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Corresponding Author:	Irene Li Environmental Health Institute Singapore, Singapore SINGAPORE
Corresponding Author's Institution:	Environmental Health Institute
Corresponding Author E-Mail:	irene_li@nea.gov.sg
Order of Authors:	Irene Li
	Keng Wai Mak
	Jeslyn Wong
	Cheong Huat Tan
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TITLE:

Using the Fluorescent Dye, Rhodamine B, to Study Mating Competitiveness in Male *Aedes aegypti* Mosquitoes

AUTHORS AND AFFILIATIONS:

Irene Li[1*], Keng Wai Mak[1*], Jeslyn Wong[1], Cheong Huat Tan[1]

[1]Environmental Health Institute, National Environmental Agency, Singapore

*These authors contributed equally.

Email addresses of co-authors:

Irene Li (Irene_li@nea.gov.sg)
Keng Wai Mak (Mak_keng_wai@nea.gov.sg)
Jeslyn Wong (Jeslyn_wong@nea.gov.sg)

Corresponding author:

Cheong Huat Tan (Tan_cheong_huat@nea.gov.sg)

KEYWORDS:

Mosquito mating competitiveness, *Aedes aegypti*, rhodamine B, Sterile Insect Technique, *Wolbachia*, fitness, population suppression

SUMMARY:

Here, we present a protocol for studying mating competitiveness of male *Aedes aegypti* using fluorescent dye as a marker. Female mosquitoes are exposed to both marked and unmarked males for copulation. Post mating, their spermathecae are examined under a fluorescence microscope to determine their mating partner.

ABSTRACT:

Incompatible and sterile insect techniques for mosquito population suppression depends on the ability of the released males to compete against their wild-type counterparts for mating with females to induce sterility in the target population. Hence, laboratory assessment of male mating competitiveness is essential for evaluating the release strain's fitness before field release. Conventionally, such an assay is performed by determining the proportion of viable eggs produced by the females after being simultaneously exposed to two sets of males (wild-type and release strains) for copulation. However, this process is time-consuming and laborious due to the need to first blood-feed the females for egg production and then hatch and enumerate the hatched eggs to determine egg viability.

Moreover, this method cannot discern the competitiveness between two sterility-inducing lines as wild-type female mosquitoes will only produce non-viable eggs in this instance. To circumvent these limitations, this paper describes a more direct method of measuring male mosquito mating competitiveness in laboratory settings using the fluorescent dye, rhodamine B (RhB). Competing

males are marked or left unmarked by giving sucrose solution containing RhB or regular sucrose, respectively. After the mating assay, the females are dissected to observe for the presence of fluorescing sperms within the spermathecae. In this manner, it is possible to directly and immediately determine which male had successfully mated with the female. This method reduces the experimental time by 90% and allows for the comparison between two sterility-inducing lines.

INTRODUCTION:

Against the backdrop of increased challenges against the effective control of dengue and other *Aedes*-borne diseases, the release of sterile and incompatible males for population suppression is actively being field-evaluated for their effectiveness in curbing the spread of these diseases or preventing outbreak incidences[1]. Male-release suppression strategies that are currently under field-test include the use of the genetic method[2], irradiation (Sterile Insect Technique, SIT)[3], endosymbiotic bacteria *Wolbachia* (Insect Incompatible Technique, IIT)[4], or a combination of the last two techniques[5,6]. The success of these approaches is largely dependent on the released males' ability to outcompete wild-type males and seek females to secure copulation. Otherwise, sterility cannot be induced in the target population.

In a classical SIT program, the optimal irradiation dosage to induce male sterility without compromising male mating fitness has to be determined by assessing the mating competitiveness of the irradiated male against the wild-type male for mating with the females under both the laboratory and semi-field conditions. Other than the irradiation dose[7-9], other factors that could potentially affect male mating fitness include the mass rearing protocol and the extent of inbreeding in the colony[10-14]. Therefore, the assessment of male mating competitiveness is a prerequisite and an essential step to evaluate the fitness of the release strain before field release. Furthermore, studies on the mating competitiveness of males in laboratory settings provide important knowledge of the mating behavioral biology of the mosquitoes for the control of the vector.

In SIT and IIT, the mating competitiveness of male mosquitoes is typically assessed through experiments, wherein wild-type females are simultaneously exposed to equal numbers of both wild-type and males meant for release (*e.g.*, irradiated or *Wolbachia*-infected)[8,11,15,16]. As copulation between wild-type females and test males should result in non-viable eggs, the hatch rate of eggs produced by wild-type females is used to estimate male competitiveness using the Fried Index[17]. Unfortunately, this method is resource-intensive and time-consuming, and the overall Fried Index can be influenced by external confounding factors affecting egg viability. For example, poor egg handling and over-desiccation can result in a low hatch rate for the compatibility cross, giving rise to an artificially low Fried Index.

Moreover, this method does not allow for the direct comparison of mating competitiveness between *Aedes* mosquitoes that are infected with different strains of *Wolbachia* or that are exposed to different doses of irradiation. Hence, a more direct method is required to address these challenges. Recent studies[18,19] have demonstrated the effectiveness of using the

fluorescent dye, RhB, to mark male mosquitoes' seminal fluid. Marked seminal fluid is transferred and stored in the female mosquitoes' spermathecae upon successful mating, allowing direct measurement of female mating interaction with marked males. Rhodamine B is a thiol-reactive fluorone dye commonly used as a biomarker for ecological and behavioral research studies in animals including insects[20]. For mosquito studies, RhB is introduced by feeding with sugar or honey water containing dissolved RhB powder[18,19,21-24]. Upon uptake, the RhB dye binds to proteins, staining body tissue with a reddish-pink stain that fluoresces bright orange under a fluorescent light source.

The strong fluorescence signal and stability of the marking, coupled with its ability to stain insect seminal fluids, allows for the monitoring of the transfer of marked seminal fluid from the labelled male to the sperm storage organs of the female insect for mating studies[18,19,21,24]. The use of RhB in a male mating competitiveness assay not only allows direct measurement of the mating interaction of females with either marked and unmarked males, but the results can also be obtained within 24 h as it obviates the process of determining the egg viability, which typically requires one week. Furthermore, this method overcomes the potential loss of data when the female mosquitoes do not blood-feed or die before ovipositioning.

This is particularly crucial because in semi-field trials, female mosquitoes are prone to damage and death during post-mating collection using a backpack or mechanical aspirator. To address the current limitations of using female fertility to indirectly measure male fitness, this paper presents an alternative method of performing a male mosquito mating competitiveness assay using RhB. The simplified and shortened workflow reduces the experimental time from two weeks to one day, allowing for more experimental replicates to be carried out. This protocol will be suitable for laboratories that are embarking on male release-based mosquito population suppression programs, as part of quality control and strain evaluation.

PROTOCOL:

1. Rearing of mosquitoes

1.1. Conduct all mosquito rearing and the male mating competitiveness assay under standard insectary conditions of 27 ± 1 °C and 75–80% relative humidity, with a photoperiod of 12 h:12 h light: dark cycles.

1.2. Designate the two sets of competing males as Set A and Set B for easy reference in the methodology described in this paper. Rear the mosquitoes under standardized conditions to ensure a fair comparison of their fitness during the assay. Rear the mosquitoes at a larval density of 500 larvae in 2 L of water and feed them with ground fish food powder *ad libitum*.

NOTE: For the generation of the representative results, Set A and Set B were the inbred and outcross males of the *Wolbachia*-infected *Ae. Aegypti*, respectively.

1.3. Sex male and female mosquitoes at the pupal stage, and contain them separately in cages (see the **Table of Materials**) of dimensions W 32.5 cm x D 32.5 cm x H 32.5 cm, with mesh size of 150 x 150 and 160 µm aperture. Maintain all adult mosquitoes with 10% sucrose solution.

2. Preparation of male and female mosquitoes

2.1. Sex the male and female mosquitoes at the pupal stage according to their size differences (male pupae being smaller than female pupae) (**Figure 1**).

2.2. For each set of mosquitoes (Set A or Set B), transfer 100 male pupae each into a prelabelled cage for sucrose feeding or RhB-sucrose feeding.

2.3. Place the female pupae in small batches of 40–50 per cage. Upon the emergence of the imagoes, check the cages for the presence of male mosquitoes.

NOTE: Adult male mosquitoes are smaller than females and have bushier and hairier antennae (**Figure 2**). Use only virgin female mosquitoes for the mating competitiveness assays. The use of pre-inseminated females will render all resulting data invalid. Thus, extreme care must be taken during sexing at the pupae stage. Do not use the female mosquitoes from a cage that has been contaminated with male mosquitoes. Extra cages of females should be prepared.

3. Preparation of 0.2% RhB–sucrose solution

NOTE: RhB is a green powder in dry form and reddish-pink in solution. Standard personal protective equipment (PPE: laboratory protection gown, nitrile gloves, and eye protection) shall be worn when handling this chemical. To avoid inhalation, weigh the RhB powder in a fume hood.

3.1. To prepare a 0.2% w/v RhB–sucrose solution, dissolve 200 mg of RhB powder for every 100 mL of 10% w/v/ sucrose solution. Mix well to ensure all the powder is dissolved.

NOTE: As RhB is light-sensitive, use amber bottles, or wrap clear bottles completely with aluminum foil.

