Journal of Visualized Experiments

Methods for reproducible microinjection of dsRNA and oviposition bioassay in mosquitoes and house flies

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58650R1
Full Title:	Methods for reproducible microinjection of dsRNA and oviposition bioassay in mosquitoes and house flies
Keywords:	RNAi; gene silencing; Aedes aegypti; Musca domestica; Diptera; Culicidae; Muscidae
Corresponding Author:	Neil Sanscrainte USDA-ARS Center for Medical Agricultural and Veterinary Entomology Gainesville, FL UNITED STATES
Corresponding Author's Institution:	USDA-ARS Center for Medical Agricultural and Veterinary Entomology
Corresponding Author E-Mail:	Neil.Sanscrainte@ars.usda.gov
Order of Authors:	Neil Sanscrainte
	Christy M. Waits
	Christopher J. Geden
	Alden S. Estep
	James J. Becnel
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Gainesville, FL USA

United States Department of Agriculture
Agricultural Research Service
South Atlantic Area
Center for Medical, Agricultural and Veterinary Entomology
1600/1700 SW 23rd Drive
Gainesville, FL 32608

June 21st, 2018

Dear Editor,

Please consider our submission "Enhanced methods for dsRNA delivery by microinjection: measuring effects of gene silencing on survival and oviposition of *Aedes* mosquitos and house flies" for publication in the Journal of Visualized Experiments. We present a robust method of inducing phenotypic response to dsRNA in adults from two families of Diptera through a detailed injection and fecundity bioassay system that we believe to be best disseminated through demonstration. While a common laboratory practice, the specifics of insect microinjection (needle preparation, insect staging, injection site location, insect recovery) are often glossed over in the literature, and with our enhancements we provide a clear and proven injection technique that can be modified to deliver various biorationals to multiple mosquito and fly species.

Thank you for your time.

Regards,

Neil Sanscrainte
USDA, ARS, CMAVE
Mosquito and Fly Research Unit
1700 SW 23rd Dr.
Gainesville, FL 32608
(352) 374-5965
neil.sanscrainte@ars.usda.gov

TITLE:

Reproducible dsRNA Microinjection and Oviposition Bioassay in Mosquitoes and House Flies

AUTHORS AND AFFILIATIONS:

Neil D. Sanscrainte[1], Christy M. Waits[2,3], Christopher J. Geden[1], Alden S. Estep[1,2], James J. Becnel[1]

[1]USDA/ARS Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, Florida, United States of America

[2]Navy Entomology Center of Excellence, CMAVE Detachment, Gainesville, Florida, United States of America

[3]Lovelace Respiratory Research Institute, Albuquerque, New Mexico, United States of America

Corresponding author:

Neil D. Sanscrainte (neil.sanscrainte@ars.usda.gov)

Email Addresses of Co-Authors:

christy.waits@ars.usda.gov
chris.geden@ars.usda.gov
alden.estep@ars.usda.gov
james.becnel@ars.usda.gov

KEYWORDS:

RNAi, gene silencing, *Aedes aegypti*, *Musca domestica*, Diptera, Culicidae, Muscidae

SUMMARY:

This protocol describes a microinjection methodology that we have standardized and used for several years to deliver specific quantities of nucleic acids directly to the hemolymph of mosquitoes and house flies. This protocol results in minimal injection mortality and allows dose correlated measurements of fecundity.

ABSTRACT:

Synthetic dsRNAs, used to induce RNA interference, may have dose dependent phenotypic effects. These effects are difficult to define if the dsRNAs are delivered using a non-quantitative method. Accurate delivery of known quantities of nucleic acids or other chemicals is critical to measure the efficacy of the compound being tested and to allow reliable comparison between compounds.

Here we provide a reproducible, quantitative microinjection protocol that ensures accurate delivery of specific doses of dsRNA, reducing the mortality typically induced by injection injury. These modifications include the addition of Rhodamine B, a graduated injection needle, and an improved recovery method borrowed from Isoe and Collins. This method allows calculation of dose responses and facilitates comparisons between compounds. Versions of this method have

been successfully used on three genera of mosquitoes as well as house flies to assess the reduction in fecundity resulting from gene silencing of ribosomal RNA transcripts.

This protocol provides strategies to reduce several challenges of small insect microinjection. Together, mechanical delivery of dsRNAs accompanied by visual verification, identification of effective locations for delivery, and inclusion of a post-injection recovery period ensure accurate dosing and low injury mortality. This protocol also describes an oviposition bioassay for uniform determination of effects on fecundity.

INTRODUCTION:

Delivering small biomolecules, such as nucleic acids, to adult dipterans has proven to be challenging in both Culicidae and Muscidae. Oral uptake of dsRNAs has been reported to produce sterility (when targeting genes exclusively expressed in testes of adults) and mortality (when targeting SNF7 and a steroid receptor coactivator) in larval *Aedes aegypti*[1,2]. Mortality has also been observed when targeting HSP70 in larval *Musca domestica*[3]. Such phenotypic effects, however, have not resulted after feeding dsRNA to adult *Ae. aegypti* in sugar meals[4,5].

Microinjection has been used to circumvent the midgut when introducing pathogens or nucleic acids, thereby inducing a systemic response[5-10]. Several microinjection techniques exist, involving equipment ranging from in-house produced apparatuses that require visual measurement of injection volume to microprocessor-controlled injectors that allow for automated volume delivery as low as 2.3 nL[5,9-11]. RNA interference (RNAi) triggers targeting ribosomal mRNAs, disrupt ovarian development in arthropods as diverse as the cattle tick *Rhipicephalus microplus*, the mosquitoes *Aedes aegypti* and *Culex pipiens*, and the house fly *Musca domestica*[5,9,12,13]. In these studies, monitoring the disruption of oviposition was essential for determining the efficacy of the inoculant as the phenotype may manifest as termination or reduction of progeny. This is a form of multigenerational lethal phenotype that is a critical and desired effect in many of the non-traditional biocontrol methods like *Wolbachia*-infected males introduction (sexual incompatibility) and RNAi induced sterility[5,9,14]. Tracking both mortality and fecundity is necessary for the characterizing and development of highly specific biorationals (naturally occurring pathogens and/or natural derivatives)[15].

This protocol presents detailed microinjection techniques for both adult mosquitoes and house flies; a process that is often not well described in the literature. In addition, oviposition bioassay methodologies to adequately evaluate dsRNA effects on adult dipterans are described. These protocols were developed specifically for *Aedes aegypti* and *Musca domestica* but can be modified for other species.

PROTOCOL:

1. Insect Preparation

1.1. Anesthetize mosquitoes by chilling them at 4 °C for several hours or by 2 min exposure to CO_2 and then holding at 4 °C prior to injections. Cold anesthetize flies at 4 °C 2–3 h prior to injections. Ensure mosquitoes and flies are mated if oviposition is a response variable.

Note: The mosquito anesthetization method depends upon the species and strain, *e.g., Culex* recover much quicker from cold anesthetization than *Aedes aegypti* and therefore CO_2 knockdown is preferable.

1.2. On ice, carefully stage mosquitoes or flies by using soft forceps to lay them ventrally exposed on microscope slides ~0.5 cm apart. Standard slides can hold two rows of 6 mosquitoes each or 6–7 house flies. Leave a 1–1.5 cm space at the left or right end of the slide clear of mosquitoes or flies to aid slide handling.

