Journal of Visualized Experiments

Induction and evaluation of inbreeding crosses using the ant, Vollenhovia emeryi

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58521R1
Full Title:	Induction and evaluation of inbreeding crosses using the ant, Vollenhovia emeryi
Keywords:	inbreeding crosses, Hymenoptera, Vollenhovia emeryi, sex determination system, diploid males, complementary sex determiner
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Question	**Response**
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TITLE:

Induction and Evaluation of Inbreeding Crosses Using the Ant, *Vollenhovia Emeryi*

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KEYWORDS:

inbreeding crosses, Hymenoptera, *Vollenhovia emeryi*, sex determination system, diploid males, complementary sex determiner

SUMMARY:

In this protocol, methods for conducting inbreeding crosses, and for evaluating the success of those crosses, are described for the ant *Vollenhovia emeryi*. These protocols are important for experiments aimed at understanding the genetic basis of sex determination systems in Hymenoptera.

ABSTRACT:

The genetic and molecular components of the sex-determination cascade have been extensively studied in the honeybee, *Apis mellifera*, a hymenopteran model organism. However, little is known about the sex-determination mechanisms found in other non-model hymenopteran taxa, such as ants. Because of the complex nature of the life cycles that have evolved in hymenopteran species, it is difficult to maintain and conduct experimental crosses between

these organisms in the laboratory. Here, we describe the methods for conducting inbreeding crosses and for evaluating the success of those crosses in ant *Vollenhovia emeryi*. Inducing inbreeding in the laboratory using *V. emeryi*, is relatively simple because of the unique biology of the species. Specifically, this species produces androgenetic males, and female reproductives exhibit wing polymorphism, which simplifies identification of the phenotypes in genetic crosses. In addition, evaluating the success of inbreeding is straightforward as males can be produced continuously by inbreeding crosses, while normal males only appear during a well-defined reproductive season in the field. Our protocol allow for using *V. emeryi* as a model to investigate the genetic and molecular basis of the sex determination system in ant species.

INTRODUCTION:

Eusocial Hymenopteran taxa, such as ants and bees, have evolved a haplodiploid sex-determination system in which individuals that are heterozygous at one or more complementary sex determination (CSD) loci become females, while those that are homo- or hemizygous become males (**Figure 1A**)[1].

Genetic and molecular components involved in the sex determination cascade have been well studied in the honeybee, *Apis mellifera*, a hymenopteran model organism[2-4]. Recent comparative genomics investigations suggest that ants and honeybees share many putative homologs in the sex determination pathway, such as the initial sex determination gene, *csd*[5]. However, evidence for the functional conservation of these homologs is still lacking in ants.

To address this problem, inbreeding lines need to be developed as they are essential for genetic mapping and molecular studies. However, it is difficult to maintain and conduct experimental crosses between these organisms in the laboratory because of the complex nature of the life cycles that have evolved.

Here, we use *Vollenhovia emeryi* as a model to investigate the genetic and molecular basis of the sex determination system in ants[6,7]. The inbreeding lines of this species were developed previously for linkage mapping of quantitative trait loci (QTL) for traits related to sex determination for the first time in ants[6]. In addition, the molecular sex-determination cascade has been investigated[7]. This species has evolved an unusual reproduction system that employs

both gynogenesis and androgenesis (**Figure 1B**)[8,9]. Most new queens and males are clonally produced from the maternal and paternal genomes, respectively. In addition, workers and some queens are produced sexually[8]. This reproduction system is particularly well suited to genetic studies because the inbreeding crosses produced using sexually produced queens and males are genetically equivalent to a classic backcross. Since sexually produced queens differ morphologically from queens produced from maternal genomes[10] (**Figure 1B**), conducting and evaluating inbreeding crosses is greatly simplified using this method.

In this article, the methods for establishment of laboratory colonies for crossing test, application of inbreeding crosses using full-sib pairs, and evaluating the success of those crosses using genotyping of colony members and dissection of male offspring genitalia are described in *V. emeryi*.

Regardless of the reproduction system employed, application of inbreeding crosses is often the essential first step in any investigation of sex determination systems in the Hymenoptera. For example in *Cardiocondyla obscurior*, the almost complete absence of diploid males after 10 generations of full-sib mating in the laboratory demonstrates absence of CSD locus[11]. It is possible to predict the number of CSD loci from the ratio of males produced in inbreeding crosses[6,12,13].

PROTOCOLS:

1. Field Collection and Maintenance of *V. Emeryi* Colonies in the Laboratory

Note: Nests of *V. emeryi* are found in rotting logs and fallen decaying tree branches in secondary forests throughout Japan. 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]. In this protocol, we collected the latter type of colonies in Ishikawa prefecture, Japan.

1.1. Collect *V. emeryi* colonies during early summer.

Note: To obtain sufficient numbers of sexual individuals during the reproductive season, colonies containing more than 300 individuals are preferred.

1.2. Transfer the ant specimens from the collected branches to an artificial plaster nest with a glass cover using an aspirator (**Figure 2**, left).

1.3. Maintain colonies in the artificial nest at 25 °C under a 16:8 h light/dark cycle. Provide tap water with a wash bottle to wet the plaster.

1.3.1. 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 (F_1 winged-queens and F_1 males) emerge.