4. Feeding of male mosquitoes

NOTE: Data from RhB–sucrose feeding optimization is presented in **Supplemental Material, section 1**.

4.1. Prepare 20 sugar feeder bottles with a wick. Add 10 mL of 10% sucrose in 10 feeder bottles and 10 mL of 0.2% RhB–sucrose solution in the other 10 feeder bottles (use amber bottles, or wrap the bottles in aluminum foil).

4.2. Place the feeder bottles in the respective male cages (5 bottles per cage) prepared in steps

2.2 and 2.3. Allow the male mosquitoes to feed for three days before the mating experiment.

5. Checking for RhB fluorescence in male mosquitoes

5.1. Aspirate RhB-sucrose-fed male mosquitoes, and observe them under a fluorescence stereo microscope to ensure that all the RhB-sucrose-fed male mosquitoes have been successfully marked with RhB.

5.2. Turn on the mercury burner lamp and the stereo microscope. Allow the light source of the mercury burner lamp to stabilize for 10 min. Set the fluorescence filters for red fluorescence protein 1 (RFP1) (excitation wavelength 540 nm, emission wavelength 625 nm).

5.3. Aspirate a small number of mosquitoes (four or five) at a time into the glass tube of the oral aspirator. Through the glass tube, observe the body of the male mosquitoes under the fluorescence stereo microscope. Exclude male mosquitoes not marked with RhB from the experiment.

NOTE: The abdomen of male mosquitoes marked with RhB will appear pink under white light (**Figure 3A**) and glow bright orange under fluorescent light (**Figure 3B**).

5.4. Transfer Set A male mosquitoes into 12 paper cups secured with netting (mesh size 150 x 150, 160 µm aperture); 6 cups each with 10 sucrose-fed male mosquitoes and the other 6 cups each with 10 RhB-sucrose-fed male mosquitoes. Repeat this step for Set B male mosquitoes.

6. Mating competitiveness assay

6.1. Set up 12 W 60 cm x D 60 cm x H 60 cm cages with mesh size 44 x 32, 650 µm aperture for the mating assay. In each of the six cages, include 10 Set A (RhB-marked) male mosquitoes, 10 Set B (unmarked) male mosquitoes, and 10 virgin wild-type female mosquitoes. In the other six cages, include 10 Set A (unmarked) male mosquitoes, 10 Set B (RhB-marked) male mosquitoes, and 10 virgin wild-type female mosquitoes. Label these cages to clearly distinguish between the two mating combinations.

NOTE: Based on experience, a 60 cm x 60 cm x 60 cm cage was used for the mating assay as a smaller cage may encourage mixed mating.

6.2. Place the respective cups of males prepared in step 5.4 into the mating cages according to the label in step 6.1. Remove the netting and gently tap the cup to jostle the males out of the cup. Carefully remove the paper cup and the netting from the cage to ensure no mosquitoes escape from the cage. Allow the male mosquitoes to acclimate in the mating cage for at least an hour.

6.3. Using an oral aspirator, transfer virgin wild-type female mosquitoes into 12 paper cups, with each cup containing 10 mosquitoes.

6.4. After the acclimation period for the male mosquitoes, transfer one cup of females into every mating cage and remove the netting. Gently jostle the cup to encourage any remaining female mosquitoes out of the cup. Carefully remove the paper cup and the netting from the cage to ensure no mosquitoes escape from the cage.

6.5. Allow mating to take place for 3 h.

NOTE: Recommended mating duration was determined through prior observations of wild-type *Ae. aegypti*. In experiments involving 10 females and 20 males being held in a 60 cm x 60 cm x 60 cm cage, 90% female insemination was achieved in 3 h (**Supplemental Material, section 2**). Do not disturb the cage during this period as agitation could lead to interrupted and mixed mating. Mixed mating (where the female has mated with both marked and unmarked males) results in a bias towards RhB-marked males as it is difficult to distinguish unmarked sperms from RhB-marked sperms under a fluorescence microscope.

6.6. To terminate the mating experiment, remove all the mosquitoes from each cage using a mechanical aspirator. Cold anesthetize the mosquitoes on ice for at least 5 min. When the mosquitoes are fully anesthetized, gently pick up the female mosquitoes and house them in a separate paper cup secured with netting (mesh size 150 x 150, 160 µm aperture). Label the paper cup by transferring the respective label from the mating cage onto the paper cup.

NOTE: It is possible to pause the experiment at this point and maintain the females with 10% sucrose solution. The RhB-marked seminal fluid will remain stable inside the female spermathecae for at least a week. It is best to keep the females alive before dissection as dead and desiccated specimens are difficult to dissect.

6.7. To score the female spermathecae, cold anesthetize the female mosquitoes on ice for at least 5 min before dissection under a stereo microscope (**Video 1**). Examine the spermathecae under a compound light microscope (magnification 100x) for their insemination status (**Figure 4**). For inseminated individuals, determine whether the spermathecae contain RhB-marked seminal fluid by examining them under the fluorescence stereo microscope equipped with an RFP1 filter and a camera imaging system.

NOTE: When using a fluorescence stereo microscope with an attached imaging system, it is recommended to utilize a prolonged exposure time (5 s) to increase the detection sensitivity. If the female mosquito has mated with a marked male, her spermathecae will fluoresce bright orange (**Figure 5A**). However, if the female mosquito has mated with an unmarked male, her inseminated spermathecae will not fluoresce (**Figure 5B**).

7. Disposal of RhB waste

7.1. Treat aqueous RhB waste with activated carbon[25] before discharging it as general waste-

water. Dispose of solid RhB waste (mosquitoes marked with RhB, paper towels, and wicks soaked with RhB) as chemical waste. Don standard PPE when handling RhB waste.

REPRESENTATIVE RESULTS:

The male mating competitiveness between the inbred and outcross lines of Singapore *Ae. aegypti* infected with the *w*AlbB strain of *Wolbachia* (*w*AlbB-Sg) was evaluated using the above-mentioned protocol. The inbred line was maintained for 11 generations in the insectary while the outcross line was generated by backcrossing the females with wild-type male *Ae. aegypti*. Both males from the inbred and outcross lines were allowed to compete for mating with wild-type female *Ae. aegypti*. The objective of this mating competitiveness assay was to determine whether there could be a loss in male mating fitness after several generations of inbreeding. Experimental triplicates of the mating competitiveness assay were conducted. The insemination data are shown in **Table 1** and **Figure 6**.

To establish if RhB had any effects on the mating competitiveness of the experimental males, the first step was to determine whether was any correlation between mark-status and mating success. **Table 1** and **Figure 6** showed that the proportion of females inseminated by the inbred or outcross males was not affected by the RhB marking. Based on these results, the next step was to analyze the overall percentage of females inseminated by the inbred or outcross males (**Table 2** and **Figure 7**). There was a significantly higher percentage of females mated with the outcross males than with the inbred males in all three replicates ($P \leq 0.05$, Mann-Whitney *U*-test), indicating that inbreeding could lead to loss in mating fitness.

FIGURE AND TABLE LEGENDS:

Figure 1: Lateral view of male (left) and female (right) *Aedes aegypti* pupae. Under the same rearing conditions, *Ae. aegypti* can be sexed at the pupal stage according to size; males are significantly smaller than females.

Figure 2: Differentiation of male (left) and female (right) *Aedes aegypti* adults. Adult male mosquitoes (left) have bushier and hairier antennae than the adult female; the red arrows indicate the antennae.

Figure 3: Rhodamine B marking of male mosquito. (**A**) Light microscopy; (**B**) fluorescence microscopy. The mosquito on the left is unmarked (fed with 10% w/v sucrose), while the one on the right is marked (fed with 0.2% RhB-sucrose). Marked mosquitoes have a visible pink abdomen under white light (the mosquito on the right in **A**), which fluoresces bright orange under fluorescence microscopy (**B**). Scale bars = 5 mm. Abbreviation: RhB = Rhodamine B.

Figure 4: Inseminated and non-inseminated female spermathecae under a compound light microscope (100x magnification). The insemination status of a female mosquito can be determined by observing its spermathecae under a compound light microscope. An inseminated female mosquito will contain at least one filled spermatheca while all three spermathecae of a

non-inseminated female mosquito will be empty. Thread-like, motile sperm will be visible in a filled spermatheca under a compound light microscope. Scale bar = 100 µm.

Figure 5: Female mosquito spermathecae inseminated with seminal fluids under a fluorescence stereo microscope. (**A**) RhB-marked and (**B**) unmarked spermathecae inseminated with RhB-marked seminal fluids will fluoresce bright orange under fluorescence microscopy. Scale bars = 100 µm.

Figure 6: Number of wild-type females inseminated by either the inbred or outcross males in the experimental triplicates with reciprocal marking. (**A**) The inbred males were marked with RhB while the outcross males were unmarked. (B) The outcross males were marked with RhB while the inbred males were unmarked. A higher number of females was observed to have mated with outcross males regardless of their marking status.

Figure 7: Proportion of inseminated females mated with inbred or outcross males in the 3 experimental replicates. For each experimental replicate, there is a significantly higher percentage of females mated with the outcross males ($P \leq 0.05$, Mann-Whitney *U*-test).

Video 1: Dissection of female *Aedes aegypti* for spermathecae under a light stereo microscope.

Table 1: Number of females mated with RhB-marked and unmarked *w*AlbB-Sg *Aedes aegypti* inbred and outcross males. A total number of 120 females were used in each replicate.

