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Small-cage laboratory trials of genetically-engineered Anopheline mosquitoes

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TITLE:

Small-Cage Laboratory Trials of Genetically-Engineered Anopheline Mosquitoes

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KEYWORDS:

Transgenic, mosquitoes, cage trials, gene-drive, population modification, malaria

SUMMARY:

The protocols reported here illustrate three alternative ways to assess the performance of genetically-engineered mosquitoes destined for vector control in laboratory-contained small cage trials. Each protocol is tailored to the specific modification the mosquito strain bears (gene drive or non-gene drive) and the types of parameters measured.

ABSTRACT:

Control of mosquito-borne pathogens using genetically-modified vectors has been proposed as a promising tool to complement conventional control strategies. CRISPR-based homing gene drive systems have made transgenic technologies more accessible within the scientific community. Evaluation of transgenic mosquito performance and comparisons with wild-type counterparts in small laboratory cage trials provide valuable data for the design of subsequent field cage experiments and experimental assessments to refine the strategies for disease prevention. Here, we present three different protocols used in laboratory settings to evaluate transgene spread in anopheline mosquito vectors of malaria. These include inundative releases (no gene-drive system), and gene-drive overlapping and non-overlapping generation trials. The three trials vary in a number of parameters and can be adapted to desired experimental settings. Moreover, insectary studies in small cages are part of the progressive transition of engineered

insects from the laboratory to open field releases. Therefore, the protocols described here represent invaluable tools to provide empirical values that will ultimately aid field implementation of new technologies for malaria elimination.

INTRODUCTION:

Strategies based on genetically-engineered mosquitoes are being pursued to control transmission of vector borne pathogens such as those that cause malaria. These include technologies 1) aimed at decreasing the numbers and densities of *Anopheles* mosquitoes (population suppression), or 2) aimed at impairing the ability of vectors to transmit parasites responsible for human disease (population modification, replacement, or alteration) wherein strains of vectors are engineered to express effector genes that prevent pathogen transmission. These genetic approaches have been bolstered by the advent of CRISPR/Cas9-based gene drives, with proofs-of-concept in parasite-transmitting mosquitoes of effective spread of payload traits as well as anti-parasitic effector molecules in caged populations.

Small laboratory cage trials represent a first step for evaluating the characteristic of transgenic strains as part of a phased approach to their further development towards field applications[2]. Specific outcome considerations include heritability of the introduced DNA in a competitive environment, penetrance and expressivity of the phenotype, and stability. Relevant experimental design features include the size of the cages, mosquito densities, number of replicates, overlapping or non-overlapping generations, age-structured target populations, single or multiple releases of engineered strains, male-only, female-only or mixed-sex releases, release ratios, blood meal sources (artificial or live animal), and screening procedures.

We describe here protocols used to evaluate strains of anopheline mosquitoes for inundative releases (no gene-drive system) and those that carry autonomous gene-drive systems mediated by Cas9 endonucleases and guide RNAs (gRNA). Applications of these protocols appear in Pham *et al.* (2019)[2], Carballar-Lejarazú *et al.* (2020)[3], and Adolfi *et al.* (2020)[4].

Inundative release trials evaluate the spreading rate of a designed transgene under Mendelian inheritance following multiple releases of a large number of transgenic mosquitoes into a wild population. Without the attachment of the transgene to a drive system, data from inundative release trial provides information regarding the fitness and dynamic of the transgene of interest in a stabilized population.

When mosquito populations contain an autonomous gene-drive system, small cage trials are designed to assess the dynamics of the spread of the desired transgene by determining the rate of dominant marker increase following a single introduction of transgenic males. Autonomous gene-drive elements carry the genes encoding the Cas9 nuclease, gRNA and dominant marker linked in such a fashion as to be active in subsequent generations.

'Overlapping' generations refer to the simultaneous presence of multiple generations in the same cage to create an age-structured continuous population, while 'non-overlapping' refers to single discrete generations in each consecutive caged population[2]. Gene-drive cage experiments can be

terminated once the initial dynamics of the drive (conversion) rate can be determined (8-10 generations depending on the construct), and while they provide information on the short-term stability of the transgene within the mosquito population, they may not reveal what happens when and if the dominant marker frequencies reach or are close to full introduction (every mosquito carrying at least one copy of the gene-drive system).

PROTOCOLS:

1. Inundative release trials on non-gene drive mosquitoes (Figure 1)

1.1. Cage setup and maintenance

1.1.1. Set up three sets of triplicate 0.216 m^3 cages by adding 60 second-instar wild-type (WT) larvae in each cage over three successive weeks.

NOTE: It is not possible to determine the sex of second-instar larvae, so the samples added to each cage will consist of both males and females.

1.1.2. At every week, provide adult females in each cage with anesthetized mice as bloodmeal source (**Figure 2A**) and an oviposition container 3 days after the bloodmeal.

NOTE: While an alternative artificial feeding apparatus can be used, providing live anesthetized mice for bloodmeals results in better mosquito feeding performance in these large (0.216 m^3) cage formats. This requires an approved animal use protocol and relevant (e.g., Institutional Animal Care and Use Committee, IACUC) approval for using mice.

1.1.3. Hatch eggs from each cage weekly and select 60 second-instar (L2) larvae at random to be returned to their respective cages to offset mortality (weeks 4-8).

NOTE: Steps 1.1.1 to 1.1.3 are necessary to establish a stable and distributed age-structured population in the cages – referred to as 'Initial Phase'.

1.1.4. At week 9, assign cages assembled in step 1.1.1 randomly in triplicates for releases of the desired male release ratio.

1.1.4.1. Designate one set of triplicate cages as controls to assess consistency throughout the experiment.

1.1.4.2. Designate one set of triplicates for each desired release ratio (for example, 1:1 or 1:0.1 transgenic:WT males)..

NOTE: This point on is referred to as 'Experimental Phase'.

1.2. Replicates and release ratios

1.2.1. Add 60 WT pupae (30 males and 30 females) to the control cages weekly.

1.2.2. To maintain a 1:1 ratio, add weekly 30 transgenic male pupae along with 60 (30 male and 30 female) WT pupae into each respective cage.

1.2.3. To maintain a 1:0.1 ratio, add weekly 300 transgenic male pupae along with 60 (30 male and 30 female) WT pupae into each respective cage.

NOTE: Continued addition of wild mosquitoes to the cages maintains the cage density, which is expected to diminish weekly due to age-related adult mortality.

1.3. Screening of phenotypes

1.3.1. Select a total of 300 larvae from each cage at random. With the use of a stereo microscope equipped with fluorescence filters, screen for the expression of the fluorescent dominant marker at the larval and pupal stages and score the sex of the resulting adults (**Figure 3**).