1.3. Keep slides of staged insects at 4 °C in large Petri dishes until ready for injection.

Note: Drilling a hole through the Petri dish lid will help prevent insects from being disturbed by air displacement when capping. Placing slides on a couple of glass capillaries secured to the dish bottom will ease removal from Petri dish.

2. Insect Injection

2.1. Prepare dsRNA as described by Estep *et al.*[5] and Sanscrainte *et al.*[9]. If free of contaminants, dsRNA can be quantified by using a spectrophotometer to measure the absorbance at 260 nm and calculating RNA concentration in µg/mL as A_{260} × dilution factor × 40.

2.2. Set up dissecting microscope and microinjector over a chill table or shallow ice bucket to perform injections on the cold-anesthetized mosquitoes and flies (see step 1.1–1.3 and **Figure 1**).

2.3. Pull glass capillaries to a fine tip with needle puller (settings: heater = 15 units, solenoid = 4 A).

Note: See **Table of Materials** for manufacturer's details.

2.4. Place the capillary needle into a microinjector as per the manufacturer's instructions and break needle tip by pinching with a pair of forceps to produce a sharp point.

Note: Suggested needle opening sizes are ~150 µm for mosquito and ~250 µm for housefly.

2.5. Set microinjector for desired delivery volume. Meniscus movement from ~50 nL aliquots is easily observable and recommended for mosquito microinjections. If available, enable fast injection settings. Rinse the needle by filling and expelling nuclease-free water 3 times.

2.6. Prepare solutions for injection at a concentration such that 100–150 nL will provide desired dose for mosquitoes and 500 nL will provide desired dose for house flies.

Note: Suggested initial doses: 1 µg for each mosquito and 5 µg for each housefly[5,9].

2.7. Optionally for mosquitoes, add Rhodamine B (to aid in visualization) to solutions at a final concentration of 3.0 µg/mL.

Note: The dye will be clearly visible by its pink color after injection through the nearly clear cuticle on the ventral surface at the abdomen/thorax intersection.

2.8. Pipette 3–4 µL of solution to inject onto a clean surface (such as a piece of paraffin film) and draw into the glass needle without sucking in any air. Depress the inject button repeatedly until liquid begins to dispense from the needle and gently wipe droplets off with a delicate task wiper.

Note: While some models of microinjectors require backfilling the syringe, the injector referenced in the **Table of Materials** does not.

2.9. Using an ultra-fine point marker, draw hash marks ~1 mm apart starting from the liquid meniscus to the needle shank. This will aid in visualizing the meniscus movement and ensure that the solution has entered the mosquito during injection.

2.10. Set the slide of mosquitoes or flies (as prepared in steps 1.2–1.3) under the needle on the chill table or ice (**Figure 1**). Ensure that field of view in the microscope is wide enough to see the solution meniscus in the needle.

2.11. For mosquitoes, align the needle with the middle one-third of the mesokatepisternum (**Figure 2A**), and while bracing the mosquito against the needle with forceps placed on the opposite side, gently puncture the cuticle with the needle tip using the microinjector micrometer.

2.12. For house flies, align the needle with the mesopleuron (**Figure 2B**) and, while bracing the fly against the needle, gently puncture the cuticle with the needle tip.

2.13. Gently slide the needle into the mosquito or fly body until the tip has passed through the midline.

Note: Inserting the needle too shallow often results in the injected liquid beading out of the insect (see step 2.15).

2.14. While watching for movement of the liquid meniscus in the needle, depress the inject button until the desired amount of liquid has been injected. For mosquitoes, if set to 50.6 nL aliquots, press 2X for ~100 nL or 3X for ~150 nL. For house flies, depress the inject button until ~500 nL has been injected (*i.e.,* with 69 nL aliquots press 7X for 483 nL).

2.15. If the meniscus does not move, slowly slide the mosquito or fly off the needle while watching for meniscus movement. If a portion of the injected solution beads out of the cuticle upon needle removal or if the meniscus fails to move, discard the insect as this injection was not successful.

2.16. Transfer successfully injected mosquitoes or house flies in groups of 10–15 to clear 3.5 oz holding cups. Cover with tulle or netting and recover at room temperature.

2.17. After mosquitoes and flies have recovered (1 – 2 h), invert each holding cup over a cotton ball soaked with 10% sucrose solution.

Note: The inversion of the cup on top of the sucrose cotton aids in survival by providing easier access to a sugar meal[10].

2.18. Rinse needle as in step 2.5 between injection solutions. When done injecting, discard used needles in an appropriate sharps container.

3. *Aedes aegypti* mortality and oviposition bioassay

3.1. For three days after injection, continue to provide mosquitoes with 10% sucrose and record the number of visibly dead mosquitoes.

3.2. Fill a ≤12-inch artificial membrane (such as collagen sausage casing) with fresh blood (*i.e.,* bovine for *Aedes aegypti*) and heat to 45 °C in hot water bath.

3.3. Briefly dry the membrane by rolling on a paper towel and lay across the tulle caps of the 3.5 oz clear holding cups. To enhance feeding, spike blood with 1 mM ATP after heating as a phagostimulant or by handle the warmed blood sausage with clean bare hands to leave human volatiles on the casing surface.

3.4. After blood feeding, replace the cotton ball soaked with 10% sucrose on top of the holding cups and allow mosquitoes to rest for ~24 h before transferring to oviposition cups.

3.5. Construct oviposition cups by filling clear 3.5 oz bioassay cups with ~30 mL deionized H_2O and placing a piece of seed germination paper (~4 cm wide and 5 cm long with ridges running vertically) at the bottom of the cup and against the side (**Figure 3A**). Cover with tulle or netting and cut a small slit (~1 cm) in the tulle or netting cap.

3.6. At 24 h post-blood feeding, cut a small slit (~1 cm) in the tulle cap of the holding cup and transfer only individual females that successfully fed — with a visible blood bolus in abdomen — by gentle mouth aspiration into oviposition cups (1 female/oviposition cup). Seal the small cut in the oviposition cap with a 10% sucrose saturated cotton ball.

3.7. Hold cups at ~27 °C for 5–7 days to allow mosquitoes to fully oviposit. Count eggs under a dissecting scope.

4. *Musca domestica* mortality and oviposition bioassay

4.1. For three days after injection, continue to provide the flies with 10% sucrose and record the number of visibly dead flies.

4.2. Three days after injection, anesthetize flies by briefly placing a polyethylene tube dispensing CO_2 over the holding cups until all flies are motionless at bottom of the cups.

4.3. Transfer flies to a clean cage comprised of a 4 L plastic jar with a stockinette sleeve covering the opening (10 cm, **Figure 3B**). Provide flies with water and a mixture of granulated sucrose, powdered milk, and dried egg yolk in an 8:8:1 ratio (by volume) for four days, recording and removing dead flies daily.

4.4. Prepare dry ingredients for fresh fly larval rearing medium by mixing 75% wheat bran with 25% pelleted livestock feed by weight. Add water to reach 62% moisture (until a drop of water can barely be squeezed out).