Table 2: Percentage of inseminated females mated with *w*AlbB-Sg *Aedes aegypti* inbred and outcross males.

Supplemental Figure S1: Comparison of workflow for RhB-based and conventional mating competitiveness assay. In comparison to the conventional mating competitiveness assay, the simplified and shortened workflow for RhB-based mating competitiveness assay significantly reduces the experimental duration.

Supplemental Figure S2: Kaplan Meier survival curves of male adult *Aedes aegypti* during and after feeding with 0.2% and 0.4% rhodamine B–sucrose feeding. Percent survival of (**A**) male wild-type and (**B**) *w*AlbB-Sg *Ae. aegypti* during and after three days of feeding on 0.2% and 0.4% RhB-sucrose, compared to controls that were fed with sucrose only.

Supplemental Table S1: Insemination rate of females in a W 60 cm x D 60 cm x H 60 cm cage (ratio of 10 females to 20 males) at 1-, 2-, and 3-h time points.

DISCUSSION:

Marking is commonly used in entomological research to study insect population dynamics, dispersal, behavior, and mating biology[26]. In the control of economically important pest insects using Sterile Insect Technique (SIT), marking is carried out to differentiate the release strain from

the field population to study their dispersal and optimize the release ratio. The marking methods used involve genetic marking[27,28], incorporating isotopes in larval food[29,30], fluorescent dust[31], and dye[32]. For the suppression of mosquito populations using SIT or IIT, fluorescent dyes have been used as markers to study mosquito mating biology[18,19].

Male mating fitness is one of the critical components of a mosquito population suppression program using the release of sterility-inducing males. Conventionally, female fertility assays are used to assess the ability of released males to find and compete with wild-type males for mates. However, this assay is time-consuming and labor-intensive due to the necessity of several post-mating procedures (**Supplemental Figure S1**). These processes include blood-feeding of the females, egg collection, hatching of the eggs, and enumeration of the proportion of hatched eggs to determine egg viability. On average, this assay requires 30 man-hours and two weeks of experimental work (starting with the setting up of the competitiveness assay cages to the final determination of male mating competitiveness).

This paper presents the use of a fluorescent dye, RhB, (fed as 0.2% RhB–sucrose to the mosquitoes, **Supplemental Figure S2**) as a marker for immediate determination of the mating interaction of females between two sets of marked and unmarked males. This protocol obviates the need to perform the time-consuming experimental procedures detailed above. On average, this RhB-based assay requires approximately 10 man-hours and a day to obtain similar data. This translates to a 90% savings in time. The time saved can then be used by researchers to perform additional replicates, providing a more robust validation of male mating fitness. In addition, this assay can be used to assess the mating competitiveness between two sterile or *Wolbachia*-infected strains of mosquitoes, as illustrated by the representative results.

The conventional female fertility assay would be unsuitable because no viable eggs would be produced in either crossing. Notwithstanding, any mixed mating in the experiment will result in bias toward the marked population as it is difficult to identify unmarked sperms in female spermathecae that contain seminal fluid from both RhB-marked and unmarked males. A similar conclusion was made in a study assessing mating competitiveness of *Anopheles gambiae* using RhB[18], whereby a greater proportion of females in the mating assay was found to be mated by marked males. As polyandry is more likely to occur in females that had previously engaged in an interrupted mating[33], the probability of this occurring was reduced in this study by using fewer mosquitoes (20 males to 10 females) in a larger cage volume (0.216 m^3) in these experiments.

The results showed no bias toward the RhB-marked population, indicating that mixed mating was limited. In summary, incorporation of RhB to mark males in a mating competitiveness assay is an economical and rapid way of evaluating male mating fitness. This method also allows for the direct comparison of mating competitiveness between males exposed to different doses of irradiation, reared in different rearing regimes, or those infected with different strains of *Wolbachia*, making it a valuable tool for the evaluation of male mating fitness for any male release-based mosquito population suppression program.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

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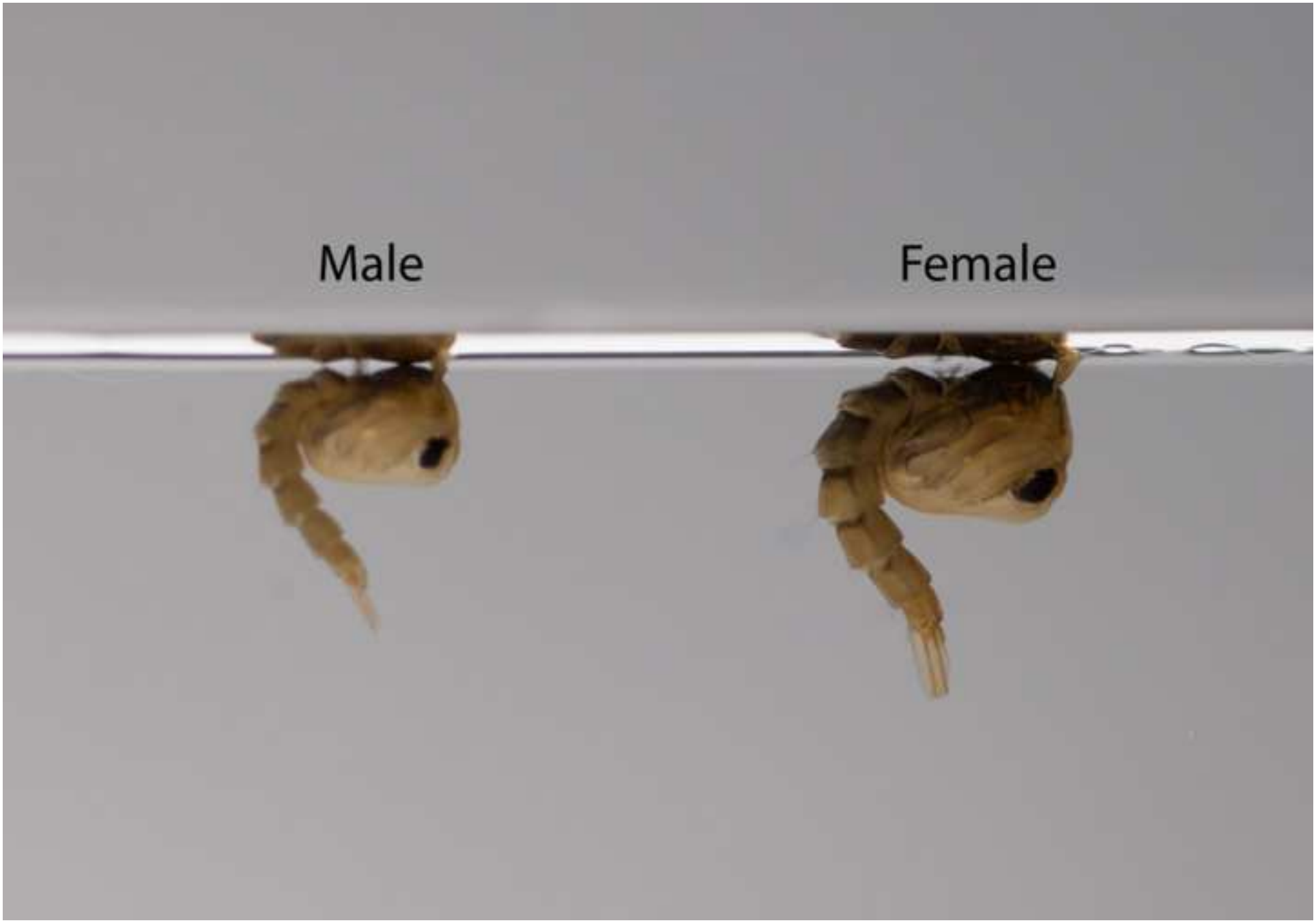

Figure 2

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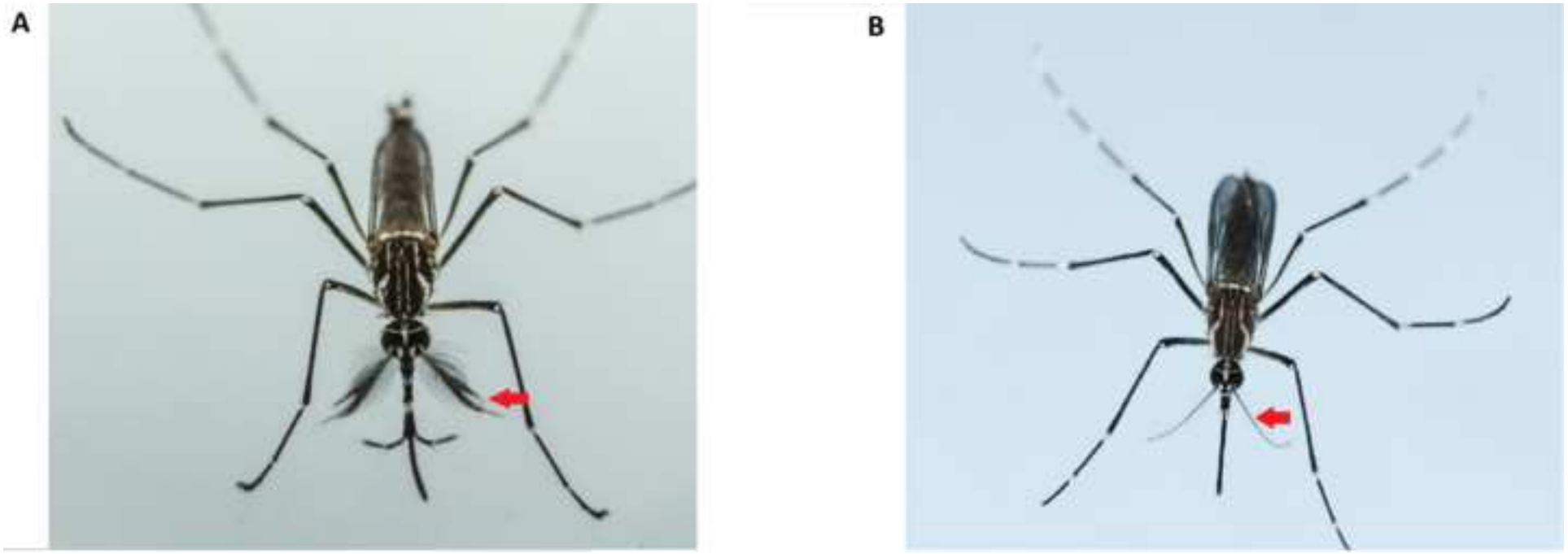

