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Determining the Egg Fertilization Rate of Bemisia tabaci Using a Cytogenetic Technique

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Corresponding Author:	Elizabeth Bondy UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	ebond@email.arizona.edu
Order of Authors:	Elizabeth Canlas Bondy
	Martha S. Hunter
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September 30, 2018

Dear Editor,

We are submitting a technique paper for you to consider as part of a video article in JoVE: "Determining primary sex ratio of *Bemisia tabaci* using a cytogenetic technique."

Our paper contributes a technique to directly determine the primary sex ratio (=fertilization rate) of these haplodiploid insects. Whiteflies in the species complex collectively known as *Bemisia tabaci*, include some of the worst cosmopolitan pests. As vectors of plant viruses, they threaten cassava production for small landholders in Sub-saharan Africa, and direct damage to cotton lint in cotton production make them the worst pests of cotton in the USA. These insects have been the subject of several international conferences (International Whitefly Symposium) and hundreds of ecology, pest management and evolutionary biology papers, including papers in Science, PNAS, Current Biology and others.

While sex allocation studies are a staple of the behavioral ecology of insects and particularly of parasitoid Hymenoptera, to our knowledge, no one has determined the primary sex ratio of whiteflies, yet being able to predict sex ratios is important for population forecasting, and being able to determine whether fertilization occurs is important for determining what type of reproductive isolating mechanisms stand among the dozens of sibling species in this complex.

As our technique is difficult and novel for whiteflies, a video medium would allow a wider audience to fully understand and perfect this method.

Thank you for your consideration,

Martha S. Hunter and Elizabeth C. Bondy

TITLE:

Determining the Egg Fertilization Rate of *Bemisia tabaci* Using a Cytogenetic Technique

AUTHORS AND AFFILIATIONS:

Elizabeth C. Bondy[1], Martha S. Hunter[2]

[1]Graduate Interdisciplinary Program in Entomology and Insect Science, University of Arizona, Tucson, AZ, USA

[2]Department of Entomology, University of Arizona, Tucson, AZ, USA

Corresponding author:

Elizabeth C. Bondy (ebond@email.arizona.edu)

Email addresses of co-author:

Martha S. Hunter (mhunter@ag.arizona.edu)

KEYWORDS:

Bemisia tabaci, sex allocation, primary sex ratio, fertilization rate, cytogenetics, DAPI

SUMMARY:

We present a simple cytogenetic technique using 4',6-diamidino-2-phenylindole (DAPI) to determine the fertilization rate and primary sex ratio of the haplodiploid invasive pest *Bemisia tabaci*.

ABSTRACT:

A few species of sap-sucking whiteflies are some of the most damaging terrestrial pests worldwide because of the crop damage they inflict and plant viruses they vector. Despite numerous studies of the biology of these species in different environments, a key life history parameter, offspring sex ratios, has received little attention, yet is important for predicting population dynamics. The primary sex ratio (sex ratio at oviposition) of *Bemisia tabaci* has never been reported but can be found by determining the egg fertilization rate of this haplodiploid insect. The technique involves the dechorionation of eggs with bleach, a series of fixation steps, and the application of the general DNA fluorescent stain, DAPI (4',6-diamidino-2-phenylindole, a DNA-binding fluorescent dye), to bind to female and male pronuclei. Here, we present the technique, and an example of its application, to test whether an endosymbiotic bacterium, *Rickettsia* sp. nr. *bellii,* influenced the primary sex ratio of *B. tabaci*. This method may assist in population studies of whiteflies, or in determining if sex allocation exists with certain environmental stimuli.

INTRODUCTION:

The study of sex allocation, or the relative investment in male and female offspring, is a cornerstone of behavioral ecology[1–3]. In addition to its power for testing adaptive models of behavior, knowing the sex allocation strategy of an organism may improve models of its population dynamics. In many species, sex allocation is controlled by mothers. To determine sex

allocation, it is important to determine the primary sex ratio or the proportion of females at the time of egg deposition. Although the sex ratio at adult emergence may provide clues to sex allocation, differential developmental mortality between male and female juveniles may commonly skew the adult sex ratio substantially. In some species of Hymenoptera, the order of insects that contains ants, bees, and wasps, the primary sex ratio has been determined with cytogenetic assays, staining the embryos to view genetic DNA. Because hymenopterans are haplodiploid, an incipient male egg is haploid and contains only the female pronucleus (n), while incipient female eggs are diploid and contain both male and female pronuclei (2n). Although Aleyrodidae, the sap-feeding family of true bugs (Hemiptera) known as whiteflies, are also haplodiploid, there has not been an established assay to find the primary sex ratio in these insects. This is perhaps surprising given the intensity of study of the few cosmopolitan serious pests in this family and the importance of sex ratios in competitive interactions of whiteflies[4–10] and in population dynamics generally. In haplodiploid insects too, sex ratios are unconstrained by sex determination systems, allowing the possibility of selective fertilization and labile sex ratios that vary with the environment[2]. Here we present a technique to determine the primary sex ratio of the species complex of whiteflies known collectively as the sweetpotato whitefly, *B. tabaci.* This one species name encompasses more than 28 species worldwide[11] and includes some of the most damaging global invasive pests[12,13]. The application of this technique to determine sex allocation patterns in *B. tabaci* and other Aleyrodidae will allow a more rigorous investigation of variables, including temperature, host plant, endosymbiotic bacteria, or plant/whitefly pathogens, that may influence whitefly primary sex ratios and whitefly population dynamics.

We are unaware of any comparable egg-staining techniques for *B. tabaci*. The protocol is convenient in comparison with staining methods used for other insect eggs[14] as it omits an overnight fixation step and, therefore, can be completed within 3 h. As one example of an application, an endosymbiotic bacterium, *Rickettsia* sp. nr. *bellii,* is associated with female bias in our laboratory lines of *B. tabaci* Middle East-Asia Minor 1 (MEAM1)[15,16]. In one *B. tabaci* MEAM1 laboratory line ("MAC1," collected from the Maricopa Agricultural Center), we test whether *Rickettsia*-infected (R^+) females fertilize more eggs than uninfected (R^-) females.

PROTOCOL:

NOTE: Ensure that all work is performed at room temperature in a well-ventilated area or under a fume hood. All 'drops' in this protocol are defined as 5–20 µL, depending on the operator's preference.

1. Initial setup

1.1. Allow female whiteflies to oviposit on clean leaves. Examples for oviposition arenas include clip cages or leaves cut to fit on agar in a Petri dish. Make a coverable hole in the clip cage or Petri dish cover to insert and remove the adults.

1.1.1. Alternatively, quickly put a vessel of collected adults on ice and, then, deposit them on the leaf. Limit the time between oviposition and egg fixation to no more than 60 min, to ensure the

observation of the sperm transition into the paternal pronucleus in eggs.

NOTE: Eggs not removed from the leaf can be reared to adulthood to record adult sex ratios.