4.5. Prepare a 2.5 cm diameter ball from a 1:1 mixture of the above fresh larval media and "used" fly larval medium (medium in which flies have previously pupated). Wrap the ball in a square of black cotton cloth, squeeze lightly until medium liquid seeps through, then use rubber bands to hold the cloth in place around the ball.

4.6. Put the ball in a 60 mL cup and place it in the fly cage for 5 h (**Figure 3B**).

4.7. Rinse eggs off the oviposition ball and ensure that the eggs are removed from under folds in the cloth. Shake eggs to disrupt clusters and transfer them to a graduated 20 mL centrifuge tube with a transfer pipet.

4.8. Wait until the eggs settle and note the volume of the settled eggs in the graduated tube. Add sufficient water to bring the volume up to 20X the volume of settled eggs. Record the total volume of water plus eggs.

4.9. While mixing the water and egg suspension with either a magnetic stir bar or vigorous stirring, use a 1 mL pipet tip (with the end of the tip cut off) to dispense 0.5 mL of the water and egg suspension onto a piece of pre-moistened black cloth in a series of lines. Count the eggs under a dissecting microscope.

4.10. Repeat step 4.9 two more times. If one of the counts is a severe outlier (*i.e.,* differs from the other two counts by >35%), make a fourth count and disregard the outlier.

4.11. Calculate the mean number of eggs per sample and multiply this value by 2 to get the number of eggs/mL of the suspension. Multiply the eggs/mL value obtained by the volume of the egg suspension from step 4.8. This is the estimate for the total number of eggs laid.

REPRESENTATIVE RESULTS:

Microinjection of dsRNA in mosquitoes

This microinjection method has been used in our laboratory to evaluate gene expression, mortality and oviposition response for over 80 dsRNA triggers across several mosquito genera (*Aedes*, *Anopheles*, *Culex*). Injecting females from the colony strain of *Ae. aegypti* (ORL1952) with 1 µg of dsRNA derived from the ribosomal transcripts RPS6 and RPL26 (see Estep *et al.*[5] for dsRNA production methods and sequence data) showed significant reduction in relative expression (RE) across multiple oviposition cycles when compared to mosquitoes injected with a control dsGFP. *Aedes aegypti* RE measured following the 2nd gonotrophic cycle showed significant reduction of RPS6 expression ($P < 0.05$) in mosquitoes injected with dsRPS6 as determined by a Student's t-test between the dsGFP control of the same group (**Figure 4**)[5].

Fecundity was assessed from individual females using the oviposition bioassay described here. After 3 days post-injection, mosquitoes were blood fed, transferred into individual oviposition containers, and allowed to lay to completion. Average clutch sizes for the first gonotrophic cycle were significantly reduced for both dsRPS6 treated *Ae. aegypti* (n = 102 females, 1.3 ± 0.8 (mean ± SE) eggs) and dsRPL26 treated (n = 86 females, 4.0 ± 1.1 eggs) when compared to dsGFP injections (n = 79 females, 49.3 ± 3.5 eggs, **Figure 5A**). Clutch assessment after a second gonotrophic cycle also showed significantly reduced oviposition for both dsRNA treated groups, but with reduced effect size. Group sizes of *Ae. aegypti* were variable and thus means separation was performed by Dunn's test[5]. Twenty-four-hour mortality was normally 3% or less.

A clear dose effect was observed when injecting dsRPS6 from 1 µg to 50 ng in female *Ae. aegypti*, and clutch size varied significantly when compared to 1 µg/female dsGFP injected controls in all but the 25 ng/female dsRPS6 dose (**Figure 5B**).

Microinjection of dsRNA in house flies

Colony *Musca domestica* (ORL normal) were injected with 5 µg of dsRNA constructs designed against the house fly RPS6 and RPL26 transcripts[9]. As was observed in *Ae. aegypti*, significant reduction of both specific transcript expression (RPS6 and RPL26) and reduction in clutch size was observed (**Figures 4 and 5A**). Significant reduction in RE was determined by Student's t-test between the dsGFP control of the same group ($P < 0.05$) and the Holm-Sidak test was used to determine significance between clutch sizes for different *M. domestica* treatment groups. Ovarian dissections showed reduced oogenesis in dsRPS6 and dsRPL26 treatments, while dsGFP fed flies had normal vitellogenin deposition as rated on the Tyndale-Biscoe scale[9].

FIGURE AND TABLE LEGENDS:

Figure 1. Microinjection setup for delivering microvolumes to adult mosquitoes and house flies. Injections are performed on cold-anesthetized insects staged on microscope slides over a chill table.

Figure 2. Microinjection sites for *Ae. aegypti* and *M. domestica*. (A) Adult *Ae. aegypti* are injected in the middle one-third of the mesokatepisternum. **(B)** Adult *M. domestica* are injected in the mesopleuron. Arrows indicate sites of injection.

Figure 3. Oviposition bioassay setup. (A) *Ae. aegypti* oviposition assay cups with seed germination paper as an oviposition substrate, capped with tulle and 10% sucrose-soaked cotton. **(B)** Adult female *M. domestica* in an oviposition assay container with food (A), water (B), and larval media (C).

Figure 4. Relative expression of RPS6 in *Ae. aegypti* and *M. domestica* injected with species-specific dsRNAs. *Aedes aegypti* and *M. domestica* were injected with 1 and 5 µg respectively of either control dsRNA (dsGFP) or dsRNA designed against RPS6 or RPL26 transcripts from each insect. Significant reduction ($P < 0.05$) of RPS6 expression was observed in mosquitoes and flies injected with dsRPS6 and by flies injected with dsRPL26 (indicated by asterisks). Error bars represent mean ± SE and number at column base indicates number of individuals examined. This figure has been modified from Estep *et al.*[5] and Sanscrainte *et al.*[9].

Figure 5. Average clutch size of *Ae. aegypti* and *M. domestica* injected with species-specific dsRNAs. (A) Egg deposition was significantly reduced ($P < 0.05$) in both *Ae. aegypti* and *M. domestica* after injection of 1 and 5 µg respectively of either control dsRNA (dsGFP) or dsRNA designed against RPS6 or RPL26 transcripts from each insect. Asterisks indicate significant differences from the dsGFP control of the same group. Error bars represent mean ± SE and number at column base indicates number of individuals examined. This figure has been modified from Estep *et al.*[5] and Sanscrainte *et al.*[9]. **(B)** Dose curve of *Ae. aegypti* injected with dsRPS6 from 50 ng/female to 1000 ng/female shows significant reductions in fecundity in comparison to dsGFP injected cohorts (50, 100, and 1000 ng/female). Error bars represent mean ± 95% CI from 36–81 individual organisms per dose. Points with letters represent significant difference between groups ($P < 0.05$) identified by ANOVA. This figure has been modified from Estep *et al.*[5].

DISCUSSION:

Microinjection is a valuable laboratory technique to ensure delivery of dsRNA or other biorationals (*i.e.,* pesticides, viruses, microsporidia). While many laboratories perform microinjection, the exact amount injected is often unclear due to technical limitations of the delivery system where delivered volume is not directly measured[11]. Delivered volume and concentration are critical parameters that allow calculation of standard toxicological measures such as EC50 or IC50 or to define minimal effective doses[5,16]. This is especially important in functional studies using RNAi in adult insects, where delivery by feeding does not always result in systemic exposure to the desired particles and the actual dose crossing the midgut is unclear[5].

