

Dear Dr. Alisha DSouza

Thank you for the opportunity to submit a revised version of our manuscript JoVE58420 “A visual guide to behavioral defenses to pathogen attack in leaf-cutting ants” by Stephen Nilsson-Møller, Michael Poulsen and Tabitha Innocent for consideration in JoVE.

We appreciate the positive and constructive comments from you and the reviewers and below we outline the changes we have made to the manuscript to accommodate all queries. In addition to these changes, we have made a number of small additional improvements to the manuscript. Line numbers indicated refer to the final revised manuscript without track changes.

We believe that we have addressed all aspects in full and hope the manuscript can be deemed suitable for publication in JoVE.

Thanks in advance for your consideration.

On behalf of all authors,

Kind regards  
Michael Poulsen

CC: [ndt144@alumni.ku.dk](mailto:ndt144@alumni.ku.dk), [tabitha.innocent@bio.ku.dk](mailto:tabitha.innocent@bio.ku.dk)

Dear Dr. Poulsen,

Your manuscript, JoVE58420 A visual guide to behavioral defenses to pathogen attack in leaf-cutting ants, has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300 dpi.

Your revision is due by **Jun 25, 2018**.

To submit a revision, go to the [JoVE submission site](#) and log in as an author. You will find your submission under the heading "Submission Needing Revision".

Best,

Alisha DSouza, Ph.D.  
Senior Review Editor  
[JoVE](#)  
617.674.1888

### Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

[We have now carefully proof-read the manuscript throughout.](#)

2. Please upload videoclips 1 and 3.

[These have now been uploaded as requested.](#)

3. Figure 3: Please change “hrs” to “h”.

[Changed as requested](#)

4. Figure 2 legend: Please define SE.

[Changed as requested](#)

5. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

[We now clearly state the overall goal of this method \(Lines 96-100\).](#)

6. Please use SI abbreviations for all units: L, mL,  $\mu$ L, h, min, s, etc.

[Checked throughout and changed as requested](#)

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

[Changed as requested.](#)

8. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

[Changed as requested.](#)

9. Lines 184-299: Please note that information provided here is a better fit for Representative Results than Protocol. Therefore please combine them into the Representative Results section. Note that Representative Results should be explained in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc.

[This is not a description of an outcome of the protocol, but rather of an essential component in the protocol, which is necessary for readers to correctly use the methods and protocol. The protocol we present is centered on how to observe, identify and define these defensive behaviors in ants, not on how to set up a behavioral experiment per se – methods for which are in widespread use in this field; as such we describe elements of the experimental set-up only as far as they are relevant to being able to observe and describe/identify the behaviors accurately, but these are not the focus. The behavioral identifications, however, are an essential part of the core protocol. We therefore have kept the description as is.](#)

10. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

[These are highlighted with notes throughout the protocol. To avoid redundancies, we have refrained from further discussion them in the Discussion section.](#)

b) Any modifications and troubleshooting of the technique

[This is discussed in Lines 489-93 \(and in notes throughout the protocol\).](#)

c) Any limitations of the technique

[This is discussed in Lines 463-468, 484-491, 493-96.](#)

d) The significance with respect to existing methods

This is discussed Lines 481-487.

e) Any future applications of the technique

This is discussed Lines 396-407, 489-496, 505-508.

11. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

We have uploaded a revised xlsx file with all essential supplies, reagents and equipment.

## Reviewers' comments:

### Reviewer #1:

Manuscript Summary:

Firstly, I would like to thank you for the opportunity to review this interesting study on prophylactic behaviour of leaf-cutting ants. Particularly, as a researcher of leaf-cutting ant behaviour for a long time, I consider this study to be indispensable. Although the terminology used to describe ant behaviour is relatively standardized, it is not certain that different researchers are naming the same behavioural act consistently. The proposal of the work is clear and objective and I believe it will be a reference for several studies on ant behaviour.

Major Concerns:

As suggestion, I think the authors should improve the quality of the images exhibiting worker behaviours. It seems to me the plastic cover of the colonies makes the image slightly blurred.