1.2. Before or during whitefly oviposition, prepare a microscope slide by cleaning it with soap and water, drying it well, and then stretching a piece of paraffin film over one end, making sure the paraffin film surface does not break (**Figure 1**).

NOTE: The paraffin film is hydrophobic and semi-opaque, allowing liquids to form drops and eggs to be more easily seen.

2. Dechorionation

2.1. With a glass Pasteur pipette, add drops of bleach solution (0.83% sodium hypochlorite) to the paraffin film.

NOTE: It is easier to keep track of eggs if only 2–3 eggs are in each drop. Alternatively, to manage a large egg number, collect the eggs in drops of 1x phosphate-buffered saline (PBS).

CAUTION: Bleach is corrosive; wear gloves when handling bleach and do not inhale.

2.2. Remove the adult whiteflies from the leaf.

2.3. Place the leaf under a microscope to see the eggs clearly and collect the eggs singly and carefully with a thin probe. To make a probe, insert a minuten nadeln pin at 45° or at a comfortable working angle into a melted pipette tip (**Figure 2**).

2.4. Transfer the eggs to bleach or 1x PBS. If using 1x PBS, remove the 1x PBS with a glass Pasteur pipette once all the eggs are collected and, then, add bleach to the eggs.

NOTE: When collecting eggs, slowly lift the egg from its base until the pedicel is removed from the leaf. The pedicel is sticky, and the egg will commonly stick to the probe tip until it is dipped into the bleach. It is recommended to use separate glass Pasteur pipettes for all reagents, and the pipettes may be modified with heat to make the tips narrower and reduce the risk of accidentally aspirating whitefly eggs. To prevent residue and contamination, clean the pipettes with deionized water after every use.

2.5. Wait for 10 min. If there is an interest in embryogenesis in eggs older than 1 h, leave the eggs in bleach for up to 15 min.

NOTE: For eggs that are up to 1 h old, 10 min is sufficient.

3. Fixation

NOTE: These steps are taken from a Hymenopteran protocol[17].

3.1. Remove the bleach (containing the chorion fragments) with a glass Pasteur pipette and discard it. Add drops of glacial acetic acid with a glass Pasteur pipette and wait 3 min.

CAUTION: Proceed with this step under a fume hood. Glacial acetic acid is corrosive; wear gloves when handling glacial acetic acid and do not inhale, especially in combination with the residual bleach.

3.2. Remove the glacial acetic acid with a glass Pasteur pipette and add drops of Clarke's solution (3:1 of absolute ethanol:glacial acetic acid) with a glass Pasteur pipette. Wait until most of the solution has evaporated (or 10 min max).

3.3. Add drops of 70% ethanol to the eggs with a glass Pasteur pipette and wait until most of the ethanol has evaporated (or 10 min max).

4. Staining

4.1. Remove any residual ethanol with a glass Pasteur pipette and add drops of 1x PBS to the eggs with a glass Pasteur pipette to get the pH close to 7.0. Set the microscope slide in a humidity chamber to prevent desiccation, for example, in an empty pipette tip box with a wet paper towel inside (**Figure 3**). Wait at least 30 min.

NOTE: This is the best point if a pause is needed, as long as the humidity chamber can prevent desiccation.

4.2. Remove the 1x PBS with a glass Pasteur pipette, and add drops of 0.1 µg/mL DAPI, a DNA fluorescent stain, with a glass Pasteur pipette. Set the microscope slide in a dark humidity chamber and wait at least 15 min.

CAUTION: DAPI is an irritant, so handle it with gloves.

5. Washing

5.1. Remove the DAPI with a glass Pasteur pipette.

5.2. Add drops of 1x TBST (5x solution made from 30 g of Tris, 43.8 g of NaCl, 5 mL of polysorbate 20, and 1.0 g of NaN_3 [pH 7.5], and brought to 1 L with PCR grade water) to the eggs with a glass Pasteur pipette. Wait 5 min before removing the 1x TBST with a glass Pasteur pipette. Repeat this step 2x.

6. Mounting

6.1. After the final wash, carefully pipette all the eggs from the paraffin film onto a clean part of the microscope slide. Remove excess 1x TBST; then, add 20 µL of mounting media (80% glycerol and 20% 1x TBST with 2% n-propyl-gallate) and place a clean cover slide on top of the eggs.

6.2. For long storage, seal the cover slide with clear nail polish and, then, either store the slide in the dark at 2 °C or immediately view it under a fluorescent microscope.

REPRESENTATIVE RESULTS:

To test whether *Rickettsia* affects the fertilization rate of *B. tabaci* MEAM1 females, we reared *Rickettsia*-infected (R^+) or uninfected (R^-) *B. tabaci* on cowpea plants (*Vigna unguiculata*) in separate cages at 27 °C, 70% relative humidity, and a 16 h light/8 h dark photoperiod. R^+ and R^- fourth instar whiteflies were carefully removed from leaves and isolated in 200 µL strip tubes. When adults emerged, they were collected in groups of 50% females and transferred to clean leaves in Petri dishes (n = ~50–100/leaf) for 4 days of mating. Groups of approximately 20 females and several males were then transferred to a clean leaf disk (one for R^- adults, one for R^+ adults) in 35 mm Petri dishes resting on 1% agar. The Petri dish lid had been cut out and the fine fabric mesh used for containment also prevented excess condensation. After approximately 45 min, all adults were removed, some of the eggs were harvested to determine the fertilization rate, and the eggs that were not collected were reared to adulthood to calculate the adult sex ratio. One cohort for a single day, for both R^+ and R^- whiteflies, was defined as a block. There were seven blocks for calculating the fertilization rate or primary sex ratio, while there were six blocks for calculating the adult sex ratio, as there were not enough leftover eggs from one block to rear to adulthood. A generalized linear model was used in the statistical package R to determine whether the fertilization rates or adult sex ratios were significantly influenced by *Rickettsia* infection and/or block. The response variables were the proportion of fertilized eggs/all eggs, or the proportion of female adults/all adults, respectively, while explanatory variables were the block and *Rickettsia* infection status.

Egg dechorionation followed by DAPI nuclear staining allowed the unambiguous assignment of fertilization (and embryo sex) when observed with a fluorescent microscope (**Figure 4**). For this experiment, 90 eggs laid by R^- *B. tabaci* MEAM1 females and 82 eggs by R^+ *B. tabaci* MEAM1 females were scored. As for eggs reared to adulthood, 60 R^- and 95 R^+ adults were scored. While a female bias in adult sex ratios has been shown consistently in earlier studies[15,18,19], in the current study, adult R^+ sex ratios (69% females, median) were female-biased compared to R^- females (50% females, median), but the sex ratios in the two treatments were not significantly different (χ^2 = 1.02, df = 1, p = 0.31; **Figure 5**). The primary R^- sex ratios (60% fertilized eggs, median) were female-biased compared to R^+ sex ratios (44% fertilized eggs, median) but were also not significantly different (χ^2 = 0.51, df = 1, p = 0.47), providing no evidence for greater fertilization rates by R^+ females (**Figure 5**). Block also did not have a significant effect on the primary (χ^2 = 0.29, df = 1, p = 0.59) or adult (χ^2 = 1.20, df = 1, p = 0.27) sex ratio.