While the microinjection procedure can be initially challenging, rapid improvements in speed and delivery accuracy are achieved with practice and patience. Mastering the injection procedure itself is a time investment, but, once accomplished, the procedure produces repeatable results and injury mortality of <3%. The ratio of successful injections will increase as proficiency increases allowing injection of 300 mosquitoes or flies per day.

Many of the challenges of microinjection are due to the needle being used. Determining the proper capillary needle break point and tip angle is a challenging aspect of the procedure and requires some trial and error to determine the most effective opening size for a given species. The fine needle most useful for mosquito microinjection (~150 µm) is too small to quickly deliver the larger volume injected into flies while conversely, the larger needle opening that works on flies (~250 µm) causes extensive injury damage to the smaller mosquitoes and unacceptable injection mortality. A needle broken with a blunt tip is often difficult to get through the cuticle without tissue damage and increased mortality. Insect scales or tissue pieces can clog needles over the course of an experiment, so it is often helpful to have several needles pulled if replacement is required. We have found that it is easier to replace a difficult needle rather than spend time to try to clear a jammed or fouled needle.

Observing the injection solution entering the insect is critical so that unsuccessful injections can be removed (see steps 2.14–2.15). Rhodamine B addition to injection solutions provides a clear visual to ensure that cold-staged insects receive proper dosing and is especially helpful while practicing (see step 2.7).

The injection and oviposition bioassay methods presented here have successfully induced gene knockdown and measurable phenotypic effects in adult *Ae. aegypti* and *M. domestica* when using dsRNAs designed against species-specific ribosomal transcripts (**Figures 4 and 5A**). Additionally, the ability of this method to accurately deliver microvolumes to individuals allows for dose response curves to be generated for small amounts of material (as low as 50 ng; **Figure 5B**). This is especially useful for methods such as screening siRNAs or dsRNAs, as producing large quantities of these molecules can be cost-prohibitive.

This method can be modified to inject biological agents (viruses, bacteria, microsporidia) or chemical pesticides, after ensuring that any delivery buffers are innocuous by injection. As delivery volume is limited by the size of the insect, producing concentrated testing solutions is of importance.

To adequately evaluate dsRNA efficacy, it is necessary to track oviposition as lethal phenotypes may only manifest in progeny. As presented here, holding female *Ae. aegypti* separately after blooding allows for individual clutch size determination and further testing on the same individuals can be done downstream of oviposition (*i.e.,* gene expression studies, additional gonotrophic cycles, tissue specific knockdown) to correlate reproductive output with other measures such as gene expression. As *M. domestica* generally oviposit in a group response, inciting isolated gravid flies to lay is very labor intensive and not practical for large numbers of individuals[17]. Therefore, a method to determine mean clutch size is presented.

The microinjection method presented here provides consistent systemic delivery to mosquitoes and house flies. Coupled with mortality and oviposition tracking bioassays, these versatile tools can be used to assess the transcriptional and phenotypic effects of small RNA molecules, biological agents, and traditional pesticides where oral delivery is not viable.

ACKNOWLEDGMENTS:

The authors thank Hanayo Arimoto, Dana Johnson, Lucy Li, and Roxie White for help with data acquisition and analysis. We also thank Drs. Pia Olafson and Ke Wu for critical review of the manuscript. Funding was provided by the USDA, the WII-141C project of the Navy and Marine Corps Public Health Center, and the Deployed War Fighters Protection Program. The funders had no role in design or direction of the study or development of the manuscript.

DISCLOSURES:

All authors are employees or contractors of the United States Department of Agriculture or the Department of Defense. This manuscript was produced during the course of official duties and is by law in the public domain. The views expressed represent the opinion of the authors and do not represent an official position or endorsement by the US government. Mention of a commercial product is not a recommendation for use.

REFERENCES:

1. Whyard, S. *et al.* Silencing the buzz: a new approach to population suppression of mosquitoes by feeding larvae double-stranded RNAs. *Parasites & Vectors*. **8** (96), (2015).
2. Das, S., Debnath, N., Cui, Y., Unrine, J., Palli, S.R. Chitosan, carbon quantum dot, and silica nanoparticle mediated dsRNA delivery for gene silencing in *Aedes aegypti*: a comparative analysis. *ACS Applied Materials & Interfaces.* **7** (35), 19530-19535 (2015).
3. Tang, T. *et al.* Stress-induced HSP70 from *Musca domestica* plays a functionally significant role in the immune system. *Journal of Insect Physiology*. **58** (9), 1226-1234 (2012).
4. Coy, M. R. *et al.* Gene silencing in adult *Aedes aegypti* mosquitoes through oral delivery of double-stranded RNA. *Journal of Applied Entomology*. **136** (10), 741-748 (2012).
5. Estep, A. S., Sanscrainte, N.D., Becnel, J.J. DsRNA-mediated targeting of ribosomal transcripts RPS6 and RPL26 induces long-lasting and significant reductions in fecundity of the vector *Aedes aegypti*. *Journal of Insect Physiology*. **90**, 17-26 (2016).
6. Wen, D. *et al.* N-glycosylation of Viral E Protein Is the Determinant for Vector Midgut Invasion by Flaviviruses. *mBio*. **9** (1), e00046-18 (2018).
7. Liu Y., Cheng G. Techniques for Experimental Infection of Mosquitoes with West Nile Virus. In *Methods in Molecular Biology*. *Vol. 1435: West Nile Virus* (ed Colpitts, T.), 151-163, Humana Press (2016).
8. Puglise, J. M., Estep, A. S., Becnel, J. J. Expression profiles and RNAi silencing of Inhibitor of Apoptosis transcripts in *Aedes*, *Anopheles*, and *Culex* mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology*. **53** (2), 304-314 (2015).
9. Sanscrainte, N. D. *et al.* Reduction in *Musca domestica* fecundity by dsRNA-mediated gene knockdown. *PloS one.* **13** (1), e0187353 (2018).

10. Isoe, J., Collins, J., Badgandi, H., Day, W. A., Miesfeld, R.L. Defects in coatomer protein I (COPI) transport cause blood feeding-induced mortality in Yellow Fever mosquitoes. *Proceedings of the National Academy of Sciences*. **108** (24), E211-E217 (2011).
11. Drake, L. L., Price, D. P., Aguirre, S. E., Hansen, I. A. RNAi-mediated gene knockdown and *in vivo* diuresis assay in adult female *Aedes aegypti* mosquitoes. *Journal of Visualized Experiments: JoVE*. **65**, doi: 10.3791/3479 (2012).
12. Kurscheid, S. *et al.* Evidence of a tick RNAi pathway by comparative genomics and reverse genetics screen of targets with known loss-of-function phenotypes in Drosophila. *BMC Molecular Biology*. **10** (1), 26 (2009).
13. Kim, M., Sim, C., Denlinger, D. L. RNA interference directed against ribosomal protein S3a suggests a link between this gene and arrested ovarian development during adult diapause in *Culex pipiens*. *Insect molecular biology.* **19** (1), 27-33 (2010).
14. Atyame, Célestine M. *et al.* Comparison of irradiation and Wolbachia based approaches for sterile-male strategies targeting *Aedes albopictus*. *PLoS one*. **11** (1), e0146834 (2016).
15. Becnel, J. J. and Floore, T. G. Introduction to "Biorational Control of Mosquitoes." *Journal of the American Mosquito Control Association*. **23** (sp2), 1-2 (2007).
16. Bolognesi, R. *et al.* Characterizing the mechanism of action of double-stranded RNA activity against western corn rootworm (*Diabrotica virgifera virgifera* LeConte). *PloS one*. **7** (10), e47534 (2012).
17. Jiang, Y. *et al.* Semiochemicals from ovaries of gravid females attract ovipositing female houseflies, *Musca domestica*. *Journal of Insect Physiology*. **48** (10), 945-950 (2002).

