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Effective Oral RNA Interference (RNAi) Administration to Adult *Anopheles gambiae* Mosquitoes

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Abstract

RNA interference has been a heavily utilized tool for reverse genetic analysis for two decades. In adult mosquitoes, double-stranded RNA (dsRNA) administration has been accomplished primarily *via* injection, which requires significant time and is not suitable for field applications. To overcome these limitations, here we present a more efficient method for robust activation of RNAi by oral delivery of dsRNA to adult *Anopheles gambiae*. Long dsRNAs were produced in *Escherichia coli* strain HT115 (DE3), and a concentrated suspension of heat-killed dsRNA-containing bacteria in 10% sucrose was offered on cotton balls *ad-libitum* to adult mosquitoes. Cotton balls were replaced every 2 days for the duration of the treatment. Use of this method to target *doublesex* (a gene involved in sex differentiation) or *fork head* (which encodes a salivary gland transcription factor) resulted in reduced target gene expression and/or protein immunofluorescence signal, as measured by quantitative Real-Time PCR (qRT-PCR) or fluorescence confocal microscopy, respectively. Defects in salivary gland morphology were also observed. This highly flexible, user-friendly, low-cost, time-efficient method of dsRNA delivery could be broadly applicable to target genes important for insect vector physiology and beyond.

Introduction

Many diseases are transmitted by mosquitoes, making the study of mosquito physiology and genetics an important undertaking. The use of RNAi in these organisms has been prominent in the last 20 years and has allowed for the functional characterization of many mosquito genes[1,2,3,4,5]. The most commonly used technique for dsRNA delivery has been microinjection, which has the drawbacks that it can injure the mosquitoes and requires significant time and effort.

Oral delivery methods for RNAi have been tested, but mainly in the larval stage of the mosquitoes[6,7,8,9]. Oral delivery of dsRNA in adult mosquitoes has not been fully explored and could be a useful tool for the study of vector biology and vector control.

Malaria is transmitted by *Anopheles* mosquitoes when an infected female mosquito takes a blood meal from an uninfected host and injects saliva containing malarial parasites[10]. To ultimately be transmitted in the saliva of a mosquito, the parasite must overcome many hurdles, including evading the mosquito immune system, traversal of the midgut barrier, and invasion of the salivary glands[11]. Mosquito salivary gland (SG) architecture is key to parasite invasion and that architecture is controlled both by key salivary gland-expressed transcription factors as well as determinants of sexual dimorphism. Several highly conserved transcription factors are required for cellular specification and homeostatic maintenance of the salivary glands and for the production and secretion of salivary proteins that function in blood-feeding[12,13,14]. Fork head (Fkh) is a winged helix transcription factor that functions as a major regulator of insect SG structure and function (based on studies in fruit flies and the silkworm moth)[15,16,17,18,19,20]. In the *Drosophila* SGs, Fkh functions with Sage, an SG-specific basic helix-loop-helix (bHLH) transcription factor, to promote SG survival and saliva production[19]. An important, positive co-regulator of saliva production in *Drosophila* is CrebA, a well-studied leucine zipper transcription factor that upregulates the expression of secretory pathway genes[21,22,23]. There is also a strong degree of morphological differentiation in female salivary glands that likely plays a key role, not only in blood-feeding but also in the ability of parasites to invade this tissue[24].

Many of the genes involved in determining salivary gland survival, structure, physiology, and sexual dimorphism have complex spatiotemporal expression profiles[25,26,27], and the traditional delivery methods of dsRNA to induce RNAi are not always efficient at targeting these kinds of genes in this or other tissues. However, oral delivery of dsRNA in the larval stage *Aedes aegypti* and *An. gambiae* mosquitoes has been used successfully to silence the female-specific form of the *dsx* gene[9,28]. Previous studies using dsRNA in mosquito salivary glands found that, although large amounts of dsRNA were required, the silencing effect was relatively long-lasting (at least 13 days)[29]. Here, the ability of heat-killed *E. coli* strain HT115 (DE3) expressing sequence-specific dsRNA for *dsx*, *fkh,* or *CrebA* to induce RNAi silencing of these genes in adult female mosquitoes was tested. Oral administration of dsRNA induced gene knockdown in *An. gambiae*, with clear reductions in mRNA levels and with phenotypes consistent with the loss-of-function of these genes. Thus, this approach will likely work to knock down the function of a variety of salivary gland genes.

Protocol

1. Cloning dsRNA into *E. coli* expression vector

1. Select the target gene sequence to insert into an appropriate vector for the expression of dsRNA. Retrieve the expression values from Vectorbase.org using the following method.
    1. Search for a gene of interest (e.g., **Table 1**) on the homepage search box.
    2. In the resulting gene page, navigate to the **8. Transcriptomics** section.
    3. Look for the listed relevant RNA-seq and microarray gene expression experiments.

4. Transcribe values of interest into the spreadsheet software and create a data table.

2. Select a commercially available plasmid with at least one T7 promoter to be used. If the selected plasmid has only one T7 promoter (as most commercial plasmids do), include a second T7 promoter in the reverse primer to be used for the amplification of the dsDNA for the gene of interest.

   NOTE: The dsRNA sequence for the target genes can be selected using the web application E-RNAi for the design of RNAi reagents[30]. Either long dsRNA (approximately 400 bp) or short-hairpin dsRNA (shRNA) can be designed based on specific gene sequences. These sequences should be amplified and sequenced for identity confirmation before cloning. The selected gene regions, plasmids, and promoters used in this study are listed in **Supplementary File 1**.