FIGURE LEGENDS:

Figure 1: Photo of a microscope slide with paraffin film and drops of bleach.

Figure 2: An example of a probe, fashioned with heat, from a pipette tip and a minuten nadeln pin.

Figure 3: An example of a humidity chamber, fashioned from an empty pipette tip box and a wet paper towel.

Figure 4: Fluorescent microscope images of *B. tabaci* eggs. (**a**) *B. tabaci* MEAM1 fertilized, incipient female eggs. (**b**) Unfertilized, incipient male eggs. The eggs were fixed at less than 1 h after oviposition and stained with DAPI. The base of each egg is fluorescing due to bacterial DNA (*Portiera*, *Hamiltonella*, and possibly *Rickettsia*) in the bacteriome progenitor cell, which is included in the laid egg[20,21]. In each egg, the female pronucleus is near the center of the egg, and in the female egg only, the sperm is visible as a bright streak near the apex of the egg near what is presumably an autofluorescing micropyle. The images are screenshots taken from a Z-stack generated video, produced by a laser scanning confocal inverted microscope. The scale bars were estimated from previous microscope images.

Figure 5: Primary and adult sex ratios of *Rickettsia*-infected or uninfected *B. tabaci*. Box and whisker plots of the primary sex ratio (percentage of fertilized eggs, or percentage of female zygotes) in black compared to the adult sex ratio (percentage of adult females) in grey, of the *Rickettsia*-infected (R^+) and uninfected (R^-) *B. tabaci* MEAM1, "MAC1" genetic line. The box and whisker plots show the median as the middle line, the mean as a plus sign, and upper and lower quartiles as the lines that make the ends of the box, and the range is represented in the outer lines extending from the box. For R^- eggs scored: $n = 90$; for R^+ eggs scored: $n = 82$. For R^- adults counted: $n = 60$; for R^+ adults counted: $n = 95$. In a logistical analysis of the primary sex ratio (proportion of fertilized eggs) performed in the statistical package R, no significant effects were found for block ($n = 7$, $\chi^2 = 0.29$, df = 1, $p = 0.59$) or for *Rickettsia* infection ($\chi^2 = 0.51$, df = 1, $p = 0.47$). Adult sex ratios, as influenced by block ($n = 6$) and *Rickettsia* infection status, were similarly analyzed. Here as well, no significant effects were found for block ($\chi^2 = 1.20$, df = 1, $p = 0.27$) or for *Rickettsia* infection ($\chi^2 = 1.02$, df = 1, $p = 0.31$).

DISCUSSION:

This protocol is the first to capture the fertilization rate or primary sex ratio of *B. tabaci*. The challenge of this protocol is that it requires researchers to learn how to handle the whitefly eggs quickly, ensuring that not more than 1 h has passed since the eggs were oviposited until they are fixed. During preliminary experiments, eggs that were fixed at 3 h or more postoviposition were too old to observe fertilization, as syngamy had occurred and mitotic divisions were underway. Between 1 to 3 h, the pronuclei took on a rounder shape. While the early presence of two nuclei indicates a fertilized egg, a bit later, the apposition of the two nuclei in preparation for syngamy can appear to be one nucleus, and later again, the two products of the first mitotic division are found in both male and female eggs. Therefore, the distinction between the sexes is not clear at these later time points, and we advise limiting the interval from oviposition to fixation to 1 h as a conservative measure. It is also challenging to learn how to be gentle with the eggs with each transfer of liquid so that they are not accidentally aspirated into the pipette. At the time of

viewing the eggs under a fluorescent microscope, a few of the eggs may have broken during the protocol, so those eggs cannot be sexed and counted as the pronuclei and yolk may have escaped. Otherwise, once the operator becomes comfortable with these steps, the protocol can be completed conveniently within 3 h, as it does not require an overnight fixation step. It is also flexible in that it can be modified to stain older eggs, for those researchers interested in capturing development.

An application of this protocol includes research on sex allocation. Although many dozens of studies of whitefly biology have reported adult sex ratios in different environments, adult sex ratios confound sex allocation of the mother with sex-specific developmental mortality of nymphs and make it impossible to determine the cause of any sex ratio pattern. The cytogenetic technique described here allows the possibility of understanding whitefly sex allocation patterns more generally. While the large dispersive populations of *B. tabaci* and other pests such as the greenhouse whitefly, *Trialeurodes vaporariorum,* might be predicted to result in 1:1 sex ratios, as often exhibited in laboratory settings[22,23], we also predict that reproductive interference, endosymbionts, and potentially host plant quality could influence primary sex ratios. That these same factors could also influence sex-specific mortality patterns during development, systematically skewing sex ratio estimates, underscores the need for a direct measure of the primary sex ratio.

While female bias associated with *Rickettsia* infection has been consistently found in the genetic line "MAC1"[15,18,19], the adult sex ratios were not significantly female biased in the current study. The primary sex ratios observed with the cytogenetics technique were not significantly biased either. We censused the primary and adult sex ratios of *B. tabaci* females on just 1 day, at the beginning of an oviposition bout, when the females were 4 to 5 days old, so the sex ratios studied may not have represented the sex ratios that we observed over longer periods. Nonetheless, the technique made it possible to determine the fertilization rate or primary sex ratio and, in this instance, showed a correspondence between the primary and adult sex ratios.

