Programmable CRISPR interference for gene silencing using Cas13a in mosquitoes

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Running title: Cas13a-CRISPRi for gene silencing in mosquitoes

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Abstract

In the CRISPR-Cas systems, Cas13a is an RNA-guided RNA nuclease specifically targeting single strand RNA. In this study we developed a Cas13a mediated CRISPR interference tool to target mRNA for gene silencing in mosquitoes and tested the machinery in two mosquito species. A *Cas13a* expressing plasmid was delivered to mosquitoes by intrathoracic injection, and *Cas13a* transcripts were detectable at least 10 days post-delivery. In *Anopheles gambiae*, *vitellogenin* (*Vg*) gene was silenced by *Vg*-crRNA injection two hours post blood meal, which was accompanied by a significant reduction in egg production. In *Aedes aegypti*, the α- and δ -subunits of *COPI* genes were knocked down by a post-blood meal crRNA injection, which resulted in mortality and fragile midguts, reproducing a phenotype reported in a RNAi mediated *COPI* gene silencing study. The silencing of mulitple genes simlutaneously is achievable when mosquitoes are given a cocktail of Cas13a construct with crRNAs targeting respective genes. This study adds a programable CRISPR tool to manipulate RNA in insects.

Keywords CRISPR-Cas13a, RNA interference, *Anopheles gambiae*, *Aedes aegypti*, gene silencing

Introduction

In the post-genomic era, mosquito studies have been greatly accelerated by the integration of functional genomics approaches. The genetic basis for various mosquito life traits are being characterized and can inform innovative control strategies through the identification of genes involved in development, host-seeking, blood feeding, digestion, fecundity, immunity against various pathogens, xenobiotic metabolism and insecticide resistance. With increased insecticide resistance, the tool box for vector control is needing new tools to combat these insect vectors of significant human disease.

RNA interference (RNAi) is a defense mechanism that eukaryotic organisms use to target invading RNA viruses. RNAi based approaches have been widely used in gene function study to identify genes that are relevant to vector competence, and recently has been developed into effectors for mosquito control [1-5]. The RNAi machinery consists of Dicer and RNA-inducible silencing complex (RISC). The machinery is trigged by double-stranded RNA (dsRNA), which can be recognized by Dicer and cleaved into small interfering RNAs (siRNAs). Guided by siRNA, the nuclease Argonaut in the RISC complex cleaves target mRNA to silence the gene. CRISPR-Cas systems are adaptive immune mechanisms prokaryotes use to fight against invading DNA and RNA [6-9]. Cas9 is a RNA guided DNA nuclease and once assembled with a CRISPR guide RNA (sgRNA), the Cas9 is able to cleave target DNA specifically. The DNA-targeting Cas9 has been harnessed for genome editing [10, 11]. Recently, CRISPR-Cas9 based genome editing tools have been developed in the mosquito research field [12-14]. Alternatively, catalytically inactive Cas9 (dCas9) was adopted for manipulation of gene expression. The dCas9 can be fused with a gene repressor or transcription activator. Guided by CRISPR RNA, such variants are able to bind target promoter or exonic DNA sequence without cleavage, and either repress transcription (CRISPR interference, CRISPRi) or activate transcription of target genes (CRISPR activation, CRISPRa) [15-17]. Recently, newly identified RNA nucleases, Cas13 systems have been repurposed for specifically targeting endogenous RNAs as well as viral RNAs [8, 18-21]. Most Cas13 proteins are single "effector" proteins with two Higher Eukaryotes and Prokaryotes Nucleotide-binding (HEPN) domains [7, 22]. Once being loaded with a target-specific crRNA, a Cas13 protein will locate target RNA and execute RNase activity to degrade the target. Unlike Cas9, no Protospacer Adjacent Motif (PAM) sequences are required for Cas13 to function. Although a Protospacer Flanking Site (PFS), A, U,

