Dear Editor

We thank you and the reviewers for their careful reading of our manuscript and their constructive criticisms. A point-by-point response to their comments is provided below with our answers in blue. We hope that the revised manuscript will now be acceptable for publication.

**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 carefully checked wards and grammar in the current manuscript.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account.

> In this paper, we used modified data and figures published in PLOS GENETICS (Miyakawa and Mikheyev 2015) and Insect biochemistry and molecular biology (Miyakawa et al 2018). According to the Licenses and Copyright in PLOS (http://journals.plos.org/plosgenetics/s/licenses-and-copyright) and Elsevier (https://www.elsevier.com/about/policies/copyright/permissions), we can reuse published data.

The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

>We wrote citation at all reused data and figures (Figure 1, 4 and 5).

3. Please revise the title to be more concise.

>We changed title: Protocols for the induction and evaluation of inbreeding crosses using ant, *Vollenhovia emeryi*

4. The current Abstract is over the 150-300 word limit. Please shorten the Abstract and rephrase it to more clearly state the goal of the protocol.

>We simplified abstract (188 wards) and emphasized goal of the protocol “Here we describe the methods for conducting inbreeding crosses and for evaluating the success of those crosses in ant *Vollenhovia emeryi*”.

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

>In new introduction, we emphasized that the goal of our protocol is to induce crosses in the laboratory, and to evaluate occurrence of inbreeding using *V. emeryi* (line 88-91).

6. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

> A space is included in all units in current manuscript except “%”.

7. 1.1: Please add more details here. For instance, where are the ants collected? Please also move information in Note to step 1.1.

> We added geographical information for ant collection in Note step 1.

8. 3.3.2, 3.4.7: Please add more details to this step. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

>We omitted “3.3 Genotyping using microsatellite markers” as reviewer #3 suggested and just cited our paper. We added step 3.3.10 - 3.3.14, which are about how to observe samples by confocal laser microscope. We added equipment into JOVE materials list.

9. 3.4.1: What is used to dissect?

> We wrote “using forceps” (step 3.1.2, 3.3.1 and 3.3.8)

10. 3.4.2: What is the incubation temperature?

>We wrote “at room temperature (15 – 25 °C ) at step 3.3.3 and 3.3.7 ”

11. 3.4.3: What volume of PBS is used to wash? Please specify throughout. How and where to mount tissue?

> We wrote volume of PBS at step 3.4.4 and 3.3.8

**Reviewers' comments:**

**Reviewer #1:**

Manuscript Summary:

The authors describe a protocol for inbreeding *Vollenhovia emeryi* ants. The manuscript seems reasonable as a companion to a visualization showing how the crosses occur. I have only a few minor comments based on the written manuscript, which I have provided below. I did not see any major concerns with what was presented and expect that visual information would be helpful to understanding the entire procedure.

Major Concerns:

My only major comment is that these procedures are likely to work in only a single species of ant. The authors explain that this is likely to be the case, as this species has a very unusual reproductive and genetic system. Regardless, I don't think this is a fatal problem. It just means that the procedures are not widely applicable.

> We agree that our protocol may be not applicable for experiments using other ant species, but it will be a hint for them to try laboratory crosses. In the current manuscript, we added detail steps for inducing inbreeding crosses in the laboratory (i.e., step 2.3).

Minor Concerns:

36 Capitalize Hymenoptera here and elsewhere in the manuscript.

> We capitalize Hymenoptera in the manuscript (line 19, 36, 95 and 256).

Fig 2 Might be helpful to have a ruler in the photo so the reader knows the size of the nest etc.

> We took a picture of artificial nests with ruler (Figure 2).

126 Removing the mid-legs of workers is interesting. Is there any evidence that this affects the viability of the workers? If not, this should be mentioned.

>We do not have evidence about worker viability, but they can survive during experiment.

138 How do you know if inbreeding has occurred?

> We added next sentence “After performing inbreeding crosses between F1 offspring, eggs can be observed under a stereoscopic microscope” (line 149-151, step 2.6).

