# Evaluation of non-invasive dsRNA delivery methods for the development of RNA interference in the Asian tiger mosquito Aedes albopictus

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**Research Article**

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# Abstract

The Asian tiger mosquito *Aedes albopictus* is one of the most invasive species and an efficient vector of several pathogens. RNA interference (RNAi) has been proposed as an alternative method to control mosquito populations by silencing the expression of genes that are essential for their survival. However, the optimal delivery method for dsRNAs to enhance an optimal RNAi remains elusive and comparative studies are lacking. We have, therefore, compared the efficiency of three non-invasive delivery methods to mosquito larvae: soaking, rehydration and nanoparticle ingestion. Each method was tested separately on four genes predicted to code non-essential proteins (*i.e. collagenase*-like, *kynurenine 3-monooxygenase*-like, *yellow*-like and *venom serine protease*-like) in order to be able to compare the importance of gene knock-down.

All tested methods successfully downregulated mosquito gene expression. However, silencing efficiency strongly varies among methods and genes**.** Silencing (95.1%) was higher for *Kynurenine 3-monooxygenase*-like with rehydration and nanoparticle ingestion (61.1%). For the *Venom serine protease*-like, the most efficient silencing was observed with soaking (74.5%) and rehydration (34%). In contrast, the selected methods are inefficient to silence the other genes. Our findings also indicate that gene copy numbers, transcript sizes and GC content correlate with the silencing efficiency.

From our results, rehydration was the most specific and efficient methods to specifically knock-down gene expression in *Ae. albopictus* larvae. Nevertheless, considering the observed variability of efficiency is gene-dependent, our results also point at the necessity to test and optimize diverse dsRNA delivery approaches to achieve a maximal RNAi efficiency.


# Key Message

- RNA interference (RNAi) of essential genes has been proposed to help basic research and insecticides production.
- Optimal noninvasive delivery methods of dsRNA to induce RNAi still needs to be determined.
- In this study we showed that an osmotic stress can increase the RNAi efficiency.
- Genes characteristics (copy numbers, transcript sizes and GC content) correlated with the silencing efficiency.

# 1. Introduction

RNA interference (RNAi) is a conserved mechanism in plants, animals and fungi that is primarily involved in innate antiviral immunity [1]. This biological process is initially triggered intracellularly by double stranded RNAs (dsRNAs), especially originating from hairpin structures, transposons or viruses [2]. In insects, three major RNAi pathways have been identified and classified: the small interfering RNA (siRNA), the micro RNA (miRNA), and the piwi-interacting RNA (piRNA) pathways [3]. An incredibly useful RNAi application, mainly based on the siRNA pathway, involves the introduction of dsRNA or shRNA sequences

homologous to endogenous transcripts to knock-down the expression of targeted genes. Once dsRNA are delivered into the insect body, the cellular uptake can be either *via* transmembrane channel or *via* clathrin-mediated endocytose [4]. After this internalization, the ribonuclease complex Dicer turns dsRNA into short fragments, the siRNA [5]. To induce gene silencing, those siRNA are cleaved into single strand RNA and conducted to the target mRNA by proteins of the RISC complex [6]. Finally, the target mRNA that has hybridized to the interfering RNA is cleaved within the hybridizing region inducing gene silencing [7]. This technic has been involved in many application fields such as agriculture, human therapy and pest management [8–10].