Determining fertilization may also be valuable in instances in which researchers might want to determine the type of reproductive isolation among whitefly populations. While it has been a matter of some controversy[12,24,25], it is now generally accepted that the name *B. tabaci* refers to tens of cryptic species, with evidence being provided by both genetic divergence and crossing tests in which few females are produced[12,25]. In these studies, it would be of interest to know where the isolation occurs. Is sperm transferred, and does heterospecific sperm fertilize eggs, or are attempted matings unsuccessful? Regarding the determination of the primary sex ratio, this technique can determine whether the primary sex ratio is influenced by endosymbionts, selfish genetic elements, or one of many other factors, including conspecifics, competitors, predators, parasitoids, pathogens, the host plant, or abiotic effects.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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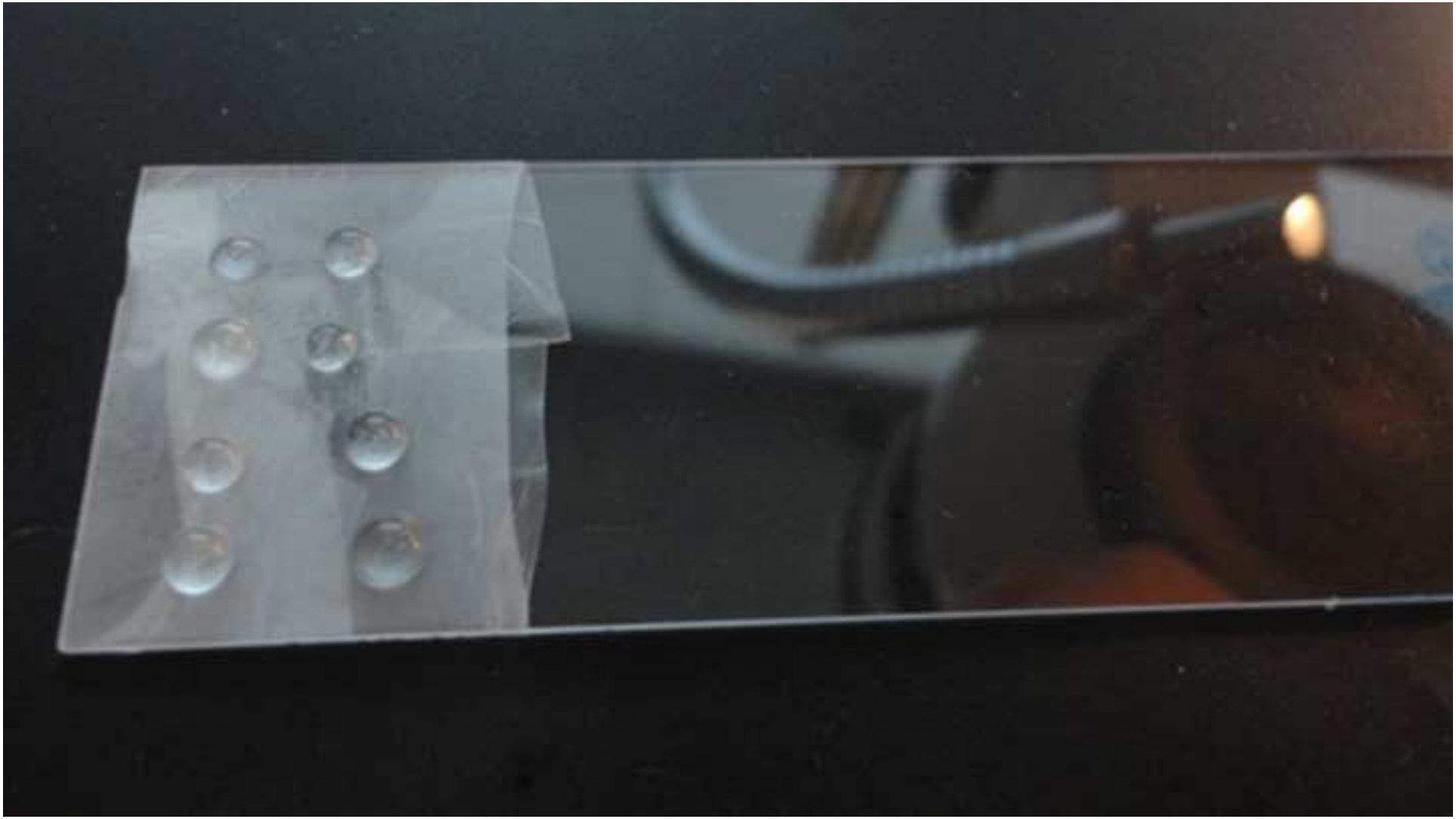