2. Experimental Laboratory Crosses

Note: New reproductives start to emerge from late summer to autumn (**Figure 3**). Long-winged queens are produced sexually, and short-winged queens are produced clonally and have the maternal genome (**Figure 1**). Use long-winged queens and males for inbreeding crosses.

2.1. To stop individuals from moving, place colonies in a constant environment room at 4 °C for 15 min.

2.2. Remove the mid-legs of 30 workers using forceps under a stereoscopic microscope and transfer them into new smaller plaster nest (**Figure 2**, right) for inbreeding crosses.

Note: The legs are removed to distinguish the present workers from the workers that will be produced by the subsequent inbreeding crosses.

2.3. Add 3-4 larvae or pupae into a plaster nest containing workers.

Note: Workers show exploratory activity in the F_0 queens-less colony. Larvae or pupae can effectively attract these workers and new reproductives in the center of the colony during

crossing test. As a result, keep conditions of the experimental colony close to the normal colony.

2.4. Transfer a long-winged queen and a male into a plaster nest prepared in step 2.3 for inbreeding crosses.

2.5. Keep colonies at 25 °C under a 16:8 h light/dark cycle with food and water provided as described in 1.3 until the queen lose her wings and lay eggs.

Note: This takes one week to a month.

2.6. Check the experimental colony everyday under a stereoscopic microscope. After performing inbreeding crosses between the F_1 offspring, eggs can be observed under a stereoscopic microscope.

2.7. After the F_1 queen starts laying eggs, remove F_1 males and larvae or pupae added in step 2.3 from the nest to avoid mixing of the F_1 generation (males and females used for inbreeding crosses) and the F_2 generation (offspring produced from inbreeding crosses).

Note: If there are few males in the colony, it is possible to induce inbreeding crosses using one male and 1 to 3 queens in the same experimental colony.

2.8. Keep colonies under the same conditions as described in 1.3, until F_2 offspring emerge.

Note: Transfer F_1 queen and F_2 offspring into new larger plaster nest (**Figure 2**, left) for long-term colony keeping.

3. Evaluation of Inbreeding Success

3.1. DNA extraction and genotyping of the parental generation (F_0)

3.1.1. Remove one leg of a F_0 queen using forceps and transfer the leg to a 1.5 mL microtube containing 100 µL of chelation agent.

3.1.2. Under a stereoscopic microscope, dissect a female abdomen in glass dish filled with 300 µL of ultrapure water using forceps and isolate the spermatheca containing the sperm from mated males.

3.1.3. Peel away the tissue of the spermatheca and isolate the sperm from the tissue of the female using insect pins.

Note: To facilitate sperm extraction from the spermatheca, store female specimens in 100% EtOH for more than one day before dissection.

3.1.4. Using a micropipette, transfer the sperm into a 1.5 mL microtube containing 100 µL of chelation agent.

3.1.5. Incubate samples of F_0 queen and sperms prepared in step 3.1.1 and 3.1.3, respectively, at 95 °C for 20 min. Flash centrifuge the microtube and store at 4 °C.

3.1.6. Genotype all samples using method described elsewhere[4].

3.2. DNA extraction from the pair of ants used for inbreeding crosses (F_1)

3.2.1. After confirming egg production by the sib mated F_1 queen, extract the DNA of the queen using her shed wings or one mid-leg and genotype using same method described in section 3.1 above.

3.2.2. Extract DNA of F_1 male using one leg and genotype them using same method described in section 3.1 above.

Note: Samples can be stored in 100% EtOH before DNA extraction, and DNA in chelation agent can be stored for two months at 4 °C.

3.3. Evaluation of male fertility in males produced from inbreeding crosses

Note: Diploid males produced from inbreeding crosses are often sterile.

3.3.1. Dissect internal reproductive organs in a glass dish with 400 µL of PBS solution using forceps.

3.3.2. Remove PBS and add 4% paraformaldehyde (PFA) using a micropipette.

3.3.3. Fix the tissue by incubating in PFA for 30 min at room temperature (15 – 25 °C).

3.3.4. Wash tissue 5 times with 400 µL of PBS using a micropipette.

3.3.5. Dilute the 4',6-diamidino-2-phenylindole (DAPI) solution to 1 µg/mL in PBS.

3.3.6. Remove PBS and add approximately 300 µL of this dilute DAPI staining solution to tissue.

3.3.7. Incubate 15 min under dark condition at room temperature (15 – 25 °C).

3.3.8. Wash tissue 5 times with 400 µL of PBS, and transfer tissue on center of slide glass using forceps.

3.3.9. Mount tissue on mounting medium containing Tetramethylrhodamine (TRITC)-conjugated phalloidin.

3.3.10. Observe samples by confocal laser scanning microscope using 20X or 63X objective lenses.

3.3.11. Use a 405 nm excitation laser and a hybrid detector at 410-530 nm for DAPI detection.

3.3.12. Use a 561 nm excitation laser and a hybrid detector at 565-650 nm for TRITC detection.

3.3.13. Use a scan speed of 400 Hz (400 lines/s) at a resolution at 1024 × 1024 pixels.

3.3.14. Capture images using a software platform.

REPRESENTATIVE RESULTS:

Results of microsatellite analysis using F_0 and F_1 generations showed that inbreeding crosses were produced successfully (**Figure 4**)[6]. As a result of inbreeding crosses, mated queens were obtained within one month of establishing the experimental crossing colonies. A quarter (27.1 ± 8.91% SD) of all offspring (F_2) from the inbreeding crosses was male, while the remainder was female (workers and a queen)[6]. QTL mapping using the offspring from inbreeding crosses showed that males produced by inbreeding crosses were diploid and homozygous at two CSD loci (CSD1 and CSD2 in **Figure 5**), while females (workers) produced from inbreeding crosses were diploid and heterozygous at least at one CSD locus[6].