Figure 1

Click here to access/download;Figure;Fig_1.tif

Figure 2

Click here to access/download;Figure;Fig_2.tif

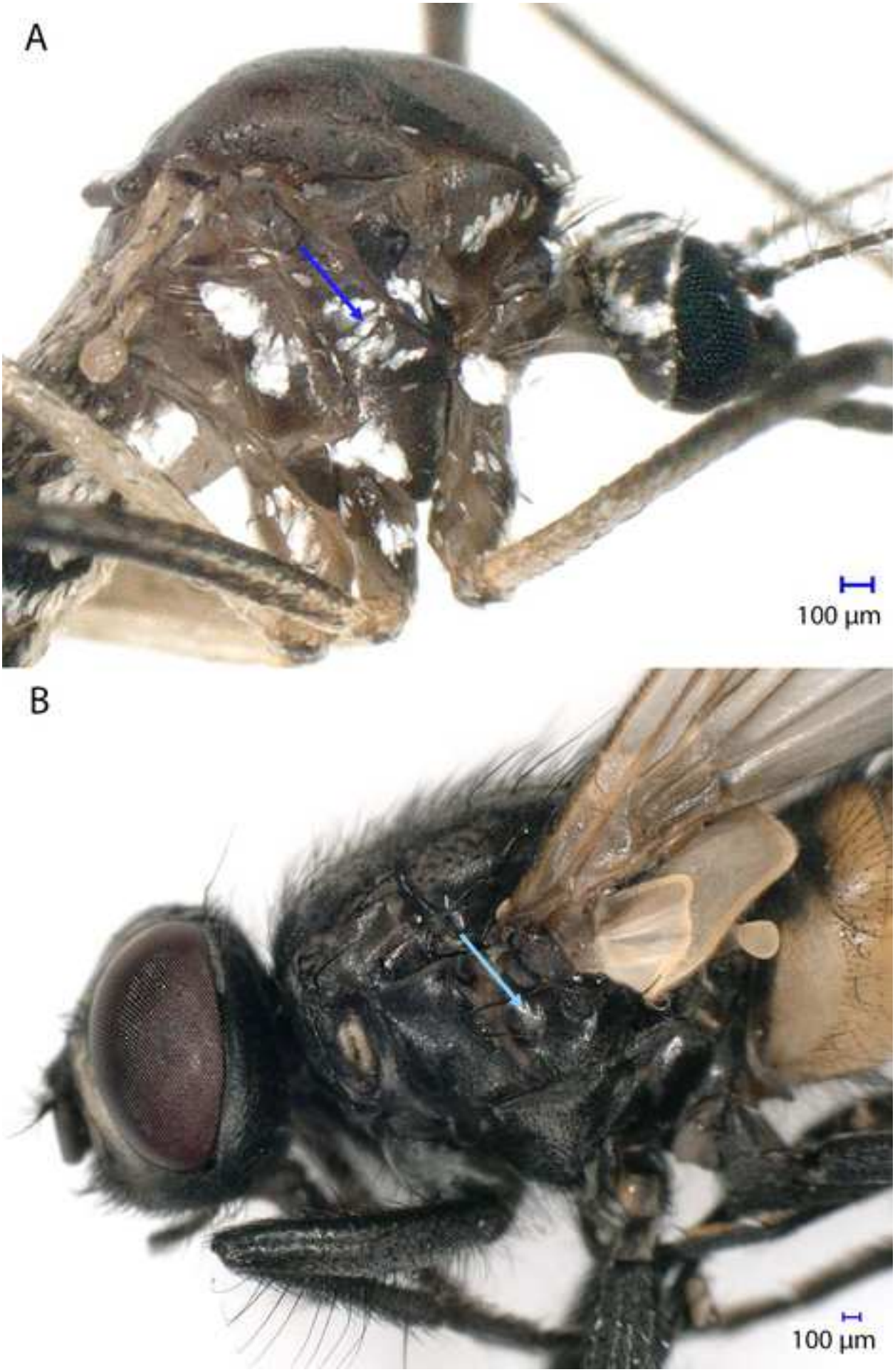

Figure 3

Click here to access/download;Figure;Fig_3.tif

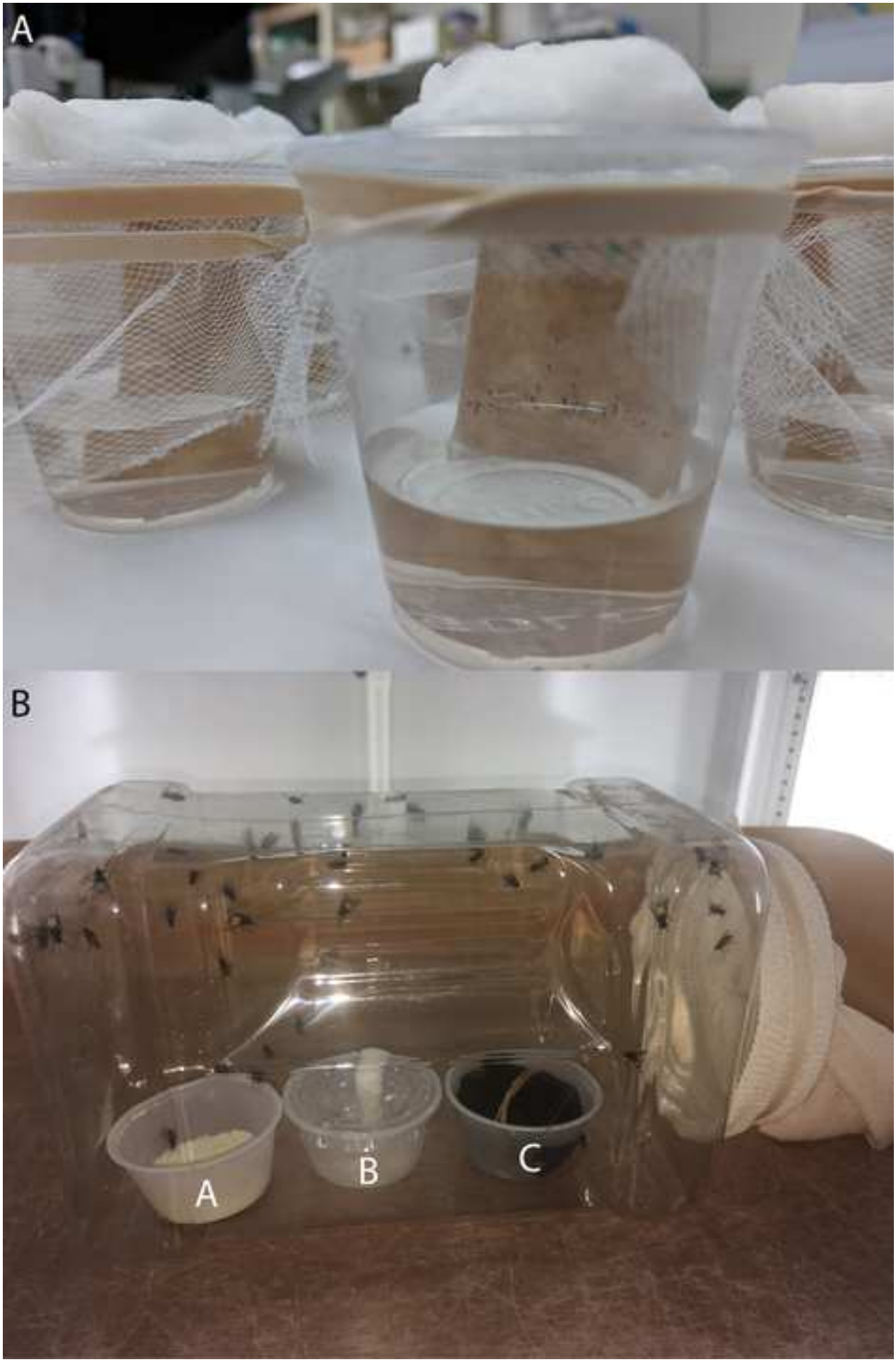

Figure 4

Click here to access/download;Figure;Fig_4.tif

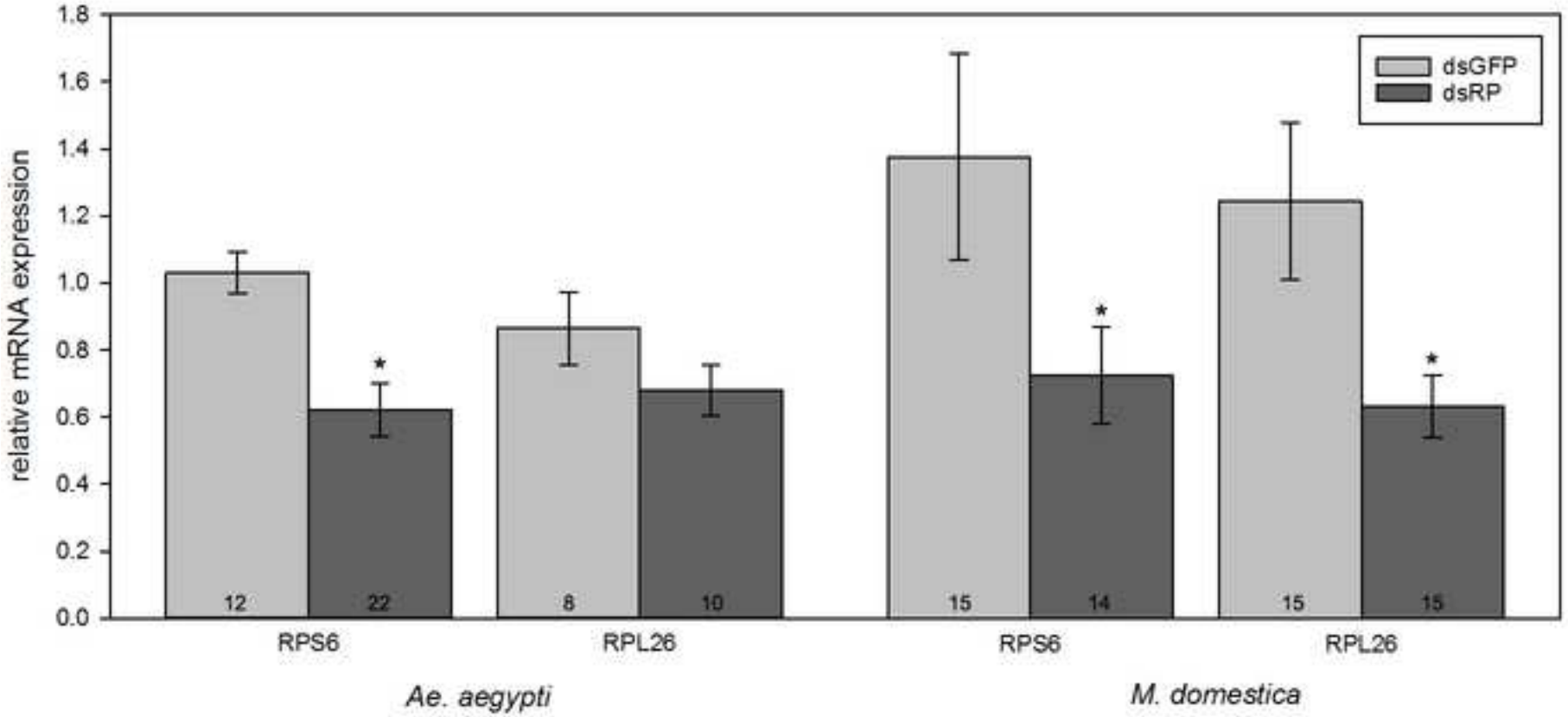

Figure 5

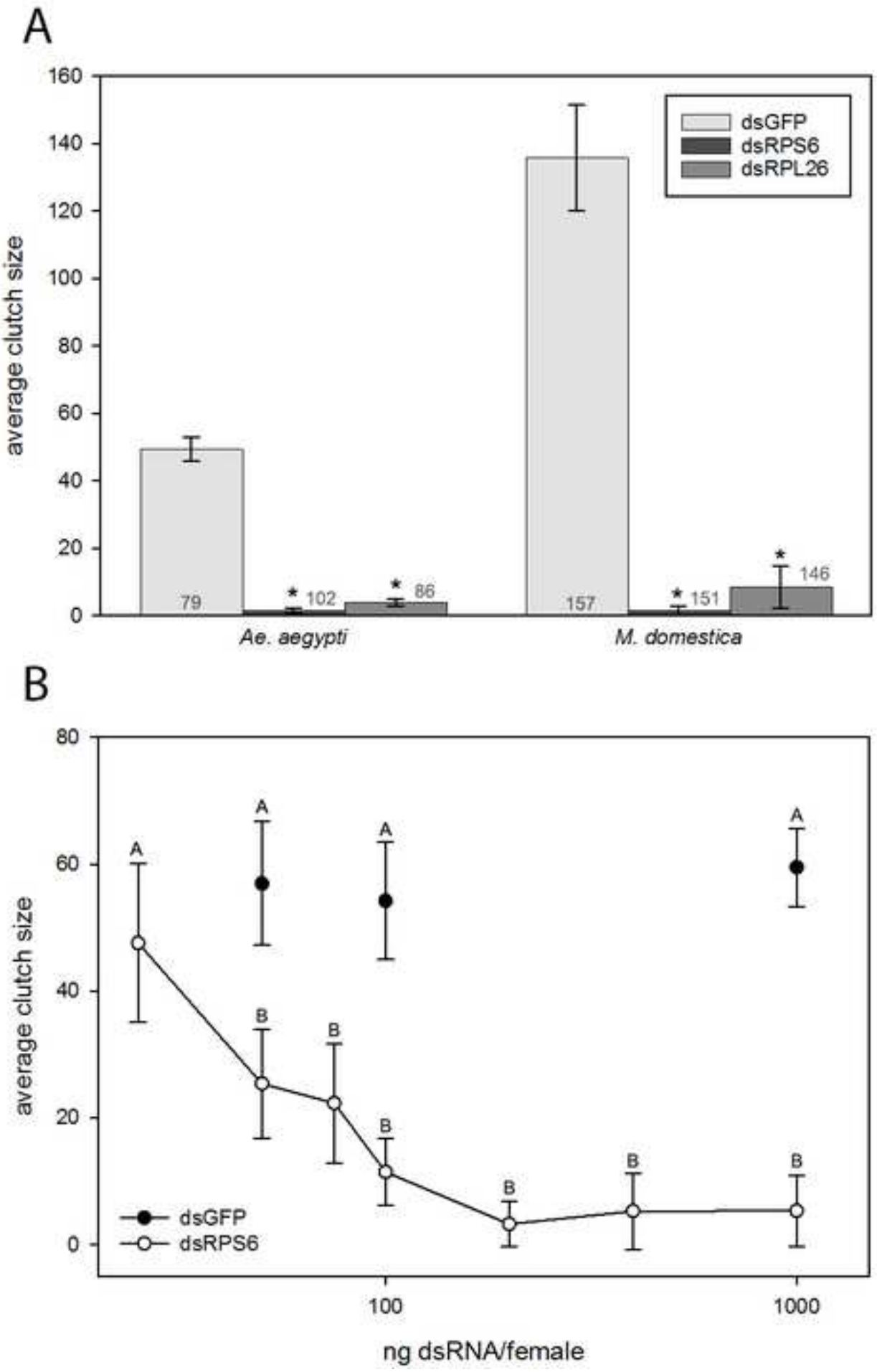

Table of Materials

Click here to access/download;Table of Materials;2018_07_26_materials_table.xlsx

Name of Material/ Equipment	Company	Catalog Number
needle pulling		
vertical pipette puller	Kopf	720
glass capillaries, 3.5" long, ID = 0.530 mm ± 25 µm, OD 1.14 mm	World Precision Instruments	504949
microinjector station		
Nanoliter 2010	World Precision Instruments	NANOLITER2010
manual micromanipulator	World Precision Instruments	KITE-L
magnetic stand	World Precision Instruments	M9
precision stereo zoom binocular microscope on boom stand	World Precision Instruments	PZMIII-BS
1.5X objective	World Precision Instruments	501377
light LED ring	World Precision Instruments	504134
laboratory chill table	BioQuip Products	1431
microscope slides	Fisher Scientific	12-544-1
Rhodamine B, 98+%	Acros Organics	AC296571000
mosquito oviposition bioassays		
large Petri dish	Fisher Scientific	FB0875714
plastic cup - 3 1/2 oz.	Dart Container Corporation	TK35
matte tulle fabric	Joanne Fabrics	1103068
blood source	locally acquired	
1 x 30mm clear edible collagen casing	Butcher and Packer	30D02-05
heavy weight seed germination paper	Anchor Paper Co	SD7615L
oral aspirator with HEPA filter	John W. Hock Company	612
house fly oviposition bioassays		
4 L plastic food storage canister	Walmart	555115143
10-inch stockinette sleeve	Medonthego.com	FS15001H
wheat bran	locally acquired	
Calf Manna performance supplement	ValleyVetSupply.com	16731
dried egg yolk	BulkFoods.com	40506
black cotton cloth	locally acquired	
60 mL cup	Dart Container Corporation	P200-N

Comments/Description
settings: heater = 15 units, solenoid = 4 amps
capillaries for pulling glass needles
microinjector
left hand KITE manipulator
holds micromanipulator
dissecting scope for microinjections
objective for microscope
light ring for injection microscope
chill table for microinjections
for staging insects while injections
for holding staging slides
mosquito oviposition bioassay cups