NOTE: The phenotypical screening will depend on the dominant marker included in the transgene construct integrated into the mosquitoes (for example, Discosoma sp. red fluorescent protein [DsRed], cyan fluorescent protein [CFP], green fluorescent protein [GFP]), and on the promoter driving its expression (the most used in mosquito transgenesis is the 3xP3 promoter driving expression in the eyes and nerve cord).

1.3.2. Follow this protocol for as many generations as required by the outcome parameters defined in the experimental design.

NOTE: The trial is usually terminated when all mosquitoes have at least one copy of the transgene (determined by the presence of the dominant fluorescent marker) or the ratio of transgenic-to-WT mosquitoes in a cage is stabilized and does not fluctuate greatly after a few (3-5) generations.

2. Overlapping generation trials of gene-drive mosquitoes (Figure 4)

NOTE: Mosquitoes carrying gene-drive systems require written and reviewed protocols and should be approved by an Institutional Biosafety Committee (IBC) or equivalent, and others where required. Mosquito containment (ACL 2^+ level) should follow recommended procedures[5–7]. Specifically, the gene drive experiments should employ two stringent confinement strategies. The first is usually physical barriers (Barrier Strategy) between organisms and the environment. This requires having a secure insectary and standard operating procedures (including monitoring) for ensuring that mosquitoes cannot escape. The second confinement strategy can be Molecular, Ecological or Reproductive[5].

2.1. Cage setup

2.1.1. Set up two sets of triplicate 0.216 m^3 cages for each desired transgenic:WT male release ratio.

2.1.1.1. To achieve a male release ratio of 1:1, add 120 transgenic males, 120 WT males and 120 WT females at the pupa stage to each replicate cage.

2.1.1.2. To achieve a release ratio of 1:10, add 12 transgenic males, 120 WT males and 120 WT females at the pupa stage to each replicate cage.

NOTE: Different release ratios can be tested (1:1, 1:3, 1:10, etc.) and the number of mosquitoes used to initiate the experiments varies accordingly. However, it is important to consider the effects of low numbers on the statistical evaluation of the data.

2.2. Population maintenance and screening

2.2.1. Provide 4-7 days old females in each cage with a blood meal using anesthetized mice (**Figure 2A**).

2.2.2. Three days after the blood meal, insert an oviposition container in each cage.

2.2.3. Hatch eggs in a larval tray, select ~240 first instar (L1) larvae at random from each cage, rear them to adulthood, and return them to their respective cages.

2.2.4. Provide additional (2-3) blood meals every 3-4 days for the newly emerged adults as described in step 2.2.1.

NOTE: No additional transgenic males are added during any of the subsequent generations.

2.2.5. Select a total of 300 larvae from each cage at random and screen them for the presence of the dominant marker phenotype at the larval and pupal stages using a fluorescence stereo microscope and score emerging adults for sex (**Figure 3**).

NOTE: As before, the phenotypical screening will depend on the dominant marker and promoter included in the gene-drive system and integrated into the transgenic mosquitoes (DsRed, CFP or GFP). If homozygous or heteroallelic disruptions of the targeted genes result in a visible phenotype (for example, genes related to eye pigmentation), screening of this trait will depend on which stage it is easiest to visualize the altered phenotype.

2.2.6. Follow this protocol for as many generations as required by the outcome parameters defined in the experimental design.

NOTE: Each generation (delimited by the blood meal) takes ~three weeks. The trial is usually terminated when all mosquitoes are deemed homozygous for the gene-drive construct or the

populations stabilize at a maximum percentage of mosquitoes carrying at least one copy of the gene-drive construct.

3. Non-overlapping generation trials of gene-drive mosquitoes (Figure 5).

3.1. Cage setup

3.1.1. Set up triplicate 0.005 m^3 cage populations for each specific release ratio of transgenics to WT males to be investigated (for example, three sets of triplicate cages each set up with 1:1, 1:3, 1:10 release ratios). Set up all cages with an equal total number of males and females.

NOTE: The **Supplementary File** is a video demonstrating the construction of the 0.005 m^3 colony cage.

3.1.1.1. Add 50 transgenic males, 50 WT males, and 100 WT females to each of three replicate cages to achieve a 1:1 male release ratio.

3.1.1.2. Add 25 transgenic males, 75 WT males, and 100 WT females to each of three replicate cages to achieve a 1:3 male release ratio.

3.1.1.3. Add 10 transgenic males, 90 WT males, and 100 WT females to each of three replicate cages to achieve a 1:10 male release ratio.

NOTE: Different release ratios can be tested and the number of mosquitoes used to initiate the experiments can vary accordingly. However, it is important to consider the impact of low numbers of mosquitoes on the statistical analyses. These are single releases; no additional transgenic males are added at any subsequent generation.

3.2. Population maintenance and screening

3.2.1. Provide the 4-7 days old females in each cage with blood meals using an artificial feeding apparatus (**Figure 2B**) on two consecutive days.

NOTE: Routine blood meals for females consist of a commercially available source of blood (e.g., calf's blood) provided from a feeding apparatus. Live anesthetized mice are used only to provide bloodmeals in larger (0.216 m^3) cage formats for better feeding performance.

3.2.2. Add an oviposition container 3 days after the second bloodmeal. After three days, remove the oviposition containers.

NOTE: At this step, 5-10 females can be selected at random from each cage and placed individually in vials to assess additional fitness parameters, such as fertility and fecundity, if needed.

3.2.3. Score by sex all adults (dead and alive) remaining in the cage and store them at -80°C for molecular analysis.

3.2.4. Hatch eggs and randomly select 200 L1 larvae from the 1:1 and 1:3 ratio cages to populate new cages for the next generation.

NOTE: Due to the low frequency of starting transgenic individual in the 1:10 ratio cages, random sampling may lead to excessive loss of transgenic progeny in the next generation to carry on the population.

3.2.5. To ensure an accurate sampling for the 1:10 cages and sufficient numbers of transgenic mosquitoes, screen all larvae for the dominant marker and select 200 larvae reflecting the observed transgene frequency to populate the new cages.

NOTE: The 1:10 cages can be maintained identically to 1:1 and 1:3 cages when they reach a transgene frequency of ≥80%.

3.2.6. Select 500 larvae from each cage at random for an in-depth analysis. Screen under a fluorescence stereo microscope for the expected marker phenotypes at the larval and pupal stages and score sex on adults (**Figure 3**).

NOTE: 'Exceptional' phenotypes can be selected to be further crossed and analyzed molecularly to monitor resistant allele formation.

