Improved transient silencing of gene expression in the mosquito female *Aedes aegypti*

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Abstract

**Gene silencing using RNA interference (RNAi) has
become a widely used genetic technique to study gene
function in many organisms. In insects, this technique
is often applied through the delivery of dsRNA. In the
adult female *Aedes aegypti*, a main vector of human-
infecting arboviruses, efficiency of gene silencing
following dsRNA injection varies greatly according to
targeted genes. Difficult knockdowns using dsRNA
can thus hamper gene function analysis. Here, by ana-
lysing silencing of three different genes in female
Ae. aegypti (*p400*, *ago2* and *E75*), we show that gene
silencing can indeed be dsRNA sequence dependent
but different efficiencies do not correlate with dsRNA
length. Our findings suggest that silencing is likely also
gene dependent, probably due to gene-specific tissue
expression and/or feedback mechanisms. We demon-
strate that use of high doses of dsRNA can improve
knockdown efficiency, and injection of a transfection
reagent along with dsRNA reduces the variability in effi-
ciency between replicates. Finally, we show that gene
silencing cannot be achieved using siRNA injection in
Ae. aegypti adult females. Overall, this work should help
future gene function analyses using RNAi in adult
females *Ae. aegypti*, leading toward a better under-
standing of physiological and infectious processes.**

**Keywords: gene silencing, dsRNA, siRNA, RNA inter-
ference, *Aedes aegypti*.**

1. Introduction

Mosquito-borne arboviruses cause a large medical, veteri-
nary and economic impact on predominately tropical and
sub-tropical regions of the world. Medically important
mosquito-borne arboviruses include chikungunya, den-
gue, Zika and yellow fever virus (Weaver and
Reisen, 2010; Weaver *et al*., 2016; Mayer *et al*., 2017;
Wilder-Smith *et al*., 2017; Paixao *et al*., 2018). The major
vector for these viruses is the *Aedes aegypti* (*Ae. aegypti*)
mosquito. Despite the huge burden these viruses cause to
the healthcare systems and the economy in endemic coun-
tries there are few effective vaccines or antivirals available
to target infections caused by these viruses. Therefore,
mosquito control remains a key strategy to limit arbovirus
transmission (Kean *et al*., 2015; Achee *et al*., 2019; Lam-
brechts and Saleh, 2019). As *Aedes* mosquitoes develop
resistance to insecticides (Dusfour *et al*., 2019), new vector
control strategies are urgently needed. Therefore, it is
important to increase our understanding of mosquito biol-
ogy and mosquito-arbovirus interactions that influence
the outcome of arbovirus infection in mosquitoes. The
major advances in the last two decades in vector biology,
genomics/comparative genomics, transcriptomics and pro-
teomics have allowed the identification of a large number of
genes and proteins putatively involved in mosquito-
arbovirus interactions. However, the functions of many of
them are still unknown.

In a model and genetically transformable species, such
as *Drosophila melanogaster*, reverse functional genomics
(from gene to phenotype) is classically achieved using
mutant lines or RNA interference (RNAi). In the latter case,
animals are genetically transformed to contain a transgene
in their genome expressing a gene fragment cloned as an
inverted repeat. This results in the expression of a hairpin
double-stranded RNA (dsRNA), which is recognized by
the small interfering RNA (siRNA) pathway. Upon recogni-
tion, the nuclease Dicer-2 (Dcr2) chops the hairpin dsRNA
into short interfering RNA duplexes (siRNAs). These smal-
ler fragments are then loaded into a multi-protein complex
called the RNA Induced Silencing Complex (RISC) where
the active component Argonaute-2 (Ago2) binds to the

siRNA and unwinds and degrades one of the strands. The remaining strand is used to target homologous mRNAs for degradation and thus gene silencing. This system can be combined with the GAL4-UAS system to drive the expression of such hairpin dsRNA, and therefore analyse gene function, in a stage- and tissue-specific manner (Dietzl *et al*., 2007; Belles, 2010).

Although good progress has been made in the last decade with mosquito transformation techniques (Nimmo *et al*., 2006; Meredith *et al*., 2011; Pondeville *et al*., 2014; Kistler et al., 2015; Li *et al*., 2017; Chaverra-Rodriguez *et al*., 2018), very few mosquito genetic tools and transgenic lines have been produced, in comparison to model organisms. In addition, establishment of mosquito transgenic lines requires specific equipment and reagents, skills and training, is time- and space-consuming, and is greatly hampered by the small number of well characterized tissue-specific promoters and the absence of tools to maintain toxic/lethal mutants and transgenes. For these reasons, the easiest method so far to study gene function without having to generate genetically modified mosquito lines, is through transient gene silencing by directly delivering *in vitro*-synthetized dsRNA into mosquitoes at the stage of interest and then analysing the resulting phenotype.

Transient RNAi in adult mosquitoes was first performed in *Anopheles gambiae* to knockdown the *defensin* gene in adult females (Blandin *et al*., 2002). In this species, transient gene silencing in adults has proved successful to knockdown genes in multiple tissues and cells involved in parasite development, including midgut, haemocytes and ovaries by injecting into the haemolymph 100–150 ng of dsRNA (Blandin *et al*., 2002; Osta *et al*., 2004; Boisson *et al*., 2006). However, RNAi efficiency can greatly vary between species, with some insect species requiring high doses of dsRNA to efficiently silence genes (Belles, 2010; Joga *et al*., 2016). Even in sensitive species, knockdown efficiency is tissue- and dose-dependent. For example, Boisson *et al*. showed that silencing gene expression in salivary glands and the muscle cells surrounding the midgut in *An. gambiae* requires high concentrations of dsRNA (Boisson *et al*., 2006). In *Ae. aegypti*, knockdown efficiency following injection of 200–500 ng of dsRNA seems to vary according to genes with some genes silenced quite efficiently (up to 80%) and other genes for which knockdown efficiency is relatively low (0–20%) (Sim *et al*., 2013; McFarlane *et al*., 2014; Anglero-Rodriguez *et al*., 2017; Barletta *et al*., 2017). Recently, we identified p400 as a positive regulator of the antiviral exogenous siRNA pathway and restricting factor of arbovirus infection in *Ae. aegypti* (McFarlane *et al*., 2020). Using classical doses (*i.e.* 500 ng per female) of dsRNA targeting p400, we were unable to achieve a sufficient and reproducible silencing efficiency. Therefore, we sought to optimize and determine

a transient silencing protocol to facilitate successful and reproducible knockdown in *Ae. aegypti* mosquitoes.