Many reports advocate the potential role of RNAi to develop novel and specific biological insecticides [11–15]. For instance, Muller et al. has recently discussed potential RNAi applications for fields control of the Asian tiger mosquito *Aedes albopictus* [16]. This species is one of the most invasive species in the world as well as a major vector of human and animal pathogens such as dengue and Chikungunya viruses or filarial nematodes like *Dirofilaria immitis* [17, 18]. Currently, those mosquito-borne diseases are a major threat to public health due to limitations in treatments and vaccines [19]. Mosquito population control remains the preferred approach to prevent disease outbreaks [20]. Management of *Ae. albopictus* populations mostly relied on the mechanic destruction of small containers of standing water that constitutes privileged larval habitats and partly on chemical insecticides [21]. However, due to the impact of insecticides on non-targeted species and the spread of resistances within mosquitoes populations (including behavioral adaptations), alternative biological and genetic approaches were developed [22]. Among those techniques, the sterile insect technique (SIT), involving the release of irradiated sterilized male mosquitoes and the Release of Insects carrying a Dominant Lethal (RIDL) involving the release of genetically modified male mosquitoes, have both already been used successfully in the field [23, 24]. Although those techniques are very promising, there is still a room for improvement because they cannot be used all over the world easily due to challenges related to the methods efficiency, acceptance of genetically modified insects and heavy production processes. The use of microbial insecticides such as the larvicidal bacteria *Bacillus thuringiensis israelensis* (Bti) [25] has also been shown as a promising alternative to chemicals but it also affects non-targeted organisms and may induce adverse effects on local biodiversity [26]. In this context, RNAi has been presented as a potential biological alternative, although many challenges still remain to be lifted up before fields applications. Indeed, the basic knowledge concerning RNAi efficiency for insects remains insufficiently documented due to the lack of comparative studies [11–15].

Currently, the RNAi efficiency for gene silencing is considered to be very variable among insect species [3]. Therefore, the literature is biased in favor of Coleoptera that are associated with a high RNAi efficiency meanwhile other insect orders such as Diptera have been less documented [27]. The first step for RNAi optimization is to identify suitable targets and to design associated dsRNA. The gene, the sequence of hybridization and the absence of off-target sequences on the host transcriptome for which unspecific hybridizations may occurs should be assessed to design dsRNA [28]. The dsRNA size varies between 150 bp and 500 bp [29]. The second step for RNAi optimization is to identify the most suitable dsRNA delivery method. Microinjection is the most widely used method across insect models [30] and involves pricking

and directly delivering dsRNA in the insect hemocoel. However, the survival rate after microinjection has been estimated to be very low for some insects, including mosquito larvae (5.08%) and pupae (3.6%), although it is more suitable for embryos (44%) and adults (60%) [31]. In addition, microinjection is not suitable for environmental applications and non-invasive methods appear to be more promising from this perspective [16]. This prompted to develop milder and non-invasive delivery methods either active such as ingestion [32], or passive such as soaking [33]. These methods can also be coupled to carriers such as liposomes, nanoparticles or microorganisms [34–37] to improve dsRNA stability and uptake.

Although RNAi delivery methods have already been investigated on mosquitoes [38], there are no comparative studies, hindering the establishment of efficient pest control methods. Here, we compared the efficiency of various non-invasive delivery methods combined with different target genes (*collagenase*-like gene, *kynurenine 3-monooxygenase*-like gene, *yellow*-like gene and *venom serine protease*-like gene). Those genes were selected to optimize the RNAi delivery method and test its influence on gene extinction since they are expressed constitutively along the mosquito development and their expression is not mandatory for the individuals survival. The first method named “soaking” consists in exposing the larvae to dsRNA by delivering the molecule within their water habitat. The second method named “rehydration” consists in performing an osmotic shock with NaCl before soaking the larvae into water supplemented with dsRNA in order to stimulate their internalization. The third method named “nanoparticles” consists in complexing the dsRNA with chitosan nanoparticles before providing the Np-dsRNA complex within the larval food. Because we wanted to quantify precisely the gene silencing efficiency in control conditions, we decided to knockdown only non-essential genes. We specifically addressed three objectives: (i) to assess non-invasive methods efficiency, (ii) to identify their potential side effects and (iii) to identify whether gene-specific characteristics (transcript size, GC content, copy number) are correlated with the RNAi efficiency.

# 2. Material and Methods

# 2.1. Mosquito rearing

All experiments were performed on a F22 mosquito laboratory strain of *Ae. albopictus* referred as AealbVB. This population derived from adult individuals collected in 2017 in Villeurbanne and Pierre-Bénite (France). Adult mosquitoes were raised at 28°C in a BSL2 insectarium with an 18h:6h light and dark photoperiod and fed 10% sugar water. Female mosquitoes were engorged on anesthetized mice (the protocol was reviewed by the Institutional Animal Care and Use Committee, acceptance reference number: Apafis #31807-2021052715018315) to allow subsequent egg-laying. Eggs were collected on a blotting paper partially immerged on water. Egg hatching was synchronized in nuclease free sterile water (GIBCO, USA) using a *vacuum* bell at -20Hg for 3h at 28°C. Experiments were conducted in 6-well plates with 5 mL of nuclease-free water. Larval food was provided in the form of a Ø1cm sterile plug made of 2.5% grinded TetraMin tropical fish food embedded in a 1% Agar gel. A total of 14 ± 2 first instar larvae were reared in each well, with 10 replicates per condition (described in the following paragraphs), randomly distributed across the wells.