We agree that this would be desirable, but because the behaviors were monitored and filmed as part of an infection experiment, we needed the lids kept on to ensure high humidity for the fungus garden. Since the slight blurriness does not prevent behaviors from being observed, and since many behaviors are so rare that capturing them on video is not trivial, we have retained the existing footage. This is thus a consequence of the inevitable trade-off between resolution and filming sub-colonies for extended periods of time, which we discussed at length to find an optimal solution prior to carrying out the experiment. We resolved this in ways that we believe provide sufficient quality videos while making the protocol accessible and suitable for researchers at large, and have now discussed this in the context of method limitations in the text (Lines 493-99).

Minor Concerns:

Line 46 - Major workers act as soldier in *Atta* species.

We have removed this specification of soldiers in *Atta*, as it is not essential to the text nor focus of the study.

Line 89 - There are other actinomycete genera isolated from leaf-cutting ants that control *Escovopsis* too.

We have rephrased this sentence to reflect that *Pseudonocardia* is not the only symbiont protecting against *Escovopsis* (Lines 83-7).

Lines 130-133 - The authors should point out the relevance of the queens in colony behaviour.

This is a good point and we now explicitly address in the manuscript that we expect, but cannot guarantee, that queenless colonies are likely to behave as queenright for the short period of time the experiment ran (Line 133-5).

Line 151 - I would consider inoculating a sterile powder (like graphite) to differentiate between an infection with a fungus and an inert agent.

This would have been an improvement to the protocol, so we suggest this now, although we did not

do it in the experiment in the current manuscript (Line 153-55).

Line 160 - The authors seem confident that this is a bacterium of the genus *Pseudonocardia*. How? Previous and recent work indicate that the symbiont population present on the cuticle of *Acromyrmex echinator* is vastly dominated by *Pseudonocardia* (Poulsen et al. Molecular Ecology 2005; Andersen et al. Molecular Ecology 2013). The presence of other secondarily acquired Actinobacteria is predicted to be likely in addition to, rather than instead of, *Pseudonocardia* (Scheuring & Yu, 2012; Worsley et al., 2018) – and, that any additional Actinobacteria are likely to also play a defensive role, and thus not change the behaviors that are focus of this study. We have clarified this in the text and added suitable additional references (Lines 83-7; 166-9; 463-5; plus above references added throughout).

Line 160 - covering more than

We have rephrased this to 'covering most of the cuticle' (Line 167).

## Reviewer #2:

Manuscript Summary:

The introduction is quite good, provides the necessary ant-specific background regarding social immunity, and clearly makes the case for it's protocol.

Major Concerns:

Order of worker introduction into the sub-colonies is unclear and potentially very problematic.

All workers and brood were introduced to sub-colonies at the same time in both control and treatment sub-colonies, and we have edited the protocol in line with the comments below to clarify this (Lines 164-74).

Minor Concerns:

There are several items that need to be addressed:

-In the protocol (B, 1.2) it states that sub-colonies are made 'consisting of 4 minor workers and four major workers' but in B, 1.8 it states that 2 brood are also added. If there is a difference in the demographics of the 0 and 72 hour observations it would require an explanation.

There are no differences in the demographics and we now – due to the comments below – remove the specifications of the demography of ants here to ensure that it is clear that the fungus is added to empty sub-colony containers and that workers and brood are only added later.

-Reading through part B sections 1.1 to 1.3 it seems that the ants are already in the box. But 1.4 to 1.5 indicate that fungus comb is added to an empty box. The order of part B would be better if it stated that comb was added to empty boxes, inoculated and then the ants added (see above comment about demographics). It would also benefit from a brief statement about spore behavior in the absence of ants and how it might influence their behavior.

We agree and have revised accordingly (Lines 142-5, 158-62).