3. Perform cloning according to a simple one-step procedure described previously[9,31]. For this purpose, purify the PCR product and ligate to the linearized plasmid DNA. Use the product of the ligation for the heat-shock transformation of competent *E. coli* cells[32]. Select the transformed cells through blue/white screening. Confirm the orientation of the insert using a T7-primer PCR and confirm the sequence using M13 primers.

   NOTE: White/blue screenings can be used when the plasmid selected for transformation carries the lacZ gene that codes for β-galactosidase. White colonies should contain the desired insert within the lacZ and can be selected to further confirm the presence and orientation of the target sequence[33].

4. Purify the plasmid from the first transformation and use it to transform competent *E. coli* HT115 (DE3) as previously described[34]. After confirmation that the plasmid with the insert is present in the competent *E. coli* HT115 (DE3), make glycerol stocks of bacteria for single use.

   NOTE: An appropriate non-related control dsRNA should be acquired or prepared to use in every experiment. In this case, the sequence for the unrelated gene *aintegumenta (ant)* from *Arabidopsis thaliana* is used.

2. Preparation of heat-killed bacteria expressing dsRNA

1. Grow a culture from a single bacterial colony of *E. coli* strain HT115 (DE3) containing the dsRNA expressing plasmid in 50 mL of Luria Broth (LB) containing 100 µg/mL of ampicillin and 12.5 µg/mL of tetracycline, on a platform shaker (180 rpm) at 37 °C for 12 h.

2. Dilute the bacterial culture (1:1000) into 2x Yeast Tryptone (2x YT) media containing 100 µg/mL of ampicillin and 12.5 µg/mL of tetracycline.

3. Induce dsRNA production by adding 40 µM (final concentration) isopropyl β-D-1-thiogalactopyranoside (IPTG).

4. When the cells reach an $O.D._{600}$ = 0.4, approximately after 2 h of induction at 37 °C with agitation at 180 rpm, prepare a concentrated suspension of heat-killed bacteria as described by Taracena et al [9]. Pellet the cells by centrifugation (4000 x *g*, 4 °C, 10 min) and wash cells in one volume of sodium phosphate buffer (PBS).

5. Spin again under the same conditions, re-suspend in PBS to 1/100 of the initial volume, and place at 70 °C for 1 h.

6. Make 400 µL aliquots of the heat-killed bacteria and store these aliquots at -20 °C until further use (do not

store for more than a week). This suspension of heat-killed bacteria contains the specific dsRNA for the RNAi experiments. Carry out this procedure both for the target-gene dsRNA-bacteria and for the un-related dsRNA-control to be used in each experiment.

3. Feeding mosquitoes with heat-killed bacteria expressing dsRNA

1. Defrost one aliquot of dsRNA (HT115 (DE3) bacteria suspension) and mix with 1.6 mL of 12% sugar solution containing 0.2% methylparaben.
2. Soak a small cotton ball in this solution and place the soaked cotton ball inside a cage containing 5-day-old mosquitoes. Ensure that the mosquitoes feed on this solution, picking up both the sugar and the dsRNA-containing bacteria simultaneously.
3. Change the cotton ball soaked in dsRNA-sugar solution every other day for 8 consecutive days.
4. Keep mosquito cages under constant conditions, i.e., 27 °C and 80% relative humidity with a photoperiod of 12 h:12 h light: dark photocycle, separated by a 30 min dawn and 30 min dusk period.

4. Assay target gene expression levels

1. Cold-anesthetize the mosquitoes by placing the container on ice for a min or until the mosquitoes stop moving. Once the mosquitoes are anesthetized, place them on a cold surface to isolate females for dissection.
2. Spray 70% ethanol to the mosquitoes and place them on a glass surface with PBS. With a pair of forceps, secure the mosquito head steady and pull the thorax very slowly, allowing the salivary glands to be released into the PBS.
3. Keep the salivary glands in ice-cold PBS until 10 individuals have been dissected. Pool Ten SGs for RNA extraction using the guanidinium thiocyanate-phenol-chloroform method. Suspend the RNA pellet in 30 µL of RNase-free water.
4. Use 1 µL aliquot of the RNA extracted from the SG in the previous step, to read absorbance at 260 and 280 nm and calculate the RNA concentration of each sample by multiplying with the dilution factor. A 260/280 ratio of ~2.0 indicates good quality RNA.
5. Use 1 µg of the purified RNA to synthesize complementary DNA (cDNA) using a commercial reverse transcription kit.
6. Make a 1:10 dilution of the cDNA to prepare an RT-PCR reaction according to the manufacturer's recommendations. For each sample, prepare a reaction for the target gene and in parallel, set up a reaction with the housekeeping (HK) gene. Set each gene reaction in a technical triplicate to eliminate the impact of random variation from the method.
   NOTE: Here, the *An. gambiae* ribosomal *S7* gene (GeneBank: L20837.1) and *actin* (VectorBase: AGAP000651) have been used as HK genes.
7. Use all primers at a final concentration of 300 nM, following the SYBR-green manufacturer's indications. Amplify with standard PCR conditions: 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C.
   NOTE: To quantify gene expression, the delta-delta-Ct method (ΔΔCt) is used. Delta Ct (ΔCt) is the difference between the Ct of the target gene and the Ct of the housekeeping gene. ΔΔCt is the difference between the ΔCt of the experimental group and the ΔCt of the control group[35].