C, may be present for the *Psp*Cas13b activity [23], no PFS is needed for *Lwa*Cas13a [8]. The Cas13s tested for human RNA knockdown thus far have demonstrated high specificity, exhibiting negligible and significantly less off-target effects compared to matched RNAi (shRNA) target sequences [8, 23]. In bacteria, Cas13 HEPN-nuclease is able to cleave not only the target-RNA *in cis* but also other non-target RNA present *in trans*. Interestingly, no collateral effect was observed in three studies of CRISPR-Cas13 using human or plant cell lines [8, 23, 24], but Cas13a associated collateral RNA cleavage was reported in human glioma cancer cells [25]. Fascinatingly, Cas13 can effectively silence serval transcripts in parallel [8, 18, 24, 26]. Taken together, CRISPR-Cas13 systems have become a new exciting version of CRISPRi [27]. In this study, we engineered a construct to express Cas13a from *Leptotrichia wadei* in mosquitoes, and demonstrated its efficacy in silencing genes in mosquito *Anopheles gambiae* and *Aedes aegypti.*

Materials and Methods

Plasmid construction

The *Cas13a* gene from *L. wadei* belongs to the class 2 type VI RNA-guided RNA nuclease[8]. It's RNA targeting effect has been demonstrated in eukaryotic and plant cells[8, 18, 25, 28]. Plasmid pAc-sgRNA-Cas9 was used as a template to engineer construct pAc-Cas13a (**Fig. 1**). Plasmid pAc-sgRNA-Cas9 was a gift from Ji-Long Liu (Addgene plasmid # 49330). The codons of *LwaCas13a* gene were optimized to *Drosophila* preference. The codon optimized Cas13a sequence was synthesized and cloned at GenScript (https://www.genscript.com). The Cas9 coding sequence in plasmid pAc-sgRNA-Cas9 as replaced by the *Cas13a* coding sequence to create the pAc-Cas13a construct. The Cas13a sequence was confirmed by sequencing. The *Cas13a* was under the control of *Drosophila*

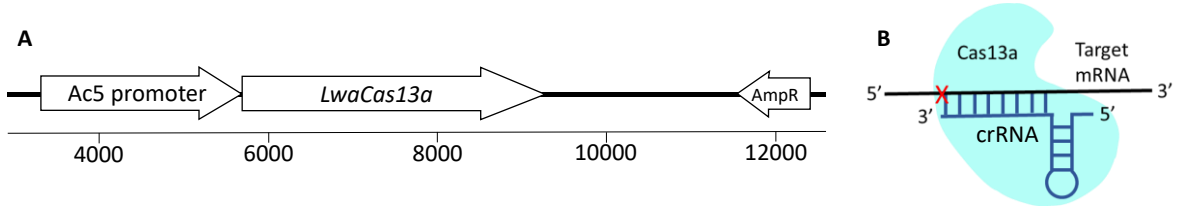

Fig. 1. Cas13a construct and function. (A) Map of pAc-*Lwa*Cas13a (partial). (B) Cas13a/crRNA mediated target RNA cleavage.

actin (Ac5) promoter for constitutive *Cas13a* expression. The Ampicillin resistance gene (AmpR) allows selection of positive clones (**Fig.1**). The plasmid was replicated in *E.coli* host cells, and extracted using a QIAprep kit (Cat No.27104, Qiagen).