caps for oviposition bioassays
typically bovine or live chickens
typically found at feed stores
pelleted livestock feed
typically in craft supplies section

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Enhanced methods for dsRNA delivery by microinjection: measuring effects of gene silencing on survival and oviposition of *Aedes* mosquitos and house flies
Author(s):	Neil D. Sanscrainte, Christy M. Waits, Christopher J. Geden, Alden S. Estep, James J. Becnel

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☒ Open Access

Item 2: Please select one of the following items:

☐ The Author is **NOT** a United States government employee.

☒ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Neil Sanscrainte
Department:	Mosquito and Fly Research Unit
Institution:	USDA-ARS-CMAVE
Title:	Molecular Biologist
Signature:	NEIL SANSCRAINTE Digitally signed by NEIL SANSCRAINTE Date: 2018.06.21 13:37:19 -04'00'
Date:	06/21/2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Editorial comments:

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

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

This has been done to the manuscript.

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

Both Estep et al. 2016 and Sanscrainte et al. 2018 are published under a creative commons license (CC BY 4.0 - https://creativecommons.org/licenses/by/4.0/**) and are therefore allowed to be re-printed. This information has been uploaded in the resubmission and all figures that use previously published data cite the original source appropriately.**

3. Figure 2: Please include a space between numbers and their units of the scale bar (i.e., 100 µm). Please use colored arrows for contrast.

These changes have been made to the figure.

4. Figures 4 and 5: Please shorten the figure legends. 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.

Figure legends have been shortened and text moved to the appropriate sections.

5. Please revise the title to be more concise.

The title has been changed to "Methods for reproducible microinjection of dsRNA and oviposition bioassay in mosquitoes and house flies".

6. Please provide an email address for each author.

Neil D. Sanscrainte - neil.sanscrainte@ars.usda.gov
Christy M. Waits - christy.waits@ars.usda.gov
Christopher J. Geden - chris.geden@ars.usda.gov