Dissection of haploid males revealed testes and sperm, as expected (**Figures 6A**-**6C**). However, in diploid males, sperms were never observed, suggesting that males produced in inbreeding crosses are sterile in *V. emeryi*[6]. In addition, testes of diploid males failed to develop (**Figures 6D**-**6E**).

Figure 1: **Typical reproductive system in (A) Hymenoptera and the atypical reproductive system involving androgenesis and gynogenesis in (B) *V. emeryi*.** Typically, females (workers and queens) develop from fertilized diploid eggs, and males develop from unfertilized haploid eggs containing half of the maternal genome (A). In *V. emeryi*, sterile workers and a few long-winged queens (LWQ) develop from fertilized diploid eggs, while short-winged queens (SWQ) develop with nearly complete maternal genomes from unfertilized diploid eggs (gynogenesis). Males never inherit maternal genomes but are clones of their fathers (androgenesis) (B). This figure has been modified from [Miyakawa *et al.* 2018][7].

Figure 2. Experimental set up of *V. emeryi* colonies. After field collection, colonies are transferred into an artificial plaster nest and kept in the laboratory. A large plaster nest (left) is prepared for maintain collected colonies, whereas a smaller plaster nest (right) is prepared for experimental inbreeding crosses.

Figure 3. New *V. emeryi* reproductives emerge during the reproductive season. Mature and well-fed colonies tend to produce long-winged queens (LWQ) with the parental genome in addition to short-winged queens (SWQ) which bear only the maternal genome (**Figure 1B**). Photo courtesy of Taku Shimada.

Figure 4. Design of inbreeding crosses and microsatellite genotypes of F_0 and F_1 generations. Using 11 microsatellite markers developed in previous studies[6,8,9], females and males of the parental generation (F_0) showed different genotypes. The genotypes of females and males used for experimental crosses (F_1) inherited the parental and paternal genotypes, respectively, indicating that females were crossed successfully with their brothers, with which the females shared half their genomes. Numbers indicate lengths of PCR products at microsatellite locus *L-5*, which is one of the markers used for genotyping[6,8–10]. This figure has been illustrated according to the data from [Miyakawa and Mikheyev 2015][6].

Figure 5. Allele patterns of two CSD loci (CSD1 and CSD2) in offspring produced by inbreeding crosses. Proportion of diploid males (about 25%) and QTL mapping using offspring produced by sib-mated queens suggest the existence of two CSD loci in *V. emeryi*. Females are heterozygous in at least one of the two CSD loci whereas males are homozygous at all loci. Genotypes are represented by letters of the alphabet. This figure has been modified from [Miyakawa *et al.* 2018][7].

Figure 6. Male internal reproductive organs of androgenetic haploid and diploid males in *V. emeryi*. Morphologies of testes and other internal reproductive organs dissected out from the androgenetic haploid males (A). Sperm (fibrous tissue) could be seen in testes of haploid males (B and C). Blue color marks nuclei stained by DAPI, and red color marks F-actin stained by Tetramethylrhodamine (TRITC)-conjugated phalloidin in B, C, and E. (a) accessory glands; (t) testes; (v) vas deferens; (g) external genitalia. Morphologies of internal reproductive organs

dissected out from the diploid males (D). Testes and sperm of diploid males were never observed (D and E, N >> 30).

DISCUSSION:

This article demonstrates protocols that can be used to induce inbreeding crosses and evaluate the occurrence of inbreeding in the ant *V. emeryi*. In the experiments, genotyping of the individuals used for crosses is necessary to ensure that inbreeding crosses were successful. However, the effectiveness of these crossing tests is clearly apparent as diploid males can be produced throughout the year, while haploid males can only be produced in autumn in both the field and the laboratory[6]. Sib-mated queens start to produce male offspring immediately after crossing. No morphological phenotypic differences were observed between diploid and haploid males in *V. emeryi*[6,7]. However, diploid male *V. emeryi* almost invariably fail to develop testes. In the absence of genetic markers for testing the genetic relatedness of pairs used for crossing tests, the reproductive potential of male offspring can be used to infer whether inbreeding has occurred. However, it should be noted that males produced in inbreeding crosses are not always sterile in other hymenopteran species[15–17].

The first critical step in the success of the protocol is the maintenance of well-fed colonies, as feeding them after field collection will increase the likelihood of obtaining sufficient numbers of reproductives for crosses. In *V. emeryi*, a positive correlation has been reported between nutrition and the production of long-winged queens, which are the queens used for the inbreeding crosses[10]. In social insects, small colonies, or colonies in poor health, tend not to produce new reproductives[18]. It is therefore important to collect mature colonies from the field and to provide them with adequate amounts of nutritious food for experiments using new reproductives.