## 2.2. Target gene selection

The target genes have been selected from available larval transcriptomes of *Ae. albopictus* [39]. Candidate genes were selected according to the following criteria: (i) no predicted vital functions, (ii) constitutive and high expression levels across mosquito larval development, (iii) limited probability to obtain off-targets. Off-targets and gene copy numbers were predicted for each targeted gene based on the NCBI genome browser using the latest *Ae. albopictus* genome version (GCF_006496715.1, released in 2019). Gene copy number refers to identical sequences identified *in silico* in different genomic loci. Off-targets are defined as a perfect 16 nt match between dsRNA and another gene transcript, or an almost perfect match (> 80% identity) for a sequence of at least 26bp [40]. Copy numbers and off-targets related to the selected genes, along with the loci number from the reference genome, are provided in **Table S1**. Since it was impossible to obtain genes with a perfect dissimilarity with the rest of the transcriptome, we selected genes for which designed dsRNA sequences would produce little off targets, but used this opportunity to then evaluate the impact of the number of off-targets on RNAi efficiency. Among all our candidate genes, we selected four genes for their broad and independent functions within the insect biology, namely *yellow*-like, *venom serine protease*-like, *collagenase*-like and the *kynurenine 3-monooxygenase*-like. Hereafter, the target genes will be referred respectively using the following abbreviations: YEL, VEN, COL and KYN, respectively.

## 2.3. RNA extraction and cDNA synthesis

Total RNA from mosquito larvae was extracted using TRIzol (Invitrogen, USA) and adapted from a previously published protocol [41]. Briefly, five larvae were placed in a 2 mL tube containing ∅1.0mm glass beads with 200 µL of TRIzol reagent before being crushed at 6 m/s for 20 s in a FastPrep-24 homogenizer (MP Biomedicals, Germany). Then, 800 µL of Trizol reagent and 200 µL of chloroform were added. Samples were vortexed for 30 s, left 5 min at room temperature, vortexed for 3 min and then centrifuged for 30 min at 13,000g. The collected aqueous phase was mixed up with one volume of isopropanol and stored overnight at -20°C. The solution was then centrifuged for 30 min at 13,000 g, then washed with 1 mL of ethanol before being centrifuged again. After removing the supernatant, dried RNA was resuspended in 40 µL of nuclease-free water. A DNAse treatment was performed with the TURBO DNAse kit (Invitrogen, USA) following the manufacturer's recommendations. After RNA was quantified using the NP80 Nanodrop (Implen, Germany), reverse transcription was performed using the iScript cDNA Synthesis Kit (Biorad, France) with 500 ng of purified RNA and 4 µL of a 5X reactions mix, 1 µL of reverse transcriptase and nuclease-free water to reach a 20 µL total volume. The mix was incubated at 25°C for 5min, 46°C for 20min and 95°C for 1min.

## 2.4. PCR reactions and dsRNA synthesis

The dsRNA synthesis for the target genes was performed using a two-step *in vitro* process based on a previously published protocol [42]. First, target genes were amplified from total mosquito larvae RNA by RT-PCR using primers flanked with the T7 promotor (see primer details on **Table S1**). The GFP dsRNA used as control was amplified from a pCR2.1 cooling plasmid. Amplifications were performed using High