Alden S. Estep - alden.estep@ars.usda.gov
James J. Becnel - james.becnel@ars.usda.gov

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

These changes have been made to the text.

8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Kimwipe, Calf Manna, etc.

These changes have been made to the text.

9. 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. Please move the discussion about the protocol to the Discussion.

These changes have been made to the text.

10. 3.2.4: Please specify the size of the small slit. How many females are transferred into one cup?

These changes have been made to the text.

11. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

These changes have been made to the text.

12. 4.1: Please break up into sub-steps.

This step has been altered and now conforms to the format.

13. 4.3.4: There is no step 6.3.3. Should it be 4.3.3?

This has been corrected.

14. 4.3.6: There are not steps 6.3.5 and 6.3.2. Should them be 4.3.5 and 4.3.2?

This has been corrected.

15. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies 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.

This has been done to the manuscript.

16. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

This has been done to the manuscript.

17. 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.

This has been done to the manuscript.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The article describes a method for the injection adults with dsRNA, for the purpose of knocking down expression

Major Concerns:

Nothing major.

Minor Concerns:

The nature of the hole for transfer of injected adults between cups would have been useful.

This change has been made to the text.

I have tried to attach an annotated PDF with comments and sections highlighted where i think small improvements and clarifications could be made - there was no facility to do so I have cut and pasted the comments from my PDF file below:

Page: 3
line 67 - could do with a bit of expansion - I doubt these applications will be familiar to most, and certainly not how this technique feeds into those

These changes have been made to the text.

line 80 'exposure to CO2' is vague, how long? what dose? In my experience excessive CO2 exposure itself can cause a reduction in egg output, at least when given to smei-gravid/gravid females.