The second critical step is to keep workers, reproductive and few larvae or pupae together during the crossing tests and to maintain the experimental crossing colony at the same state as that of a normal colony until crossing is completed (for a week to a month). It is difficult to maintain colonies that are to be used for crossing tests without workers for more than 3 days because males are unable to feed themselves and must be fed by workers. Under such unnatural conditions, the success rate of inbreeding crosses was extremely low[6].

There are two limitations regarding the application of these protocols to other ant species. First, the cues for inducing crosses are species specific. It is relatively easy to induce laboratory crosses in *V. emeryi* since intra-colony mating without flight occurs in nature. However, many ant species have evolved mating rituals that involve nuptial flights during which new queens and males mate during or after flight[19]. It is therefore important to elucidate the triggers that induce crossing in each species in a laboratory setting. For example, in the parasitic ant *Acromyrmex ameliae*, the main stimulus for triggering nuptial flights appears to be light[20]. The second limitation is, in some cases, the diploid males produced by inbreeding crosses cannot be collected as they are inviable or killed by workers because they do not work and/or have no or low reproductive potential, and are thus a major cost to the colony of the objective species[21–23]. Fortunately, diploid *V. emeryi* males are not killed by workers and they live until they die naturally, which suggests that they are not frequently encountered in nature and a strategy to exclude diploid males from colonies has not evolved in this species.

Compared to other ant species that employ arrhenotokous parthenogenesis to reproduce (**Figure 1A**), we can assume that there are certain advantages to producing experimental inbreeding crosses using *V. emeryi* by androgenesis as the inbreeding crosses are genetically equivalent to a classic backcross. Indeed, the system has enabled us to design experiments to investigate the sex-determination genes, molecular mechanisms, and perform functional studies in this species[6,7].

In summary, methods to conduct inbreeding crosses and to evaluate the success of the resulting crosses have been described in *V. emeryi*. These protocols are essential for experiments directed at understanding the genetic and molecular basis of sex determination systems in the Hymenoptera.

ACKNOWLEDGMENTS:

This project was funded by the Japan Society for the Promotion of Science (JSPS) Research Fellowship for Young Scientists (16J00011), and Grant in Aid for Young Scientists (B)(16K18626).

DISCLOSURES:

The authors have nothing to disclose.

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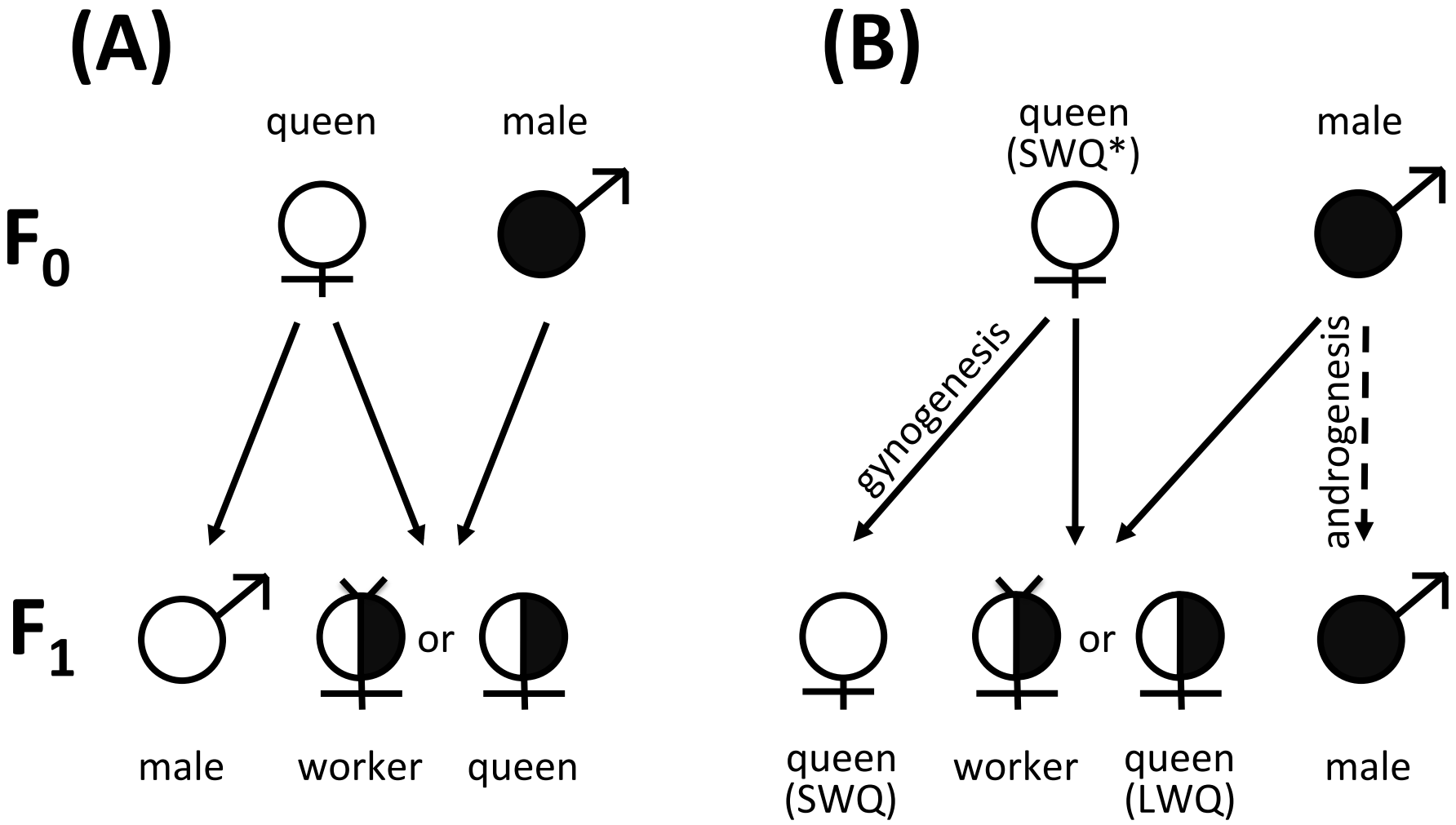