Synthesis of crRNA

A crRNA consists of a 36-nt repeat sequence and 28-nt target RNA specific sequence (N_{28}), which is an anti-sense sequence complementary to the target RNA sequence. T7 promoter sequence was added to the 5' end to enable crRNA synthesis using T7 RNA polymerase *in vitro.* The crRNA template is AGTTAATACGACTCACTATAGGGATTTAGACTACCCCAA AAACGAAGGGGACTAAAAC(N_{28}). The target specific sequences (N_{28}) used in this study are shown in Table 1. The selection of crRNA is straightforward, *Lwa*Cas13a does not require PFS for activity[8], which makes no limitation to choose a crRNA in a target gene. For each target gene (except for *Vg*, which had only one crRNA), two specific N_{28} sequences were designed and used together for gene silencing. Template DNA duplex of the crRNAs were synthesized at IDT Inc. (https://www.idtdna.com). The crRNAs were synthesized using T7-RNA polymerase (RPOLT7-RO ROCHE, Sigma-Aldrich). The crRNA synthesis reactions were set up in 40 μl by adding template DNA duplex (1μg), 1 mM each of nucleotides ATP, GTP, CTP and UTP, 10X reaction buffer, T7 RNA polymerase 40U, RNase inhibitor 20U and H_2O. The reactions were incubated overnight at 37°C and stopped by heating the mixture at 65°C for 5 minutes. The crRNAs were treated with Turbo DNase I Kit (AM1907, ThermoFisher) to remove template DNA. The crRNA yield was quantified using a NanoDrop and stored at -20 ºC until use. Control crRNA consisted of a randomly scrambled N_{28} nucleotide sequence, which had no homologous hit in the genomes of *An. gambiae* and *Ae. aegypti*.

Table 1. The crRNA (N_{28}) sequences

Gene	GenBank accession	crRNA sequence (N_{28})
Vg	XM_313104	CACCTGCACCTTCACGCTGTCACCAGCC
COPI-δ	XM_001663354	TGATAGACATACCGCACGGAATCTGTCT
COPI-α	XM_001663259	ACCGCCTCTGCTTGTAGTTCCACACCT
Cactus-1	XM_001650217	ATCACCGTCGTCGTTCTGGTGGAAGTACT
Cactus-2	XM_001650217	TGATGCACAGGTCGTCCACCTTCATCGGA

Caspar-1	XM_021842247	TCAACGCCGGACTCGGCCAGTGTCGTACT
Caspar-2	XM_021842247	TACCACTGCCACCGGCGGACGATCTCTGT
Control	N/A	GACGCACATTCATAGTCTTCATCTGAGT

Construct and crRNA Delivery

An. gambiae G3 strain and *Ae. aegypti* Puerto Rico strain were obtained from MR4 BEI and maintained using rearing conditions described previously [29, 30]. The pAc-Cas13a construct (0.5μg/μl) was delivered into one-day old female mosquitoes by intrathoracic injection. To aid construct delivery into the cell, the construct was mixed with a transfecting agent FuGENE HD (E2311, Promega) at concentration of 1.6μl of FuGENE reagent with 10μg construct DNA in 20 μl. Gene specific crRNAs were either delivered with the construct or separately at a later time point. For blood inducible genes, *Vg* and *COPI*, female mosquitoes were given a blood meal three days post construct injection. The corresponding crRNAs (0.5μg/μl, prepared in FuGENE as described above) were injected into mosquito hemocoel at two hours post blood meal.

RNA isolation, cDNA synthesis and PCR

Total RNA was isolated using Trizol (Invitrogen) following the manufacturer's instruction. The RNA was treated with Turbo DNase I Kit to remove genomic DNA contamination, and then 1μg RNA was converted to cDNA using Protoscript II RT (M0368S, New England Biolabs) following the manufacturer's instruction. The PCR assays were performed using 1μl 1:5 diluted cDNA as template, 0.2 μM primers (primer sequences are presented in Table S1) and 2 × PCR Master mix (M0482S, NEB), with the following cycling parameters: 35 cycles of denaturing at 95°C for 15 seconds, annealing at a temperature optimal for the amplicon (Table S1) for 15 seconds, and extension at 68°C for 20 seconds with an extra 5 min in the last cycle for final extension.

Statistical analysis

In the gene *Vg* knockdown experiment, eggs were dissected from ovaries at day 3 post blood meal. The egg counts were compared between the *Vg* crRNA and control crRNA cohorts. The non-parametric Mann-Whitney test was used for statistical comparison of the egg numbers. In the *COPI* gene knockdown experiment, a survival curve was plotted, and Mantel-Cox analysis was performed to compare the survival between the *COPI* crRNA and control crRNA cohorts using GraphPad Prism.