Our findings presented here confirm that silencing efficiency is indeed gene dependent. The ability of the dsRNA to produce a significant knockdown was not due to the target functioning as part of the siRNA pathway itself or to the length of the dsRNA but is likely gene- and sequence-dependent. We show that different dsRNA sequences for a single target gene can lead to different knockdown efficiencies. However, some genes are more difficult to silence independently of the dsRNA sequence. For those genes, we show that increasing the injected dsRNA quantity can improve gene silencing. Importantly, including a transfection reagent increases the reproducibility of silencing levels across replicates and thereby enhances the quality of experiments, without adversely affecting the survival of the mosquitoes. Finally, we show that while delivery of siRNA can be used to efficiently knockdown genes in cultured mosquito cells, injection of siRNA in adult females did not result in target gene silencing. Overall, our results indicate that in *Ae. aegypti* females the sequence of the dsRNA and nature of the target mRNA are the major determinants of a successful knockdown, and that for genes whose expression is not significantly affected by classical dsRNA doses, silencing can be improved by injecting higher amount of dsRNA and transfection reagent together.

2. Results

2.1 Effect of two different transfection reagents on mosquito survival and knockdown efficiency

A previous study showed that cationic liposomes can increase delivery and expression of plasmid DNA in *Ae. aegypti* and *An. gambiae* mosquitoes (Cheng *et al*., 2011). We therefore hypothesized that injecting dsRNA along with a cationic liposome could also enhance cellular delivery of dsRNA, and thereby could increase knock down efficiency in *Ae. aegypti* adult females. To test this hypothesis, mosquito females were injected with 2 µg of dsRNA targeting *p400* (dsp400-1) or *ß-galactosidase* gene (dsLacZ, control) with a cationic-lipid transfection reagent or without (same final volume injected, adjusted with S2 media). As transfection reagents can have different efficiencies in different cell lines and some have deleterious effects on mosquito survival (Cheng *et al*., 2011), two different reagents were tested, Cellfectin II and Dharma-FECT2. Mosquito survival was monitored for three days post injection (dpi) and knock down efficiency was assessed in females at four dpi, a time at which gene silencing by dsRNA can generally be observed (Blandin *et al*., 2002; Boisson *et al*., 2006). Injection of dsRNA with Cellfectin II did not influence mosquito survival while injection of dsRNA with DharmaFECT2 resulted in a 10%

reduction compared to injection with Cellfectin II or without transfection reagent, although this was not significant (Fig. 1A). While *p400* knockdown was detectable with transfection reagent or without, inclusion of Cellfectin II led to a greater knockdown efficiency and reduced the variability (Fig. 1B). Taken together, these data indicate that Cellfectin II aids knockdown and reproducibility without affecting mosquito survival. Therefore, Cellfectin II was selected for further experiments.

2.2 dsRNA injection with Cellfectin II improves knockdown efficiency

To test whether the addition of Cellfectin II could allow a reduction of the dsRNA quantity needed to knockdown *p400*, female mosquitoes were injected with varying quantities of dsp400-1 or dsLacZ (500 ng, 1 μg and 2 μg) in the presence or absence of transfection reagent and silencing was determined by RT-qPCR at four dpi. Consistent with previous results, when 2 μg of dsRNA was injected, the inclusion of Cellfectin II improved the knockdown efficiency of *p400* and reduced the variability across replicates (Fig. 2A). However, the addition of the transfection reagent did not improve knockdown efficiency of the lower quantities (1 μg and 500 ng) of dsRNA injected (Fig. 2A). The length of dsRNA has been shown to influence the efficacy of the RNAi with longer dsRNA being more effective to knockdown gene expression (Miller *et al*., 2012). To ensure that the difficulty in achieving a significant knockdown of p400 was not due to the short size of the dsRNA (dsp400-1 is 149 bp in length), we designed a longer dsRNA targeting *p400* mRNA (dsp400-2, 317 bp;
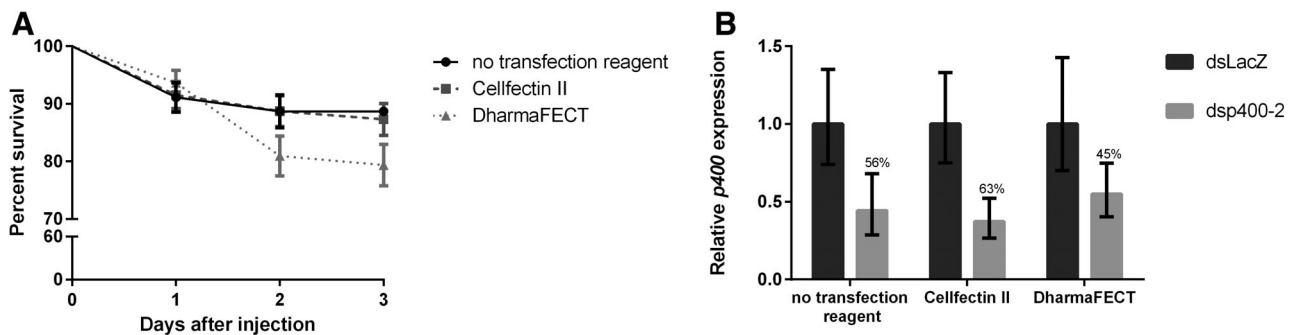