142 How does one "induce inbreeding crosses"? I suppose this will be clear from the video.

> We just keep the experimental colony and wait mating there. It is difficult to observe inbreeding crosses since *V. emeryi* avoid light. We can indirectly know the occurrence of inbreeding by other evidences, such as shed wings of queens, eggs produced by mated queens, and genotyping of offspring.

157 I don't know if you will have images of how you dissected the reproductive parts of the ants. But this would be useful.

> We will describe how to dissect reproductive organ in our video.

Fig 5 This figure should have some numbers on the y axis.

> We omitted this figure and wrote only result (line 243).

**Reviewer #2:**

Manuscript Summary:

This manuscript describes an efficient protocol for conducting inbreeding experiments for the ant *Vollenhovia emeryi.* Such experiments can be used for genetic studies, especially QTL and other genetic mapping studies. The methods here are also broadly useful for other ant species that can mate in the lab, with perhaps minor species specific modifications.

I only have some minor comments for clarification.

Minor Concerns:

1) Lines 65-68. This is only applicable for those species employing complementary sex determination (csd). Some Hymenopteran species do not do this, e.g. Nasonia (Verhulst et al 2010 Science). See also Heimpel and de Boer 2014 Annu Rev Entomology.

> We rephrase that “In eusocial Hymenopteran taxa, such as ants and bees” (line 57).

2) Line 95-96. "… inducing inbreeding crosses is an essential first step…". I would qualify with "often" because other methods may work, such as genome wide association mapping.

>We rephrase that “application of inbreeding crosses is often essential first step in any investigation of sex determination systems in the Hymenoptera” (93-95).

3) Lines 97-98. "… existence of a CSD locus in the objective species". Objective sounds awkward; suggest directly expanding the sentence to explain the experiment and species.

>We wrote example, “For example in *Cardiocondyla obscurior*, the almost complete absence of diploid males after 10 generations of brother-sister mating in the

laboratory demonstrates absence of CSD locus (line 95-97) .

4) Lines 138-140. Unclear. Exclude which males? Do you mean remove the F1 males (the one used for the cross) or remove future males that emerge, or perhaps something else? Clarification needed.

> We wrote that “remove F1 males” (line 153).

5) Lines 151-152. "… because they lost their wings when the colonies were collected in the field." Suggest rephrase because this sentence could be interpreted as the F0 queen only lose their wings during the collection (in contrast to losing the wings after mating, so were already without wings for a long time).

>To avoid confusion, we deleted this sentence in current manuscript. F0 queens are easily distinguishable from F1 queens because they have existed in the colony before new winged- reproductives arise.

6) Lines 154-155. "flash mixing". May need to elaborate on this term - is this flash freezing in liquid nitrogen and then homogenizing in a bead shaker?

>We rephrase that “Flash spin down the microtube and store them at 4 °C” (line 184-185, step 3.1.5)

7) Line 167. "Follow step 3.1.2 for DNA extraction." Is this "3.2.1"?

> We omitted this step in the current manuscript.

8) Line 226. "As a result of dissection…" What about rephrasing to "Dissection of haploid males revealed testes and sperm, as expected." Or similar?

>Thank you for your suggestion. We rephrase them as you suggested (line 250).

9) Line 271. "Testes and sperm [singular] of diploid males were never observed (D and F)." Would it be possible to provide a sample size (or perhaps N>>###).

> We added sample size (N >> 30) in line 299.

10) Lines 288-290. Fire ant diploid males can sometimes be fertile (not sterile). See Krieger et al 1999 Heredity.

>Thank you for your suggestion. We added this citation (line 318).

11) Line 318. "lethal", perhaps "inviable" would be better?

>We use “inviable” instead of lethal as you suggested (line 346).

**Reviewer #3:**

Manuscript Summary:

This manuscript describes experimental protocols for rearing and crossing the ant *Vollenhovia emeryi*. The manuscript is well-written and easy to follow. While this particular cross is of interest to a fairly niche group of scientists, crossing ants is generally quite difficult, and I suspect that a detailed protocol for how to accomplish it in Vollenhovia would inspire and help other ant biologists to attempt such crosses for other species. That being said, the level of detail in the manuscript, and the focus, needs substantial revision before it can be published, for reasons I will go into below.