Figure 2

Figure 3

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Figure 4

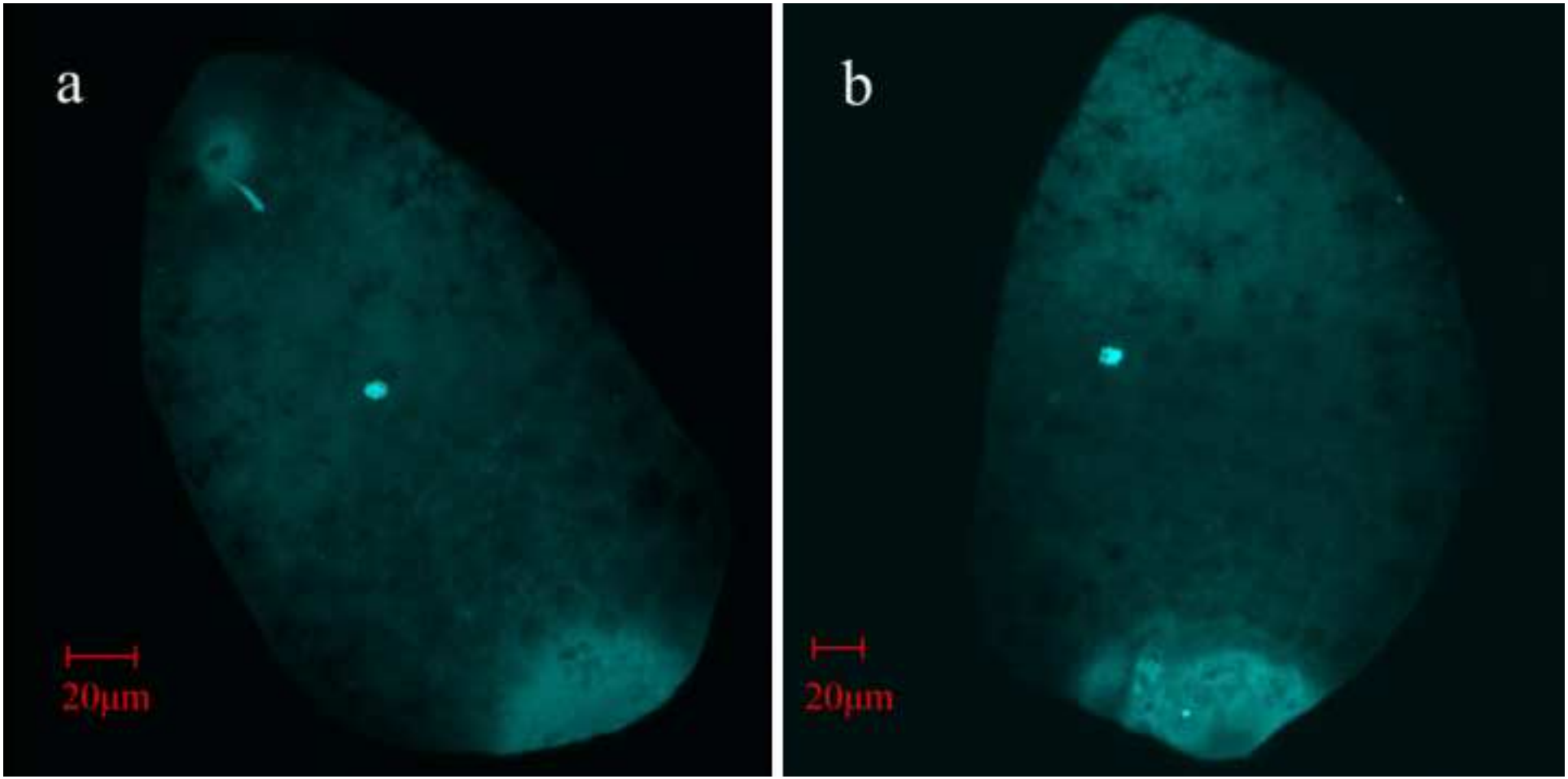
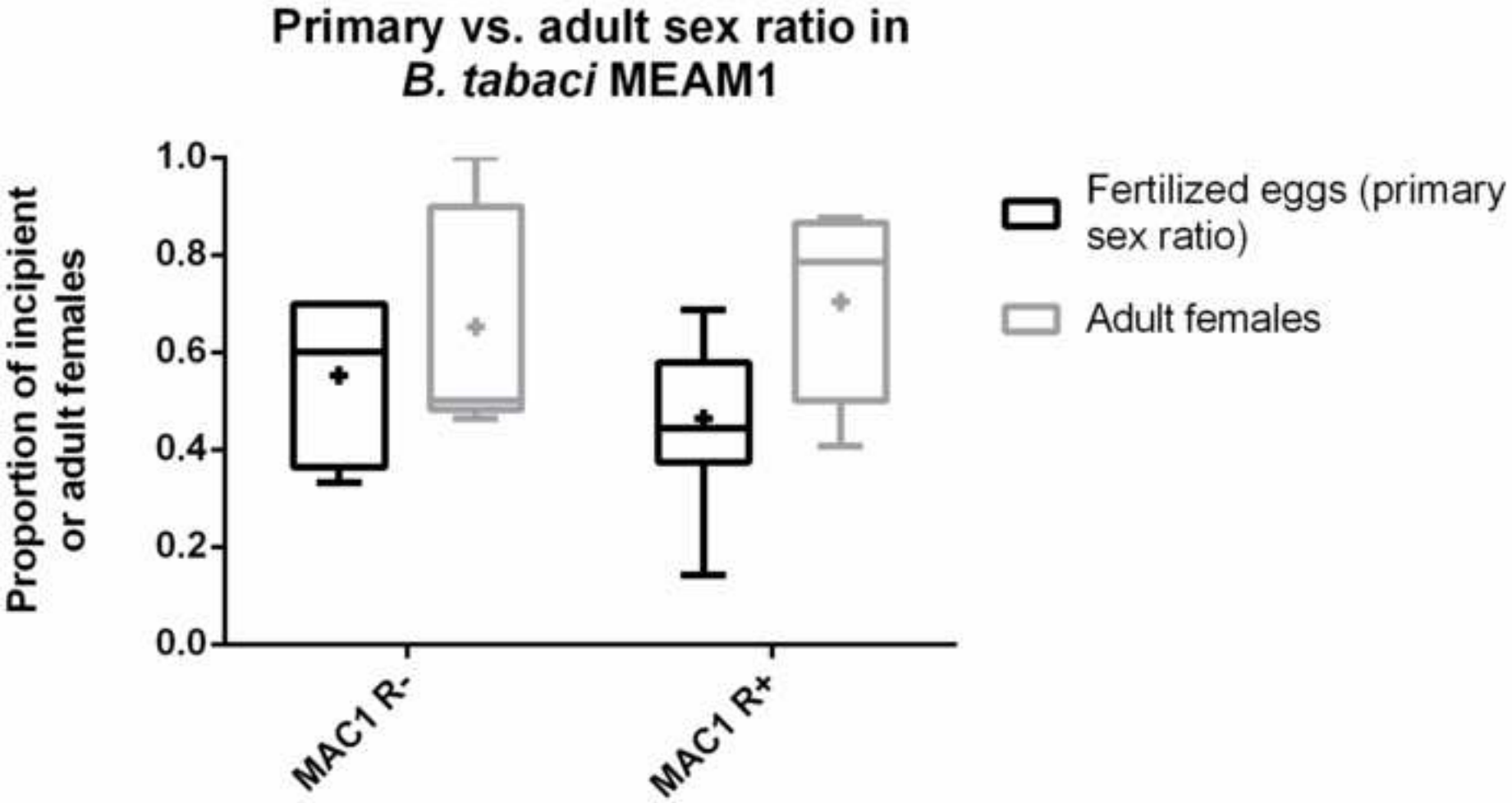


Figure 5



Name of Material/ Equipment	Company	Catalog Number
1XPBS	Any	
1XTBST	Any	
Bleach	Clorox	
Clear nail polish	Any	
DAPI dilactate	Santa Cruz Biotechnology	sc300415
Ethanol	Any	
Fluorescent microscope	Nikon	
Glacial acetic acid	Mallinckrodt	UN2789
Glycerol	Any	
Microscope	Wild	
Microscope slide covers	Any	
Microscope slides	Any	
Minuten nadel pins	BioQuip	1208SA
NaCl	Any	
NaN_3	Any	
n-propyl-gallate	Sigma/Santa Cruz Biotechnology	P3130/sc-250794
Parafilm	Bemis	

Pasteur pipettes	Fisher Scientific	13-678-20A
PCR grade water	Any	
Pipette tips	Any	
Small dropper bulb	Any	
Tris	Any	
Tween-20	Any	

Comments/Description
5X solution made from 30g Tris, 43.8g NaCl, 5 mL Tween-20 and 1.0g NaN_3 pH7.5, and brought to 1L with PCR grade water
Any household bleach will work as long as it can be diluted to 0.83% Sodium hypochlorite
Dilute to 70% EtOH
Nikon Eclipse 50i was used in this experiment, but any fluorescent microscope with 340/380 nm excitation filter and at least 4-10X magnification can be used
A Wild M5A microscope was used for this experiment, but any microscope where the operator can clearly see the whitefly eggs can be used
Methods are for 18mm x 18mm sized slide covers. More mounting media will need to be added for larger slide covers.
Minuten nadel pins are optional for fashioning as probes with pipette tips

Fisherbrand Disposable Borosilicate glass Pasteur pipettes 5.75 in. A Bunsen burner may also be needed if operator would like to lengthen and narrow pipettes
Pipette tips are optional for fashioning as probes with minuten nadel pins
Must fit on Pasteur pipette

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CORRESPONDING AUTHOR

Name:	Elizabeth Bondy
Department:	Entomology
Institution:	University of Arizona
Title:	M.S.
Signature:	EBondy
Date:	30 Sep 2018

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Dear Editor and Reviewers,

We would like to sincerely thank you for your time and great attention to detail when providing feedback to our manuscript, now renamed "Determining egg fertilization rate of *Bemisia tabaci* using a cytogenetic technique" [JoVE59213]**.** We have addressed your concerns here using blue font.

Editorial comments:

"Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 x 1080 pixels or 300 dpi."
-Done

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
-Done

2. Please expand your Introduction to include the advantages over alternative techniques with applicable references to previous studies.
-We are unaware of any similar studies with which to compare ours to. We have added this additional clarification (lines 74-76):
"We are unaware of any comparable egg-staining techniques for *B. tabaci*. The protocol is convenient in comparison with staining methods used for other insect eggs [Giorgini et al. ref], as it omits an overnight-fixation step and therefore can be completed within three hours."