Figure 1. Test of different transfection reagents on mosquito survival and knock down efficiency. Female mosquitoes were injected with 2 μg dsRNA targeting *p400* (dsp400) or *LacZ* (dsLacZ, control) and with different transfection reagents, Cellfectin II and DharmaFECT, or without (same volume injected, adjusted with S2 media). (A) Mosquito survival for 4 days post dsRNA injection. Female mosquitoes injected with the same transfection reagent but different dsRNAs were pooled together to assess survival linked to transfection reagent. Survival was not significantly different using a Log-rank Mantel-Cox test. (B) Relative *p400* expression knockdown efficiency was assessed in female mosquitoes injected with either Cellfectin, Dharmafect, or without transfection reagent, and compared to dsLacZ 4 days post injection by RT-qPCR. Results were analysed as previously described (Taylor *et al*., 2019). The group geomean of control *LacZ* was set to 1 and the geomean of *p400* calculated relative to *LacZ*. Error bars show the RQ min and RQ max (n = 3 biological replicates; 10 females pooled per condition and replicate). Percentages show the reduction in transcript expression. Significance was determined by an unpaired student's *t*-test.
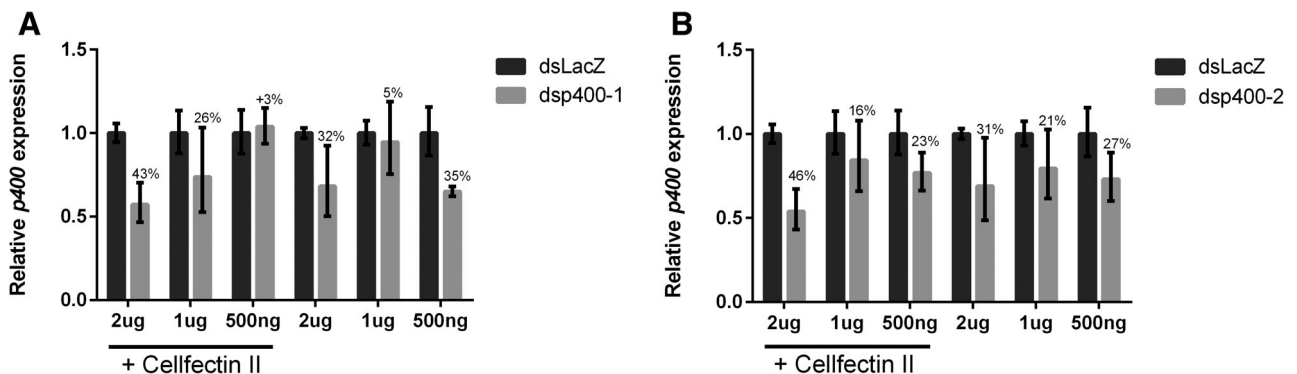

Figure 2. Optimization of p400 knockdown. Various quantities of dsRNA targeting *p400* was injected into female mosquitoes in the presence or absence of Cellfectin II. Knockdown efficiency was determined by RT-qPCR 4 days post injection and results analysed by the method outlined by Taylor *et al*. 2019 (Taylor *et al*., 2019). The group geomean of control *LacZ* was set to 1 and the geomean of *p400* calculated relative to *LacZ*. Error bars show the RQ min and RQ max (n = 3 biological replicates; 10 females pooled per condition and replicate). Percentages show the reduction in transcript expression. Significance was determined by an unpaired student's *t*-test. Two different dsRNAs were tested (A) a short version and (B) longer version of dsRNA.

dsRNA used in (McFarlane *et al*., 2020); Fig. 2B). Again, the presence of Cellfectin II increased the efficiency of knockdown and its reproducibility, however, a high quantity of dsRNA was still required to achieve a detectable knockdown.

2.3 Silencing efficiency is gene and sequence dependent

We previously showed that p400 is a positive regulator of the siRNA pathway in *Ae. aegypti* (McFarlane *et al*., 2020). As gene knockdown relies on the siRNA pathway activity, we therefore wondered whether the difficulty in knocking down *p400* was related to its role in the siRNA pathway function. To investigate this, dsRNA targeting two other genes were tested. A dsRNA targeting *ago2*, which is a critical effector of the exogenous siRNA pathway (Donald *et al*., 2012; Blair and Olson, 2015; Samuel *et al*., 2018), as well as a dsRNA targeting *E75*, a gene that does not have a role in the siRNA pathway but which is a nuclear receptor involved in the steroid response during reproduction in

