the use to fipronil and three neonicotinoids has been restricted in the European Union according to EU legislation and this ban is not restricted to Brussels sprouts. In the text we refer to the relevant legislation documents.

L195: How much ethanol? RESPONSE: as the concentration of ethanol is negligible in the dilution series (in the highest applied dosage, the stock solution has been diluted 200000 times), no ethanol has been added.

L241: Here you write tissues were frozen but on L161 you write samples were stored at 20°C – both these sections refer to the plants grown for the whole plant experiment so please clarify which is correct. RESPONSE: we have removed the text about the sampling in the section where we describe the bioassay and mention sampling and processing of the samples only in the section on fipronil analysis. Tissues were stored at 20°C before they were further processed, i.e., freeze-dried. This has been clarified in the text. We don't know the effect of freeze-drying on fipronil, but we assumed it is minimal.

L236-260: What is the reason that the leaf tissue samples were dried by two different methods? Is it known if these different methods will impact the concentrations of fipronil detected? RESPONSE: due to time constraints, the leaf discs were dried in an oven which is a slightly faster method. The results showed that the measured concentration differed only marginally from the applied concentration. This suggests that this method has little effect on fipronil.

L278: 'recovery of pupae' – does this mean survival? RESPONSE: yes, this can be interpreted as a measurement of survival. This has been clarified in the text.

L402 & 448: Your finding that exposure to fipronil during larval development primarily affected the adult stage was also found by Whitehorn et al. (2018), who looked at the effects of the neonic imidacloprid on the same species of butterfly:

Whitehorn, P.R., Norville, G., Gilburn, A. and Goulson, D., 2018. Larval exposure to the neonicotinoid imidacloprid impacts adult size in the farmland butterfly *Pieris brassicae*. *PeerJ*, 6, p.e4772.

RESPONSE: the results of the Whitehorn et al study found that duration of pupation was reduced when reared on fipronil treated plants and that adult butterflies were smaller, so there results contrasts with the results presented here. We mention these results now in the discussion,

Very minor corrections:

L32: probability to pinpoint → probability of pinpointing. RESPONSE: corrected.

L70: Perhaps better to start a new sentence after [20]. In contrast, neonicotinoids....RESPONSE: OK, done.

L345: 'been' should be 'between'. RESPONSE: corrected.

L467: check formatting of name of ref [2]. RESPONSE: corrected.

Referee: 2

Comments to the Author(s)

Dear Authors,

Good work in modifying your manuscript.

I have one concern - the dose-response curve in Fig 3. The shape is now much better. Two things need fixing:

(a) The 'zero reproduction' points at 32 ppb need to display on the figure otherwise the tail looks like a complete guess, when it isn't.

RESPONSE; we have added the 'zero reproduction in Fig 3.

(b) You should delete the statistical testing for Fig 3's sigmoidal regression; you do not appear to have replication at each dose because you pooled the subjects. It doesn't matter - your other experiment shows that fipronil reduces reproduction and nobody is going to contest that fipronil is dose-dependent reprotoxic - the only thing that matters is the fit. The fit looks great, but I can't find the r-squared in text or figure legend (please add). You can test the decrease in reproduction with dose using the group means if you want but you don't have many doses; but you really, really mustn't use psuedoreplicates as independent data in stats testing as their inter-relationships invalidates the p-value. But I'm perfectly happy that you use the pseudoreps to fit the sigmoidal, as the only assumption is least-squares. You'll need to justify this in methods somewhere.

RESPONSE: we have not pooled data here. Each point used in the analysis and shown in Fig. 3 is a true independent replicate where each replicate is the average performance of three females. For each concentration we have five independent replicates, except for the 4  $\mu\text{g kg}^{-1}$  DM dosage, which had only two replicates. When analyzing data with a logistic regression, an equivalent statistic to R-squared does not exist. The model estimates from a logistic regression are maximum likelihood estimates which are obtained through an iterative process.