5. Phenotypic evaluation: successful blood-feeding

1. To evaluate the ability to blood-feed, set groups of 15 female mosquitoes treated with target and control dsRNA on small cages (12 cm diameter) and starve them for 4 h.
2. Using a circulating water bath set to 37 °C, glass mosquito feeders (24 mm diameter) and parafilm membrane, offer defibrinated sheep blood to the mosquitoes.

   NOTE: Blood can be acquired from a commercial vendor who aseptically draws it from healthy, donor animals of U.S. origin and manually defibrinates without anticoagulants or additives.
3. By direct observation, count and record the number of probing attempts to successfully acquire a blood meal from the first five females to become fully engorged in each group.

   NOTE: To avoid significant metabolic changes in the mosquitoes, that could interfere with energy resources impacting blood-seeking behavior, starvation was kept to the minimum (4 h). As a result, not every mosquito would avidly seek the blood-meal and we limited the count of the engorged females to five (a third of each group's total), to reduce the effect of time variables such as exposure to human odor, temperature change between the chambers and the feeding surfaces, etc.

6. Phenotypic evaluation: Salivary gland morphology and down-regulation of relevant proteins

1. Isolate fresh tissue in 1x Phosphate-buffered saline (PBS) as described in step 4.2 and fix in ice-cold acetone for 90 s. Rinse several times in 1x PBS after removing the acetone. Incubate with primary antibodies overnight at 4 °C with antiserum (see **Table of Materials**) diluted into 1x PBS.

   NOTE: See **Table of Materials** for identification of the primary antibodies used for saliva proteins (*Anopheles* anti-platelet protein, AAPP; Mucin 2, MUC2), SG transcription factors (Fork Head, fkh; Sage, sage; Cyclic-AMP response element-binding protein A, CrebA), and a marker of secretory vesicles (Rab11). These antibodies are used as readouts for SG form and function. However, any antibody suitable for immunofluorescence should be suitable for this protocol.
2. Wash in 1x PBS several times. Add secondary antibodies (fluorescent) diluted in 1x PBS, and incubate in the dark at room temperature for 2 h. Add any counterstain [such as 4′,6-diamidino-2-phenylindole (DAPI; DNA), wheat germ agglutinin (WGA; for chitin), phalloidin (for F-actin), and/or Nile Red (for lipids)] 30 min before the end of the 2 h incubation.
3. Wash three times in 1x PBS. Then, mount the tissues in 100% glycerol on a standard microscope slide with a 1 mm thick coverslip and store at -20 °C until imaging using a fluorescence confocal microscope.

   NOTE: To obtain quantitative data, imaging settings must be held constant. Here, only maximum intensity projection images through the entire 3D volume of the tissue were included, and all image quantification was normalized between treatments (within an experiment) based on DAPI signal in non-SG tissue remnants (fat body, cuticle, or head) also present on the slide.

Representative Results

To begin, microarray expression data from VectorBase was used to scan potential targets across developmental

stages[36,37] to determine the expression status of all genes relevant to the current study (**Table 1**). As expected, all our chosen target genes showed expression in adult SGs. Levels of *aapp* and *sage* were particularly high (**Table 1**). Also of note were the high levels of expression of *f-Agdsx* in adult female SGs[9].

Specific segments from each gene were evaluated for use as dsRNA using the web application *E-RNAi for the design of RNAi reagents*[30]. The ~400 bp regions containing sequences unique to each target gene were then cloned (**Figure 1A**), transformed into the appropriate bacterial strains, and used to prepare suspensions of heat-killed bacteria, which were induced to produce dsRNA. Adult mosquitoes were fed for 8 days on the sucrose-soaked cotton balls containing the bacterial suspensions of dsRNA for *f-Agdsx,fkh*, or *ant* (the unrelated negative control).

For the analysis of RNAi feeding of female mosquitos, it was first determined whether *f-Agdsx* or *fkh* dsRNA-feedings induced gene silencing. A 98.8% reduction (±2.1) in *fkh* transcript levels was observed in the group fed with *fkh*-dsRNA (**Figure 1B**), indicating that the dsRNA very effectively reduced the abundance of *fkh* transcripts in SGs. Surprisingly, *fkh* mRNA levels were reduced by 82.0% (±18.9) in the mosquitoes treated with dsRNA for *f-Agdsx,* which had an 89.86% (±4.48) of *f-Agdsx* reduction, suggesting that *fkh* could be a target of F-Dsx in the salivary gland. Concomitant with the significant reduction in *fkh* expression levels, the *fkh*-knockdown mosquitoes exhibited a significant increase in the number of probing attempts needed to blood-feed. These mosquitoes exhibited, on average, five times more feeding attempts than the control group or *f-Agdsx* dsRNA fed mosquitoes to be completely engorged with blood (**Figure 1C**). This led to asking whether the *fkh* knockdown RNAi treatments caused changes in localization and/or distribution of key transcriptional regulators (SG TFs Sage and CrebA) (**Figure 2**), secreted proteins (AAPP and mucin) (**Figure 3**), and secretory machinery [Nile Red (lipids) and Rab11 (secretory vesicles)] (**Figure 4**). Importantly, substantial differences in staining intensity were observed across different lobe regions, lobes, and individual SGs.