Ae. aegypti (Cruz *et al*., 2012). These dsRNA were designed to have roughly the same size as dsp400-1 (143 bp for dsago2 and 130 bp for dsE75-1) to exclude any size effect. Different quantities (2 µg, 1 µg and 500 ng) of dsRNA targeting *ago2* were injected into adult female mosquitoes in the presence or absence of Cellfectin II and silencing measured four dpi by RT-qPCR (Fig. 3A). The expression of *ago2* was successfully knocked down with all concentrations we tested irrespective of whether Cellfectin II was included in the injection mix. In contrast to *ago2*, a very low knockdown of *E75* expression was obtained with dsE75-1 irrespective of the addition of Cellfectin II and dsRNA quantities injected, although Cellfectin II did reduce variability between experiments (Fig. 3B) as observed for *p400*. We next tested a second dsRNA sequence targeting another region of E75 (dsE75-2, 108 bp). Using this dsRNA, knock down efficiency was improved (Fig. 3C). Inclusion of Cellfectin II did not enhance knockdown, however, it reduced variability between experiments.
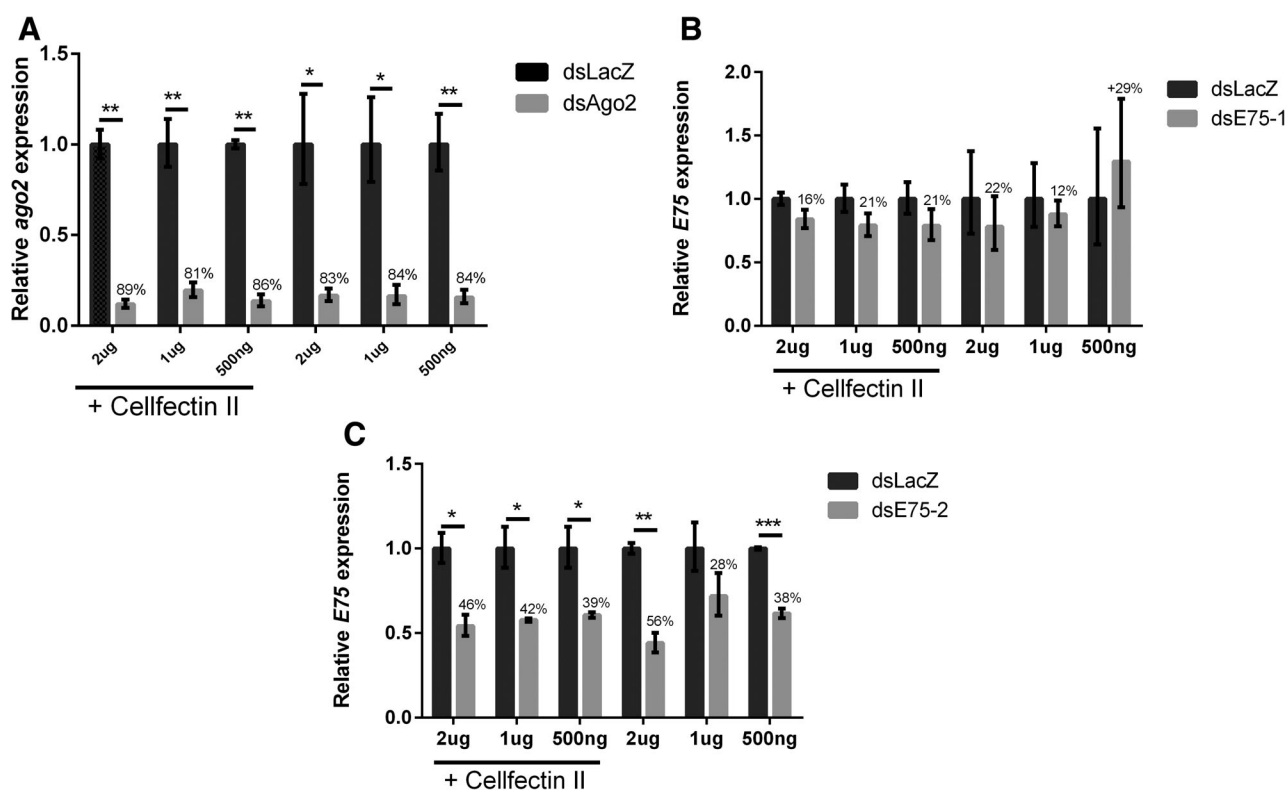

Figure 3. Optimisation of *ago2* and *E75* knockdown. (A) Various quantities of dsRNA targeting *ago2* was injected into female mosquitoes in the presence or absence of Cellfectin II. Knockdown efficiency was determined by RT-qPCR 4 days post injection and results analysed as previously described (Taylor *et al*., 2019). The group geomean of control *LacZ* was set to 1 and the geomean of *ago2* calculated relative to *LacZ*. Error bars show the RQ min and RQ max (n = 3 biological replicates; 10 females pooled per condition and replicate). Percentages show the reduction in transcript expression. Significance was determined by an unpaired student's *t*-test (*P = <0.05; **P = <0.001). (B) and (C) Various concentrations of two different dsRNAs targeting *E75* were injected into female mosquitoes in the presence or absence of Cellfectin II. Knockdown efficiency was determined by RT-qPCR 4 days post injection and results analysed as previously described (Taylor *et al*., 2019). The group geomean of control *LacZ* was set to 1 and the geomean of *E75* calculated relative to *LacZ*. Error bars show the RQ min and RQ max (n = 3 biological replicates; 10 females pooled per condition and replicate). Percentages show the reduction in transcript expression. Significance was determined by an unpaired student's *t*-test (*P = <0.05; **P = <0.001; ***P = <0.0001).

As these are high concentrations of dsRNA, we wanted to test silencing efficiency using a reduced concentration of dsRNA. As *ago2* silencing was highly efficient we reduced the concentration tested in the presence or absence of Cellfectin II (Fig. 4). Female mosquitoes were injected with 100 ng, 200 ng or 300 ng of dsRNA with or without transfection reagent and silencing determined four dpi by RT-qPCR. Reduced quantities of dsRNA targeting *ago2* still resulted in a highly significant knockdown in *ago2* mRNA levels, although the percentage of knockdown was lower compared to experiments with 500 ng to 2 μg (Fig. 3A). Altogether, the results of these experiments indicate that for genes that are difficult to knockdown: (1) increasing the quantity of dsRNA improves silencing, (2) addition of a transfection reagent enhances silencing efficiency by reducing variation between independent replicates and (3) that design of the dsRNA has a greater impact on the effectiveness of the dsRNA than the function of the targeted mRNA.

2.4 siRNA can knockdown expression *in vitro but not in vivo in* Ae. aegypti

Due to the time-consuming nature of production of dsRNA and the possibility of generating "off targets" (Kulkarni *et al*., 2006), we sought to characterize the silencing activity of siRNAs *in vivo*. We first wanted to determine the best purification method for siRNA production, for standard or
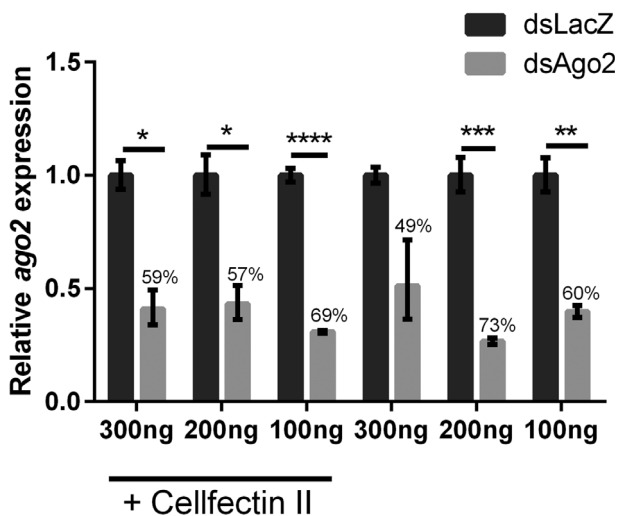