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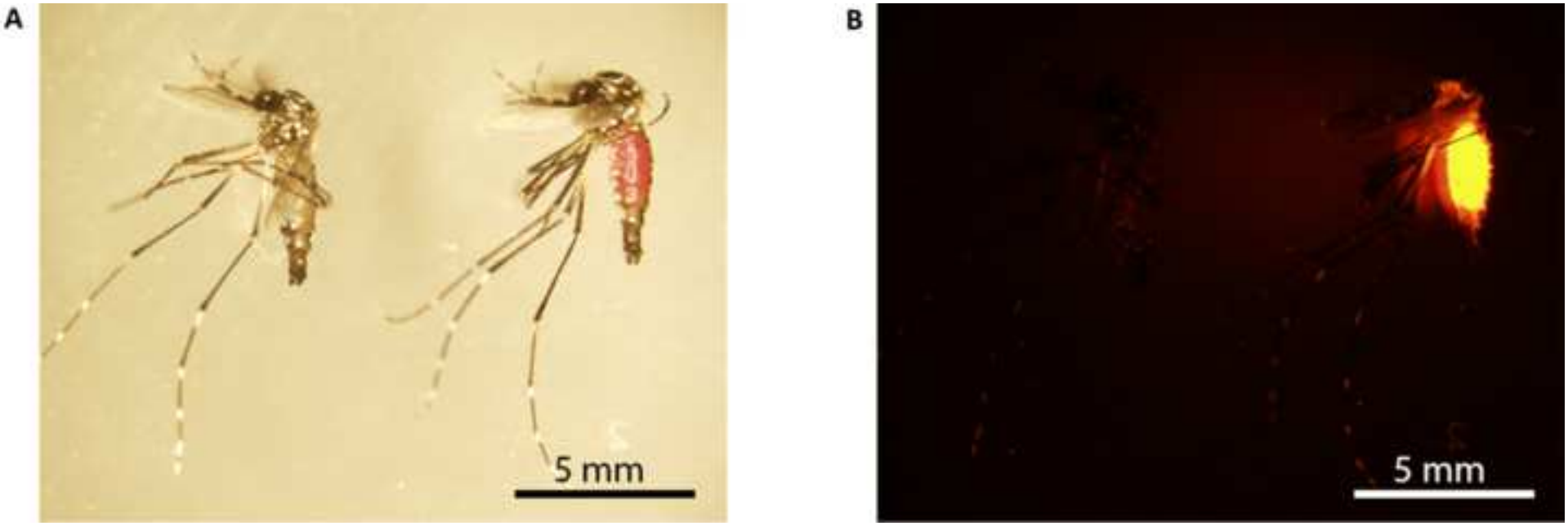

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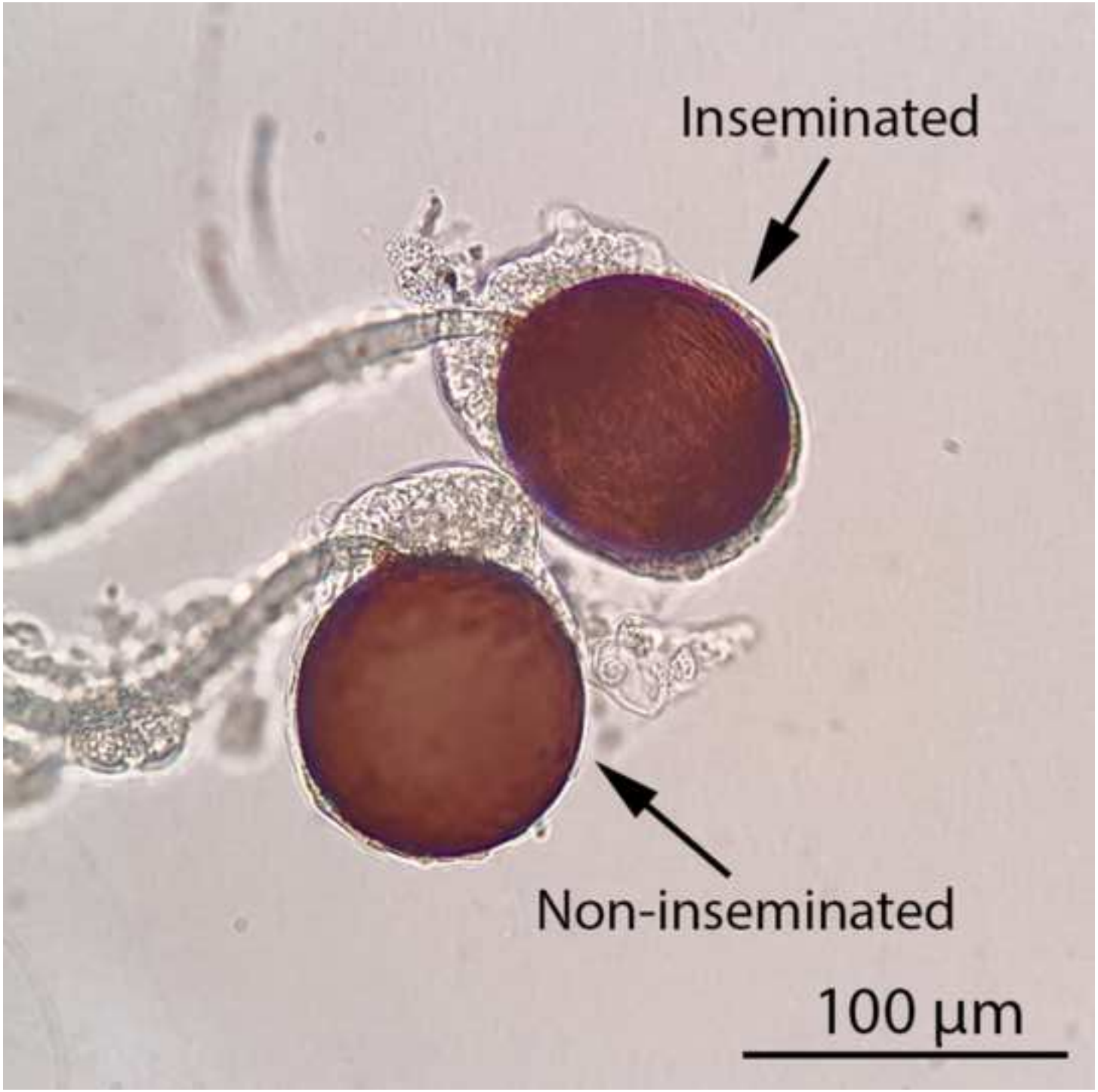