Fidelity PCR EcoDry (Takara, USA) and conducted on the T100 Thermal Cycler (Biorad, USA) with the following cycle: 95°C for 1 min, 35 cycles at 95°C for 30 sec and 68°C for 1 min, and finally 68°C for 1 min. PCR products were separated on a 1% agarose gel and purified with the QIAquick Gel Extraction Kit (Qiagen, Germany) following manufacturers recommendations and quantified with the NP80 Nanodrop. Afterwards, dsRNAs were synthesized with the MEGAscript RNAi Kit (Invitrogen, USA). Briefly, from 500 ng to 1 µg of purified PCR products were mixed with 2 µL of each dNTP (75 mM), 2 µL of T7 enzyme mix, 2 µL of 10X transcription buffer and nuclease-free water up to 20 µL total volume. The mix was incubated at 37°C for 16 h for transcription, then at 75°C for 5 min. The dsRNAs were then treated with DNAse for 1h at 37°C, then purified on silica columns following manufacturers recommendations and quantified using the NP80 Nanodrop. Double-stranded RNA integrity was assessed on a 1% agarose gel electrophoresis by loading 5 µL of the diluted (1/100) synthesis product. Finally, the purified dsRNAs were stored at -80°C until use no later than one week after being prepared.

# 2.5. dsRNA delivery methods

Three different dsRNA delivery methods were tested and adapted from previously published protocols [43–45]. The experimental design is summarized in Fig. 1. Protocols have been adapted to expose larvae to the same dsRNA quantity (2 µg/mL) for each method and to fit *Ae. albopictus* rearing requirements. The first protocol (soaking) was conducted by adding dsRNA directly into the larval rearing water before inoculating larvae into the well. For the second protocol (rehydration), first-instar larvae were exposed to an osmotic stress before dsRNA was added into the larval rearing water. To that end, larvae were immerged 30min in a 1.5% NaCl solution. The last protocol (nanoparticles) was performed by complexing dsRNA with chitosan nanoparticles through electrostatic interactions. Chitosan from shrimp shells (Sigma-Aldrich, USA) was dissolved in sodium acetate buffer (0.1 M) to make a 0.02% suspension, while dsRNA was separately mixed with sodium sulfate buffer (50 mM) to make 1 mL of a 0.032% suspension. Both suspensions were then mixed equally (to provide the adequate dsRNA quantity), heated at 55°C for 1 min and immediately vortexed for 30s. The mixture was then centrifuged at 13,000g for 10min and the nanoparticles were obtained from the pellet. dsRNA-chitosan coupling efficiency was measured through dsRNA quantification of the supernatant and showed an average of 92.7% coupling efficiency (between 88.7% and 98.1%). Resulting nanoparticles were then incorporated into the preparation of food plugs. Each method was tested on 10 biological replicates (pool of five larvae from the same cohort) for each target, a negative control (GFP dsRNA) and a blank condition ($H_2O$ without dsRNA).

# 2.6. Gene silencing estimation by qPCR

The gene silencing was estimated through RT-qPCR, using two reference genes for normalization (RPS17 and RPL32) previously identified in *Ae. albopictus* larvae [46]. The target genes were quantified using different primers (see **Table 1** for details) designed either in the dsRNA site or outside. Total RNA from larvae was converted into cDNA as previously described [41] and samples were then diluted with 4 volumes of ultrapure water. Finally, mixtures of 12 µL reaction volumes were obtained with 1 µL of sample, 1 µL of each primer (10 µM) and 9 µL of SYBR Green iTaq (Bio-Rad). All qPCR reactions have

been performed in the MicroAmp Optical 384-wells using the QuantStudio 7 Pro Real-Time PCR machine (Thermo Fisher Scientific, USA) with the following cycle: a 3 min initial denaturation at 95°C, then 40 cycles with 5 s of denaturation at 95°C and 1min of hybridization and amplification at 60°C. The gene silencing measurement was finally estimated using the $2^{-\Delta\Delta Ct}$ method adjusted with PCR efficiency [47].

