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DsRNA-mediated targeting of ribosomal transcripts RPS6 and RPL26 induces long-lasting and significant reductions in fecundity of the vector *Aedes aegypti* ☆

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ABSTRACT

Ribosomal transcripts produce critical proteins that are involved in most cellular production processes. Targeting ribosomal transcripts has produced mortality in mites and ticks but the effect of ribosomal transcript knockdown has not been thoroughly examined in mosquitoes. We examine the effects of triggers targeting four ribosomal proteins (RP) transcripts. Although no significant mortality was observed after dsRNA microinjection and subsequent blood feeding, significant contrasts were observed on fecundity. Triggers targeting RPS6 and RPL26 effectively reduced gene expression but more importantly, reduced reproductive output by more than 96% and 91% at the first oviposition while triggers targeting RPL1 and RPS2 did not cause a reduction although gene expression was reduced. Significantly reduced fecundity continued through a second oviposition cycle in dsRPS6 and dsRPL26 cohorts, although the effect was not as strong. Relative gene expression levels confirmed specific transcript knockdown up to 20 days post-injection in mosquitoes that did not oviposit or produced reduced clutch sizes. Dissections at 36 h post-blood meal indicated defects in oocyte provisioning. The strong phenotype produced by dsRPS6 allowed us to examine the effects in various tissues as well as the dose response, trigger format, delivery method and trigger specificity in *Aedes aegypti*. Strong knockdown was observed in the abdomen and the ovaries. Greater than 50 ng of dsRPS6 significantly reduced fecundity but not when delivered in a sugar meal or as an siRNA. Similar bioassays with mutated dsRPS6 triggers indicates that up to three mismatches per possible siRNA are still effective in reducing fecundity. These studies indicate that while active and effective triggers can be developed for vector species, the lack of an efficient delivery method is the biggest barrier to use as a potential control method.
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1. Introduction

The discovery of a natural antiviral response system in *Caenorhabditis elegans* (Fire et al., 1998) essentially homologous to plant-based post-transcriptional gene silencing (PTGS) ushered in a new era of functional genomics studies as well as hopes for agricultural and public health pest control (Huvenne and Smagghe, 2010). It was noted that exogenous RNA, formatted as siRNAs or dsRNAs to trigger the native RNAi machinery, could possibly be developed against targeted species but would remain relatively harmless to non-target and beneficial organisms. Multiple reviews discuss the potential uses and pitfalls of RNAi-based therapeutics as a means of pest control (Perrimon and Mathey-Prevot, 2007; Huvenne and Smagghe, 2010; Zhang et al., 2013; Baum and Roberts, 2014).

A critical first step requires identification of effective RNAi triggers for a pest species. Relatively quick progress has occurred for agricultural pest insects due to the burden of economic injury and the potential market for the resulting control products. Baum et al. (2007) screened almost three hundred individual

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dsRNA triggers and identified a subset that resulted in significant larval mortality when fed to the western corn rootworm (*Diabrotica virgifera virgifera*). DsRNA-mediated RNAi has also produced mortality in the Colorado potato beetle (*Lepitnotarsa decemlineata*) (Baum et al., 2007; Zhu et al., 2011), the lepidopteran pest, *Helicoverpa armigera* (Jin et al., 2015) and numerous chelicerate arthropods including predatory mites (Wu and Hoy, 2014), *Varroa destructor* (Garbian et al., 2012) and ticks (de la Fuente et al., 2006; Hatta et al., 2007; Kurscheid et al., 2009). The difficulty of identifying lethal phenotypes and the slow induction of critical deficits in adult insects has caused some groups to use dsRNA to target genes that induce or reduce susceptibility to pathogens (Campbell et al., 2008; Maori et al., 2009) or pesticides (Bautista et al., 2009; Mao et al., 2007, 2011) which can lead to increased insect death or inability to host vectored pathogens.

By comparing the results of these studies, it is clear that effective triggers and targets in one particular organism may not be effective in another due to differences in specific life stage, physiology of the organism or ability to take up the RNAi triggering molecule. It appears that while larval life stages of insects are generally more susceptible to RNAi (Huvenne and Smagghe, 2010), the ability to effectively import RNA-based triggers into tissues is variable within some orders and with the presence or absence of specific dsRNA transporters like SID1 (Terenius et al., 2011; Feinberg and Hunter, 2003). Additionally, RNA-directed RNA polymerases (RdRP) are present in the genomes of chelicerate arthropods, viruses, and nematodes but not insects, and are responsible for secondary amplification of the RNAi response (Nishikura, 2001; Pak and Fire, 2007). Effective RNAi has been successfully initiated by triggers fed directly to some organisms or expressed in bacteria and plants (Zhu et al., 2011; Jin et al., 2015) but feeding triggers to hematophagous vectors is difficult although it has been reported.