Figure 5

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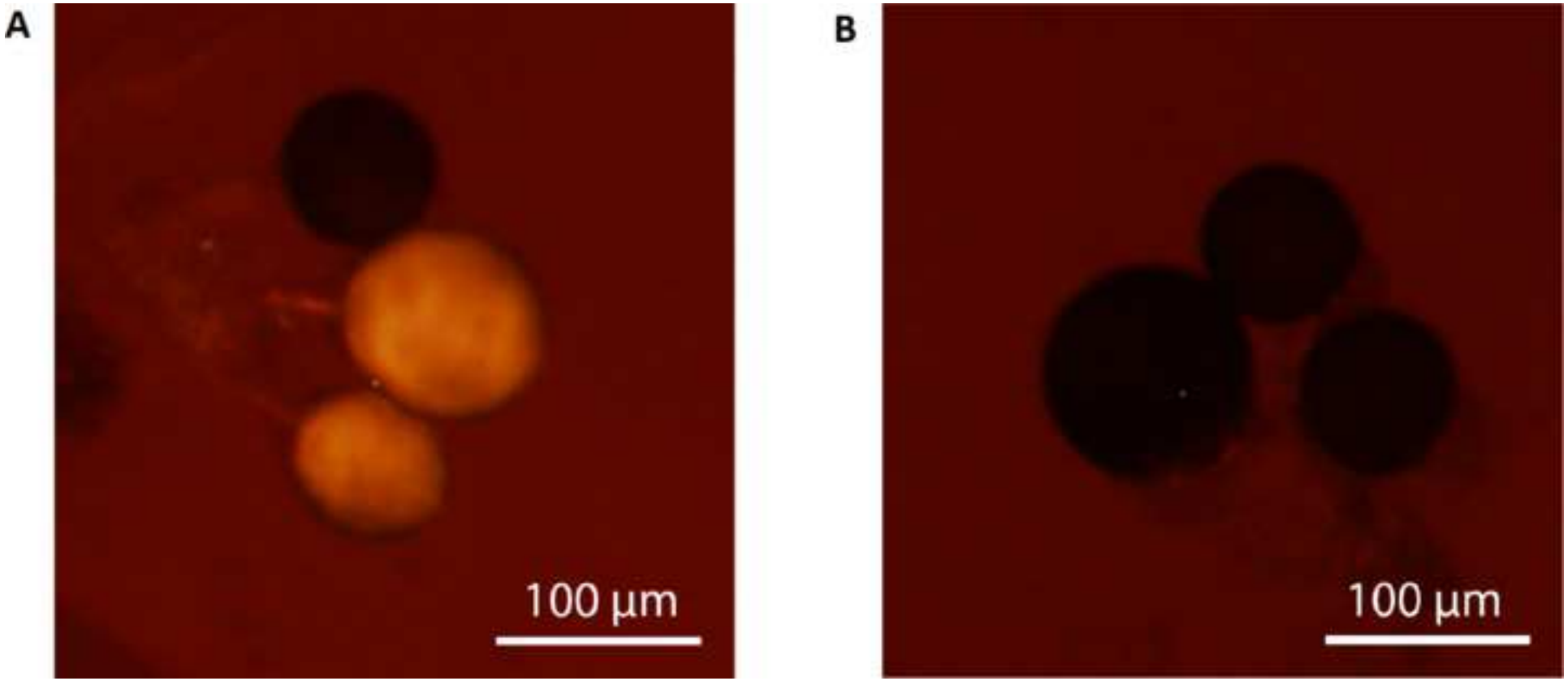

Figure 6

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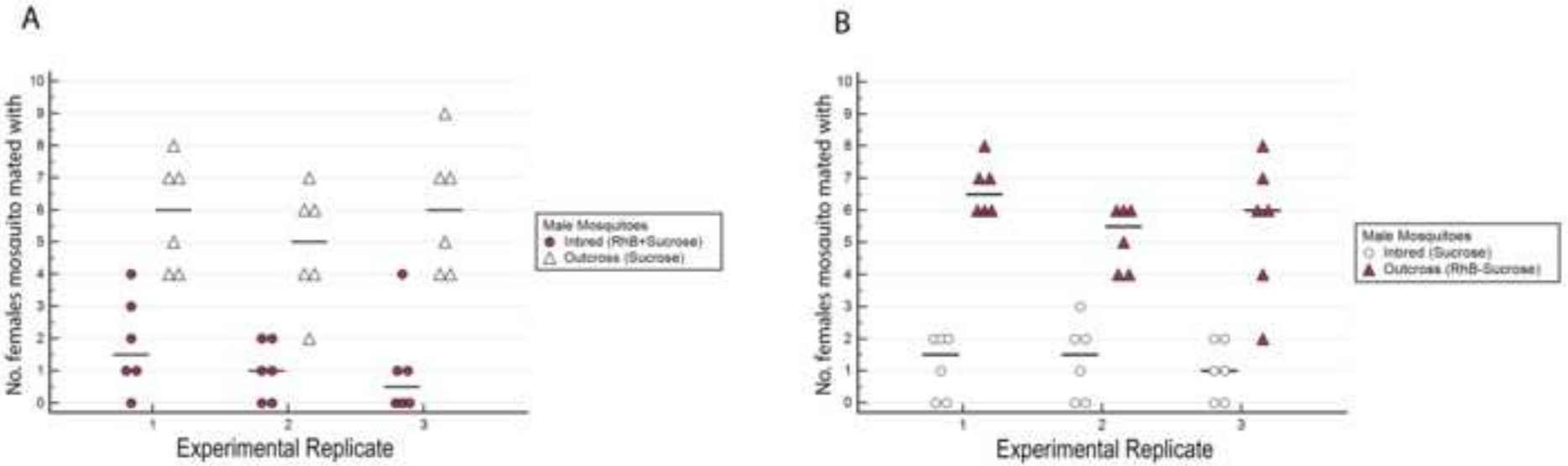

Figure 7

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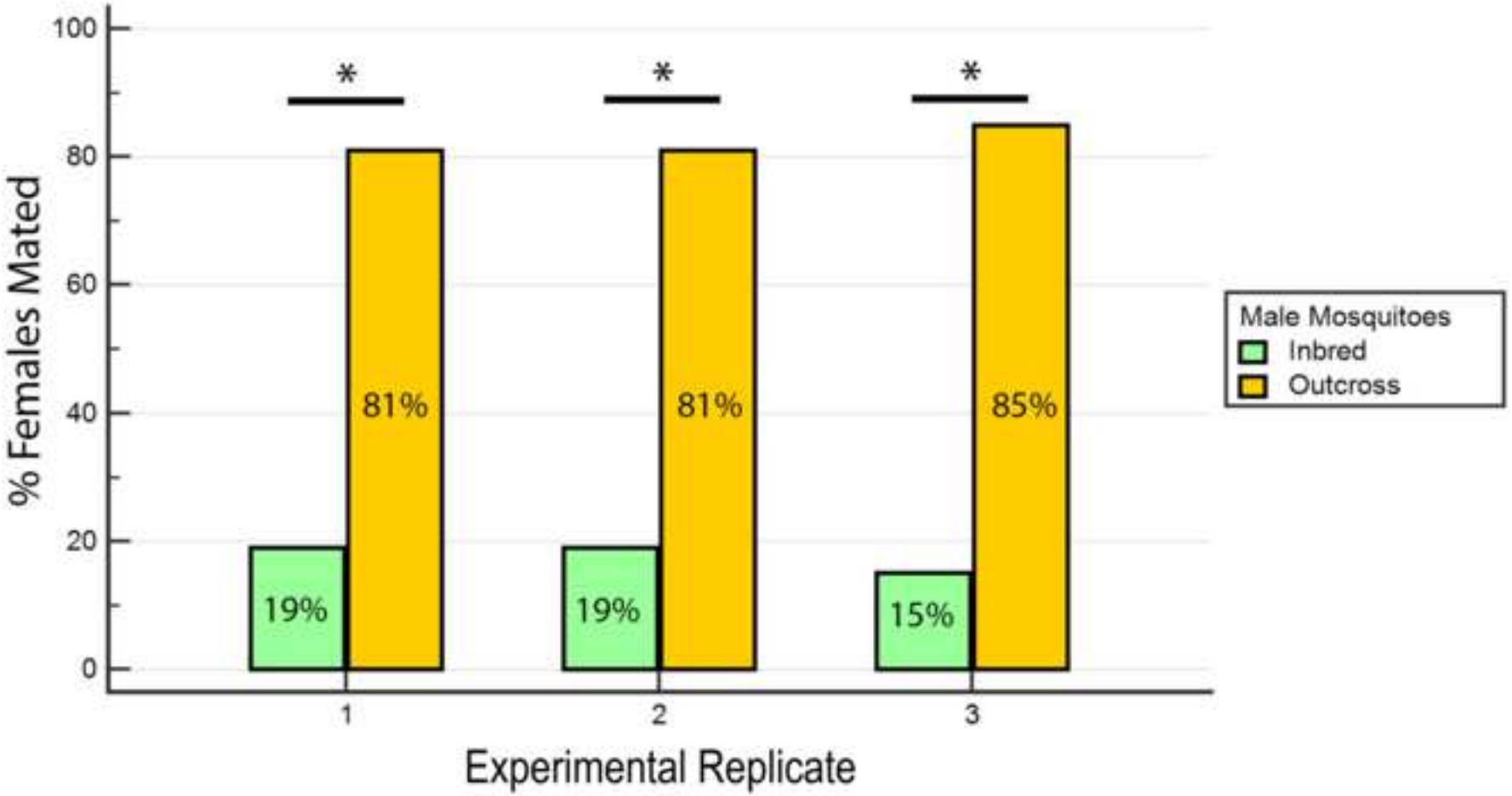
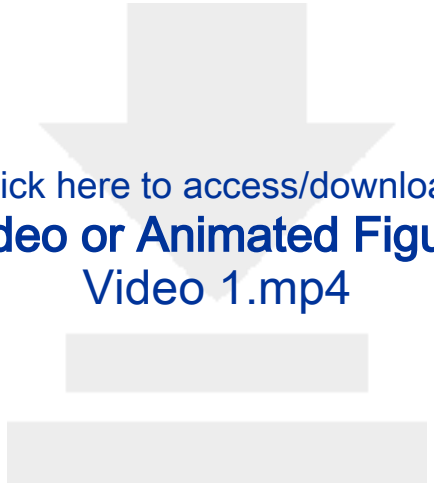


Video 1



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Video or Animated Figure

Video 1.mp4

Table 1

Click here to access/download;Table;Table 1.xlsx

	♀ x Inbred (RhB) ♂ [a]	♀ x Outcross (unmarked) ♂ [b]	♀ x Inbred (unmarked) ♂ [c]
Replicate 1	11	35	7
Replicate 2	6	29	8
Replicate 3	6	36	6