3. Please define all abbreviations before use.
-Done

4. Please include single-line spaces between all paragraphs, headings, steps, etc.
-Done

5. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.
-Done

6. Please describe how to make the probe.
-Done. In lines 114-116, we have added:
"Collect the eggs singly and carefully with a thin probe. To make a probe, insert a minuten nadel pin at 45° or a comfortable working angle into a pipette tip that has been partially melted with heat (Figure 2)."

7. Please indicate the approximate volume of glacial acetic acid added in this step.

-This sentence was added to the beginning of protocol (line 85):
"All 'drops' in this protocol are defined as 5-20 µL, depending on the operator's preference."

8. Please provide the composition of TBST.
- Done. Lines 166-167 now contain the composition:
"Add drops of 1XTBST (5X solution made from 30g Tris, 43.8g NaCl, 5 mL Tween-20 and 1.0g NaN_3 pH7.5 and brought to 1L with PCR grade water) to the eggs […]"

9. Figure 4: Please change "20µM" to "20 µm" for correct unit and format.
-Done.

10. Figure 5: Please define error bars in the figure legend. Please shorten the figure legends.
-Sex ratios are proportions and proportions are not normally distributed, such that standard errors are not meaningful. In the box and whiskers plot shown the distribution of the data is described by the median, quartile lines and the range described by the "whiskers" extending from the box. This is explained in the figure caption.
We have also shortened the figure 5 legend (lines 235-246):
"Box and whisker plots of primary sex ratio in black (% fertilized eggs, or % female zygotes) compared to adult sex ratio (% adult females) in grey of *Rickettsia*-infected (R^+) and uninfected (R^-) *B. tabaci* MEAM1, "MAC1" genetic line. Box and whisker plots show the median as the middle line, the mean as a plus sign, upper and lower quartiles as the lines that make the ends of the box, and the range is represented in the outer lines extending from the box. For R^- eggs scored, n = 90, for R^+ eggs scored, n=82. For R^- adults counted, n=60, and for R^+ adults counted, n=95. In a logistical analysis of primary sex ratio (proportion of fertilized eggs) performed in the statistical package R, no significant effects were found for block (n=7, χ^2=0.29, df=1, p=0.59) or for *Rickettsia* infection (χ^2=0.51, df=1, p=0.47). Adult sex ratios as influenced by block (n=6) and *Rickettsia*-infection status were similarly analyzed. Here as well, no significant effects were found for block (χ^2=1.20, df=1, p=0.27) or for *Rickettsia* infection (χ^2=1.02, df=1, p=0.31)."

The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.
-Done. An additional paragraph in the results section clarifies our methods, and a former part of the Figure 5 legend is now at the end of that paragraph in lines 197-201:
"A generalized linear model was used in R to determine whether the fertilization rates or adult sex ratios were significantly influenced by *Rickettsia* infection and/or block. The response variables were the proportion of fertilized eggs/all eggs, or the proportion of female adults/all adults, respectively, while explanatory variables were block and *Rickettsia*-infection status."

11. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.
-We have added additional discussion of troubleshooting thanks to the reviewer suggestions. The following has been added to the discussion (lines 250-263):
"The challenge to this protocol is that one must learn how to handle the whitefly eggs quickly, ensuring that one hour has not passed since the eggs were oviposited until they are fixed. During preliminary experiments, eggs that were fixed at three hours or more post-oviposition were too old to observe fertilization, as syngamy had occurred and mitotic divisions were underway. Between one to three hours, the pronuclei took on a rounder shape. While the early presence of two nuclei indicated a fertilized egg, a bit later the apposition of the two nuclei in preparation for syngamy can appear to be

one nucleus, and later again, the two products of the first mitotic division are found in both male and female eggs. Therefore, the distinction between the sexes is not clear at these later time points, and we advise limiting the interval from oviposition to fixation to one hour as a conservative measure.

It is also challenging to learn how to be gentle with the eggs with each transfer of liquid so that they do not get accidentally aspirated into the pipette. At the time of viewing the eggs under a fluorescent microscope, a few of the eggs may have broken during the protocol, so those eggs cannot be sexed and counted, as the pronuclei and yolk may have escaped."

12. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.
-Done

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Elizabeth C. Bondy and Martha S. Hunter reported a cytogenetic technique to determine the primary sex ratio of Bemisia tabaci. It is a really timely report! I believe this technique will be very helpful to understand the reproductive regulation of the whiteflies. The manuscript is written very well. I try to find the error in the article but indeed it is perfect for the publication.

-Thank you!

Major Concerns:
None

Minor Concerns:
None

Reviewer #2:
Manuscript Summary:
This manuscript applies a cytogenetic protocol to score fertilized/unfertilized eggs of an important invasive pest, Bemisia tabaci. The authors use a protocol already described for Hymenoptera, adapting it to whiteflies. This protocol could be useful to investigate the primary sex ratio, compared to the adult sex ratio, in order to understand the origin of sex ratio bias, if present.

Major Concerns:
line 134: experimental design: usually when you show your results and the relative statistics, you have to explain the experimental design. How many females? how many replicas? and so on. Can you please add few more details? May be with more information also the data showed at line... will be more clear.

-An additional paragraph has been added to the representative results for clarification (lines 183-201). Thank you, we realize that we had been too brief here.
"To test whether *Rickettsia* affects fertilization rate of *B. tabaci* MEAM1 females, we reared *Rickettsia*-infected (R^+) or uninfected (R^-) *B. tabaci* on cowpea plants (*Vigna unguiculata*) in separate cages at 27°C, 70% relative humidity, and a 16 h light/8 h dark photoperiod. R^+ and R^- fourth instar whiteflies were

carefully removed from leaves and isolated in 200µL strip tubes. When adults emerged, they were collected in groups of 50% females and transferred onto clean leaves in petri dishes (n~50-100/leaf) for four days of mating. Groups of approximately 20 females and several males were then transferred onto a clean leaf disk (one for R^- adults, one for R^+ adults) in 35 mm Petri dishes resting on 1% agar. The Petri dish lid had been cut out and the fine fabric mesh used for containment also prevented excess condensation. After approximately 45 minutes, all adults were removed, some of the eggs were harvested to determine fertilization rate and the eggs that were not collected were reared to adulthood to calculate adult sex ratio. One cohort for a single day, for both R^+ and R^- whiteflies, was defined as a block. There were 7 blocks for calculating the fertilization rate or primary sex ratio, while there were 6 blocks for calculating the adult sex ratio, as there were not enough leftover eggs from one block to rear to adulthood. A logistic regression was used in the statistical package R to determine whether the fertilization rate or adult sex ratios were significantly influenced by *Rickettsia* infection and/or block. The response variable was the proportion of fertilized eggs/all eggs or the proportion of female adults/all adults, while explanatory variables were block and *Rickettsia*-infection status."

