

Comments to paper previously entitled “**Role of pollen flavonoids in the heather-bumble bee-parasite interactions**” (New title: “**Heather pollen is not necessarily a healthy diet for bumble bees**”) submitted to PCI Ecology

05.07.2023

(Line numbers refer to the document in track change mode)

### **Reviewer 1 general comment:**

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In the manuscript “Role of pollen flavonoids in the heather- bumble bee-parasite interactions”, the authors tested the effect of heather pollen in microcolonies success, with and without the presence of a pathogen whose effect has been shown to diminish with heather nectar. I find the manuscript to be easy to read, the experimental design is appropriately controlled and coherent with the goals and hypotheses and the results appear to be correctly interpreted. The figures are appropriate and in line with the text. Both methods and discussion are the appropriate length and depth. Both data and scripts for analyses are freely available and uploaded.

In general, I find the paper timely and of interest to bumblebee conservation or rearing, and ready for publication in a peer reviewed journal should the authors decide so.

### **Authors’ response:**

We warmly thank the reviewer for this positive feedback and the time he devoted to reading and evaluating our manuscript. We have now revised the manuscript in response to your comments. The title has been improved and additional information on the role of flavonoids in this context has been added to the manuscript.

### **Reviewer 1 minor comment 1:**

I would recommend some minor changes, like a more appealing title (maybe stating the main results as heather pollen being detrimental to bumblebee colony development) and checking some sentences for English language (e.g., “Evidence is...” should better be “There is evidence that...”).

### **Authors’ response:**

Thank you for your valuable advice. The title has been changed to emphasise the impacts on bumblebees of the heather pollen (Lines 1 to 4). Several sentences have been modified to improve clarity for the reader and to check the English (e.g., Line 28).

### **Reviewer 1 minor comment 2:**

Also the introduction would profit from the inclusion of a bit more literature about the different effects from flavonoids that have been found so far (every time it was mentioned that some results are “controversial”, it would be nice to have a brief summary of the contradicting results found so far).

**Authors' response:**

Thank you for your comment, we concur that the introduction would benefit from more information on the importance of flavonoids on bee-plant interactions. More information has been added to the corresponding section in the manuscript (Lines 93-102).

## **Reviewer 2 general comment:**

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The paper entitled “Role of flavonoids in the heather-bumble bee-parasite interactions” by Tourbez et al. is an interesting study that highlights the need to consider both nectar and pollen when addressing medicinal effects of a plant towards pollinators. Overall, the questions asked in this study are interesting, important and topical, and in general the methods are adequate, and the results clearly presented. However, in my opinion, some parts of the paper need clarification by the authors. I have a few suggestions that I hope can be useful to improve the manuscript.

### **Authors' response:**

We would like to express our gratitude for the constructive and relevant comments you have made on our manuscript as well as for the time devoted to evaluating this work. We have now revised the manuscript in response to your comments and hope that it will meet your expectations. Clarification of the methodology details and limitations of our experiments have been added to the manuscript.

### **Reviewer 2 minor comment 1:**

Line 112: The use of willow pollen as “control” needs further explanation by the authors. Given that bumblebees normally collect pollen from diverse floral resources, wouldn't have been more appropriate to use a polyfloral pollen blend as the control pollen? Also, since willow pollen contains flavonoids, and that these are heat-sensitive and soluble in organic solvents, did the authors consider performing any treatment to decrease the concentration of flavonoids in the control pollen?

It would also be very useful to have a comparative table of flavonoids present in heather and willow pollen. Is the value supplied in Appendix A the concentration of flavonoids measured in the willow pollen? Please clarify this issue.

### **Authors' response:**

Thank you for your comment, the choice of control diet does indeed need more explanation that were added in the manuscript (Lines 118-120). We chose a control pollen that did not contain the flavonoids present in heather pollen so that we could describe their effects. Using multifloral pollen would have increased the probability of having these flavonoids, besides, these multifloral pollen can contain heather pollen which would not have allowed to compare its impact. The ideal would have been to use an artificial pollen substitute not containing flavonoids to study the effect of the presence/absence of all flavonoids, but these diets do not allow the development of *Bombus terrestris* microcolonies (Gekière et al., 2022. *Journal of Economic Entomology*, 115(5), 1423-1431). We therefore opted for a willow control which is suitable for the growth of microcolonies and contains none of the flavonoids present in heather pollen. Willow pollen was not treated to reduce these flavonoids, but we thank you for this recommendation which we will try to apply in future experiments.