♀ x Outcross (RhB) ♂ [d]	Overall insemination rate (a+b+c+d/120)
40	77.5% (93/120)
31	61.7% (74/120)
33	67.5% (81/120)

Click here to access/download;Table;Table 2.xlsx

	Percent inseminated females	
	Inbred males	**Outcross males**
Replicate 1	19% (18/93)	81% (75/93)
Replicate 2	19% (14/74)	81% (60/74)
Replicate 3	15% (12/81)	85% (69/81)

Click here to access/download;Table of Materials;Table of Materials revised.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Compound light microscope	Olympus	CX23	To score for spermathecae insemination
Dissection forceps	Bioquip	Rubis forceps (4524)	
Fluorescence stereo-light microscope with RFP1 filter	Olympus	SZX16	To check for Rhodamine B fluorescence signal
Mosquito cages	Bugdorm	4F3030	W 32.5 cm x D 32.5 cm x H 32.5 cm; mesh size of 150 x 150; 160 µm aperture For holding of male and female adult mosquitoes prior to mating assay
		6M610	W 60 cm x D 60 cm x H 60 cm; mesh size of 44 x 32; 650 µm aperture For mating competitiveness assay
Mosquito netting			150 x 150, 160 µm aperture
Rhodamine B	Sigma Aldrich	R6626	≥95% (HPLC)
Stereo-light microscope	Olympus	SZ61	For spermathecae dissection
Sucrose	MP Biomedicals	SKU 029047138	Food grade
TetraMin tropical flakes	Tetra	77101	Fish food for feeding larvae

Click here to access/download;Rebuttal Letter;response to reviewers.docx

Authors'comments in green

Editorial comments:

Editorial Changes
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.
2. Consider changing the title to e.g., "Studying mating competitiveness of male Aedes aegypti mosquitoes using fluorescent dyes."
3. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"
4. Details about the mosquito cages: e.g., mesh size, dimensions?
5. Line 130: How should the male and female adult mosquitoes be differentiated?
6. Line 136: How is the sucrose solution presented for feeding? Quantity?
7. Line 140: Add this as a "note".
8. Line 176: What is the mesh size of the netting?
9. Line 164: What is the mercury burner?
10. Line 216, 224: How long should the mosquitoes be placed on ice?
11. Please highlight complete sentences (not parts of sentences), ensuring that the highlighted part of the step includes at least one action that is written in imperative tense. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
12. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
13. Please sort the Materials Table alphabetically by the name of the material.

All editorial comments have been addressed in the revised manuscript.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
Author's conducted a study to illustrate the feasibility of using the dye Rhodamine B as a method of marking both the body and seminal fluid to allow the mating competitiveness of two Wolbachia or sterile lines to be assessed. Both stained and unstained males were allowed to compete for wild females during a short window. Females were then subsequently dissected and the spermathecae checked under a fluorescence microscope for the presence of stained sperm to determine mating status and preference. Results indicated no fitness impact by the addition of Rhodamine B to the sucrose solution. Furthermore, it successfully determined that females prefer to mate with outcrossed males as opposed to inbred, indicating a potential loss of male fitness. This study illustrates the potential of Rhodamine B to reduce labour costs when investigating the mating competitiveness of either sterile or Wolbachia lines of Ae. aegypti.

Major Concerns:
* It is a relatively straight forward study that could be further supported with additional data (see comments later in this section). The figures and attached video are simple yet effective at conveying the results and showing a visual illustration at key steps of the experimental work flow. I was hoping

to see some Rhodamine B illumination in the video, it may be too late now to add but I was waiting on it switching to the fluorescence shot and the video ended. I was a tad disappointed.

Sorry for the disappointment. Video 1 covers solely on the dissection of female spermathecae. The observation of the fluorescence in the spermathecae will definitely be included in the final Jove video.

* Rhodamine B has huge potential for use in male mosquito release programmes, either IIT, SIT or combined. You do mention this towards the end of the introduction, but I think it could be highlighted more, or specifically in the context of SIT. Instead of comparing between lines, it could be used to test sterile vs control males with the same experimental setup as illustrated in this manuscript, thus drastically reducing the laborious task that is traditional semi-field mating competitiveness studies.

Included an elaboration (line 58 to 61) in the introduction of the revised manuscript.

* I assume that there has been preliminary work carried out to identify the correct volume of Rhodamine B to add to the sucrose solution. In the Johnson et al paper he tried 0.1, 0.2, 0.4 and 0.8%. What is the reasoning behind your selection of 0.2%? Did you conduct initial trials? What were the results? There is also no mention of whether or not there is any impact upon survival following 3 days of feeding with Rhodamine B. I suspect if you had fed then 0.8% at any point you will have noticed this higher dose of Rhodamine B does negatively impact survival. It may not be necessary to add, but you may want to consider including a table with survival data between stained and unstained lines. More data would just add more weight to your conclusion.

Yes, preliminary work has been carried out to determine the lowest rhodamine concentration which could be used for sufficient male staining and yet elicit less fitness impact on the males. Based on Johnson's paper, 0.4% is the recommended concentration. Hence, in our preliminary work, we followed the survivability of wild-type *Ae. aegypti*, and the *w*AlbB strain of *Wolbachia*-carrying *Ae. aegypti* upon feeding with 0.2% and 0.4% rhodamine B. Data for this work is presented in Supplemental File 1.

* I also think your title could be improved. Maybe something like "Assessing the efficacy of Rhodamine B as a measure of successful mating in Aedes aegypti" or "Investigating the use of Rhodamine B marking as a proxy of male mating competitiveness in Aedes aegypti". Or you could elude to the fact that it can differentiate between Wolbachia or sterile lines.

The title has been revised to "The use of Rhodamine B fluorescent dye for studying mating competitiveness in male *Aedes aegypti* mosquitoes".

o The discussion is very light on references (only reference 28 is new). It could be worth discussing other studies (such as the Blanco paper) where Rhod B has been used for marking. Are there any other papers where marking techniques have helped to drastically reduce labour costs, for comparison?

Included more discussion on the use of RhB for marking in mosquito mating studies. However, we couldn't find any publication which marking has helped to reduce labour cost.

* A final note is that this manuscript would benefit from a native English speaker giving it the once over.

As advised, the revised manuscript has been proofread again.

Minor Concerns:

Thank you Reviewer #1 for your comments and suggestions. All minor concerns have been addressed in the revised manuscript.

* Abstract/ Intro
o L29: "leverages the ability of"… I would change this to "depends on the ability of"…
o L31: "targeted" should just be "target"
o L33: place "an" before "assay"
o L54: it should read "a wide range of insecticides…"
o L61: "For male release-based mosquito population suppression program".. either insert a before "male" or change "program" to the plural
o L67 and 69: "Hence" and "Besides" could be replaced to read clearer.
o L69: Change "besides" to "furthermore"
o L80: "egg" viability not "eggs"
o L86-87: Remove "for measuring mating competitiveness" it is a repeat of what's just been said.
o L97: Although reference has been made to the Blanco et al paper in which Rhod B is used in Tobacco budworm, it may be of use to acknowledge that it has been used in a recent study with Anopheles gambiae in addition to the paper by Johnson et al - (Aviles, E.I., Rotenberry, R.D., Collins, C.M. et al. Fluorescent markers rhodamine B and uranine for Anopheles gambiae adults and matings. Malar J 19, 236 (2020). https://doi.org/10.1186/s12936-020-03306-5) – Reference added

* Methods
o What are these densities? If they are noted in a previous citation, add it here, if not then state them. Same for the feeding regime

Included in the revised manuscript

o L132: just a note for future experiments that circumvents any chance of male contamination when the use of virgin females is require - tube female pupae individually, visually inspect upon emergence and then aspirated desired number into a Bugdorm

Noted with thanks.

o L209: what is the justification for only 3 hours of mating? To reduce the possibility of multiple matings?

Yes, as there might be higher possibility of multiple mating if the mating duration is increased. Preliminary work has been done to determine the mating duration needed to achieve high insemination rate of ten wild-type female adults with twenty wild-type male adults in a cage size of 60cm x 60cm x 60cm. Data for this is presented in Supplemental File 2.

* Results
o L246: this is the first mention in the manuscript of what Set A and Set B represent. This should clearly be stated in the methodology.

As advised, Set A and B is described in line 112 in the protocol section of the revised manuscript.

* Discussion
o L315-317: Surely there must be a reference for this? How do you know this cost otherwise?
The cost of rhodamine B is based on quotation from Sigma Aldrich, the company which we procure the chemical from. For blood and membrane, the cost is based on our Institute's procurement of high health status pigs' blood, and specific pathogen free mouse skin. These were purchased for our routine mosquito colonization and research work. However, upon deliberation, we decided to remove this paragraph as different institute may have different source of animal blood and membrane for mosquito blood feeding. Hence, cost comparison between rhodamine B and blood feeding may not be appropriate.

Reviewer #2:
Manuscript Summary:
The manuscript offers a simple protocol to assess the mating competitiveness between 2 sets of males, even if they are both sterile. Although this method is not new, and the use of rhodamine B in mating competitivenss studies has been described previously, the clear and simple protocol is very useful for many scientists- particularly those involved in insect mating studies, and the SIT and related techniques. It is a promising and efficient alternative to competitiveness studies based on the Fried Index, and also to using expensive stable isotopes, which also requires expensive equipment for analysis.