3.2.7. This protocol can be followed for as many generations as required by the outcome parameters defined in the experimental design.

NOTE: Each generation is delimited by the bloodmeal and takes ~three weeks. The trial is usually terminated when all mosquitoes are deemed homozygous for the gene-drive construct or populations stabilize at a maximum prevalence of transgenic mosquitoes. And as before, screening for phenotypes will depend on the dominant markers and promoter integrated in the transgenic mosquitoes (for example, DsRed, CFP, GFP) or in the targeted genes if they present a visible phenotype (for example, genes related to eye pigmentation).

REPRESENTATIVE RESULTS:

Transgenic anopheline mosquitoes generated to bear non-gene drive or autonomous gene-drive modifications are set up for cage trials as described in the Protocols section. The representative results shown here depict the phenotype dynamics of the best-performing replicates of each of the cage trials experiments performed by Pham *et al.* (2019)[2] for *Anopheles stephensi* mosquitoes. The three trials (1 – 3, respectively: inundative non-gene drive, overlapping gene-drive and non-overlapping gene-drive) varied in different parameters, such as the size of the cage (0.216 m^3 vs 0.005 m^3), whether or not the target population was age-structured, source of blood meal (mice or artificial feeder) and release ratios. As a means of representation, **Figure 6** displays the

observed data selected from the same release ratio (1:1) for all three protocols used, on the course of seven generations.

The 1:1 non-drive release reaches >80% transgene introduction within 6-7 generations. For gene-drive transgenic cage trials, the 1:1 releases in both the non-overlapping and overlapping protocols reach this level within 3-4 generations, thus, validating the expectation that a single release of a gene drive system can be more efficient than non-drive inundative releases for transgene introduction. The faster trajectory can also be confirmed by the slope of the trendlines. Both gene-drive protocols, despite different set ups, present similar angles and slope trends. At the end of observation, non-drive cages achieve ~80% of individuals bearing the transgene, while cages with gene-drive individuals reach complete (or near complete) introduction. Complete data and processing details on individual experiment results using the protocols described here can be found in Figures 1-3 of Pham *et al.* (2019)[2], Figures 2-3 of Carballar-Lejarazú *et al.* (2020)[3] and Figure 3 of Adolfi *et al.* (2020)[4].

FIGURE AND TABLE LEGENDS:

Figure 1. Non-drive inundative release trial schematic.

Nine 0.216 m^3 cages are set up with 60 wild-type second-instar (mixed-sexes) larvae added to each. Beginning week 3, females are provided a bloodmeal weekly and eggs are collected and hatched. Until week 8, 60 larvae are randomly selected and returned to their respective cages weekly to create an age-structured population in the cages (initial phase). Beginning week 9, the nine cages are randomly assigned in triplicate according to their transgenic:wild-type male release ratios (experimental phase). Cages A (Control) have no transgenic pupae added. Females are provided a bloodmeal weekly and eggs are collected, hatched, and reared to pupae. 30 male and 30 female wild- type pupae are added back to their cages. Cages 1:1 have an additional 30 transgenic male pupae added. Cages 1:0.1 have an additional 300 transgenic male pupae added. 300 larvae from each of the 9 cages are selected randomly and screened for the fluorescent marker. This procedure was repeatedly weekly until transgene fixation (stabilized ratio of transgenic-wildtype mosquitoes after a few generations). Adapted from Pham *et al.* (2019)[2].

Figure 2. Blood feeding of cage populations.

(A) Anesthetized mice or **(B)** Hemotek blood feeders are offered for blood feeding female mosquitoes on the 0.216 m^3 cages or the small 0.005 m^3 cages, respectively.

Figure 3. Screening phenotypes for non-drive, overlapping gene-drive and non-overlapping gene-drive cage trials.

Fluorescent images of a larva, pupa and adult of transgenic or wild-type phenotypes. In this example, *An. stephensi* individuals were screened for the DsRed marker driven by the 3xP3 promoter in the eyes (DsRed+ or DsRed-), visible in all three stages, and adults were screened for sex (♀ or♂). Note the background fluorescence in wild-type larvae associated with the food bolus in the midgut.

Figure 4. Overlapping gene-drive cage trial schematic.

Six 0.216 m^3 cages are set up in triplicate according to their gene-drive:wild-type male release ratios. 120 wild-type males and 120 wild-type females were added to each cage. Cages with a 1:1 gene-drive male release ratio had an additional 120 transgenic males added. Cages with a 1:10 male release ratio had an additional 12 transgenic males added. Until full introduction of the transgene, every 3 weeks, adult females are provided bloodmeals and eggs are collected and hatched. A total of 240 larvae were selected randomly and returned to their respective cages. Three-hundred (300) larvae are selected randomly and screened for the dominant marker. They are later screened as pupae and adults for eye-color and sex. No additional transgenic males are added to the original cages. Adapted from Pham *et al.* (2019)[2].

Figure 5. Non-overlapping gene-drive cage trial schematic.
Nine small 0.005 m^3 cages are set up in triplicate according to their gene-drive:wild-type male release ratios. Cages with a 1:1 male release ratio have 100 wild-type females, 50 wild-type males, and 50 gene-drive males added. Cages with a 1:3 male release ratio have 100 wild-type females, 75 wild-type males, and 25 gene-drive males added. Cages 1:10 male release ratio have 100 wild-type females, 90 wild-type males, and 9 gene-drive males added. Females are provided a blood meal and eggs collected and hatched. For 1:1 and 1:3 cages, 200 larvae are selected randomly and used to populate new cages, separate from that of their parents, for the next generation. An additional 500 larvae are selected randomly and reared to pupae, when they are screened for the dominant marker gene. The 500 pupae are then reared to adults and scored by sex. All remaining larvae are screened for the marker. For the 1:10 cages, all larvae are scored in generations 1–12 and 200 larvae reflecting the existing transgene frequency are used to populate new cages. Beginning at generation 13, these cages are set up identically to the 1:1 and 1:3 cages. Adapted from Pham *et al.* (2019)[2] and Carballar-Lejarazú *et al.* (2020)[3].