Progress with using RNAi mechanisms against public health pests has been much slower with two critical limitations. There is no reliable method for easy delivery to adult vectors although larval mosquitoes seem to be more susceptible (Zhang et al., 2010; Whyard et al., 2015). The second challenge is to identify effective targets and although several publications have indicated mortality due to dsRNA treatments; they have not been widely replicated in other laboratories (Pridgeon et al., 2008; Walshe et al., 2009; Isoe et al., 2011; Puglise et al., 2016). However, non-lethal effects that reduce fitness have been consistently identified (Gulia-Nuss et al., 2011; Isoe et al., 2011; Sim and Denlinger, 2009).

A small body of work targeting ribosomal transcripts indicates they may be attractive targets for RNAi-mediated knockdown because of the importance of the ribosome in most cellular processes. In *Drosophila melanogaster*, mutated ribosomal genes resulted in inhibition of oviposition in adults or mortality in larvae among other abnormalities (Kay and Jacobs-Lorena, 1987; Cramton and Laski, 1994). Individually knocking down ribosomal transcripts in the chelicerates *Rhipicephalus microplus* and *Metaseiulus occidentalis* also resulted in a reduction in oviposition (Kurscheid et al., 2009; Wu and Hoy, 2014). During diapause studies in *Culex* mosquitoes, dsRNA-mediated targeting of RPS3a caused a short term reduction in the ability to provision eggs (Kim et al., 2010).

In this study, we examine the effects of knockdown of several ribosomal transcripts in the mosquito *Aedes aegypti*. We examined the effects on fecundity through multiple oviposition cycles and analyzed the relative expression levels of specific ribosomal transcripts. Using the strong dsRPS6 phenotype, we investigate the effects of dose, trigger format, delivery method and siRNA mismatches on efficacy.

2. Material and methods

2.1. Mosquito colonies and rearing

Aedes aegypti (Orlando strain) have been in continuous colony at the Center for Medical, Agricultural, and Veterinary Entomology (CMAVE) since field collection near Orlando, Florida in 1952. The *Ae. aegypti* rearing protocol is highly standardized and has been described previously (Pridgeon et al., 2009). Three one-hundredths of a milliliter of eggs are placed into 100 mL of deionized water along with about 50 mg of finely ground alfalfa/pig chow mixture. The neonates are placed into a tray containing 3 L of deionized water with 0.5 g of brewers yeast:liver powder (3:2). Trays are maintained at 27 °C in an insectary under a 14:10 L:D cycle. Trays are fed 1 g of 3:2 food on the day following hatch and then again 2 days later. Collected pupae are placed into a screened colony cage with 10% sucrose soaked cotton balls *ad libitum*. Under these rearing conditions, early larval instars are completed daily, the 4th instar requires 2 days, and pupal development takes two days. Mosquitoes emerge at the end of the seventh day after hatching and females weigh 2.5 ± 0.3 mg (mean ± SD) each. Colonies were provided manually-defibrinated bovine blood as the protein source for colony maintenance. Mosquitoes used for injection experiments were 3–6 days post emergence and had not been blood-fed.

2.2. Synthesis of RNAi triggers

Five dsRNA triggers were produced for initial oviposition bioassays in this study (Tables 1 and 2). The native transcript sequences for RPS6 (AAEL000032B, XM_001647882.1), RPL26 (AAEL005817, XM_001651406.1), RPL1 (AAEL004643, XM_001649463), and RPS2 (AAEL008582, XM_001653266) from the Liverpool genome Assembly L1.1 (www.vectorbase.org/aedes_aegypti) were used for an initial *in silico* analysis to identify splice junctions and

Table 1
Primers for dsRNA construction and qPCR analysis.

Name	Forward primer	Reverse primer	Amplicon size[a]
T7-RPS6	GTCCTGACCAACACCCGT	CCCTTCTTGACGACGATCAG	192
T7-RPL26	CAAGAGCCGTAAGCGACATT	ACCTGGACCACCTTACCGAC	223
T7-RPL1	CCGGTAAAATTCCGGCTTTA	AGCCTTCTCACGGGTTCCCT	203
T7-RPS2	TGTGAAATTGGTTGATGTTC	AGCCAATGTTGATAGACACC	225
qRPL24	AATGAAGATCGGCCTTTGC	AGGACGGTCCACTTCACC	162
qRPS6	CTCGGCGAGTGTATGGAAAT	CGTAGAAGTGACGCAGCTTG	116
qRPL26	CCCTCTTTCCTTCCGACATC	GGCGAGACGAGGAAACATT	105
qRPL1	CGCAAGGCCAAAGTCAAGAA	CTGACCCAGCAGTTGTCCAC	146
qRPS2	CCGACTGCAGCAATATCCGA	GTACCCTCTTTGTGGCCCAA	128

[a] Amplicon size of templates for dsRNA includes 40 bases contributed by the T7 promoters of the fusion primers that are not present in the final dsRNA product. Primers for dsRNA templates include a minimal T7 promoter sequence (taatacgactcactataggg) appended which is not shown.

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