Results

Cas13a expression in mosquitoes post intrathoracic delivery

A construct was engineered to express *Cas13a* gene by modifying the plasmid pAc-sgRNA-Cas9, which was successfully used to transfect *Drosophila* cells for targeted genetic mutagenesis [31]. As shown in **Fig. 1**, Cas13a coding sequence is under the control of constitutive promoter Actin 5C. To deliver the construct to mosquito hemocoel, the plasmid was prepared with transfection reagent FuGENE HD and injected into thorax. We examined *Cas13a* transcription by RT-PCR in mosquitoes after receiving the construct. In *An. gambiae*, *Cas13a* transcripts were detected at three days post construct injection (**Fig. 2A**). No non-specific amplifications were observed in the controls that did not receive the construct. In *Ae. aegypti*, the construct was delivered to one-day old mosquitoes, and the *Cas13a* transcripts were detected on day 2, 5, 7 and 10 post construct delivery (**Fig. 2B**).

Cas13a mediated *Vitellogenin* gene silencing in *An. gambiae*

In mosquitoes, yolk protein precursor vitellogenins (Vg) are required for the vitellogenic stage in oogenesis after blood feeding [32]. To test Cas13a/crRNA mediated *Vg* silencing, the Cas13a

Fig. 2. Detection of *Cas13a* transcript in pAC-Cas13 injected mosquitoes. (A) RT-PCR of *Cas13a* in *An. gambiae* mosquitoes that received the construct (+) vs. control (-) at day 3 post plasmid delivery. NRT: No RT control; NTC: no template control. The *rpS5* was used as a loading control. (B) Expression pattern of *Cas13a* transcript over a period of 10 days in *Ae. aegypti*, the *rpS7* was used as a loading control.

construct was delivered to one-day old *An. gambiae* (N = 120). Three days later, the mosquitoes were given a blood meal to induce *Vg* expression (N = 96). To activate *Vg* knockdown, *Vg* crRNA (N = 41) or control crRNA (N = 40) were injected into the blood engorged females at 2 hr post feeding. The *Vg* RT-PCR was used for verification of *Vg* knockdown. As shown in **Fig. 3**, the abundance of *Vg* transcript was reduced in females that received *Vg*-crRNA as compared to females that received control-crRNA. As expected, successful *Vg* knockdown resulted in reduction in egg production. The *Vg*-crRNA treated mosquitoes produced on average 39 ± 25 eggs/female

(N = 33), while control mosquitoes produced 64 ± 23 eggs/female (N = 32). The difference was statistically significant (Mann-Whitney test, P<0.001). A second experimental replicate generated data with similar pattern showing significant reduction in egg numbers (Mann-Whitney test, P<0.01, **Fig. S1**). The Vg proteins are produced by fat body and secreted into hemolymph, and subsequently deposited in developing oocytes via receptor-mediated endocytosis. The results suggest that the Cas13 construct was successfully delivered into fat body cells, where the *Cas13a* was transcribed and translated into functional Cas13a proteins. Post blood feeding, *Vg*-crRNAs were delivered into cells, where they were assembled with Cas13a to form a target specific RNA nuclease complex, which cleaved the *Vg* mRNA effectively.

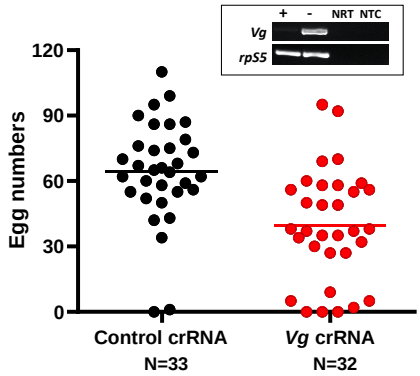

Fig. 3. *An. gambiae Vg* knockdown reduced egg production. Mosquitoes treated with Cas13a/*Vg-crRNA* had less egg counts as compared to the cohort treated with Cas13a/ctr-crRNA (P<0.001). Reduction of *Vg* transcripts was confirmed by RT-PCR (insert panel). NRT: No RT control; NTC: no template control. The *rpS5* was used as a loading control.