Major concerns:
no major concerns

Minor Concerns:
The issue of the possible occurrence of double mating is mentioned briefly, and is of some concern. additional experiments to evaluate the frequency of double mating in such studies, and their significant (or not) impact on results would strengthen the study and remove doubts. However, the fact that mosquito densities were kept low, and that the results stayed consistent regardless of marking status indicates that this method seems to be reliable in this case.
The manuscript needs extensive editing and is poorly written. however the information is of interest and the basic information is present.

Thank you Reviewer #2. Yes, double mating can occur when there is interrupted mating. In our experience, when there is excessive external disturbance to the mating cages, or when we increase the mosquito density per cage volume, these factors encouraged flying of the mosquitoes in the confined cage. The female wing beat frequency encourage courtship by the males. Hence, polyandry can occur if a second insemination occurs prior to the formation of mating plug from the first insemination. Hence, for this protocol, the mosquito densities were kept low, a bigger cage space of 60cm x 60cm x 60cm was used, and the mating duration of the competitiveness assay was kept minimal.

Noted on the language issue. The revised manuscript has been proof-read.

Reviewer #3:
Manuscript Summary:
Review on JoVE62432 "Conduct of male Aedes aegypti mosquito mating competitiveness study using fluorescent dye rhodamine B"
Suggestion:
Minor revision

General comments
Male mating competitiveness is one of the critical parameters of SIT or IIT, or SIT/IIT combination, for mosquito population control. Therefore, mating competitiveness of the released males should be monitored. The traditional competitive mating experiment is time-consuming and the results are highly varied between replicates, thus it is required to develop a fast and effective method to assess the mating competitiveness of the released males, especially for mosquitoes. In this study, the authors develop a rhodamine B-based marking method to assess the mating competitiveness of two sterile mosquito strains, which has reduced 90% man-hour compared to the traditional method. Using marking method to study the mating competitiveness of sterile males is not new in the fruit flies SIT, as the mating period is long between male and female flies. Thus it is possible to collect the files during mating and observe directly that the females mated with the marked or unmarked males. However, this method is infeasible for mosquitoes as the mating period is short and they are not easy to capture. The developed rhodamine B-based marking method is worth of investigation in field of mosquito SIT or IIT, or the SIT/IIT combination.
In my opinion, the title and abstract is appropriate for this methods article. All the materials and equipment needed have been listed in the table except for the forceps for dissection . The steps listed in the procedure have been clearly explained and it is feasible lead to the described outcome. There is no any important step missed in the protocol. The study has been set up appropriate controls and all the critical steps have been highlighted. The results are reasonable, and useful to readers.

Major Concerns:
No

Minor Concerns:
However, I have some minor comments or questions on the current manuscript.
1.It is important to present the information on proportion of RB staining on male mosquitoes after three days' feeding.

This data is now included in Supplemental File 1 (line 313 to 321 in revised manuscript).

2.As RB is toxic, the author has to mention how to deal with the remaining RB-source and the RB mated females after experiments. In addition, goggles is also required during handling RB.

Included in the protocol section on disposal of RB waste (line 237 to 240 in revised manuscript). Goggles is added as part of the PPE when handling RB.

3.How long can the RB solution be maintained under room temperature? –

We have not tested the stability of RB solution at room temperature. However, the fluorescence in the spermathecae of females mated with RB-marked males can still be observed one week after copulation.

4.A flow chart on date to describe the whole experiment is recommended. And compare with the traditional method for conducting the competitive experiment to explain the 90% reduction on man-hour.

Flow chart is included in Supplemental File 3 as Figure S2.

5.How many cages and mosquitoes totally are required for one competitive experiment?

Cage	Purpose	Quantity	No. mosquitoes
32.5cm x 32.5cm x 32.5cm	Sucrose feeding for Set A male mosquitoes	1	100 per cage, out of which **60** are aspirated for competitiveness cage
	RhB-sucrose feeding for Set A mosquitoes	1	100 per cage, out of which **60** are aspirated for competitiveness cage
	Sucrose feeding for Set A male mosquitoes	1	100 per cage, out of which **60** are aspirated for competitiveness cage
	RhB-sucrose feeding for Set A mosquitoes	1	100 per cage, out of which **60** are aspirated for competitiveness cage
	Holding virgin female mosquitoes	3	50 females per cage. Additional cages were prepared in case there was male contamination in the female cages. **120 females** were required for one competitiveness experiment (12 mating cages, 10 females per cage)
60cm x 60cm x 60cm	Mating competitiveness assay	12	6 cages – Each cage contains 10 Set A (RhB-marked) males + 10 Set B (unmarked) males + 10 virgin females 6 cages - Each cage contains 10 Set A (unmarked) males + 10 Set B (RhB-marked) males + 10 virgin females

6.Use amber bottles or wrap bottles in aluminum foil?

Yes, amber bottles or bottles wrapped in aluminium foil can be used for holding RhB-sucrose as RhB is light sensitive. Is there any picture on explanation this? See picture below. The final Jove video will also illustrate the RhB-sucrose feeding.

7.How long can we keep the female mosquitoes which have mated with RB males before the dissection of the spermathcaes? Is it possible to perform the dissection the next several days after the termination of mating experiment without affecting the sperm staining?

Yes, as long as the females are kept alive, RB marking in their spermathcae can still be observed. We have tested females which have been kept alive for one week after mating with RB-marked males, and the RhB signal in their spermathecae under fluorescence microscope is still visible.

What kind of the forceps the authors used to dissect the spermathecaes of female mosquitoes?

Any forceps can be used, as long as it is sharp and fine enough to hold on to the last segment of the mosquito abdomen. We used the Rubis forceps (catalog number 4524) from Bioquip. Information is included in the Table of Materials.

8.A table should be presented in the main text to show the exact number of female mosquitoes mated with either inbred or outcross males, and then the process of data, to make the readers understand the figures 5 and 6 clearly. As I think no all of the females have inseminated during 3 hours' mating experiment. The author should present the insemination rate as well.

As suggested by reviewer, the data is presented in Table 1 and 2.

9.The authors have to discuss the marking method used in the fruit files SIT project in the discussion part.

Added in discussion. (line 342-347)

Supplemental Material
Click here to access/download;Supplemental File (Figures, Permissions, etc.);62432_R1_Supplemental.doc

Supplemental Material

Using the Fluorescent Dye, Rhodamine B, to Study Mating Competitiveness in Male *Aedes aegypti* Mosquitoes

AUTHORS AND AFFILIATIONS:
Irene Li[1*], Keng Wai Mak[1*], Jeslyn Wong[1], Cheong Huat Tan[1]

[1]Environmental Health Institute, National Environmental Agency, Singapore

*These authors contributed equally.

Email addresses of co-authors:
Irene Li (Irene_li@nea.gov.sg)
Keng Wai Mak (Mak_keng_wai@nea.gov.sg)
Jeslyn Wong (Jeslyn_wong@nea.gov.sg)

Corresponding author:
Cheong Huat Tan (Tan_cheong_huat@nea.gov.sg)

1. Optimization of RhB-sucrose feeding for the effective marking of male adult *Ae. aegypti* and transfer of marked seminal fluids to females after copulation.

In a previous study conducted by Johnson et al., 0.4% RhB–sucrose was used to mark male *Ae. aegypti* for a mating competitiveness experiment. As a higher RhB concentration could be toxic to mosquitoes, the survival of male wild-type and *w*AlbB-*Ae. aegypti* was compared after feeding with 0.2% and 0.4% RhB-sucrose solutions, relative to controls that were fed with 10% sucrose (**Supplemental Figure S2**). The mosquitoes were fed for three days and thereafter, the sucrose was replaced with water for the next six days. Mosquito mortality was monitored daily for these nine days. Higher mortality was observed for both wild-type (**Supplemental Figure S2A**) and *w*AlbB-*Ae. aegypti* (**Supplemental Figure S2B**) fed with 0.4% RhB–sucrose.

As mosquitoes fed with 0.2% RhB–sucrose showed higher survival, the next step was to determine the marking rate of male *Ae. aegypti* using 0.2% RhB–sucrose. Two hundred wild-type male *Ae. aegypti* imagoes were allowed to emerge in a W 32.5 cm x D 32.5 cm x H 32.5 cm cage. Upon emergence, five bottles, each containing 10 mL of 0.2% RhB–sucrose, were placed at the four corners and the center of the cage. Fifty mosquitoes randomly sampled from the cage at the 24-, 48- and 72-h mark upon placing the RhB–sucrose, were checked for their RhB marking under a fluorescence stereo microscope. All mosquitoes sampled at these time points had been successfully marked with RhB.

As male mosquitoes used in most male-release suppression programs were between two to three-day-old, a three-day RhB–sucrose feeding period was selected. To ensure that seminal fluid from male mosquitoes marked with 0.2% RhB–sucrose was visible in the female

spermathecae under the fluorescence stereo microscope, 50 pairwise (marked male with virgin female) mating trials were conducted. After 24 h, the spermathecae from female adults were removed and observed for RhB signal under the fluorescence stereo microscope. All females from the 50 replicates had fluorescing spermathecae, indicating that males marked with 0.2% RhB were able to transfer marked seminal fluids to the females.