We have identified and quantified the flavonoid profile of willow and heather pollen. However, these results have been incorporated into another paper that focuses more on chemical analysis

and is currently in the process of being published. This paper deals with the variation of specialised metabolites throughout the plant and the impact of these different flavonoids on plant-herbivore interactions. I have added some results below but unfortunately, they cannot be included and discussed in this manuscript. We clarified the table in Appendix A (Lines 732-733).

**Table S2.** Flavonoid identified in willow and heather pollen.

Pollen type	Elemental composition	Full name	$\Delta$ ppm
Willow pollen	C <sub>27</sub> H <sub>30</sub> O <sub>17</sub>	Quercétine-7-O-dihexoside	0,3
	C <sub>28</sub> H <sub>32</sub> O <sub>17</sub>	Methoxykaempférol-3-O-dihexoside	0,9
	C <sub>26</sub> H <sub>28</sub> O <sub>16</sub>	Quercétine-3-O-hexosylpentoside	2,2
	C <sub>28</sub> H <sub>32</sub> O <sub>16</sub>	Isorhamnétine-3-O-hexosylrhamnoside	0,8
	C <sub>33</sub> H <sub>40</sub> O <sub>20</sub>	Kaempférol-3-O-dihexosylrhamnoside	4,8
	C <sub>27</sub> H <sub>30</sub> O <sub>15</sub>	Kaempférol-3-O-hexosylrhamnoside	1
Heather pollen	C <sub>27</sub> H <sub>30</sub> O <sub>15</sub>	Kaempférol-7-O-hexosylrhamnoside	1
	C <sub>30</sub> H <sub>26</sub> O <sub>13</sub>	Kaempférol-O-coumaroylhexoside	7
	C <sub>39</sub> H <sub>32</sub> O <sub>15</sub>	Kaempférol-O-dicoumaroylhexoside	0,4
	C <sub>36</sub> H <sub>36</sub> O <sub>18</sub>	Kaempférol-O-coumaroyldihexoside	1,2

### Reviewer 2 minor comment 2:

Line 120: The commercial bumblebee colonies very often carry pathogens, so bumblebees may have been infected by some parasite/s before starting the experiment. According to the information provided in the methods, the purchased colonies were not checked for the presence of potential pathogens before starting the experiment. Unless the supplier provided a guarantee that they were parasite-free colonies, the possible influence of other pathogens in the results cannot be discarded, and this should be mentioned in the discussion.

### Authors' response:

Many thanks for spotting this gap in information that was crucial to our study. We analysed the faeces of the parent bumblebee colonies from which we obtained our individuals. These analyses enabled us to confirm the absence of *Nosema spp.*, *Crithidia spp.* and *Apicystis spp.* However, we are not certain of the absence of other pathogens not visible in the faeces in these colonies. Viruses such as DWV could be present in our colonies, but we have not seen any symptoms of such viruses in the individuals sampled or in those left in the parent colonies. The supplier guarantees the absence of such parasites in its colonies. With this statement and our controls, we are confident of the absence of parasites in our bumblebees at the start of the experiment. Some information has been added to the manuscript (Lines 128-130).

**Reviewer 2 minor comment 3:**

Lines 138-141: Honeybee collected pollen can also carry some pathogens. As mentioned above, if there is no guarantee that pollen pellets were parasite-free, this should be mentioned in the paper.

**Authors' response:**

Thank you for pointing out this limitation which was mentioned in the manuscript (Lines 147-149). However, our uninfected colonies were subjected to faeces check at the end of the experiment (Lines 211-212). Since we were able to prove the absence of parasites in these colonies, we believe that the diet was indeed parasite free otherwise we would probably have observed parasites in the faeces of these bumblebees after 35 days.