Cas13a mediated *COPI* gene silencing in *Aedes aegypti*

The coatomer complex I (COPI) proteins are involved in blood digestion in *Ae. aegypti* [33]. The COPI complex consists of α, β, β', γ, δ, ε and ζ subunits, each encoded by separate genes. The production of COPI proteins is blood inducible between 18-36 hr post blood meal in fat body and 24-48 hr post blood meal in ovaries [33]. The dsRNA mediated knockdown of the genes encoding all but the ε subunit led to blood meal-induced mortality [33]. Therefore, we targeted the *COPI* genes to test the gene silencing efficacy of the Cas13a machinery in *Ae. aegypti*. The Cas13a construct was injected into one-day old mosquitoes (N = 90). At day 3 post construct delivery, the mosquitoes were given a blood meal. The engorged mosquitoes (N = 75) were split into two

cohorts, one was injected with the crRNAs specific for α and δ*COPI* genes at 2 hr hours post blood meal (N = 27). The other cohort (N = 29) was injected with the control crRNA. A subset of mosquitoes (N = 5) in each cohort was sampled for knockdown validation by RT-PCR at 20 hr post blood meal. Reduced abundance of the *COPI* transcripts was observed in the knockdown group (**Fig. 4**). The survival curves over 9 days post blood meal exhibited difference between the *COPI* knockdown (N = 22) and control cohort (N = 24) (**Fig. 4**). The difference was statistically significant as determined by Mantel-Cox test (P<0.001). In the *COPI* knockdown cohort, mosquitoes continue to exhibit mortality till day 11 with 0% survival as compared to 27% survival in the control cohort. It has previously been shown that *COPI* knockdown makes midgut fragile [33]. Consistently, we observed that the midguts in the *COPI* knockdown mosquitoes were apt to break and leak during dissection, while the midguts of control mosquitoes were in good shape with intact blood bolus (**Fig. 4**). These data represent the findings from a single experiment. A second experimental replicate showed a significant reduction in the survival of the *COPI* knockdown cohort by day 5 (Mantel-Cox test, P<0.01, **Fig. S2**).

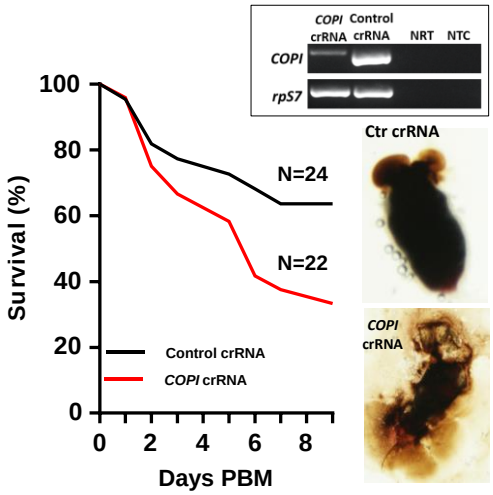

Fig. 4. Phenotypes of *Ae. aegypti COPI* knockdown. Cas13a/*COPI*-crRNA resulted in mortality post blood meal (PBM) and fragile midgut. Reduction of *COPI* transcripts was confirmed by RT-PCR (insert panel). NRT: No RT control; NTC: no template control. The *rpS7* was used as a loading control. PBM: post blood meal.

Cas13a mediated double gene knockdown

To determine the potential for silencing multiple genes, a cocktail of the Cas13a construct and crRNA against genes *Cactus* and *Caspar* or *Cactus* and *COPI* were delivered into *Ae. aegypti.* As expected, the target gene transcripts were reduced effectively by the machinery (**Fig. 5**).