Figure 4. Further optimization of *ago2* silencing. Reduced concentrations of dsRNA targeting *ago2* were injected into female mosquitoes in the presence or absence of Cellfectin II. Knockdown efficiency was determined by RT-qPCR 4 days post injection and results analysed as previously described (Taylor *et al*., 2019). The group geomean of control *LacZ* was set to 1 and the geomean of *ago2* calculated relative to *LacZ*. Error bars show the RQ min and RQ max (n = 3 biological replicates; 10 females pooled per condition and replicate). Percentages show the reduction in transcript expression. Significance was determined by an unpaired student's *t*-test (*$P = <0.05$; **$P = <0.001$; ***$P = <0.0001$; ****$P < 0.00001$).

in vivo applications. To test this, we purchased LacZ-targeting siRNAs synthesised and purified either by the standard or *in vivo* purification protocol. siRNAs resuspended in PBS or PBS alone as control were injected into females (2 μg per female) and survival monitored for three days. Although the injection of siRNAs purified for *in vivo* application resulted in a slightly better survival compared to injection of siRNAs purified for standard application, this was not significant (Fig. S1A). Considering the higher cost of siRNAs purified for *in vivo* applications compared to standard applications, standard purification was therefore chosen for further experiments. We then wanted to ensure that the resuspension solution we used did not adversely affect mosquito survival. PBS is more commonly used for *in vivo* experiments compared to water, however, resuspension of the siRNAs in water allowed us to determine the concentration of the siRNAs to ensure precise and reproducible calculations for our injection mix. We therefore injected adult female mosquitoes with either PBS or water and monitored their survival (Fig. S1B). No difference in survival was detected between conditions, therefore, siRNA resuspension with water was chosen.

Next, we sought to confirm that siRNAs could knockdown transcripts *in vivo*. For this we used siRNA sequences that were previously published (Varjak *et al*., 2017). We first confirmed that the siRNAs could function *in vitro* in our hands. *Ae. aegypti*-derived Aag2 cells were transfected with *ago2*- or control *LacZ*-targeting siRNAs. One day post transfection, cells were lysed and RNA extracted. The level of gene silencing was determined by RT-qPCR. As expected, transfection of *ago2* siRNAs resulted in a 73% knockdown of *ago2* transcript expression (Fig. 5A). As the siRNAs were designed based on the *ago2* sequence deposited on VectorBase, we next confirmed the *ago2* siRNA sequence targeted the *ago2* transcript present in our *Ae. aegypti* Liverpool strain mosquitoes. As the siRNAs worked well *in vitro* and the sequence matched our mosquito colony, we then went on to test the siRNAs *in vivo*. Female mosquitoes were injected with 2 μg of *ago2*- or control LacZ- targeting siRNAs or dsRNAs along with Cellfectin II transfection reagent. Four dpi, mosquitoes were lysed in TRIzol and RNA extracted. Gene expression was determined by RT-qPCR. While the expression of *ago2* was successfully knocked down using dsRNAs, the siRNAs did not silence expression of *ago2 in vivo* (Fig. 5B). We also confirmed that siRNAs did not silence *ago2* expression *in vivo* in the presence of DharmaFECT2 (data not shown).

3. Discussion

Together with genome sequencing, the advent of RNAi opened the door to loss-of-function analyses in many non-model insects for which genetic transformation was
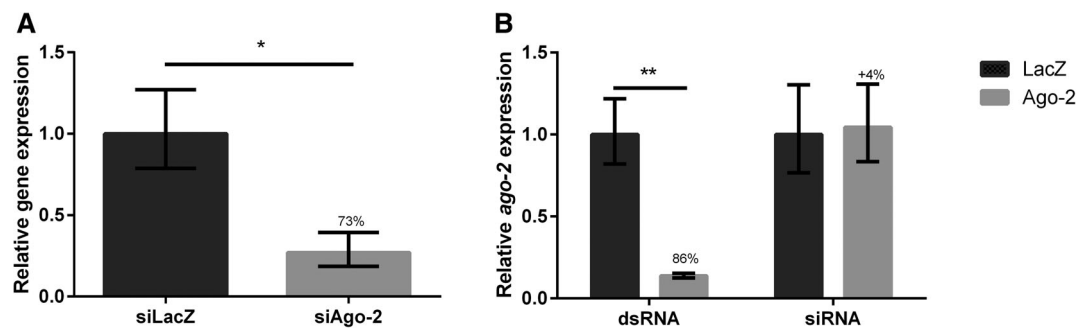
Figure 5. Testing of siRNA-induced silencing. (A) Aag2 cells were transfected with *LacZ*- or *ago2*-targeting siRNAs and the level of silencing determined after 24 h. Knockdown was determined by RT-qPCR and results analysed by the method outlined by Taylor *et al.* 2019 (Taylor *et al.*, 2019). The group geomean of control *LacZ* was set to 1 and the geomean of *ago2* calculated relative to *LacZ*. Error bars show the RQ min and RQ max (n = 4 biological replicates; 3 wells pooled per condition and replicate). Percentages show the reduction in transcript expression. Significance was determined by an unpaired student's *t*-test (*P = <0.05). (B) Female mosquitoes were injected with either siRNA or dsRNA targeting *ago2* or control *LacZ*. Silencing was measured 4 days post injection. Silencing was determined by RT-qPCR and results were analysed as previously described (Taylor *et al.*, 2019). The group geomean of control *LacZ* was set to 1 and the geomean of *ago2* calculated relative to *LacZ*. Error bars show the RQ min and RQ max (n = 3 biological replicates; 10 females pooled per condition and replicate). Percentages show the reduction in transcript expression. Significance was determined by an unpaired student's *t*-test (**P = <0.001).