Major Concerns:

The manuscript borrows liberally from two published works be the first author, without properly acknowledging what is original and what is cited, which amounts to self-plagiarism. For example, lines 54-57 in the abstract state "In this study, using offspring produced by inbreeding crosses, linkage mapping of quantitative trait loci (QTL) for traits related to sex determination was performed for the first time in ants. In addition, the molecular mechanisms underlying sex determination were investigated." As far as I can tell, this actually refers to prior work the protocols for which are being described in greater detail here. This happens at other points in the manuscript, where data form previous studies are presented as original data (e.g., Figures 5, 6 and possibly 7 unless it is modified to include details of dissection/staining). Very clear distinctions need to be drawn between published work and protocol description. The authors spend far too much time talking about what can be done with the protocol, which has already been published, versus the protocol itself.

> We carefully checked manuscript including abstract and figure legends. We cited published works at all necessary points. We rephrased “This figure has been modified from [Miyakawa *et al*. 2018]” in Figure legends. We omitted Figure 5, and wrote only average of proportion of diploid males produced by each inbred queens (line 243).

It is first time to show the image of Figure 6 (it was Figure 7 in first submitted document). In Miyakawa and Mikheyev 2015, we showed that diploid males do not produce sperms. After publication of Miyakawa and Mikheyev 2015 however, we recognized that testes of diploid males do not develop. Therefore, we showed images of whole internal reproductive organ of haploid and diploid male in this JOVE paper.

The protocols themselves are not very well described. Although a video may help this, the base level of details is typically insufficient and does not exceed the level of previously published detail. For example, Miyakawa and Mikheyev describe the laboratory rearing conditions as follows: "Experimental colonies were provided dry crickets, sugar water, and distilled water every other day. These colonies were kept in artificial plaster nests at 25 C, 50-60% humidity and a 12-hour light/dark cycle." The current manuscript provides this level of detail: "Maintain colonies in the artificial nest at 25°C under a 16/8 h light/dark cycle. Provide tap water, dry crickets and brown sugar every other day until new reproductives (queens and males) emerge." As you can see, the level of detail is essentially the same, except for changes to the light/dark cycle. Important details that could be expanded upon, such as how many crickets, how much water, etc. are omitted.

> As you suggested, we added details in protocol (line 118, step 1.3) and in Figure 2 about method to maintain colonies in the laboratory as follows, “Maintain colonies in the artificial nest at 25 °C under a 16/8 h light/dark cycle. Provide tap water by a wash bottle to wet the plaster. Add about 100 mg of dry cricket powder wrapped in aluminum foil and a brown sugar water-filled tip (20 µl tip) every other day until new reproductives (F1 winged-queens and F1 males) emerge”.

In general, the manuscript can't quite decide what methods it wants to present. It veers into microsatellite genotyping, and even presents some results, but referring to original work for the details. I strongly suggest getting rid of all that superfluous material and focusing on describing the crosses themselves in much greater detail.

> We agree. In new manuscript, we minimized protocols and results of DNA extraction and genotyping. We added effective steps and NOTEs for inducing inbreeding crosses, such as step 2.3. As other reviewer suggest, we also added steps 3.3.10 - 3.3.14 to indicate how to observe male internal organ using confocal microscope.

Minor Concerns:

The ant is repeatedly referred to as the "Japanese ant *Vollenhovia emeryi*". This is incorrect, because this species is found in much of Asia, and "Japanese ant" is not its common name. In fact, the manuscript needs to address taxonomy of the ant used in study, particularly the short- and long-winged forms, and whether the current protocol is suited for both of them or not.

>We omitted “Japanese” from new manuscript. We described about wing morphs of *V. emeryi* in NOTE of protocols step 1 as follows, “This species shows two types of colonies, i.e., (1) colonies producing only long-winged queens and (2) colonies mainly producing short-winged queens in addition to small number of long-winged queens 8, 14”.