As predicted, levels of *sage* and *CrebA* staining were markedly reduced in all SG lobes following *fkh* RNAi (**Figure 2B**) compared to *ant* control RNAi (**Figure 2A**). Reductions in both the highest maximum intensity values (red dashed lines and numeric labels) and lowest maximum intensity values (blue dashed lines and numeric labels) in line scan profiles suggested reductions in areas of both high and low signal within the tissue (**Figures 2A,B**). These data suggest that *An. gambiae fkh* RNAi is effective and that *fkh* regulates the production and/or stability of the SG TFs *Sage* and *CrebA* in *An. gambiae*, analogous to their genetic relationship in *Drosophila* SGs[19,38,39].

When considering highly abundant saliva-component proteins, levels of *Anopheles* anti-platelet protein (AAPP)[40,41] were reduced in all three SG lobes following *fkh* RNAi, compared to control RNAi treatment (**Figure 3A,B**; green). On the other hand, no changes in levels of Mucin were observed (**Figure 3A,B**; purple). These data suggest that Fkh contributes differently to the expression of different saliva protein genes.

Finally, two markers of secretion were observed (**Figures 4A,B**): Rab11 (vesicles associated with apical recycling endosomes)[42] and Nile Red (lipids). Reduced Rab11 fluorescence was observed in distal lateral (DL) lobes following *fkh* RNAi treatment (**Figure 4A** v vs. **4B v**; green). However, increased Rab11 signal in the medial (M) and

proximal lateral (PL) lobes (**Figure 4A vii, ix** vs. **4B vii, ix**; green) also occurred. No discernible difference was observed in Nile Red signal (**Figures 4A,B**; purple) after *fkh* RNAi compared to the control RNAi treatment. These data suggest that *fkh* reduction may alter some secretory machinery action in a complex manner that differs between SG lobes.

		Dataset:	*Goltsev*	*Neira Oviedo*	*Neira Oviedo*	*Baker*	*Baker*	*Baker*	*Baker*
gene symbol	**function**	**AGAP ID**	**embryo (25 hr.)**	**L3 larvae**	**L3 SG**	**adult female body (3 day)**	**adult male body (3 day)**	**adult female SG (3 day)**	**adult male SG (3 day)**
AAPP	saliva protein	AGAP009974	3.92	4.38	4.33	3.81	2.46	11.92	2.69
CrebA	txn factor	AGAP001464	6.28	5.22	5.92	2.99	2.96	3.27	3.13
"	txn factor	AGAP011038	4.50	4.46	5.23	2.96	2.86	3.05	2.88
dsx	txn factor	AGAP004050	4.91	5.39	5.55	3.72	4.00	4.57	4.01
fkh	txn factor	AGAP001671	5.18	4.67	5.25	2.99	3.09	3.21	3.05
MUC2	saliva protein	AGAP012020	4.59	5.53	5.63	2.96	3.07	3.08	3.26
Rab11	vesicular trafficking	AGAP004559	10.21	7.47	8.60	4.90	3.79	3.38	2.96
sage	txn factor	AGAP013335	5.32	5.96	8.89	3.40	3.33	7.37	7.23

Table 1: Mean log2 microarray expression profiles for *An. gambiae* genes of interest. Shown are gene names, functional category, Vectorbase (AGAP) identifiers, and mean log2 microarray expression data gathered from Vectorbase. These data indicate that our genes of interest (involved in salivary gland (SG) cell biology and secretion) are expressed and enriched in larval stage 3 (L3) and adult SGs, as compared to whole individuals.