not possible or could not be routinely achieved (Belles, 2010; Mito *et al.*, 2011). In the last two decades, transient gene silencing has been instrumental to identify many factors involved in mosquito-pathogen interactions such as immune pathways. While transient gene silencing to study gene function in *Ae. aegypti* mosquitoes still remains easier and faster to perform than genome editing, some genes appear to be more difficult to knockdown than others, and this can hamper phenotype analysis. Here we have shown that for some genes that are difficult to silence using classical doses of dsRNA, increasing the injected quantity of dsRNA up to 2 μg per female can improve silencing efficiency. Addition of a cationic lipid transfection reagent does not necessarily enhance knockdown but does improve the efficiency by reducing the variation between replicates. Importantly, inclusion of the transfection reagent, here Cellfectin II, did not significantly reduce mosquito survival. There was, however, a small reduction in survival when another transfection reagent, Dharma-FECT 2, was included. This reduction was not significant and would not have prevented the inclusion of this reagent in transfection mixes if it had performed better in knockdown studies. As injection of some transfection reagents, such as FuGENE6, can be lethal for *Ae. aegypti* mosquitoes (Cheng *et al.*, 2011), this shows that reagents should be tested before use, to ensure a beneficial impact which is not negated by any small reduction in mosquito survival.

Our results show that the difficulty to knock down p400 was likely not due to its involvement in the siRNA pathway, which is exploited to silence genes. Indeed, targeting of another RNAi pathway component, *ago2*, resulted in significant knockdown with relatively small dsRNA quantities (down to 100 ng), consistent with previous studies using dsRNA targeting *ago2* and *dcr2* in *Ae. aegypti* and *An. gambiae* (McFarlane *et al.*, 2014; Carissimo *et al.*, 2015).

A non-RNAi pathway-related gene, E75, was also difficult to knockdown confirming that gene involvement in the siRNA pathway is not a determining factor in its silencing efficiency. siRNA produced from dsRNA sequences can have different efficiencies (Horn and Boutros, 2010). Different dsRNA sequences designed using E-RNAi (Horn and Boutros, 2010) were tested for *p400* (three sequences, one not shown) and *E75* (two sequences). We could improve efficiency for *E75* silencing (using dsE75-2 compared to dsE75-1), showing that knockdown efficiency can be sequence dependent. The predicted number of efficient siRNAs that can be produced from the dsRNAs (104 for dsE75-1 and 90 for dsE75-2) cannot explain a better efficiency of dsE75-2. However, as E-RNAi uses score methods developed from studies in human cells, it cannot be excluded that there are differences in the parameters influencing siRNA efficiency between organisms. Alternatively, the dsRNA structure could affect Dcr2 specificity and efficiency resulting in different siRNA production yields (Vermeulen *et al.*, 2005). Since we did not sequence small RNAs produced in injected mosquitoes, it is not possible to know if dsE75-2 gives rise to more siRNAs or more potent siRNAs compared to dsE75-1.

It is likely that difficulty in knocking down some genes is not only linked to the dsRNA sequence as even using different sequences, knockdown efficiency could not be greatly improved. Different factors have been previously identified or hypothesized to affect RNAi efficiency in insect species, such as the presence of nucleases, the length and concentration of dsRNA, and dsRNA uptake (Belles, 2010; Joga *et al.*, 2016). The presence of dsRNAses in the haemolymph can decrease silencing efficiency and explain the RNAi insensitivity of some insect species (Garbutt *et al.*, 2013; Shukla *et al.*, 2016; Wang *et al.*, 2016). However, considering that some genes, such as *ago2* in this

study, can be silenced using low quantities of dsRNA, it seems unlikely that some dsRNAses highly degrade dsRNA in the haemolymph in *Ae. aegypti* adult females. The length of the dsRNA was also reported to be a potential determinant of whether a dsRNA functions efficiently (Miller *et al*., 2012; Joga *et al*., 2016). Although there had been few studies looking at the dsRNA length requirement in insects, design of long dsRNAs (between 100 and 700 bp) is generally preferred as this increases the probability of producing efficient siRNAs generated by Dcr2. However, longer dsRNA could potentially lead to more off target effects and therefore false phenotypic effects (Kulkarni *et al*., 2006). It has been shown that shorter dsRNA are more easily taken up into cells than longer strands, however, this does not mean that the RNAi pathway is efficiently activated, in contrast, it was reported that longer dsRNA provided a stronger and more prolonged activation of the RNAi response in *Tribolium castaneum* (Miller *et al*., 2012). The length of dsRNA was not found to be important for the silencing efficiency in this study. Indeed, a similar *p400* silencing rate was obtained using dsRNA of different sizes (149 and 317 bp) and using dsRNA of roughly the same size to target *p400* (149 bp) and *ago2* (143 bp), respectively, led to different gene silencing efficiencies.

The difference between gene knockdown efficiency could instead be due to differences in tissue-specific expression of targeted genes. Indeed, RNAi can be inefficient in some tissues and can vary according to developmental stage and species (Belles, 2010). For instance, injection of dsRNA in *D. melanogaster* larvae does not trigger RNAi in most tissues except the haemocytes (Miller *et al*., 2008). It is possible that the RNAi machinery is not highly expressed in some tissues, as observed in *An. gambiae* salivary glands (Boisson *et al*., 2006), and this may affect the silencing efficiency of transcripts according to the tissues in which they are expressed. However, studies in *D. melanogaster* highly suggest that the poor efficiency of RNAi in some tissues is rather due to a low penetration of dsRNA (Miller *et al*., 2008). Similarly, contrary to midgut cells and ovaries, salivary glands could not uptake siRNAs in *An. gambiae*, suggesting that this tissue could also be more resistant to dsRNA uptake, consistent with the requirement to inject high quantity of dsRNA to achieve gene silencing in this tissue (Boisson *et al*., 2006). We have previously shown that *p400* is highly expressed in ovaries and p400 protein is highly detected in tracheae as well as in the germline cells within the germarium (McFarlane *et al*., 2020). Therefore, it is possible that silencing in those particular cells may be more difficult to achieve due to a weak dsRNA uptake, even in presence of liposomes. As some dsRNAses can be expressed in a tissue-specific manner and limit RNAi efficiency in *Ae. aegypti* larvae (Giesbrecht *et al*., 2020), it is also possible that genes

difficult to knockdown are expressed in adult female tissues expressing dsRNAses.