A change has been made to the text. In this procedure, only non-gravid mosquitoes and flies are knocked down with CO2.

Page: 4
line 101 this needs more detail - make or type of puller at a bare minimum. Can it be made more generic for use with other types of puller? What are the desirable physical characteristics of the pulled needle -OK, scap this comment, i've now seen the table at the end. Maybe just refer here in the first instanc eto the presnce of the table describing all equipment

This change has been made to the text.

what does this mean? meniscus movement of the liquid in needle?

Yes, meniscus movement of the liquid in the needle. This has been clarified in the text.

Page: 6
lines 231 and 232 these should say 4.3.5 and 4.3.2 (not 6.3.5 etc.)

This has been corrected.

Reviewer #2:
Manuscript Summary:
In the article titled "Enhanced methods for dsRNA delivery by microinjection: measuring effects of gene silencing on survival and oviposition of Aedes mosquitoes and house flies," Sanscrainte and colleagues provide a detailed protocol for injecting dsRNA into the hemolymph of Aedes aegypti mosquitoes and Musca domestica flies, as well as methods for oviposition assays to assess effectiveness when using RNAi targeting genes that influence ovarian development. Although other articles published in JoVE have presented microinjection protocols, this topic is worthy of additional detailed methodology in the literature given the difficulty of protocol transfer between labs. Moreover, I believe this is the first JoVE protocol to include microinjection of house flies and the first to describe on oviposition bioassay, which adds novelty.

In general, the manuscript was well-written and free of grammatical errors, and the authors did a nice job explaining important details that are often overlooked. Nevertheless, I recommend that the authors make several changes that would make the paper stronger and more useful to its target audience. I outline my recommendations below by section.

Major Concerns:
PROTOCOL
*Lines 96-127: In steps 2.1-2.4.3 of the protocol, there is no information about dsRNA preparation or quantification. These steps are critical to successful RNAi and should be described in sufficient detail to

complete an experiment. Otherwise the authors' claims in the abstract that they are providing a "quantitative microinjection protocol that ensures accurate delivery of specific doses of dsRNA…" are misleading.

The design and production of dsRNA for microinjection into dipterans has been detailed in [Estep et al.][5] and [Sanscrainte et al.][9]. References to this as well as a description of how to quantify dsRNA have been added as 2.1.

Minor Concerns:

ABSTRACT

*Line 47: the word "most" should be removed. The authors identify effective locations for delivery, but did not compare injection sites, so there is no data supporting that the injection sites are the "most effective" ones.

This change has been made to the text.

INTRODUCTION

*Lines 53-55: In the sentence starting with "Oral uptake of dsRNAs…" information on the genes targeted in the cited studies would be appreciated. In line 55 please edit the last part of the sentence to say "…after feeding dsRNA to adult Ae. aegypti in sugar meals." When I first read the sentence, I interpreted it to mean that the phenotypic effects of RNAi in larvae vanished in the adult after sugar feeding.

These changes have been made to the text.

*Line 69: Please briefly define "biorationals." Some readers will be unfamiliar with the term.

These changes have been made to the text.

PROTOCOL

*Lines 96-127: When describing needle/solution preparation the authors do not mention backfilling their needle. If their system does not require that step, perhaps a sentence or two addressing backfilling is warranted since some commonly used microinjectors have unusable dead space between the plunger and the injection solution that must be filled with oil (which won't mix with the injection solution).

This change has been made to the text.

*Lines 113-114: Recommendations or examples of "desired dose" with citations would be worthwhile, particularly for labs wanting to attempt the protocol for the first time.

This change has been made to the text.

*Line 133: The word "mosquitos" should be "mosquitoes."

This change has been made to the text.

*Line 135: The placement of "(Fig 2A)" should be earlier in the sentence after "mesokatepisternum." In its current location, I expected to see an image of the needle tip puncturing the cuticle. Similarly, "(Fig 2B)" should be moved to follow "mesopleuron."

These changes have been made to the text.

*Line 139-140: I just wanted to make sure that I understood this correctly. Are the authors saying to insert the tip of the needle through the midline of the insect? That seems deep. In my experience, shallower injections in mosquitoes lead to better survival. However, I noticed below that the authors typically get > 97% survival, which is impressive. Do the authors see variation in survival depending on species of mosquito injected and or depth of injection? Perhaps a sentence or two about injection depth are warranted, particularly if the authors find that deeper injections improve knockdown of expression.

The reviewer is correct as to the depth of injection; shallow injections frequently result in the

injected solution beading back out of the insect. A note has been added to the method to ensure that the importance of depth of injection is understood.

*Lines 143-144: Do the authors find that for mosquitoes multiple injections of lower concentration dsRNA get better results than single injections of more highly concentrated dsRNA? If so that would be useful information.

The use of multiple pulses (during a single injection event) is simply to deliver a desired dose based on our starting concentration. We have no data as to the effect of further concentrating the dsRNA and reducing the net injection volume, but no reason to suspect it would not be as effective.

*Line 153: After the word "tulle" please add "or netting." Some readers will have no clue what tulle is.

This change has been made to the text.

*Lines 153-155: For the sentence beginning with "After mosquitoes and flies…", is the cup inverted for a reason rather than the cotton ball being placed on top of the tulle? An image would be helpful to understand what the authors mean.

The inversion of the cup on top of the sucrose cotton attempts to aid in survival by providing easier access to a sugar meal (see Isoe et al. 2011). A note has been added to the method to make this clear.

*Line 206: Is this supposed to say black cloth instead of "black cotton?"

This has been corrected.

*Line 209: An image at the end of the sentence would be appreciated.

A new image has been added and called out as suggested.

*Line 225: The sentence should read "Repeat step 4.3.3…" Also in that line, the authors should specify quantitatively what they mean by "severe outlier."

This change has been made to the text.

*Line 231: The sentence should read "…value obtained in step 4.3."

This change has been made to the text.

DISCUSSION

*Lines 312-313: The sentence beginning with "Delivered volume…" should be edited to say "Delivered volume and concentration are critical parameters that allow …"

This change has been made to the text.

United States Department of Agriculture

Agricultural Research Service

South Atlantic Area
Center for Medical, Agricultural and Veterinary Entomology

1600/1700 SW 23rd Drive
Gainesville, FL 32608

July 19, 2018

Dear Editor,

Figures from the Estep et al. 2016 publication "DsRNA-mediated targeting of ribosomal transcripts RPS6 and RPL26 induces long-lasting and significant reductions in fecundity of the vector *Aedes aegypti*. *Journal of Insect Physiology*. 90, 17-26" and the Sanscrainte et al. 2018 publication "Reduction in *Musca domestica* fecundity by dsRNA-mediated gene knockdown. *PloS one*. 13 (1), e0187353" are published under a creative commons license (CC BY 4.0 - https://creativecommons.org/licenses/by/4.0/) and are therefore allowed to be modified and re-printed.

Regards,

Neil Sanscrainte
USDA, ARS, CMAVE
Mosquito and Fly Research Unit
1700 SW 23rd Dr.
Gainesville, FL 32608
(352) 374-5965
neil.sanscrainte@ars.usda.gov