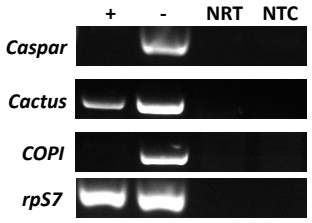

Fig 5. RT-PCR verification of double gene knockdown in *Ae. aegypti* that received a cocktail of Cas13a construct with either *Cactus*-and *Caspar*- or *Cactus*- and *COPI*-crRNAs. (+): target-crRNA; (-) ctr-crRNA; NRT: No RT control; NTC: no template control. The *rpS7* was used as a loading control.

Discussion

In this proof of concept study, we demonstrate the effectiveness of CRISPRi that is mediated by CRISPR-Cas13a/crRNA machinery. The *LwaCas13a* is derived from *L. wadei*, and under control of *Actin* promoter (**Fig.1**). The construct is delivered into the hemocoel of adult mosquitoes by intrathoracic injection, and *Cas13a* is transcribed constitutively (**Fig. 2**). Likely, the construct enters into the nucleus where the *Cas13a* gene is transcribed, and then the mRNA comes into the cytoplasm where the protein is translated. The construct remains active to transcribe *Cas13a* for at least 10 days post-delivery (**Fig. 2**), which makes it temporally flexibile to administer crRNAs targeting various genes that are expressed at different time points during a mosquito's life span. Target gene specific crRNAs can be delivered either with the construct together, or at a distinct time point as appropriate to the experimental design. The system can silence highly abundant transcripts, as demonstrated by targeting *Vg* transcripts in *An. gambiae* (**Fig. 3**) and *COPI* transcripts in *Ae. aegypti* (**Fig. 4**). In both cases, the target genes are induced by blood meal with a high transcriptional abundance. In addition, we have tested the system on genes *Cactus*, *Caspar* in *Ae. aegypti* (**Fig. 5)**. Taken together, these data conclusively demonstrate that the Cas13a-CRISPRi machinery is functional in *An. gambiae* and *Ae. aegypti* mosquitoes we tested in this study. This tool may work well in other mosquito species.

The Cas13a-CRISPRi has certain advantages over the dCas9-CRISPRi for repressing gene expression [16, 34]. First, the dCas9-CRISPRi machinery acts at DNA level while Cas13a targets mRNA directly. RNA-guided binding of dCas9 to a specific promoter or coding sequence can block transcription. This mode of action is efficient in bacteria, but not often very efficient in

eukaryotic cells [16]. The dCas9 fusion proteins with a repressive domain have been developed for transcriptional repression, such as dCas9-KRAB (Krüppel associated box) in mammalian cells [15], but it is challenging to develop a fusion dCas9 with universal applicability. In addition, target specific sgRNA selection may be limited by the PAM that is required for the Cas9 activity. On the contrary, no PFS is required for target RNA cleavage by *Lwa*Cas13a in eukaryotic cells [21]. The action mode of Cas13 is simple and programable, and the efficacy has proven high in mammalian cells [8, 23] and in mosquitoes in the current study. RNAi mediated gene silencing is a very common practice in mosquito gene function studies. Recently, application versions have been developed for mosquito vector control [4, 5]. In these application cases, dsRNA is used to trigger RNAi machinery. In dsRNA mediated RNAi, the effective siRNA sequences sometimes are difficult to predict, therefore, large dsRNA fragments are often used to increase chances to generate effective siRNA by Dicer. However, this strategy is accompanied with a higher chance to produce siRNA with off-target potentials [35, 36]. In addition, efficacy of dsRNA-RNAi varies in various contexts, optimal outcomes often result from an empirical process [37].