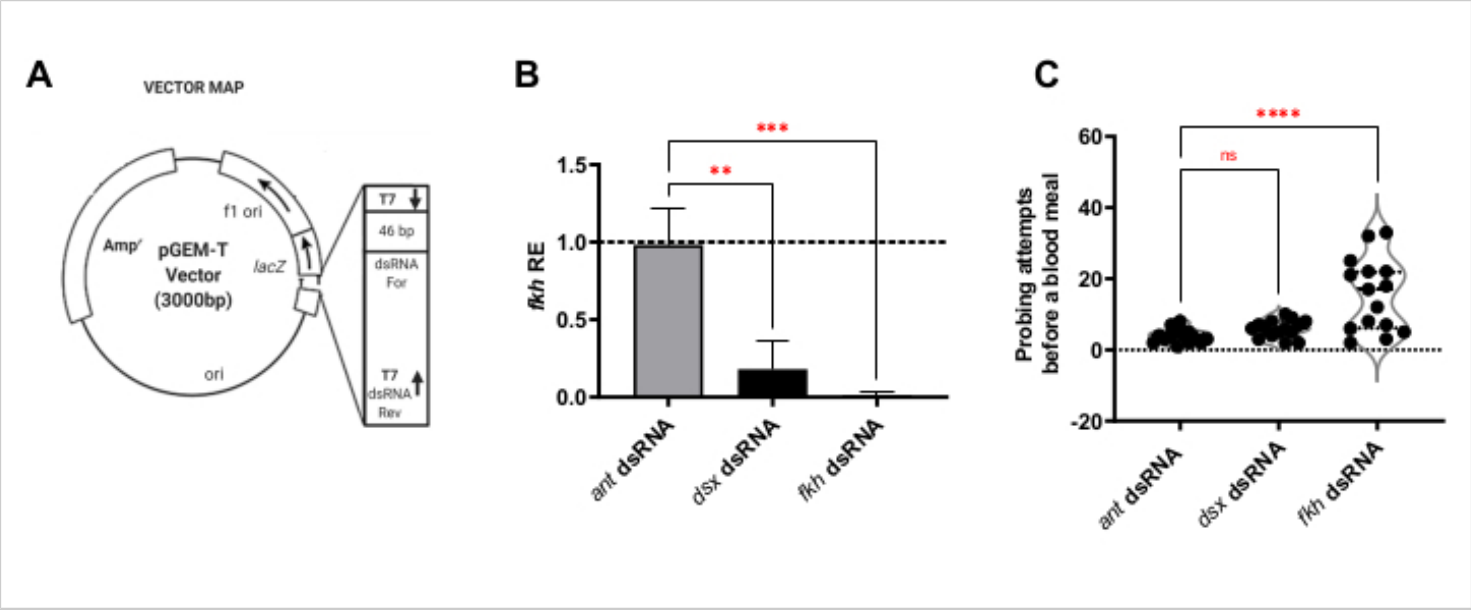

Figure 1: *f-Agdsx* and *fkh* knockdown in adult *An. gambiae* reduces *fkh* mRNA levels in the SGs and affects the female ability to blood-feed. (**A**) Representative image of the plasmid design utilized for dsRNA production in this methodology. The second T7 promoter sequence is added to the plasmid by including it in the 3' primer used to amplify the insert to be cloned into the pGEMT plasmid. The plasmid is then transformed into *E. coli* HT115 (DE3) bacteria and a feeding solution is made of a suspension of induced heat-killed bacteria in 10% sugar water. (**B**) Animals fed with a dsRNA feeding solution for either *f-Agdsx* or *fkh,* showed significantly lower levels of *fkh* transcripts (one-way ANOVA with multiple comparisons; n=15). However, only the group fed with *fkh* dsRNA (**C**) showed a significant difference in the number of biting attempts needed to acquire a blood meal. Mosquitoes in this group needed, on average, five times the number of probing attempts to obtain a successful blood meal than needed by the control or the *dsx*-dsRNA fed groups (one-way ANOVA with multiple comparisons; n=15). Error bars indicate the Standard Error of the Mean (SEM). Each experiment was conducted in three separate biological replicates. Please click here to view a larger version of this figure.