*F_0 queens lost their wings when the colonies were collected in the field.

Figure2

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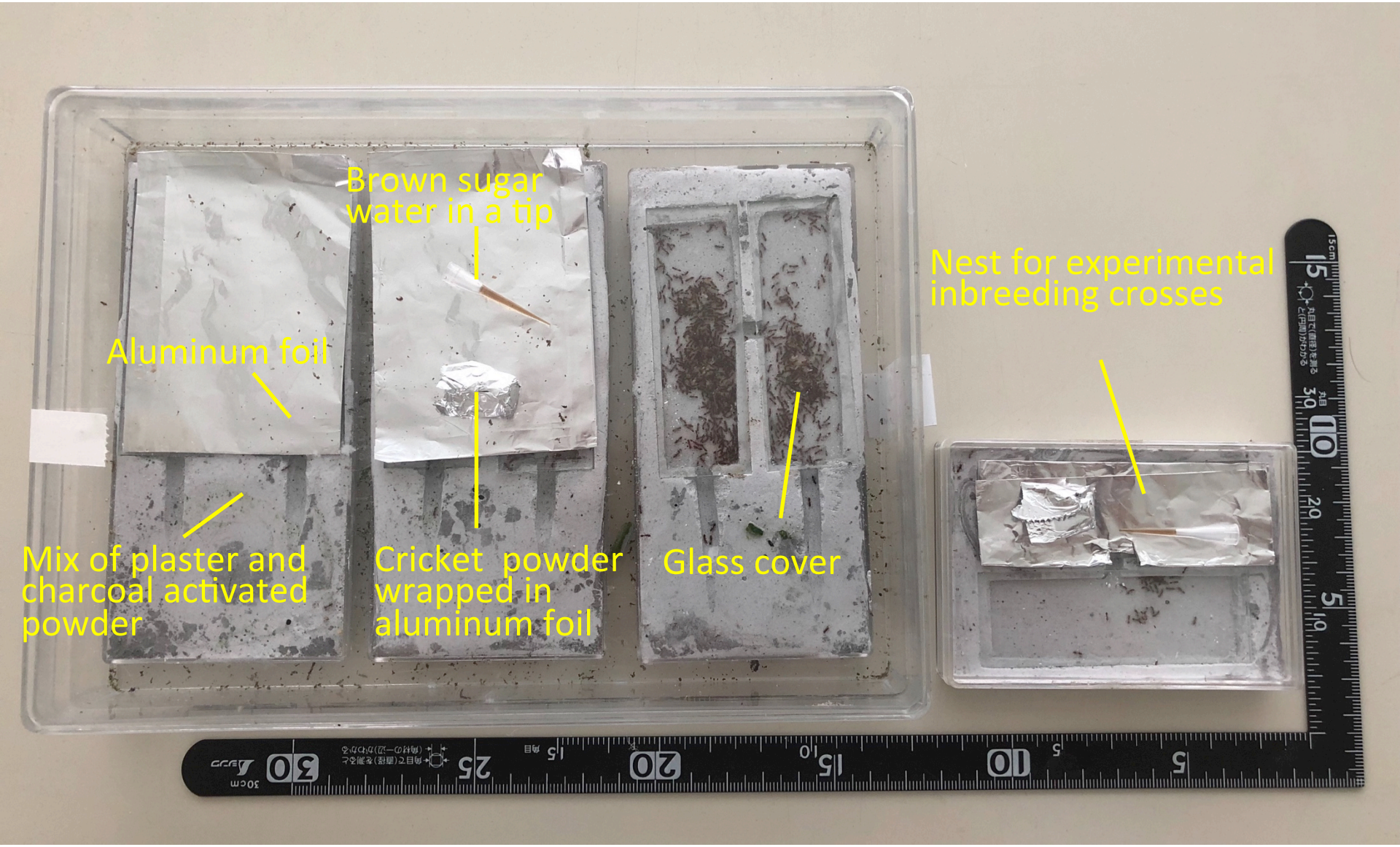