Moreover, results on female bias are lacking, you only compare Rickettsia infected and not infected. Please highlight the absence of female bias, showing statistic tests confirming the non significativity of the differences (if present)

-We have updated the results, and we have included the (non) significance of block effects as well (lines 203-213):

"Egg dechorionation followed by DAPI nuclear staining allowed unambiguous assignment of fertilization (and embryo sex) when observed with a fluorescent microscope (Figure 4). For this experiment, 90 eggs laid by R^- *B. tabaci* MEAM1 and 82 eggs by R^+ *B. tabaci* MEAM1 were scored. As for eggs reared to adulthood, 60 R^- and 95 R^+ adults were scored. While female bias in adult sex ratios had been shown consistently in earlier studies[15,18,19], in the current study, adult R^+ sex ratios were female-biased (69% females, median) compared to R^- females (50% females, median), but the sex ratios in the two treatments were not significantly different (χ^2=1.02, df=1, p=0.31, Fig. 5). The primary R^- sex ratios were female-biased (median of 60% fertilized eggs) compared to R^+ sex ratios (median of 44% fertilized eggs) but were also not significantly different (χ^2=0.51, df=1, p=0.47), providing no evidence for greater fertilization rates by R^+ females (Figure 5). Block also did not have a significant effect on primary (χ^2=0.29, df=1, p=0.59) or adult (χ^2=1.20, df=1, p=0.27) sex ratio."

Minor Concerns:

line 41: it is not clear the sense of "function" (referred to?)
-Some organisms are hermaphroditic (e.g. some plants) and so "female function" can pertain to that part of one organism that is allocated to investment in their role as a female. This is an unnecessary detail here, however, so we have omitted that here. We now have (lines 47-48):
"The study of sex allocation, or the relative investment in male and female offspring, is a cornerstone of behavioral ecology."

lines 51-52: male embryo .... female embryo... I would say "incipient male egg" and "incipient female egg" as said at line 151, rather than embryo
-Done

lines 51-51: erase (1N) and (2N) (it is redundant, and it should be written n and 2n if referring to ploidy)

-Done

line 68: "MEAM" the first time you mention it you should write also the extended form (Middle East Asia Minor)
-Done

line 73: "at room temperature in a well-ventilated area" may be it would be better "at room temperature in a well-ventilated area or under a fume hood"
-Done

line 75: describe the arena
-Description of possible arenas were added to the protocol in lines 89-91:
"Examples for oviposition arenas include clip cages, or leaves cut to fit on agar in a petri dish. A coverable hole can be made in the clip cage or petri dish-cover to insert and remove the adults."

line 80: instead of "for the first mitotic division" write "for syngamy"
-Done
line 82: instead of "of the sperm as it transitions" write "of the sperm transition"
-Done

line 88: it could be useful to explain why this step is necessary
-We have added to lines 100-102:
"The parafilm is hydrophobic and semi-opaque, allowing liquids to form drops and the eggs to be more easily seen."

line 91: instead of "if more time is required" say "to manage a large egg number"
-Done

line 93: this tip goes following 2.2
-Done

line 93: alternatively use p10 plastic tips?
-I attempted to use plastic tips when first developing this protocol. There was too much static, the whitefly eggs would attach to the inside of the plastic tips and I had no luck in getting them out.

line 94: instead of smaller use narrower; instead of "less capable" use "with a minor risk"
-Done

line 104-106: can you justify this sentence? a 1 hour chorion is softer than a 3 hours one?
-Yes, the chorion hardens, and one can even observe a difference in color from clear to light yellow. After one day, the chorion will darken further.

line 108: rephrase "Remove the bleach into a liquid waste dish" with "Remove the bleach (containing the chorion fragments) with a Pasteur pipette and discard it. "
-Done

line 110: steps with glacial acetic acid should be carried out under a fume hood; "acetic acid" is "glacial acetic acid"
-Done

line 112: glacial acetic acid
-Done

line 117: instead of "add 1x" say "add drops of 1x"
-Done

line 119: "for as long as the" remove "for"
-Done

line 128: when you say "Remove excess 1xTBST" clarify the way you do it (by pipetting?, with filter paper?)
-All liquid removals are done through glass Pasteur pipettes. This has now been clarified in all the steps. Thank you.

line 129:misspelled world: propyl-galate is propyl-gallate
-This has been corrected. Thank you

line 131: instead of "if storing" use "for long storage"
-Done

line 137: instead of "the sample data" use "this experiment"
-Done

line 138: are you talking of rickettsia infected Bemisia? please say better
-Yes, this has been clarified with an additional paragraph quoted above to address the major concern

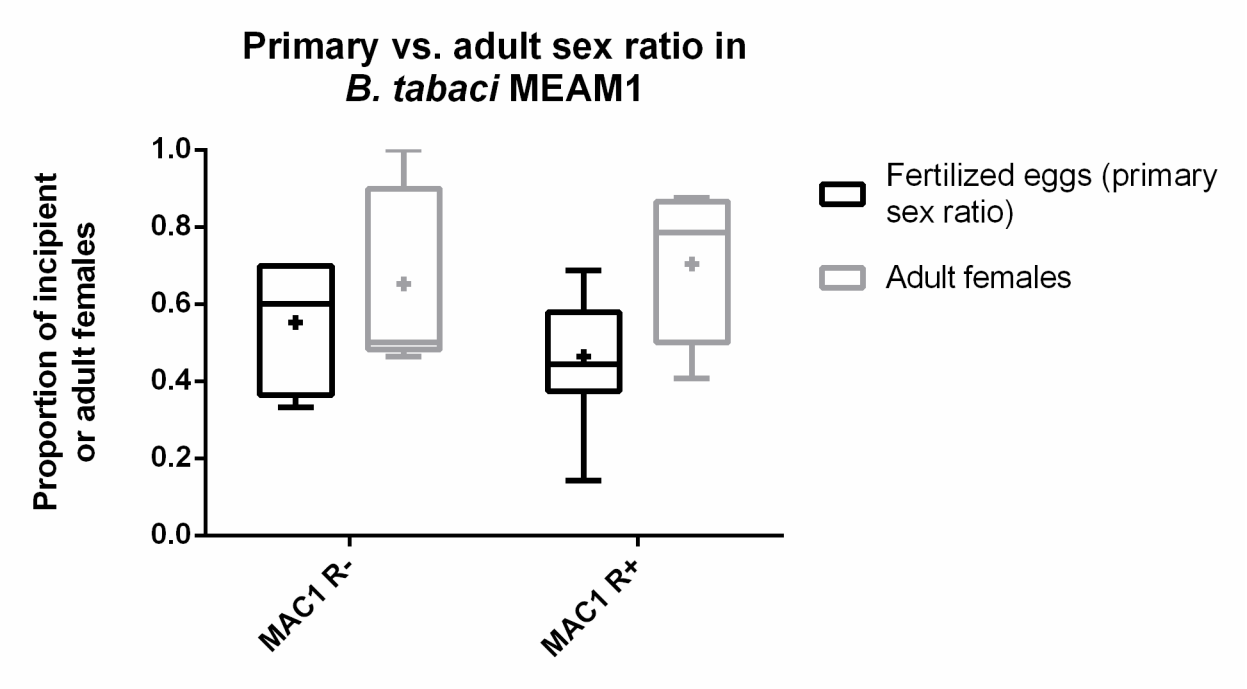
line 139: remove (it is redundant) "primary sex ratios and adult sex ratios did not differ significantly with Rickettsia infection" and substitute "in the current study. Adult R+" with "in the current study, adult R+"
-Done