The difficulty to observe a satisfying silencing four days post dsRNA injection for genes such as *p400* and *E75* could also be linked to efficient feedback mechanisms regulating gene expression. As a consequence, it would reduce the long-lasting effect of gene silencing as observed in *Blatella germanica* when targeting a lipophorin receptor (Ciudad *et al*., 2007) and also in *Ae. aegypti* when targeting *Cactus* (Raquin *et al*., 2017). It would thus be interesting to analyse transcript expression levels of targeted genes at earlier and later time points post dsRNA injection to assess whether silencing efficiency could change over time.

Finally, the ability of siRNA to silence genes in *Ae. aegypti* females was studied because if demonstrated to be effective, siRNA-induced silencing could prove to be a more time- and cost-effective method of transient silencing. Unfortunately, in our hands the siRNA targeting *ago2*, even when injected along with a transfection reagent, did not silence their target *in vivo* while it did *in vitro*. Our results contrast with reports of successful gene silencing using siRNA injection in *Ae. aegypti* pupal brain (Tomchaney *et al*., 2014) and embryos (Clemons *et al*., 2011). Absence of silencing using siRNA was also shown in *T. castaneum* (Miller *et al*., 2012) and the nematode *Caenorhabditis elegans*. As previously observed in *T. castaneum* (Miller *et al*., 2012) and in *An. gambiae* salivary glands (Boisson *et al*., 2006; Miller *et al*., 2012), there may be a tissue permeability issue where the shorter dsRNAs/siRNAs are not taken up by the target tissues. This would explain why siRNAs can efficiently silence genes in embryos (injection of siRNAs before cellularization) or in pupae (destruction of larval tissues, formation of adult ones and transformation of larval to adult tissues). Alternatively, the lack of silencing could be due to the degradation of the siRNAs by stage- and/or tissue-specific RNAses. Loss of nucleotides from the siRNA would impact recognition and/or uptake by Ago2 and therefore the siRNA pathway would not be activated.

Transient knockdown of target transcripts is an important method of investigating gene function. In this study, we found that transient knockdown using siRNA was not possible in adult mosquitoes. While for some targets, successful and significant knockdown can be achieved using dsRNA, some others can be harder to silence. In case, a dsRNA injected at a classical concentration (*e.g.* 500 ng) does not result in a satisfying silencing efficiency, we propose a set of guidelines to improve silencing efficiency. First, a second dsRNA should be designed and further tested. Second, higher doses, up to 2 μg per female should be tested. Finally, a cationic transfection reagent can be added to enhance knockdown through reduction in variation between replicates. Although transient gene silencing

by RNAi is not tissue-specific nor permanent compared to genome editing, until genetic tools and reagents are developed with the same ease and availability as for *D. melanogaster*, this technique remains the easier and faster option for identification and functional characterization of mosquito genes. This technique is particularly advantageous to screen many genes for their involvement in arbovirus infection in mosquitoes. Therefore, these guidelines will benefit such analyses to increase our knowledge of mosquito-arbovirus interactions.

4.   Experimental Procedures

4.1   Mosquito rearing

Ae. aegypti Liverpool strain (a gift from E. Devaney, University of Glasgow, UK) was reared at 28°C and 80% humidity with a 12:12 light photoperiod. Larvae were reared in water and fed on dry cat food from larvae hatching to the pupal stage. The emerging adult mosquitoes were transferred in cages with unlimited access to 10% weight/vol sucrose solution. Female mosquitoes were fed on heparinised rabbit blood (Orygen Antibodies Ltd) using a Hemotek system (Hemotek Ltd, UK).

4.2   Cell culture

Ae. aegypti-derived Aag2 cells (a kind gift of P. Eggleston, Keele University, UK) were maintained in Leibowitz L-15 medium supplemented with 10% fetal bovine serum (FBS, Gibco), 10% tryptose phosphate broth (TPB, Gibco) and penicillin–streptomycin (Gibco). Mosquito cells were maintained at 28 °C.

4.3   dsRNA design, synthesis and purification for mosquito injection

Total RNA was extracted with TRIzol (Thermo Fisher Scientific) from whole NBF *Ae. aegypti* females according to manufacturer's instructions with 1-bromo-3-chloropropane (Sigma) in place of chloroform and including DNase treatment (TURBO DNase, Ambion). cDNA was generated from 1 μg of total RNA using MMLV retro-transcriptase (Thermoscientific) and random hexamers pDN6 (Invitrogen). cDNA was used for production of the dsRNAs targeting *p400*, *E75* and *ago2* genes and the plasmid template *Drosophila*act5C-βGal (Stock number 1220 obtained from DGRC) was used for dsLacZ (used as control dsRNA). dsRNA were produced as previously described (McFarlane *et al*., 2020) using gene-specific primers with T7 RNA polymerase promoter sequence (Table S1). dsp400-1, dsago2, dsE75-1 and dsE75-2 (both targeting all E75 isoforms) were designed using the E-RNAi webservice (https://www.dkfz.de/signaling/e-rnai3/0) and to roughly have the same size to exclude size effect.

dsp400-2 was designed previously (McFarlane *et al*., 2020).

4.4   siRNA design, production and preparation

The siRNA targeting *ago2* (siago2) was designed based on the previous study in *Ae. aegypti*-derived cell line (Varjak *et al*., 2017). The siRNA targeting βGal (siLacZ, used as control) was designed by Dharmacon (# D-002000-01-20). Sequences of siRNAs can be found in Table S2. siRNAs were produced and purified for either standard or *in vivo* application (Dharmacon). siRNA for standard application were provided as desalted duplex ready to use after resuspension. siRNA for *in vivo* application were processed by counter-ion (Na+) exchange, desalted, filtered sterilely and tested for endotoxin. siRNAs were resuspended in nuclease-free PBS or water following Dharmacon's instructions, aliquoted and stored at −20 °C.