Figure 6. Predicted transgene fixation dynamics for the different population replacement cage trials.
Representation of the expected phenotype dynamics of the best-performing replicates for each of the cage trials experiments performed by Pham *et al.* (2019)[2], monitored over 7 generations. Experiments set ups are described in the Protocols. The predictions are based on data from all 9 experiments on the 1:1 release models (triplicate replicates for each of the three different cage trial protocols). The X-axis is the generation number after initial introduction and the Y-axis is the proportion of larvae showing the DsRed marker phenotype (DsRed+) over time. Dashed lines represent linear trendlines of the data. The DsRed+ phenotype results from having at least one copy of the modified allele. Hence the results reflect the spread of the transgene, expedited in the gene drive system, reaching (near) full introduction at the end of the observation. For the variability between replicates and full detailed data on the experiments, please refer to Pham *et al.* (2019)[2], Carballar-Lejarazú R et al. (2020)[3] and Adolfi A et al. (2020)[4]. Images adapted from Pham TB et al. (2019) Experimental population modification of the malaria vector mosquito, *Anopheles stephensi*. *PLOS Genet* 15(12): e1008440. doi: 10.1371/journal.pgen.1008440, Adolfi A et al. (2020) Efficient population modification gene-drive rescue system in the malaria mosquito *Anopheles stephensi*. *Nat Commun* 11(1): 5553. doi: 10.1038/s41467-020-19426-0 and Carballar-Lejarazú R et al. (2020) Next-generation gene drive for population modification of the

malaria vector mosquito, *Anopheles gambiae*. *Proc Natl Acad Sci USA* 117(37):22805-22814. doi: 10.1073/pnas.2010214117.

Supplemental File: The construction of the 0.005 m^3 colony cage.

DISCUSSION:

Genetically-engineered mosquitoes that have pathogen blocking ability or bear sterility genes constitute new tools to control vector-borne diseases. Given the multiplicity of parameters that comprise these alternative approaches, a critical step in their research consists of laboratory-confined experimental evaluations that allow a fast and safe prediction of the potential outcomes of a synthetic transgene release for control purposes[1].

Because the monitoring of the transgene dynamics in caged populations can extend for several months, one of the central aspects of the protocols is the consistency in experimental design between replicates (including mosquito rearing, cage size, age-structured populations, fixed release ratios, stable blood meal sources and minimally invasive screening procedures).

Male-only releases are considered ideal because male mosquitoes neither transmit pathogens nor feed on humans, therefore they can safely introduce heritable characteristics into wild populations. In laboratory cage experiments, it is possible to detect transgenic strains with reduced male mating competitiveness and other fitness loads associated with transgene integration. However, direct and specific experiments, such as those conducted in large cages[10], can be conducted to properly analyze male competitiveness, as well as female fecundity in more natural mosquito densities[2]. Furthermore, empirical data from the cage trials can be used to parameterize models of cage population dynamics, including resistant allele formation, and provide useful information on effectiveness and possible adjustments in the proposed technology.

The protocols described here can be easily adapted to other experimental designs as required, with minimal requirements regarding regular insectary infrastructure and conditions. In addition, except for the commercial cages and microscopes, most of the materials are inexpensive and allow low-cost multiple replicates and iterations of the trials. Notably, this also allows multiple transgenic strains to be pre-screened in small cage trials in order to prioritize best-performing candidates to be moved forward in the phased testing pathway and to suspend testing on those showing sub-optimal performances.

Finally, concern regarding the use of genetically modified organisms motivates the elaboration of frameworks for the development, evaluation, and application of genetic strategies for prevention of mosquito-borne diseases[5, 8, 9]. The relevance and execution of the protocols defined here are consistent with these guidelines.

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DISCLOSURES:

The authors have no disclosures.

REFERENCES:

1. Carballar-Lejarazú, R., James, A.A. Population modification of Anopheline species to control malaria transmission. *Pathogens and Global Health*. **111** (8), 424–435, doi: 10.1080/20477724.2018.1427192 (2017).
2. Pham, T.B. *et al.* Experimental population modification of the malaria vector mosquito, Anopheles stephensi. *PLOS Genetics*. **15** (12), e1008440, doi: 10.1371/journal.pgen.1008440 (2019).
3. Carballar-Lejarazú, R. *et al.* Next-generation gene drive for population modification of the malaria vector mosquito, Anopheles gambiae. *Proceedings of the National Academy of Sciences*. **117** (37), 22805–22814, doi: 10.1073/pnas.2010214117 (2020).
4. Adolfi, A. *et al.* Efficient population modification gene-drive rescue system in the malaria mosquito Anopheles stephensi. *Nature Communications*. **11** (1), 5553, doi: 10.1038/s41467-020-19426-0 (2020).
5. Akbari, O.S. *et al.* Safeguarding gene drive experiments in the laboratory. *Science*. **349** (6251), 927–929, doi: 10.1126/science.aac7932 (2015).
6. Benedict, M.Q. *et al.* Recommendations for Laboratory Containment and Management of Gene Drive Systems in Arthropods. *Vector-Borne and Zoonotic Diseases*. **18** (1), 2–13, doi: 10.1089/vbz.2017.2121 (2018).
7. CDC/NIH Biosafety in Microbiological and Biomedical Laboratories. (6th Edition), 1–574, at <https://www.cdc.gov/labs/pdf/SF__19_308133-A_BMBL6_00-BOOK-WEB-final-3.pdf> (2020).
8. Adelman, Z. *et al.* Rules of the road for insect gene drive research and testing. *Nature Biotechnology*. **35** (8), 716–718, doi: 10.1038/nbt.3926 (2017).
9. Long, K.C. *et al.* Core commitments for field trials of gene drive organisms. *Science*. **370** (6523), 1417–1419, doi: 10.1126/science.abd1908 (2021).
10. Facchinelli, L., North, A.R., Collins, C.M. et al. Large-cage assessment of a transgenic sex-ratio distortion strain on populations of an African malaria vector. *Parasites Vectors* **12**, 70 (2019).