-In addition to the order of operations, I am concerned that there isn't a proper control. While it is important to have the 'spore application control' there is also the basic fact that the ants are removed from their parent colony, transferred, and afforded an opportunity to experience a new setting. This can influence behavior, especially grooming and fungal comb tending, and I would like to see some clarification or assurance that this isn't influencing the results and recorded behaviors.

This is a very relevant point and we now acknowledge in the text that we cannot rule out that behaviors may differ in miniature sub-colonies compared to whole colonies or responses in nature (Lines 170-74, 133-35). This is a general shortcoming of any laboratory experimentation, but does not negate that insights can be obtained. We should also note that we performed the experiment to provide the visual representation of behaviors in the right context (i.e., experimental infection

scenario). We now discuss this explicitly in the manuscript (Lines 481-91).

-There are a couple of points regarding what data was included that require explanation. One is lines 310 - 312. It is unclear what this means. Did you leave out colonies? Are the recordings mainly from 0 hour sub-colonies? Is this why the bottom of figure 3 only has 14 colonies? It is also then curious why the authors feel the need to total the recording time (136hr) because it seems that there isn't this much. These items need clarifying in order for the protocol to have a secure experimental basis and thus application to the broader entomological and ethological communities. We now clearly state which sub-colonies had to be excluded (Lines 183-6) and the consequences for the specific results section and figures (Lines 320-24).

### **Reviewer #3:**

#### Manuscript Summary:

In the article "A visual guide to behavioral defenses to pathogen attack in leaf-cutting ants", the authors produced a library of behaviors of leaf-cutting ants related to fungal cultivar cleaning after an infection with a pathogenic fungus. To do so, they made subcolonies from 3 colonies of *Acromyrmex echinatior* collected in the field. Then they infected half of the subcolonies with a pathogenic fungus of the ants' crop (with two times of infection) and the rest of the subcolonies were kept as controls.

They observed 7 behaviors, which are documented with author produced video clips.

Although they expected to find more cleaning behaviors in infected subcolonies than in the control ones, most statistical analyses gave non-significant results, probably due to the low replication (as mentioned by the authors in the discussion). However, they find tendencies in that direction, with different responses depending on worker size.

I found the clips useful to accurately define the observed behaviors. However I have some comments and suggestions regarding the videos and the methods.

#### Major Concerns:

-Parts A and B of the protocol (which are not highlighted in yellow) seem to be relevant to include in video clips since are important steps in the article. I think that doing so will also help researchers to perform these assays in an easy and standardized way. In addition, they will help to connect more the methodological and visual parts of the article with the results of the contamination with *Escovopsis*, which otherwise are not very relevant in the article due to the non-significance of the differences between the treatment and the control. It would also help to show in the video how leaf-cutting colony rearing is performed in the lab, though that may make it too long.

We agree and have therefore highlighted more of the parts A and B protocols (pages 3-4). We have also clarified in the text that our primary aim was to create the catalogue of behaviors for wider use, and that results are merely representative, to demonstrate how the definitions may be used within an experimental set-up (Lines 94-104, 501-508). We will furthermore discuss with the JoVE film crew what options are there for including video documentation of how laboratory colonies and sub-colony are set up.

-Statistics: mixed ANOVA is not clearly explained. Are original colonies IDs included as the mixed variable? the only fixed factor seems to be the treatment vs control, but what about the worker size factor and its interaction with treatment? In addition, why are statistics reported only for some behaviors and not for others? It would be better to statistically test all behaviors separately or with a MANOVA.

To make this clearer, we include a new Table 2, which provides the test results and the overview of factors included in the ANOVAs.

Minor Concerns:

-L. 51-2: this is a relative sentence and no point for comparison was given (ie, they have less diversity regarding other ants? o insects?). In addition, some leaf-cutting ants have genetic variability due to mating with several males (Hughes & Boomsma 2004. *Evolution*, 58, 1251-1260). In addition, this sentence and those till L. 57 are not relevant to the article and can be deleted or reduced, since authors do not mention the entomopathogenic fungus again.

We agree that this is not crucial for the current study, nor extensively-enough described, so we have removed the sentence and trimmed the introduction to entomopathogenic fungi.