**Reviewer 2 minor comment 4:**

Line 146: As far as I understand from the table in Appendix A, the control willow pollen, and the heather pollen was spiked with a similar amount of ethanol than the flavonoids-supplemented willow pollen. This information would be useful here, just a sentence mentioning it, to help repeatability of the assay.

**Authors' response:**

Indeed, we agree that this can help repeatability and this has been added to the manuscript (lines 159-160).

**Reviewer 2 minor comment 5:**

Lines 150-160: I found the identification of *Crithidia bombi* by microscope problematic. At least four different trypanosomatids (with similar morphology) have been detected in *Bombus terrestris* (Bartolomé et al., 2021. Environ Microbiol. 2021 Jan;23(1):478-483). Unless the source of *C. bombi* pool was analysed by a PCR at some stage, it can't be proved that the trypanosomatid inoculated and observed under the microscope was *C. bombi*. If the authors kept the faeces samples or the inoculated dead bumblebees, I would recommend using molecular tools to corroborate the identity of the pathogens inoculated. If doing this additional analysis is not feasible, the possible misidentification of the trypanosomatid used in this study should be mentioned somehow in the discussion.

**Authors' response:**

Thank you for bringing this problem to light. We are not certain that the parasite observed is indeed *Crithidia bombi* and we no longer have any material enabling us to carry out a genetic analysis of the parasite pool used. It is indeed possible that we are dealing with *C. expoeki*, a cryptic species or other species of *Crithidia sp.* indistinguishable of *C. bombi* by microscopy. Despite *C. bombi* being by far more abundant in wild populations (Shykoff, J. A., & Schmid-Hempel, P. (1991). *Apidologie*, 22(2), 117-125; Popp, M., et al. (2012). *MicrobiologyOpen*,

1(4), 362-372; and referred in the article you mention here), we admit that we cannot differentiate *Crithidia* species and have modified the manuscript by referring to the parasite as *Crithidia sp.* throughout (see similar article Gekière, A. et al., 2023. Comptes Rendus. Chimie, 26(S2), 1-15.). Clarifications about this possible misinterpretation have been made in the manuscript (Lines 162-165).

**Reviewer 2 minor comment 6:**

Line 158: To help reproducibility of the experiment, it would be useful to describe the method used to supply the 10uL inoculate to the bees.

**Authors' response:**

More details on the parasite inoculation method were added to the manuscript (Lines 173-175).

**Reviewer 2 minor comment 7:**

Lines 337-341: In the paper it is not mentioned if the males were analysed for pathogen presence, but here in the discussion, it is assumed that they were infected just by developing in microcolonies with inoculated workers. I find the hypotheses described here plausible and interesting, but it should also be considered that maybe not all males, or even neither of them, got infected, and thus, the differences found in fat body content between experimentally inoculated workers and non-inoculated males.

**Authors' response:**

Transmission of *Crithidia spp.* from one member of the brood to another is fairly easy through contact with faeces-contaminated brood material (Schmid-Hempel, P. (2001). *Naturwissenschaften*, 88, 147-158). We have not tested this in the context of heather pollen, but we have already demonstrated and quantified male infection in exactly the same experimental design when studying sunflower pollen (Gekière et al., (2022). *Biology*, 11(4), 545). These results were not published, but analysis of the faeces of mature males confirmed infection in 90% of males in microcolonies where workers had been parasitised. We therefore consider that the presence of the parasite in males is highly likely (more information added Lines 360-362).

**Additional recommender comment:**

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**Additional comment 1:**

I would also recommend focusing the results around effect sizes, rather than on p-values. There are several ways to do so. First, estimates (for categorical factors those are usually mean and SE differences between factors) can be provided in a table in the appendix. This is important specially for researchers doing further meta-analysis. Extracting those from figures is painful. Second, this same estimates can be added when relevant in text, for example, instead of stating a significant increase in parameter X, you can mention which is the raw of percentual mean increase in this parameter. I think this will reinforce some of your results, as highlighting the effect sizes (e.g. a 20% increase in parameter X) will help the readers.

**Authors' response:**

Thank you for your comment. We have added the tables with the estimates of our variables as appendices to make it easier to re-use our data (Lines 256-257, 737-739) and have reinforced some of our results by quantifying significant variations (e.g., Line 255).