Figure 1

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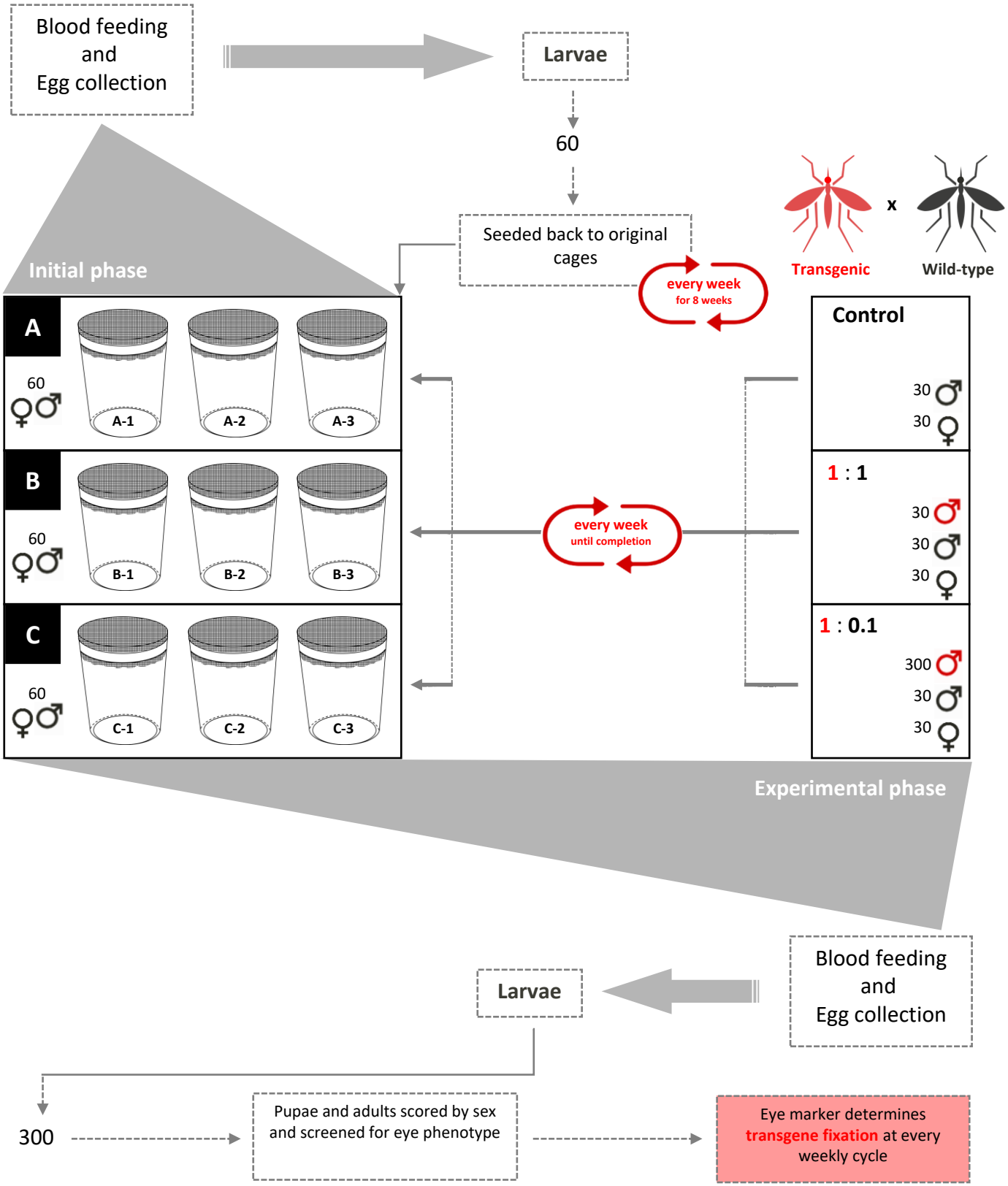

Figure 2

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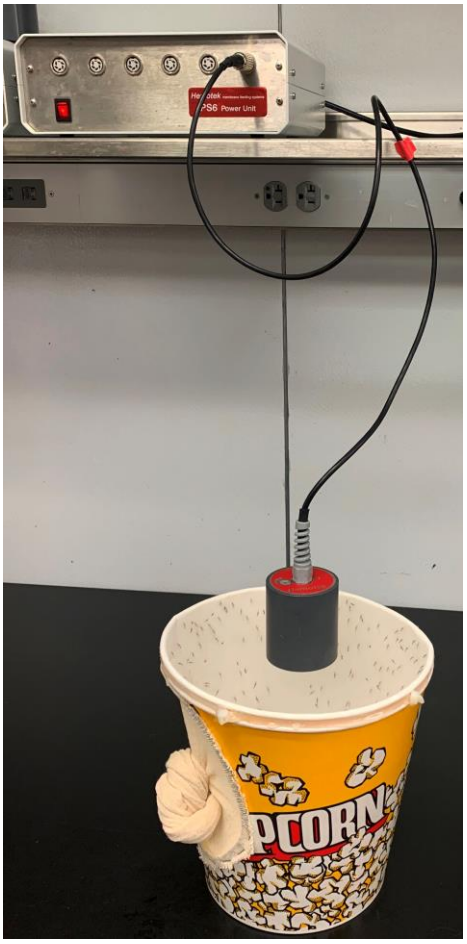

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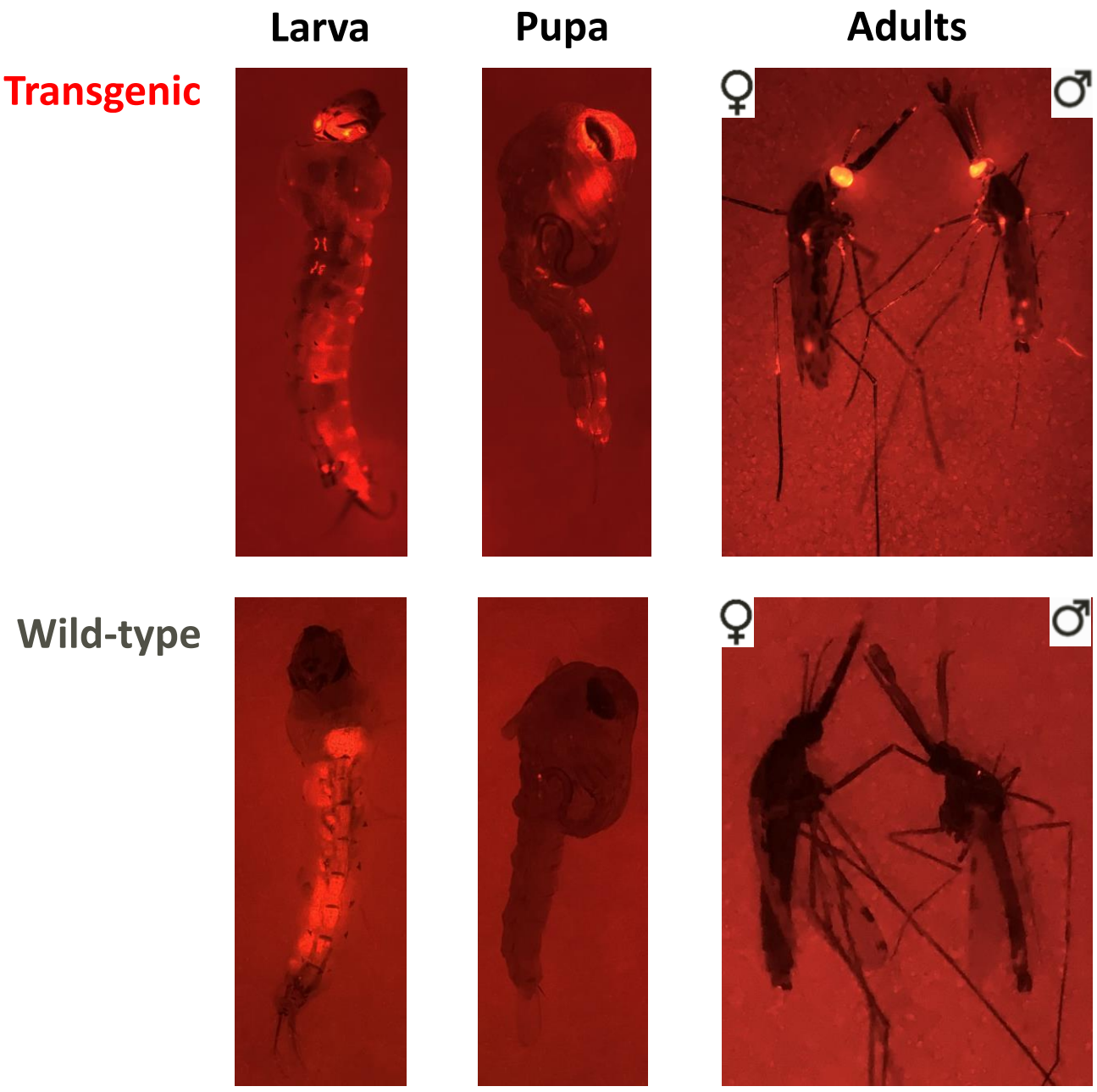