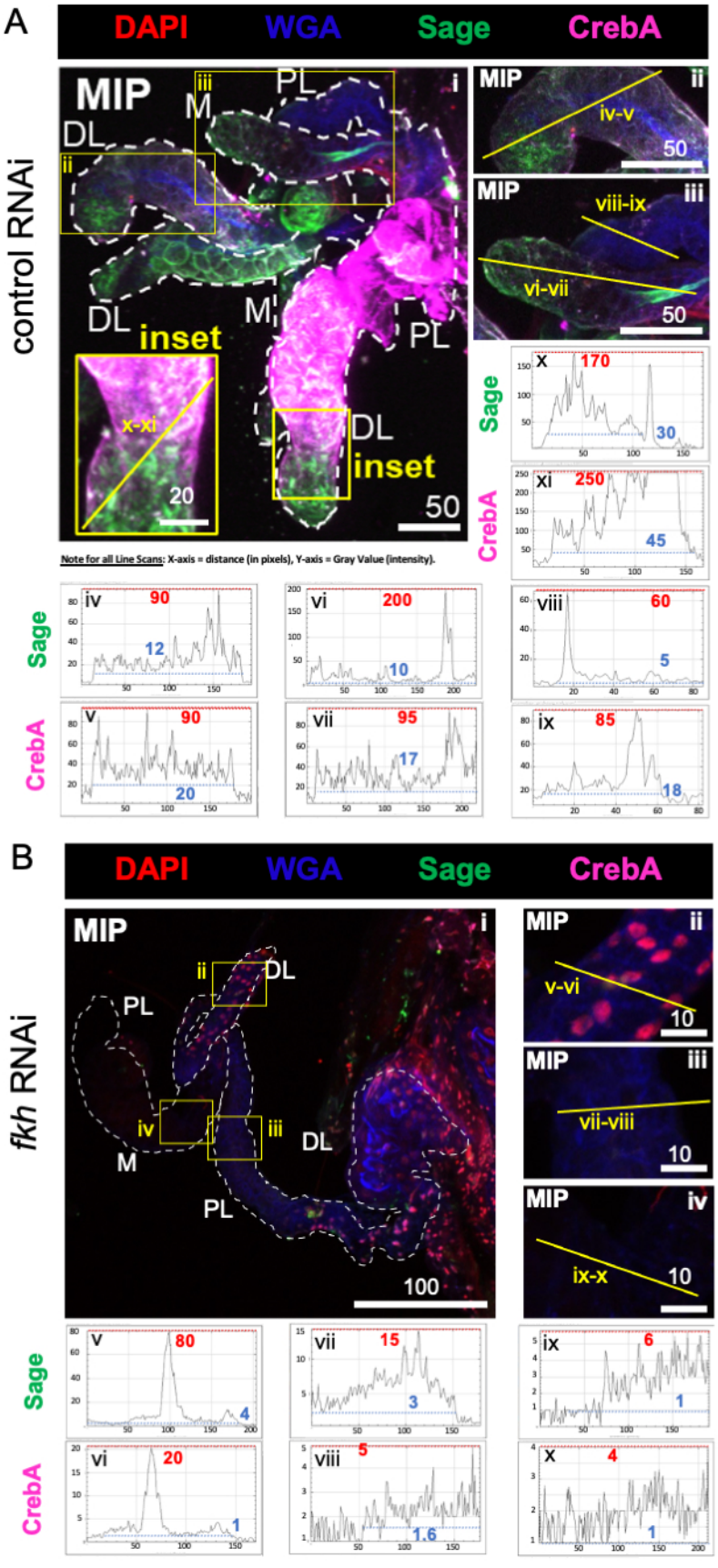

Figure 2: *fkh* knockdown in adult *An. gambiae* salivary glands reduces SG transcription factor levels. Shown are representative images from day 13 adult female *An. gambiae* SGs after 8 days (days 5-13) of oral exposure to either (**A**) non-related dsRNA control (*ant*) or (**B**) dsRNA targeting the SG TF *fork head* (*fkh*, AGAP001671) in 10% sucrose stained with the dyes DAPI (DNA; red), labeled wheat germ agglutinin (WGA, chitin/ O-GlcNAcylation; blue), antisera against the SG TFs Sage (green) and CrebA (purple). Scale bar lengths shown are microns. SGs (i) are outlined with white dashes. Yellow lines in zoomed lobe images (of the regions enclosed by yellow boxes, and labeled "inset") indicate where the line scans of signal intensity were conducted. Green and purple channel intensities corresponding to line scans for each zoomed lobe are plotted (always from left to right in the SG) in the graphs below the images; X-axis = distance (in pixels) and Y-axis = gray unit (pixel intensity). The pixel intensity's dynamic range is delimited by red (maximum) and blue (minimum) dotted lines and the corresponding values are shown on each graph. MIP = maximum intensity 3D projection through the entire SG depth. DL: distal lateral lobe; M: medial lobe; PL: proximal lateral lobe; SD: salivary duct. Please click here to view a larger version of this figure.