Figure3

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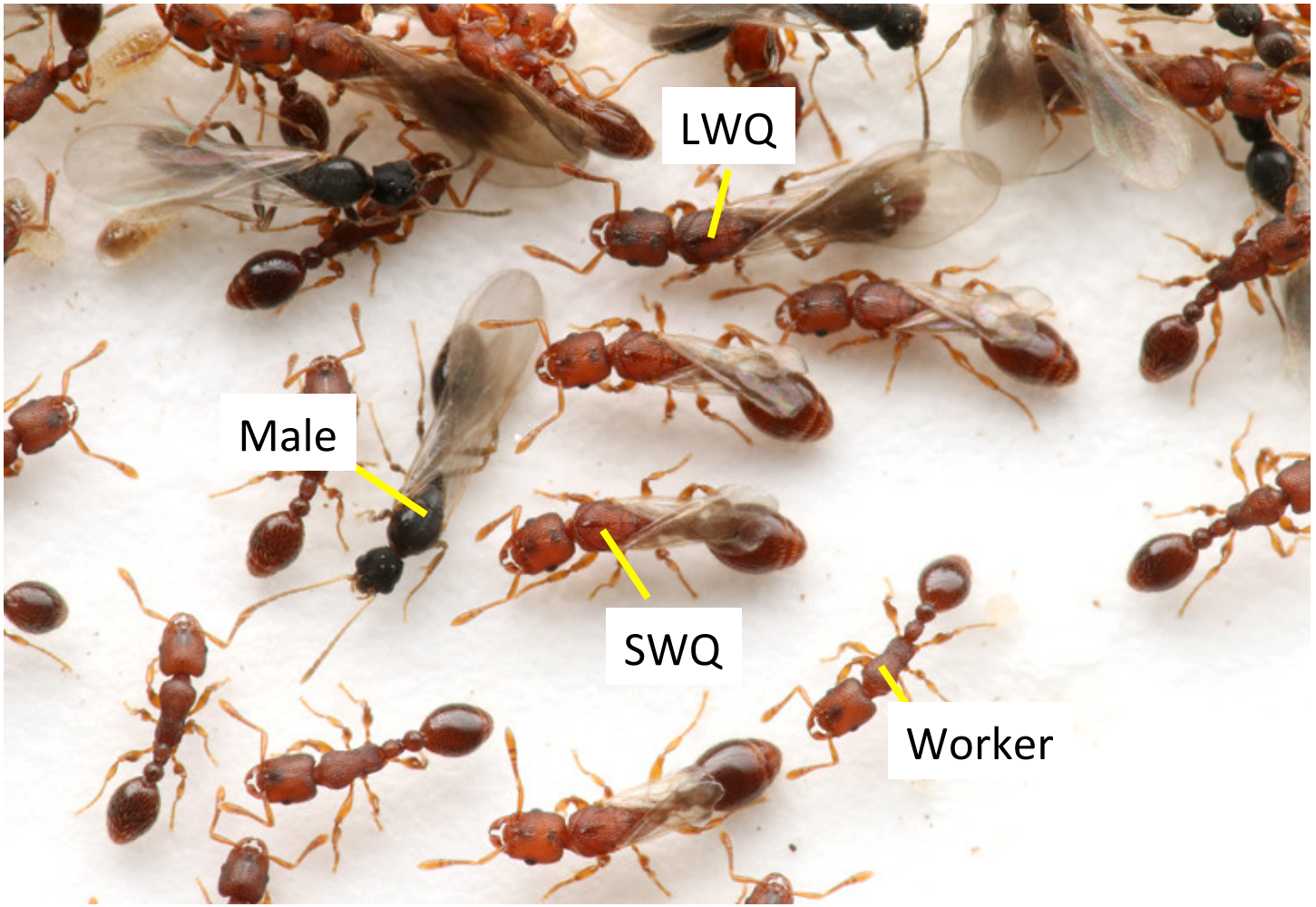