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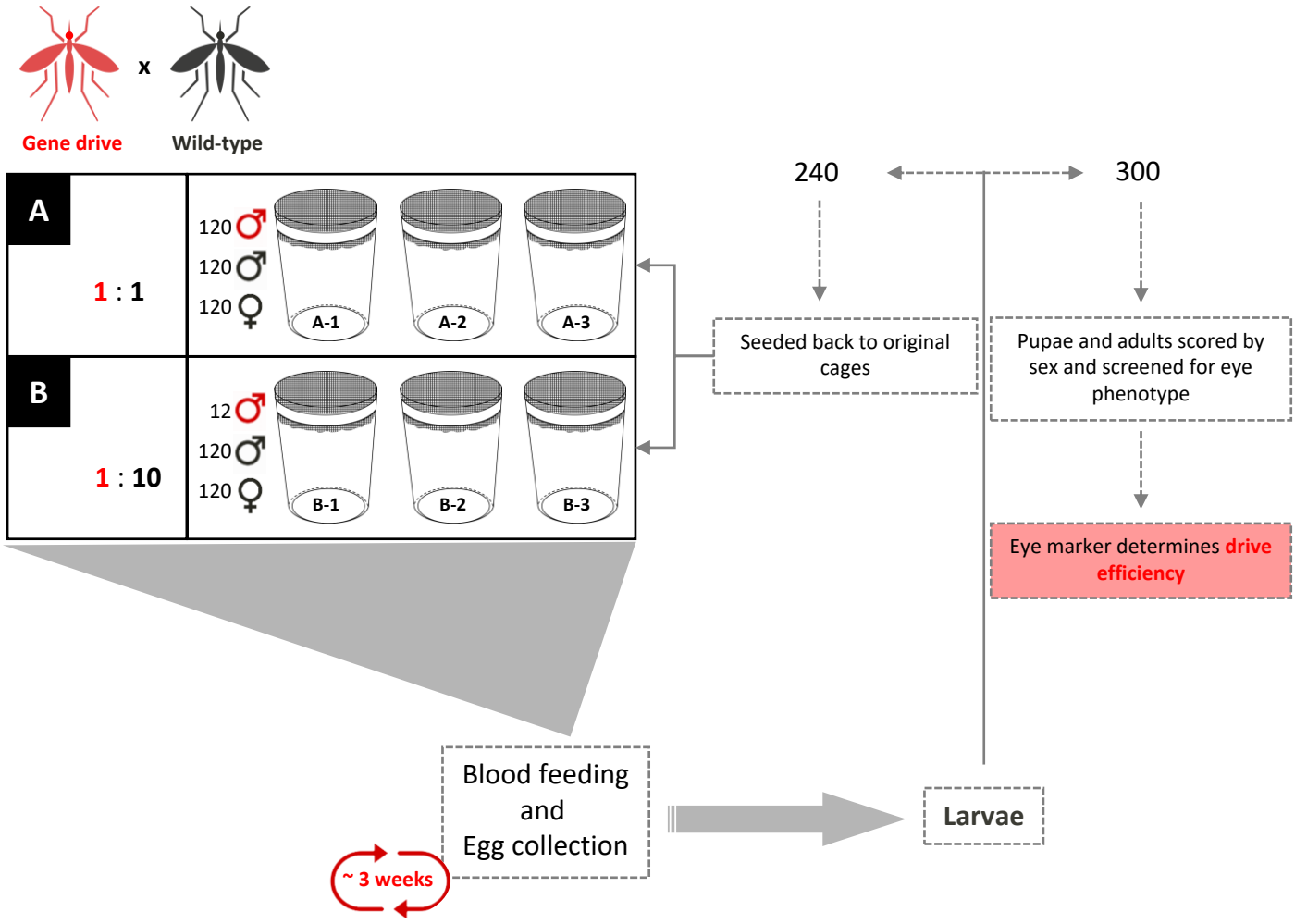