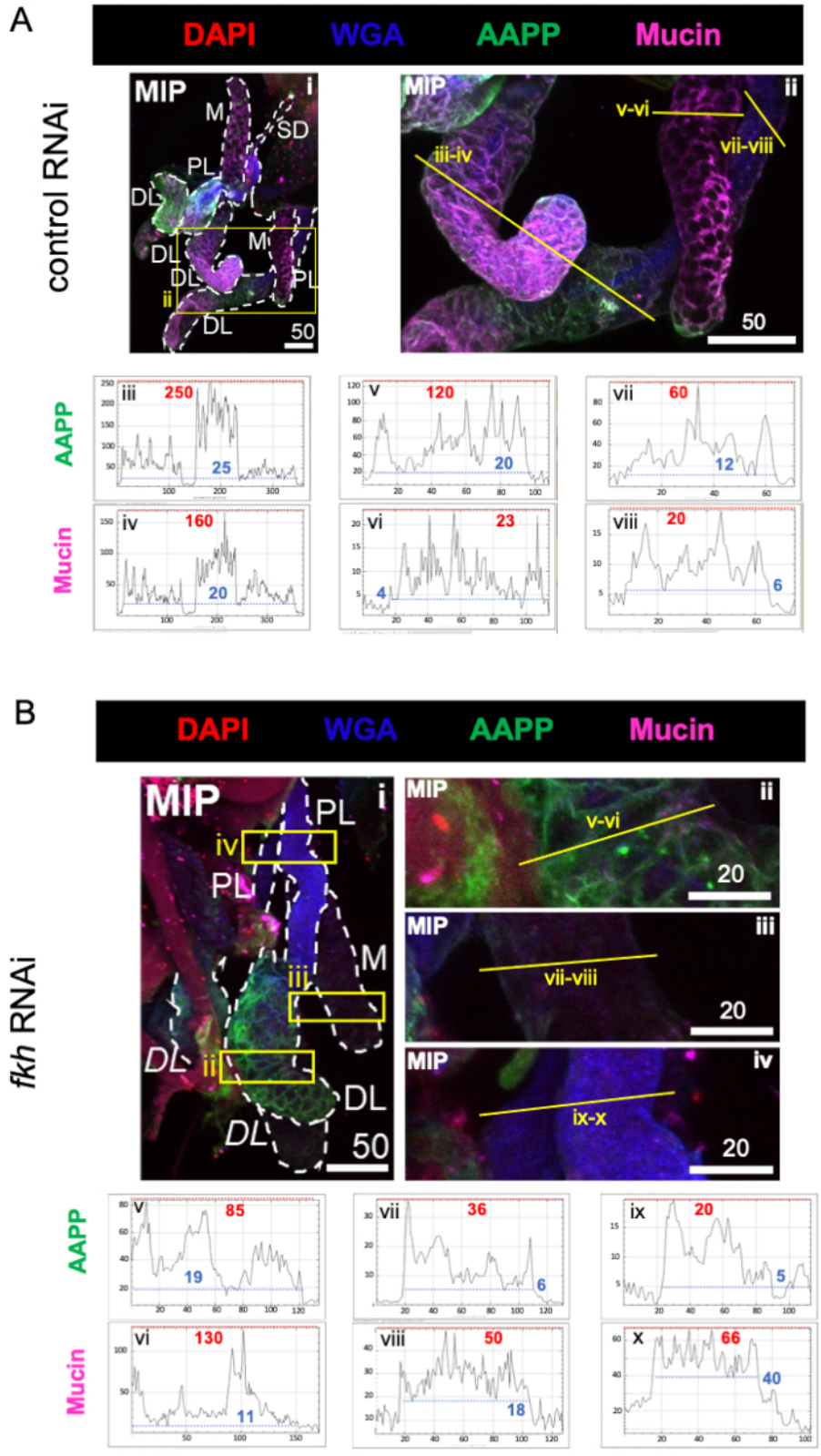

Figure 3: *fkh* knockdown in adult *An. gambiae* salivary glands reduces SG secreted protein levels. Shown are representative images from day 13 adult female *An. gambiae* SGs after 8 days (days 5-13) of oral exposure to either (**A**) non-related dsRNA control (*ant*), or (**B**) dsRNA targeting the SG TF *fork head* (*fkh*,AGAP001671) in 10% sucrose stained with the dyes DAPI (DNA; red), labeled wheat germ agglutinin (WGA, chitin/ O-GlcNAcylation; blue), and the saliva proteins AAPP (green) and Mucin (MUC2, purple). Scale bar lengths shown are microns. SGs (i) are outlined with white dashes. Yellow lines in zoomed lobe images (of the regions enclosed by yellow boxes) indicate where the line scans of signal intensity were conducted. Green and purple channel intensities corresponding to line scans for each lobe are plotted (always from left to right in the SG) in the graphs below the images; X-axis = distance (in pixels) and Y-axis = gray unit (pixel intensity). The pixel intensity's dynamic range is delimited by red (maximum) and blue (minimum) dashed lines and the corresponding values are shown on each graph. MIP = maximum intensity 3D projection through the entire SG depth. DL: distal lateral lobe; M: medial lobe; PL: proximal lateral lobe; SD: salivary duct. Italic "*DL*" labels (Bi) indicate two visible regions of the same DL lobe. Please click here to view a larger version of this figure.