lines 140-142: what does values represent? mean, median or total? Please, add F and p for primary sex ratio
-We have changed the calculations of the proportions from pooled data to the median values of the blocks as represented in Figure 5, and have corrected the paragraph to include χ^2 and p values (lines 203-213, above in previous answer)

line 150: Are these eggs from uninfected Bemisia? Rickettsia are not visible in the pictures
-We don't see a difference in the eggs of R^+ and R^- eggs with DAPI stain, while we know that intracellular symbionts in egg cytoplasm are stained with DAPI. We assume that the *Rickettsia* co-localize with the *Portiera* and *Hamiltonella* in the symbiont cell at the basal pole of the egg.

line 153: move "(Portiera, Hamiltonella)" after bacterial DNA
-Done

line 160: if possible add box and whisker plot for adults sex ratio
-Done. New Figure 5:



line 163:"Primary sex ratios were 56.7% R- females (n=90) and 51.2% R+ females (n=82)." Please specify you are talking about eggs: R- female eggs and r+ female eggs

-Done (lines 209-211):

"The primary R^- sex ratios were female-biased (median of 60% fertilized eggs) compared to R^+ sex ratios (median of 44% fertilized eggs), but were also not significantly different (χ^2=0.51, df=1, p=0.47) […]"

line 164: please write numbers (of the results) with the same decimals

-Done

line 166: not clear what is the block made of

-Clarified by additional paragraph in results, quoted above:

"One cohort for a single day, for both R^+ and R^- whiteflies, was defined as a block."

lines 168-170: why freedom degree are different from the number of blocks? More in general none of the df are clear.

-Thank you for picking up on this. Although the results do not change, we mistakenly included the statistics for a larger model (with more factors) for this analysis. We have corrected this – in the simpler analysis reported here, the model is not overdispersed and the test statistic is chi-square distributed (with 1 df in each case). This has been corrected in the caption for Fig.5.

line 188: why predicted? aren't these results of reported papers? Could you rephrase this paragraph?

-The "prediction" here refers to the sex allocation predictions for large panmictic, dispersive animals, and yes, there's support from the literature in laboratory settings. We've changed the sentence to include this (lines 273-276):

"While the large dispersive populations of *B. tabaci* and other pests such as the greenhouse whitefly, *Trialeurodes vaporariorum,* might be predicted to result in 1:1 sex ratios as exhibited in some laboratory settings[22,23], we might also predict that reproductive interference, endosymbionts, and potentially host plant quality could influence primary sex ratios."

line 195: "remove studied here"
-Done

line 196: "not surprising". Why is it "not surprising"? you have just told that there are some factors influencing primary sex ratio and/or mortality (and mentioned endosymbionts among these). Better rephrase
-You are correct. We have removed this contradiction.

lines 198-199: is a repetition of lines 194-195, please remove
-Done

lines 203-204: rather than "was successful" I would say "permitted to determine".I think that to say that a technique is successful would require validation tests (e.g cytometry)
-Done

line 283: "Pest Management Science" write in italic
-Done

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2nd pag:
row1: NaN3 subscript 3
"brought to 1L" with what? Specify
-with PCR grade water

row 3: NaCl and not Nalco
row5: NaN3 subscript 3
row7: misspelled world: propyl-galate is propyl-gallate
-Corrected typos and included diluent. Thank you

Reviewer #3:
Manuscript Summary:
In this manuscript, authors describe a novel method for observing the primary sex of a haplodiploid insect Bemisia tabaci using a cytogenetic technique. The protocol is adequately detailed and can be feasibly conducted. Thus, this method provides a new technique for observing sex allocation by whitefly female adults and potentially for observing sex allocation by other haplodiploid insects. However, the data obtained using this method may not always reflect the primary sex ratio due to the following factors.

Major Concerns:
The first issue is about timing of the egg fertilization. We do not know whether both sperm transformation and the first mitotic division always occur in the insect eggs within 60 minutes post-deposition. As authors mentioned in the manuscript, both paternal pronucleus transition and mitotic division can result in the presence of two nuclei, and this complexity may add uncertainty to detection of fertilization. This complexity may be relaxed with information on the timing of the first mitotic division in the egg. Such information may be gained by observing mitotic division in eggs deposited by unfertilized female adults. In this case, the egg contains only one maternal pronucleus before its first mitotic division, and two nuclei following the first mitotic division.
-Yes, this is a good point. Although calculating a confidence interval of time for the first mitotic division would be useful, it is outside of the scope of our study. Instead we focused on an

interval before the sperm has been remodeled and changes shape from its very distinctive streak or lozenge shape to a more typical round shape of the paternal pronucleus at karyogamy. Because we stayed within this time interval (under an hour) when the sperm is a distinctive shape, we can be confident that we are not mistakenly interpreting the products of the first mitotic division for a maternal and paternal pronucleus.

The second issue is about the criteria for judging egg fertilization. The presence of sperm attached to an egg or even inside the egg, as is shown in Fig. 4a, cannot be taken for granted as occurrence or success of fertilization.
-Fertilization is generally defined as the fusion of the egg and sperm, so we would argue that the presence of the paternal pronucleus in the cytoplasm of the egg is the most direct measure one can get of fertilization. We agree that there still could be something that interrupts karyogamy (fusion or parallel mitosis of the two nuclei), but one could argue that something could go wrong at any time beyond this moment, and those events are not what we are trying to measure. Further, since in this study we saw that the fertilization rate approximately equaled the adult sex ratios, that gives us some reassurance that our estimate was fairly accurate, and that in this instance, there weren't events affecting sexes differentially.

The third issue is about uncertainty that may occur using this method. Because whitefly eggs are extremely small (ca. 0.15 mm X 0.25 mm), and consequently the observation is challenging. It may happen that sperm or even nucleus is missed in the observation. I wonder whether this happened to the authors, i.e. when neither nucleus nor sperm could be found in an egg.
-Indeed, there were eggs from which we could not collect data. E.g. if the egg broke during preparation, the pronuclei and yolk could seep out, but we did not include any damaged eggs in the data. In intact eggs, we did not see any in which no nuclei were visible - intact eggs show surprising consistency with bright, visible pronuclei and sperm. Since we think it is a reasonable assumption that fertilized and unfertilized are equally likely to break during clumsy handling, we do not think our results are biased in this way.

Minor Concerns:
Line 141: Read "Fig. 5" for "Fig. 2".
-Done