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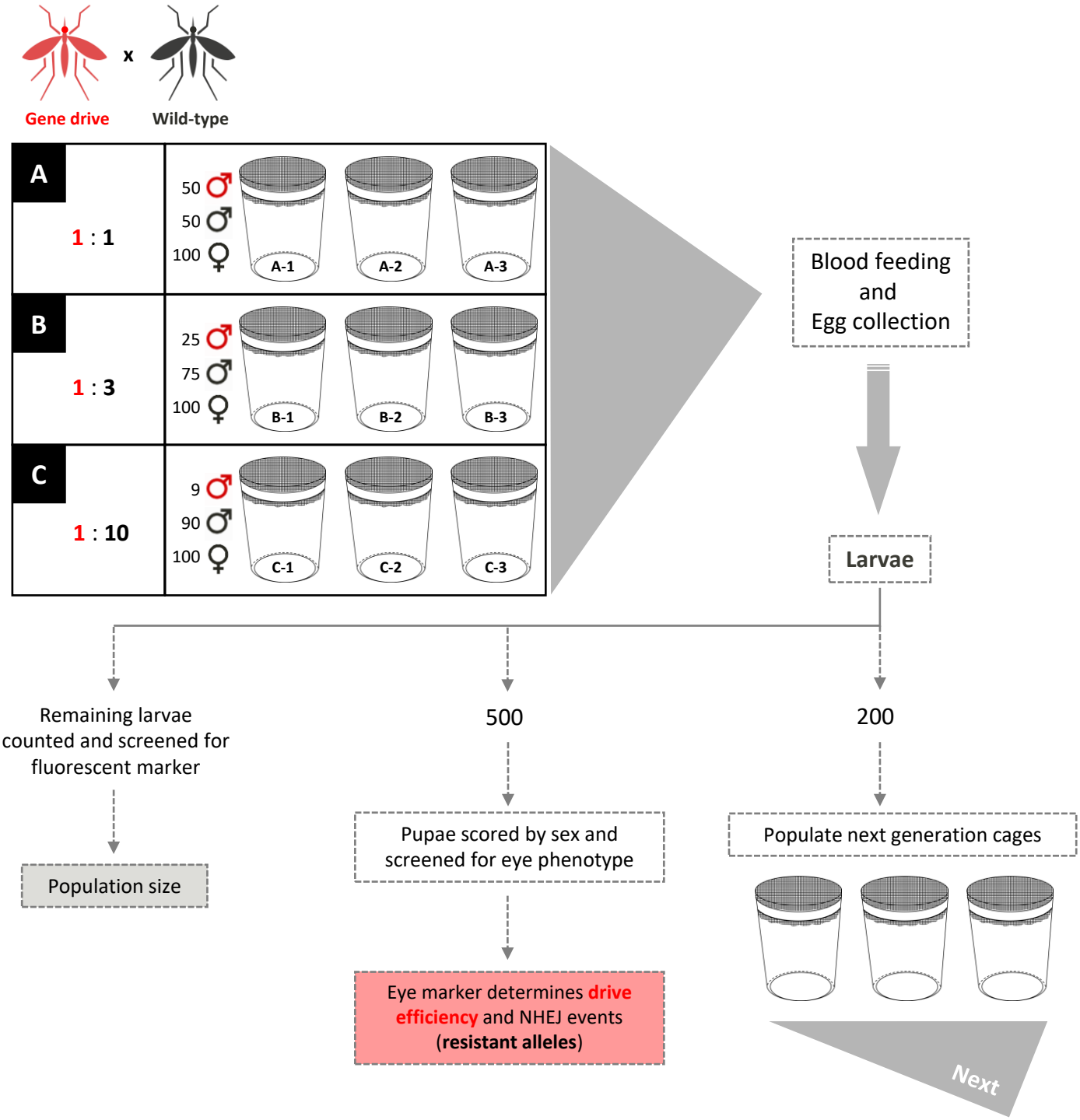

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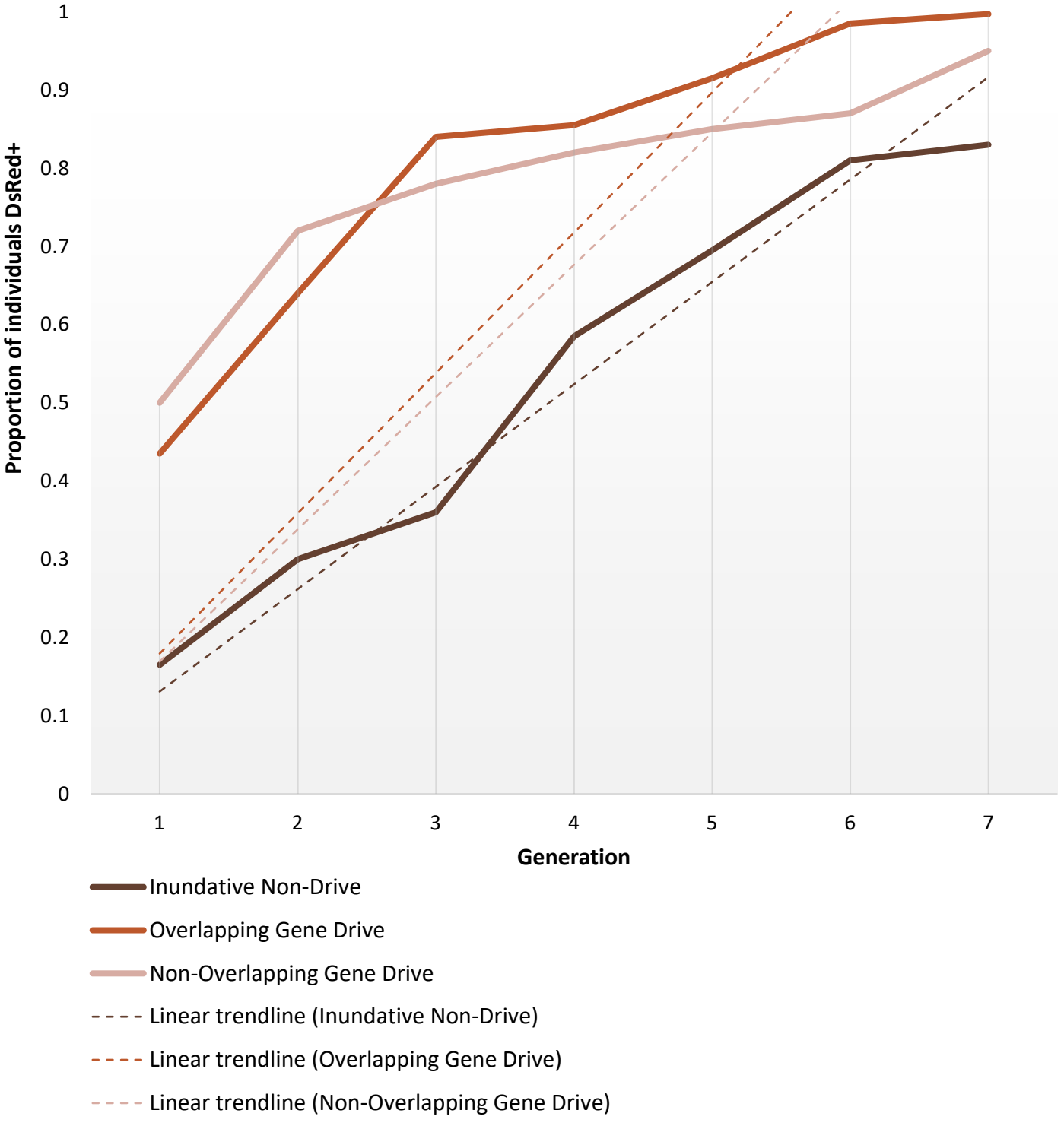