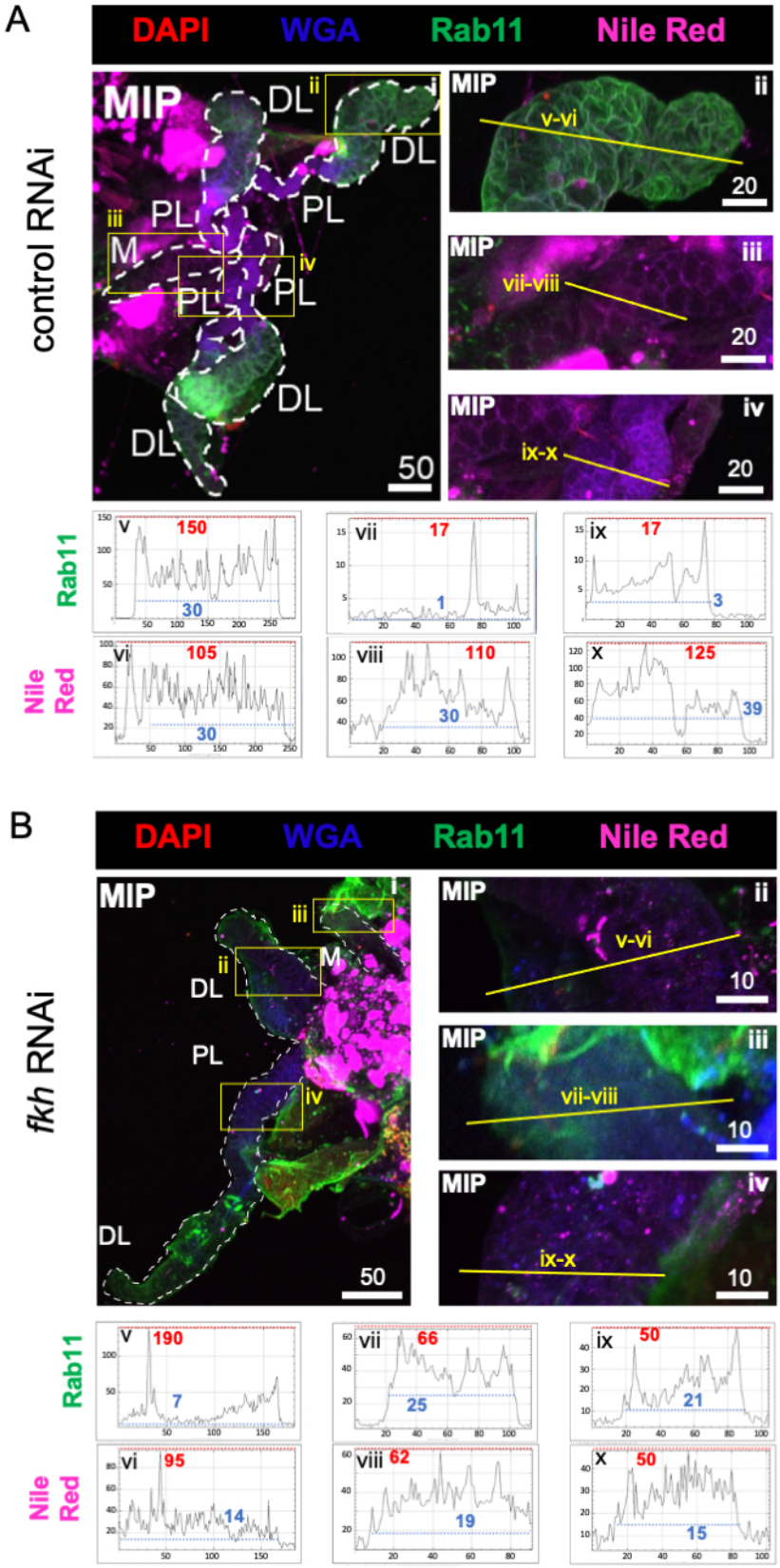

Figure 4: *fkh* knockdown in adult *An. gambiae* salivary glands reduces SG secretion markers. Shown are representative images from day 13 adult female *An. gambiae* SGs after 8 days (days 5-13) of oral exposure to either (**A**) non-related dsRNA control (*ant*), or (**B**) dsRNA targeting the SG TF *fork head* (*fkh*, AGAP001671) in 10% sucrose stained with the dyes DAPI (DNA; red), labeled wheat germ agglutinin (WGA, chitin/ O-GlcNAcylation; blue), Nile Red (lipids; purple), and antisera against the recycling endosome vesicle marker Rab11 (green). Scale bar lengths shown are microns. SGs (i) are outlined with white dashes. Yellow lines in zoomed lobe images (of the regions enclosed by yellow boxes) indicate where the line scans of signal intensity were conducted. Green and purple channel intensities corresponding to line scans for each lobe are plotted (always left to right in the SG) in the graphs below the images; X-axis = distance (in pixels) and Y-axis = gray unit (pixel intensity). The pixel intensity's dynamic range is delimited by red (maximum) and blue (minimum) dashed lines and the corresponding values are shown on each graph. MIP = maximum intensity 3D projection through the entire SG depth. DL: distal lateral lobe; M: medial lobe; PL: proximal lateral lobe; SD: salivary duct. Please click here to view a larger version of this figure.

Supplementary File 1. Please click here to download this File.

Discussion

The ability to effectively deliver dsRNA to *An. gambiae* mosquitoes by oral feeding has broad implications for studies of vector biology both in the laboratory and in the field. Microinjection has long been accepted as the preferred mode of delivery of chemicals, antibodies, RNAi, and genetic modification strategies in mosquitoes[43,44]. The consequence of substantial physical manipulation, cellular damage, and stress can be avoided by the use of oral delivery, which could also be potentially suitable for large-scale or field applications. Previous work has suggested that RNAi acts ubiquitously within an individual adult mosquito[29], allowing for effects in all tissues, including salivary glands. By feeding mosquitoes with large numbers of dsRNA-expressing *E. coli* that are digested asynchronously over a long timeframe, one can potentially achieve consistent and uniform exposure to the RNAi across all individuals in a cage. This method allows to feed large numbers of mosquitoes and analyze potential variability of the resulting phenotypes depending on the target gene. However, one important consideration is the possibility of heterogeneous distribution of the bacteria, and hence dsRNA, in the cotton fiber. The 400 µL of bacteria used daily for mosquito sugar-feeding would contain approximately ≤4.6 µg of dsRNA, as described and calculated previously[9] but the amount of dsRNA ingested by each mosquito was not individually determined. If building dsRNA constructs becomes routine, this simple treatment protocol allows for rapid assimilation of this technique by any mosquito researcher. *A priori*, the time expenditure during treatment (30 min per day) is trivial compared to the time taken to learn and apply microinjection to similar sample sizes.

Feeding dsRNA is routinely used for reverse genetics studies in the model organism *Caenorhabditis elegans*[45]. This heavy level of use underscores the value of the oral delivery approach. Construction of an *An. gambiae* genome-wide library in transformed *E. coli*, similar to that which exists in *C. elegans*[46,47], would allow for rapid reverse genetic screening in mosquitoes at an increased scale. However,

it is important to note that the efficiency of the method depends in great measure on the endogenous levels of transcript and if the expression is not limited to the target tissue but expressed more broadly[4,8,44]. Additionally, there is evidence that some insecticides could induce behavioral avoidance from mosquitoes[48], and feeding with bacteria that potentially induce adverse effects in them could trigger similar patterns of avoidance. In the controlled setting of the laboratory, where the mosquitoes did not have an alternative food source, they did not have a choice to avoid the sugar water with *E. coli* and the need for a nutritious source would probably override the instinct to avoid the bacteria. However, this should be considered if the strategy were meant to be used in less controlled settings.

It may be possible to target multiple genes simultaneously (using one construct, multiple constructs, or a mixture of transformed bacterial isolates), but further studies are needed to assess effectiveness. Another important consideration to this point is the evaluation of possible off-target or synergistic effects when using single or multiple targets. The establishment of appropriate control genes and groups is an important part of the experimental design. Further, it is tempting to speculate that this approach could be used to target other pathogens or viruses[49]. Previous work toward RNAi induction in mosquitoes was performed under conditions where the reagent was directly injected, so *E. coli* were not present. The *E. coli* may provide a protective compartment allowing for the slower release of dsRNA over time, ensuring that exposure is more or less continuous over a much longer period[29].

Finally, these results show that the effects of this technique are tunable by adjusting the time frame (length and starting day) of exposure and the quantity of *E. coli* used. This feature allowed us to study the functions of essential genes (*dsx* and *fkh*) by identifying optimal knockdown conditions by trial and error. This greatly enhances the likelihood that target genes of interest can be investigated using this technique.

In summary, it was found that oral delivery of RNAi to adult mosquitoes can be simple, versatile, and a powerful approach to studying mosquito gene function and for the creation of novel and malleable tools for vector control of mosquito-borne diseases.

Disclosures

The authors report that they have no conflicts of interest to disclose.

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the authors and do not necessarily represent the views of the CDC. Use of trade names is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention, the Public Health Service, or the US Department of Health and Human Services.

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