2. Optimization of mating duration for the mating competitiveness assay

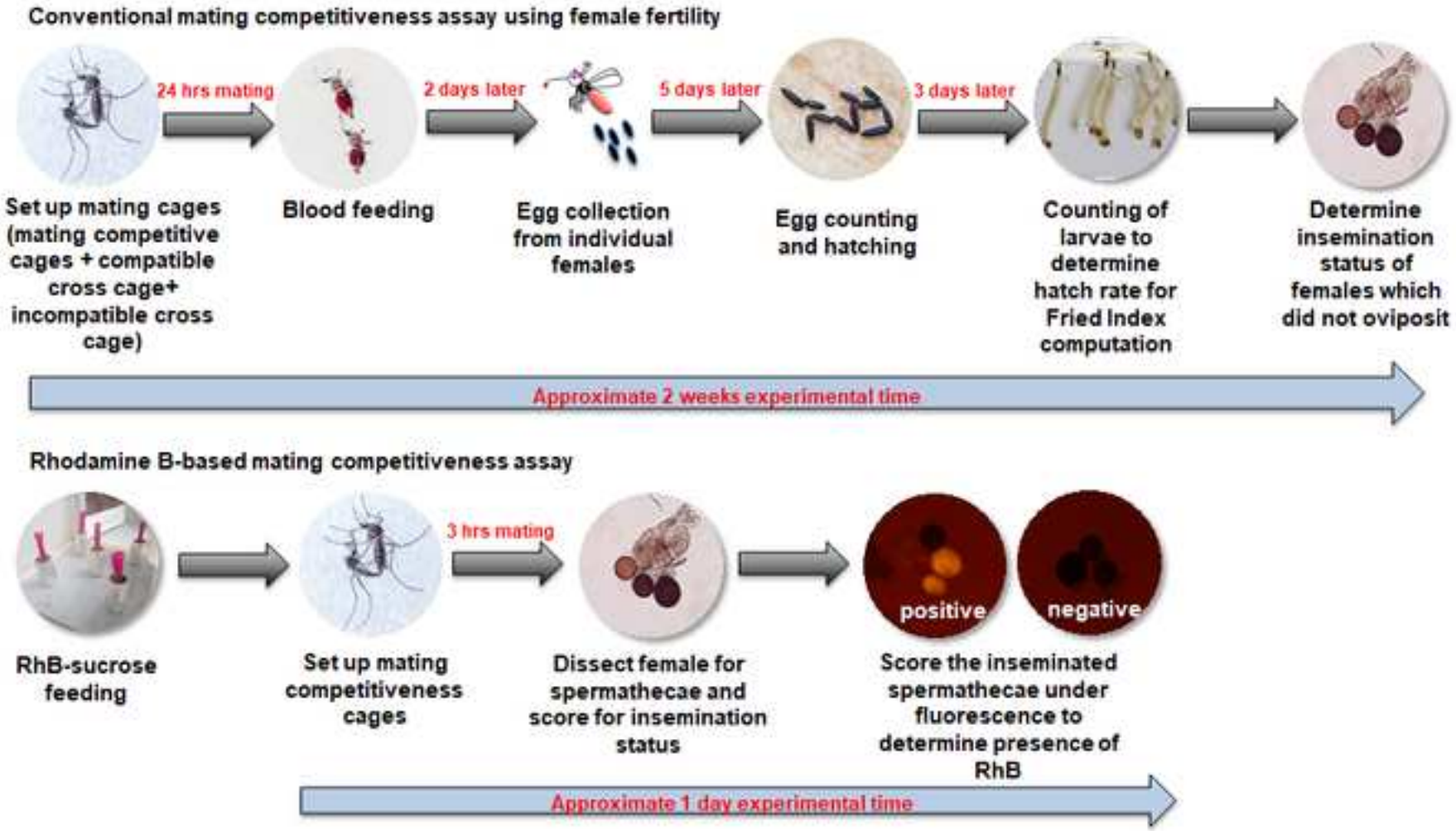
To reduce the chance of mixed mating, a W 60 cm x D 60 cm x H 60 cm cage was used with a mosquito density of 10 females to 20 males. As the mating duration should also be reduced to minimize the chance of mixed mating, the next step was to determine the minimum mating duration required to achieve 90% insemination rate. Nine mating assay cages (each with 10 female and 20 male wild-type *Ae. aegypti*) were set up. The female insemination rate from three replicate cages was determined at 1-, 2- and 3-h time points (**Supplemental Table S1**). As over 90% insemination rate can be achieved in 3 h, the mating duration for the mating competitiveness assay was set at 3 h.

3. Comparison of workflow and experimental duration required for RhB-based and conventional mating competitiveness assay

Supplemental Figure S1 illustrates the simplified workflow and shortened experimental duration to perform the male mating competitiveness assay using RhB, in comparison to the conventional method using a female fertility assay.

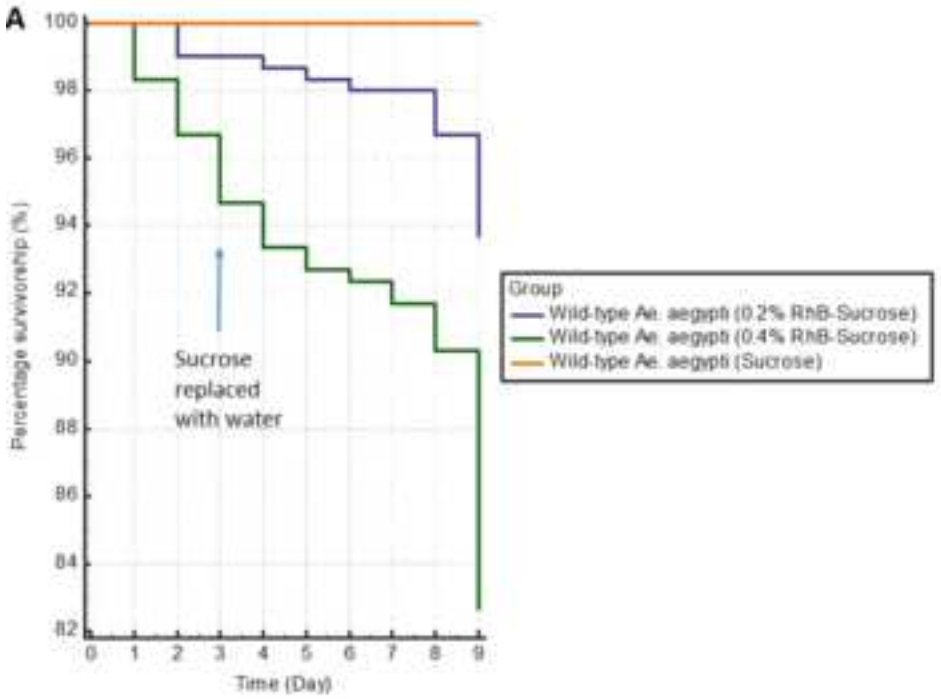
Supplemental Figure S1

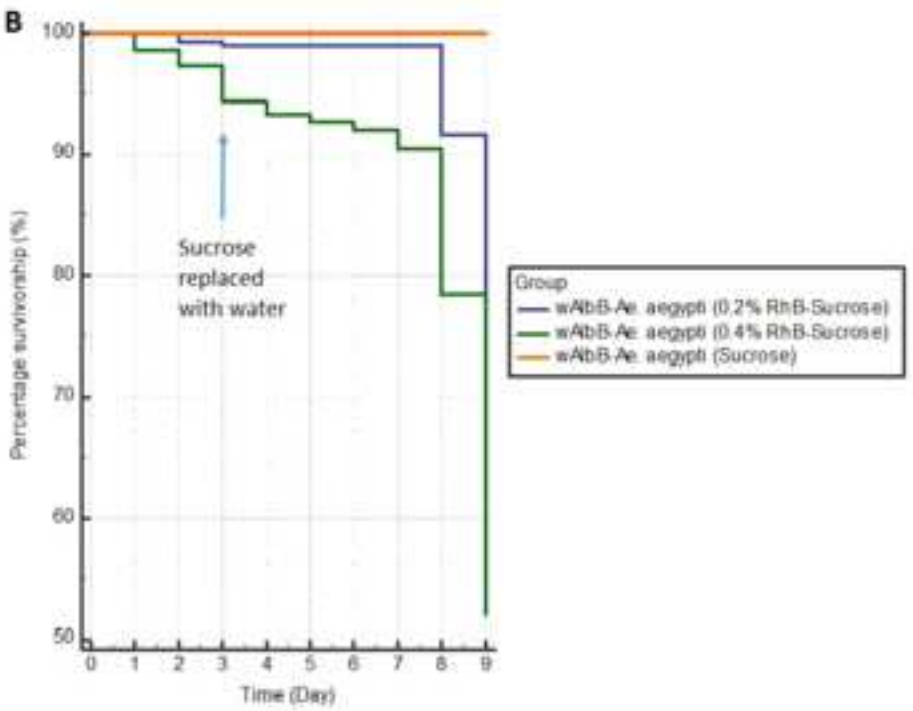
Click here to access/download;Supplemental File (Figures, Permissions, etc.);Figure S1.tif



Supplemental Figure S2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Figure S2.tif





Supplemental Table S1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Table S1.xlsx

Mating duration (h)	Insemination rate		
	Replicate 1	Replicate 2	Replicate 3
1	30% (3/10)	50% (5/10)	50% (5/10)
2	70% (7/10)	70% (7/10)	80% (8/10)
3	100% (10/10)	90% (9/10)	90% (9/10)