Table of Materials

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Name	Company	Catalog Number	Comments
Artificial feeders	Hemotek	SP6W1-3	Starter pack – 6 feeders with 3ml reservoirs
Cage, commercial	BioQuip	1450D	Collapsible Cage, 24 X 24 X 24" - 0.216 m^3 (60 cm^3)
Cage tub (popcorn)	Amazon.com	VP170-0006	0.005 m^3 (170 fl oz)
Dissecting microscope with fluorescence light and filters	Leica	M165FC	
Glue sticks	Michaels	88646598807	Gluesticks 40 pk, 0.4X4”
Hot glue gun	Woodwards Ace	2382513	Stanley, 40 watt, GR20
Nylon screen (netting)	Joann.com	1102912	Tulle 108" Wide x 50 Yds - ~35.6 cm^2 (14 in^2)
Oviposition cups	Fisher	259126	Beaker PP grad 50 mL
Razor cutting tool	Office Depot	487899	Box cutters
Scissors	Office Depot	978561	Scotch Precision Ultra Edge Titanium Non-Stick Scissors, 8"
Stapler	Office Depot	908194	Swingline Commercial Desk Stapler
Surgical sleeve (stockinette)	VWR	56612-664	~48 cm (19”) cut from bolt ~15 cm (6”) X ~23 m (25y)
Zip ties	Home Depot	295715	Pk of 100, 14” cable ties - 35.6 cm (14 in)

RESPONSES TO EDITORIAL AND REVIEWER COMMENTS

Editorial comments:
Changes to be made by the Author(s):

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

2. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.
New text included.

3. Please ensure the Introduction include all of the following with citation:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application
Revised in response to outline.

4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).
Resolved

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.) from the beginning to the end. 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." Also only one note can follow one step.
Changes made.

6. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
Changes made for clarification.

7. Please include an ethics statement to show that approvals were sought from the institutional committee for the use of the animal as a food source for mosquitoes. Please place this ethics statement before the start of the protocol.
Ethics statement added.

8. Do you include anesthetized/euthanized mice or include blood from mice as a food source for the mosquitoes?
Included an extra note for clarification (lines 225-227).
Clarified.

9. 1.1.1: So the cages contain only female larvae? Or do you remove the males from the cage? Please provide better clarity on this.
Anopheles larvae cannot be sexed visually at the larvae stage, so the term 'mixed sex' is appropriate. Added Note for explanation.

10. 1.1.4: Are these different cages than the ones used for females? Do you provide any special conditions for the eggs to hatch?
The cages are the same ones established in step 1.1.1: mixed-sexed, age-structured and stabilized to offset mortality. An additional clarification was included in the text. No special conditions were used to hatch the eggs.

11. What is the phenotype of the mosquitoes added to the cage?
The phenotype of the mosquitoes added to the cage are referred to as 'transgenic' or 'WT' in the text. The specific phenotype of the mosquitoes (i.e. fluorescent, eye color) depends on the nature of each experiment. Here, we only lay out the general framework of the number of mosquitoes added and either they are WT or transgenics.

12. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 action sentences per step. e.g., Lines 120-133, etc.
Resolved

13. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identify the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Revised

14. If you want to include the video with the submission, please upload and name it as a supplementary file. If you would want to use it for filming purposes, please include in the text how to do so and time stamp the protocol steps.
VIDEO SIZE WAS TO LARGE TO UPLOAD TO WEBSITE. PLEASE ADVISE

15. Please discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.
N/A

16. Please ensure there is a figure or a table in the Representative Results showing the effectiveness of your technique backed up with data.
Figure 6 shows the Representative Results

17. Please expand the description in the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title.
Figure 6 is now explained in sufficient detail

18. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
Done. Acknowledgements are consistent with the most stringent requirements of the journals.

19. Please remove the embedded table from the text manuscript and upload it separately as a .xlsx file.
Deleted from text and submitted as required

20. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6

paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Done

21. Please cite a minimum of 10 references.
Resolved

Reviewer comments:

Reviewer #1:
Manuscript Summary:
The manuscript "Small-cage laboratory trials of genetically-engineered Anopheline mosquitoes" submitted to JoVE described 3 lab experiments to assess efficacy of transgenic mosquito release for vector control. The experiments were well designed with linear structure and illustrations. The publication will provide visualized methods to people who need to start similar experiment and can be easily adopted for other genetically engineered insect research, therefore, I recommend for acceptance.

Minor Concerns:
74 1.1.2. At every week, provide the females in each cage with anesthetized mice as bloodmeal source (Figure 2A). &
207 3.2.1. Provide to the 4-7 days old females in each cage bloodmeals via artificial feeding apparatus
Indicate why 2 different blood meals are used in different experiment. Are they interchangeable?
Included an extra note for clarification.

I would like to see how the data is analyzed (not in great detail but at least mention what method was used to determine significance) because the experiment is not complete without data processing.
For the representative data plot present in Figure 6, observed data were selected from the same shared release ratio (1:1) for the three different protocols discussed in this manuscript, originally present in Pham et al. 2019. The X-axis represents the generation number after initial introduction of transgenic individuals and the Y-axis represents the proportion of larvae progeny showing the DsRed marker phenotype (DsRed+) over time. These data are collected respectively on steps 1.3.1, 2.2.5 and 3.2.6 of the protocols. Trendlines illustrate the overall curve fitting (slope) using linear regression but no statistical analysis was deployed here. Data description (*`Representative Results'*) was altered for clarification (lines 286 and 290). Please see source material (Pham et al. 2019) for details of the data processing used in each individual experiment.

Last page: Table of materials
The table seems to be disoriented and need to be redone if it is needed. However, this table has been presented before and therefore, is a kind of redundancy. Suggest to put the link "Click here to access/download;Table of Materials;table of materials.xlsx" together with the previous table on line 271.
Table deleted from text and supplied as requested by the journal

Reviewer #2:
Minor Concerns:
#1: General comment overall, the paper is very well written. It addresses an important aspect in the implementation of new strategies in the fight against malaria i.e to assess the performance of genetically-engineered mosquitoes in laboratory-contained small cage trials. I think the author need to outline the limitation

of this study. We agree with the authors that the Small-cage laboratory trials are best ways to assess the performance of genetically-engineered mosquitoes destined for vector control, but How did you decide on the size of the cages you used in your experiment? Please provide all the information regarding these cages.

We used large metal cages for the overlapping generation inundative release experiments because dead mosquitoes can be removed to keep the cage clean and new mosquitoes can be add every generation/week. For non-overlapping trial, we used the disposable cages so that it is straightforward to set up a new cage every generation. The sizes of cages depend on the material available commercially.

#2: this section is not super clear, Lines 357-360.Can you be much more specific on the life traits that might be affected by the small cages?
Altered text for better clarification.

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