4.5   Sequencing

To check that the siago2 sequence matched the *Ae. aegypti* Liverpool strain used in this study, cDNA from whole *Ae. aegypti* females was used to amplify the corresponding gene region by PCR using KOD HOT Start master mix (Sigma) and specific primers (Table S1). The PCR fragment was gel purified using QIAquick gel extraction kit (Qiagen) and ligated in pJET plasmid (Thermo Scientific) and transformed in JM109 competent bacteria (Promega). Transformed bacteria were cultured overnight in LB media supplemented with ampicillin. Plasmid DNA was extracted from colonies using QIAquick miniprep kit (Qiagen). Sequencing of the insert was performed by Source Bioscience. Sequence matching with the siRNA sequence was analysed on Serial Cloner software v2.6.1.

4.6   dsRNA and siRNA injection in mosquitoes and sampling

At 1–2 days after emergence, cold-anesthetized female mosquitoes (n = 15–20 per condition and replicate) were injected using a nanoinjector (Nanoject II, Drummond Scientific). Cellfectin® II (Thermofisher) or DharmaFECT2 (Horizon Discovery) transfection reagent was mixed with S2 medium (Life Technologies) (1:1, vol:vol). After 5 min of incubation, this mix was added to dsRNA/siRNA solution (1:1, vol:vol) previously adjusted with S2 medium to give the required quantity of dsRNA/siRNA per female/injection volume. The injection solution was then incubated for 15 min at room temperature before injection. For conditions without transfection reagent, the dsRNA solution was adjusted with S2 medium to give the required quantity of dsRNA/siRNA per female/injection volume. After injection, mosquitoes were left to recover in netted cardboard boxes with cotton balls wet with 10% sucrose. To decrease bacterial infection risk post-injection, injected mosquitoes were

left at 21 °C and 80% of humidity for 24 h and then moved to 28 °C and 80% of humidity. Four days post-injection, 10 mosquitoes were homogenized in 1 ml of TRIzol reagent (Thermo Fisher Scientific) with glass beads using the Precellys 24 homogenizer (Bertin Instruments) before RNA extraction.

4.7   Analysis of mosquito survival

The effect of injection with different reagents on mosquito survival was studied by injecting 40–50 mosquitoes per condition and replicate. The same final volume (414 nl) was injected for all conditions. When using dsRNA or siRNA, the same quantity (2 μg) was also injected for all conditions. Survival was monitored for 3 days by counting and removing by aspiration dead mosquitoes.

4.8   Cell transfection and sampling

1.7×10^5 Aag2 cells were plated out in 24 well plates. The following morning, *LacZ* and *ago2* targeting siRNAs (5 ng/μl final) were transfected into the Aag2 cells using Dharma-fect2 (Horizon Discovery) according to manufacturer's protocol. siRNA-treated cells were sampled 1 day after transfection. Cells were centrifuged at 4 °C and 400×*g* for 10 min. Cell media were then replaced with 1 ml of TRIzol (Thermo Fisher Scientific) for further RNA extraction.

4.9   RNA extraction and reverse-transcription

Total RNA from siRNA-treated cells and whole females was extracted with TRIzol (Thermo Fisher Scientific) following manufacturer's instructions with 1-bromo-3-chloropropane (Sigma) in place of chloroform and a DNAse treatment (TURBO DNase kit, Ambion). cDNA was generated from 1 μg of total RNA in 40 μl through reverse-transcription (RT) using the MMLV Reverse Transcriptase (Thermoscientific) and random hexamers pDN6 (Invitrogen) according to manufacturer's protocol. For each sample, 3 independent RTs (further pooled) and 1 negative reverse-transcription control (NRT) were prepared. The NRT includes all reagents except the reverse-transcriptase which was replaced by water. cDNAs were stored at −20 °C into 2.8 μl aliquots for further qPCR analysis.

4.10   qPCR analysis

qPCR was performed using 2.8 μl cDNA aliquots with FAST SYBR Green master mix (Applied Biosystems) according to manufacturer's instructions and using the 7500 Fast PCR Machine (Applied Biosystems). Primers used to check the knock down efficiency were designed to be outside of the dsRNA regions to avoid dsRNA amplification and are listed in Table S1. Results were treated with the 7500 Software v2.0.6 and analysed as described previously (Taylor *et al.*, 2019). The ribosomal S7 transcript

was used as reference to normalized expression values, and dsLacZ control group geomean was set at 1. The RQ minimum and maximum were calculated from the geomean and displayed as error bars. For each experiment, data were obtained from 3 biological replicates and 10 females pooled per condition and replicate.

4.11   Statistical analysis

Statistical analysis was conducted using Graph Pad v7.02 software. Mosquito survival analyses were conducted with a survival curve and Log-rank Mantel-Cox test. Analysis of qPCR data was conducted using unpaired and two-tailed *t*-tests on Log2-normalized expression values according to Taylor's method (Taylor *et al.*, 2019).

Author Contributions

M.M: Data curation, Formal Analysis, Investigation, Methodology, Supervision, Validation Visualization, Writing – original draft, Writing – review & editing. M.L: Data curation, Formal Analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. T.L: Data curation, Formal Analysis, Investigation, Validation. S.T: Resources. A.K: Funding acquisition, Writing- review & editing. E.P: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing.

Conflicts of Interest

The authors declare no conflict of interest.

Data Availability

The datasets generated and analysed during the current study are available in the University of Glasgow repository (https://doi.org/10.5525/gla.researchdata.1082).

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1: Analysis of female survival. (A) Female mosquito survival for 4 days after injection of either siRNA lacZ purified for standard or *in vivo* application or PBS. Survivals were not significantly different using a Log-rank Mantel-Cox test. (B) Female mosquito survival for 4 days after injection of either PBS or nuclease free water. Survivals were not significantly different using a Log-rank Mantel-Cox test.

Table S1: Primers used for dsRNA synthesis, to check knockdown efficiency and to sequence *ago2* region targeted by siago2.

Table S2: siRNA sequences and position within target gene.