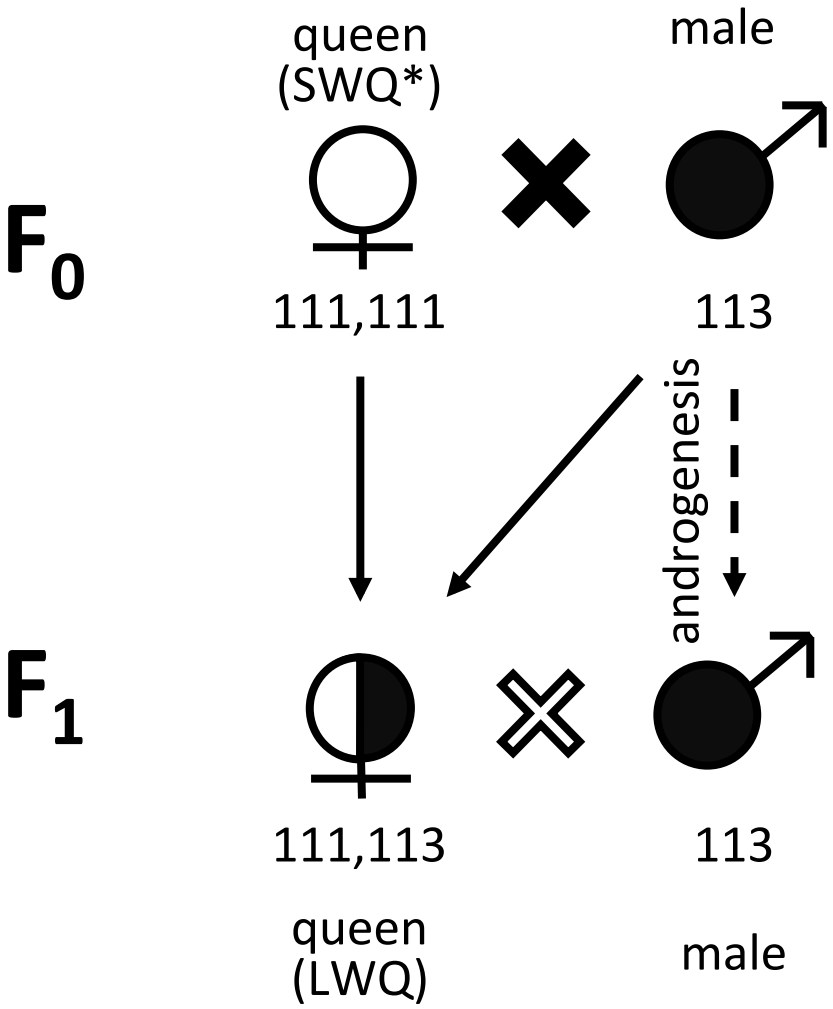

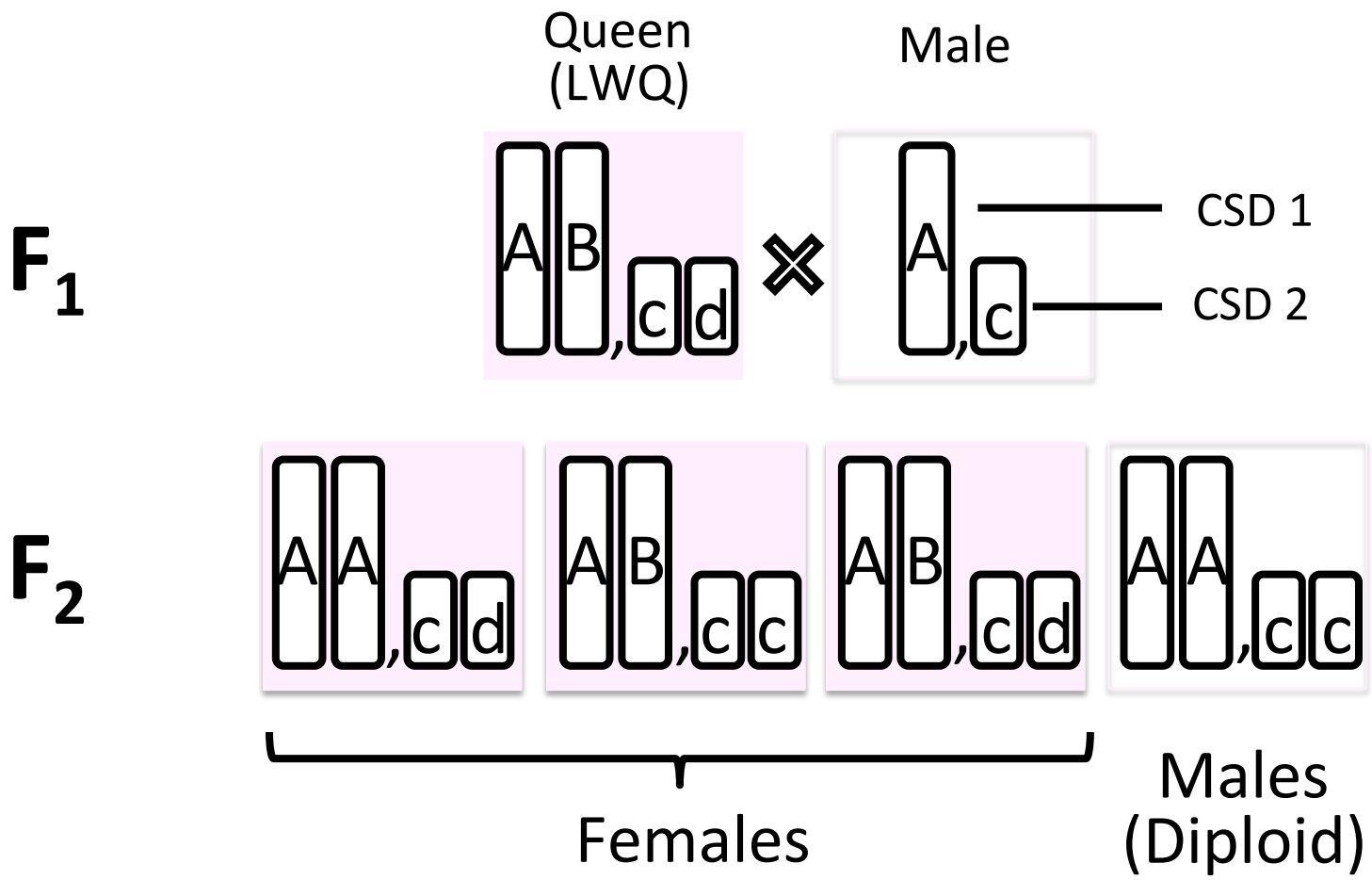

Figure6

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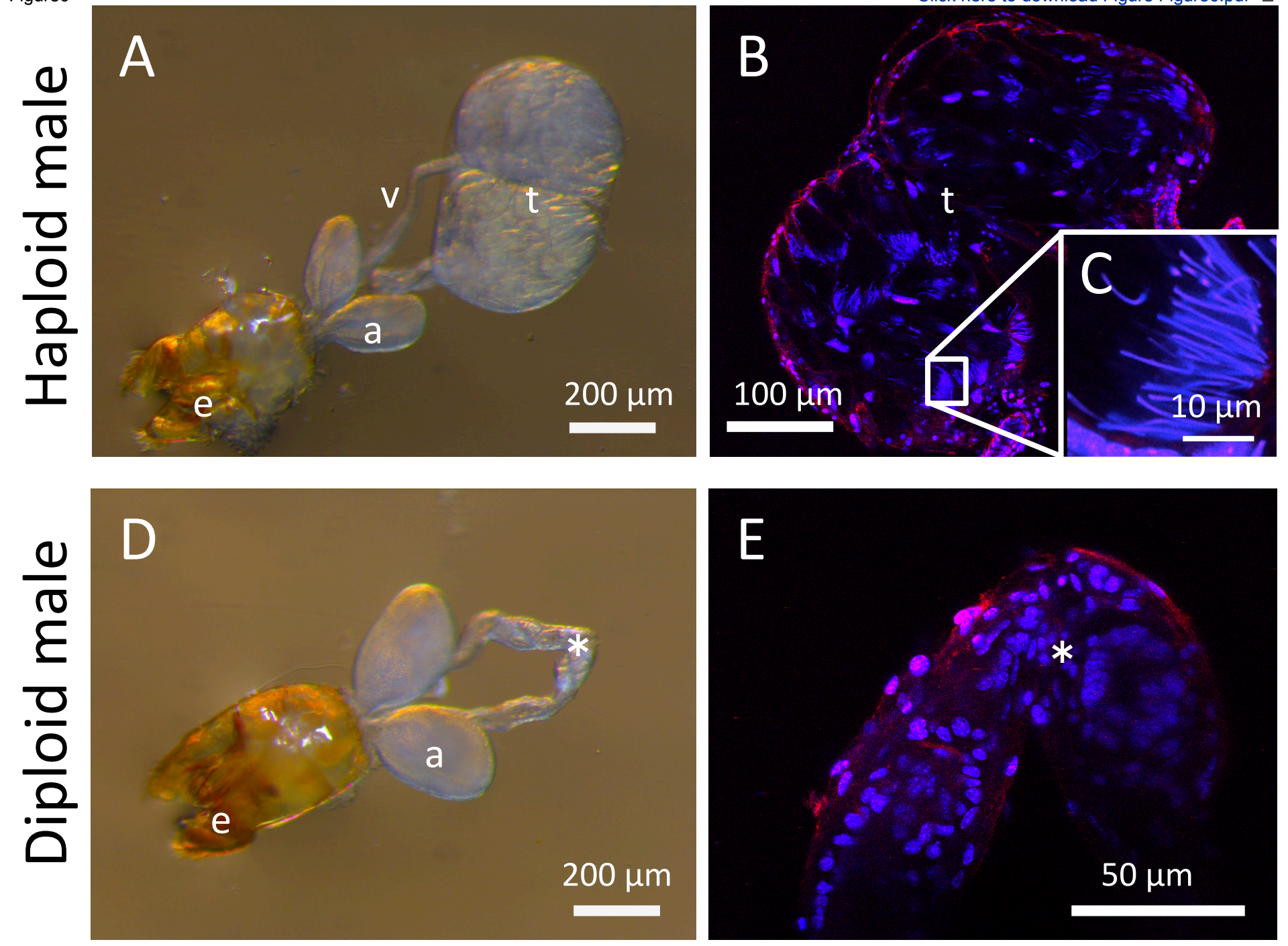

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
Plaster powder	N/A	N/A
Charcoal, Activated, Powder	Wako	033-02117,037-02115
Slide glass	N/A	N/A
Dry Cricket diet	N/A	N/A
Brown shuger	N/A	N/A
Styrene Square-Shaped Case	AS ONE	Any size
Incbator		
Aluminum block bath Dry thermo unit DTU-1B	TAITEC	0014035-000
1.5mL Hyper Microtube,Clear, Round bottom	WATSON	131-715CS
Ethanol (99.5)	Wako	054-07225
Stereoscopic microscope	N/A	N/A
Forseps	DUMONT	0108-5-PO
Chelex 100 sodium form	SIGMA	11139-85-8

Phosphate Buffer Saline (PBS) Tablets, pH7.4	TaKaRa	T9181
Paraformaldehyde	Wako	162-16065
-Cellstain- DAPI solution	Dojindo Molecular Technologies	D523
VECTASHIELD Hard・Set Mounting Medium with TRITC-Phalloidin	Vector Laboratories	H-1600
ABI 3100xl Genetic Analyzer	Applied Biosystems	
Confocal laser scanning microscope Leica TCS SP8	Leica	
HC PL APO CS2 20x/0.75 IMM	Leica	
HC PL APO CS2 63x/1.20 WATER	Leica	
Leica HyDTM	Leica	
Leica Application Suite X (LAS X)	Leica	

Comments/Description
Any brand can be used
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Any brand can be used
Size varies by number of ants
Any brand can be used
Any brand can be used

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Title of Article: Application of experimental inbreeding crosses for genetic and molecular studies of Japanese ant, Vollenhovia emeryi

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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.

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> 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 F_1 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 F_1 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. F_0 queens are easily distinguishable from F_1 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 (F_1 winged-queens and F_1 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]”.