There is a concern about the potential of collateral cleavage with Cas13a, in which non-specific RNA sequences can be cleaved by Cas13a in bacteria [8]. Interestingly, this promiscuous RNA degradation activity was not observed in several studies in mammalian and plant cells [8, 23, 24]. These data have warranted its safety to be used as an effector to target against RNA viruses that infect humans [21]. However, a Cas13a/crRNA associated collateral cleavage was shown recently in human cancerous U87 glioblastoma cells [25]. In the study, exogenous gene *GFP* or *EGFRVIII* were overexpressed and targeted by Cas13a/crRNA. In this context, a partial degradation of ribosomal RNA was observed, and the abundance of non-target transcripts of *GAPDH, HOTHAIR* and *L3MTL1* was reduced as well [25]. Furthermore, the RNA integrity was compared between the LN229 glioma cell line and HEK293T cells after treatment with the Cas13a/crRNA, the LN229 cells tended to be more sensitive to the collateral effect than the HEK293T cells [25]. In our study, we used genes encoding ribosomal protein *rpS5* in *An. gambiae* and *rpS7* in *Ae. aegypti* as non-target gene for control; we did not observe reduced abundance of these two control transcripts in the experiments in which the effective Cas13/crRNA target gene silencing occurred (**Fig. 3-5**). Although our data showed no effect on those two non-target genes, we could not rule out the possibility of collateral cleavage of other RNAs. Additional studies are required to examine non-target RNA cleavage by the Cas13/crRNA system in mosquitoes.

The Cas13a-CRISPRi system as a new tool holds promise for robust and flexible programming to silence one or more genes simultaneously in mosquitoes, with potential applications in other arthropods.

Author Disclosure Statements

No competing financial interests exist.

Authorship Confirmation Statement

JX conceived the idea for the project and devised the study. JX, AK, KAH designed experiments, AK, WY, ASM, AP conducted experiments. AK, JX, KAH wrote manuscript. The authors confirm that all co-authors have reviewed and approved the manuscript. The authors affirm that the paper is original with unpublished findings, not under consideration by any other journals.

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Table S1. Primer sets used in the study

Primer	Sequence	Amplicon Size
Aa_Cactus_F	AGACAGCCGCACCTTCGATTCC	232 bp
Aa_Cactus_R	CGCTTCGGTAGCCTCGTGGATC	
Aa_Caspar_F	GAATCCGAGCGAGCCGATGC	270 bp
Aa_Caspar_R	CGTAGTCCAGCGTTGTGAGGTC	
Aa_rpS7_F	AAGGTCGACACCTTCACGTC	252 bp
Aa_rpS7_R	TCTTGTCCCTCCGTTTGGTG	
Aa_COPI_F	GACGTTGCGCATATCAGACG	256 bp
Aa_COPI_R	CAGCATTCTCAGAGGGCCAA	
Cas13a_F	TCCGCCAACAAGGAAGAGAC	442 bp
Cas13a_R	CCGATGGCCTTCTCGTACTC	
Ag_Vg_F	ACTTCTTCCAGGGCAAGCAC	250 bp
Ag_Vg_R	CACAGCGCAAGATGGATGGT	
Ag_rpS5_F	CCATGTCACGTCTCGTCACT	104 bp
Ag_rpS5_R	CGAAAACCATCCACACACAC	

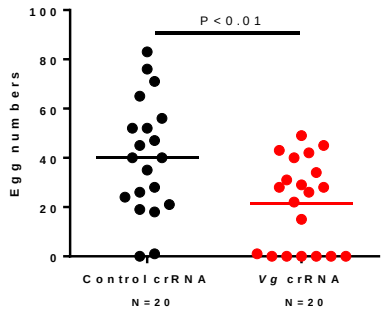

Figure S1. The *Vg* crRNA/Cas13a treatment significantly reduced egg production in *An. gambiae*. Mann-Whitney test, P<0.01.

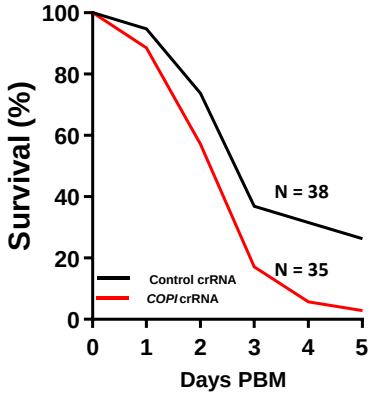

Figure S2. The *COPI*-crRNA/Cas13a treatment resulted in a significant higher mortality post a blood meal, Mantel Cox test, P<0.01.