-L. 130: using only 8 workers (4 majors and 4 minors) seems too little for a colony that can contain hundreds of workers. Won't this reduce interactions among workers (ie. allogrooming)?

We now acknowledge that this may affect behavioral responses, and argue for why it is nevertheless a relevant setup (Lines 170-74, and 481-91).

-L. 132: explain why 72 h after infection and not 24h maybe for avoiding the risk of contamination, as mentioned later in the article (L. 310-1).

This is a very good point and waiting 72h certainly poses a risk of contamination/growth of other fungi. However, we chose this time frame to increase the probability that *Escovopsis* would have germinated, as we expected based on unpublished work (Lines 158-62).

-L. 172: why 136h? is not 144h=36 subcolonies\*4h? or am I misunderstanding?

We now specify that two sub-colonies (one control and one treatment) after 72h were excluded, due to heavy infections with fungi other than *Escovopsis*, removing 8hrs of footage (Line 183-86).

-For video clips in general: it would be necessary to highlight the focal ant (maybe with an arrow at the beginning of the clip), since there are more than one ant in most clips. In addition, slow motion would help to clearly see some particular behaviors, after they are played at the normal time frame. Video quality (illumination specially) was not good in the videos.

We fully agree and have planned to work with the JoVE staff to encircle or put arrows within the video frames. The limitations our experimental approach placed upon video quality were discussed above.

-L. 184 and 211: video clips 1 and 3 were not available for review.

These have now also been uploaded for review.

-L. 208: this part is not very clear in the video. Maybe you can add another video with high magnification

We agree, but this is again a result of the necessary trade-off between resolution and using multiple cameras for extended periods of recording, and not feasible within this study or approach.

-L. 256-9: this behavior is not recorded in the clip, it will be interesting to also show that behavior

We agree, but this is not possible in the current setup, as distinct waste piles were not visible in the sub-colonies. However, we now specify that this would be a suitable extension of the current protocol (Lines 269-70).

-L. 272-2: this behavior is not recorded in the clip, it will be interesting to also show that behavior

As above, we now specify that this would be a suitable extension of the current protocol (Lines 287-88).

-L. 310-1: I think that the fact that treatment was cross contaminated is not justifying that authors should not include these results since they are investigating the behaviors to fungal infections in general. Thus, the fact that they were contaminated is not justifying not including those results, but maybe discussing that the behaviors at 72h are not caused only by *Escovopsis* (as was in part mentioned in L. 387-90).

We agree that risk of other fungal infection is possible, and that *Escovopsis* is not the only fungus that could provoke a behavioral response. We have clarified this and the specific sub-colonies that

were excluded from analysis in the protocol and results (Line 183-6, 320-24). We also make clearer our main objective, to create a catalogue of behaviors as a tool for future research, while acknowledging the limitations of the small, illustrative behavioral experiment we use to demonstrate how this might be used in other studies (Line 481-499).

-L. 313: not clear what "timing" means in this context, please, specify

We have rephrased this to 'time after infection' (Line 325).

-Table 2: it is redundant with Table 1, and I suggest deleting it

We agree and have merged the previous Tables 1 and 2 into a single new Table 1.

-Figure 3: y axis should say "mean frequency" instead of "mean number"

Changed as requested.

-L. 423-5: not clear what "nest border" means, and the sentence is not very clear. Please, clarify.

The same for the next sentence.

We have rephrased this to "edge of the fungus garden" for clarity (Lines 433-37).

-L. 439: differences between Pseudonocardia carrying workers and not Pseudonocardia-workers (two of each in every subcolony) were not reported or analyzed. Probably this added noise to statistical results when analyzing grooming. I suggest considering this factor in ANOVAs

We did not distinguish behaviors between these worker groups, because the presence/absence of Actinobacteria was not treated as a factor but merely done to include several worker groups (minors, younger majors, older majors) in the sub-colonies. It could however be included as an extension of the current protocol, which we now specify (Lines 170-74 and 491-93).