## 2.7. Statistical analyses

All the statistical analyses have been conducted using the R software v.3.3.0. Larval survival was analyzed using non-parametric Kruskal-Wallis test and a pairwise Mann-Whitney Wilcoxon *post-hoc* analysis with the Bonferroni correction for multiple comparisons. Expression of target genes was compared between larvae not exposed to dsRNA, exposed to GFP dsRNA or exposed to specific dsRNA (see **Table 2** for details). The effect of non-invasive delivery of dsRNA on gene expression levels was estimated by comparing exposure of larvae to GFP dsRNA (dsRNA control) to larvae that were not exposed to dsRNA ($H_2O$ control). A linear model involving additive effects and interactions was performed using the *lme4* R package. Gene expression level (defined with the $2^{-\Delta\Delta Ct}$ method) was considered as the response variable and dsRNA delivery method, genes as well as dsRNA treatments were defined as explanatory variables. The influence of fixed factors was evaluated with a type II ANOVA followed by Tukey-HSD pairwise *post-hoc* tests implemented in the *car* and *emmeans* R package. When appropriate, the effect size was provided as a t-ratio that represents the difference in means between two groups divided by the standard errors of the difference. Finally, RNAi efficiencies were correlated with either the relative gene expression, predicted off-target number, mRNA size or the gene copy number, using a non-parametric Spearman correlation test.

# 3. Results

## 3.1. Involvement of the target gene and the delivery method in RNAi efficiency

After dsRNA provision targeting the selected four genes, COL, KYN, YEL and VEN, efficiency of the different dsRNA delivery methods was estimated though RT-qPCR using primers designed in the dsRNA hybridizing domain (Fig. 2.**A**). Only one of the sixteen tested conditions successfully induced knockdown, namely KYN, with nanoparticles delivery ($p = 0.0294$) although a strong tendency was also observable when the rehydration delivery approach was used ($p = 0.0510$). Surprisingly, in the soaking delivery condition 3 out of the 4 target genes were overexpressed in comparison with dsGFP ingestion. Based on those results, we hypothesized that this apparent overexpression could result from a bias due to dsRNA detection in RT-qPCR. To overcome this technical difficulty, we decided to test primers that hybridizes out of the dsRNA hybridizing domain (Fig. 2.**B**). Hereafter, 4 conditions successfully induced knockdown and all delivery methods were able to knockdown at least one target gene. A statistical model showed that qPCR primer design, type of dsRNA and delivery methods significantly influence the gene expression level, and all those factors interact between each other (**Table 2**). The maximum gene silencing (95.1%, $p < 0.0001$, t-ratio = 4.234) was observed for KYN through rehydration. The same gene was also silenced

(61.1%, $p = 0.0018$, t-ratio = 2.936) significantly through nanoparticle ingestion although soaking delivery was also almost significant (36.9%, $p = 0.0664$, t-ratio = 1.506). The second most efficient gene silencing (74.5%, $p = 0.0077$, t-ratio = 2.435) was observed for VEN through soaking. For this gene, silencing was also obtained through rehydration (34.1%, $p = 0.0201$, t-ratio = 2.059) while nanoparticles coupled dsRNA did not succeed to decrease the expression levels ($p = 0.9992$, t-ratio = -3.181). Finally, the efficiency of RNAi strongly varied depending on the targeted genes since none of the delivery methods was found to efficiently silence the *collagenase*-like and *yellow*-like genes. Altogether, these results suggest that the optimal method for gene silencing is gene-dependent and that some genes cannot be silenced by any of the tested methods. Surprisingly, we also observed that the use of primers out of the dsRNA hybridizing domain was not sufficient to explain the overexpression of the YEL and COL genes as this pattern was conserved despite primer replacement (Fig. 2.**B**).

## 3.2. Non-invasive delivery methods of dsRNA affect gene expression

Based on our previous observations, the impact of dsRNA delivery methods on mosquito physiology was measured through different experiments. Firstly, we estimated the mortality of the different methods on larva mortality after treatment. The salt dose for rehydration experiments was estimated before the RNAi experiment and a sublethal dose (killing no more than 15% of the larvae) was used for the knockdown experiment (**Figure S1.A**). After the different treatment, with or without dsRNA delivery, we did not report any significant impact of the delivery method on larval living frequency (**Figure S1.B**). Secondly, the impact of the method on the gene expression was investigated (Fig. 3, **Table 2**). Contrary to our expectation, provision of dsGFP coupled with nanoparticles, resulted in higher relative expression of VEN compared to the control performed without dsRNAs ($p < 0.0001$, t-ratio = -13.459) and YEL ($p = 0.0277$, t-ratio = -2.209) when measured with primers outside of the dsRNA site (Fig. 3B). The same pattern was observed for YEL in the presence of nanoparticles for primer annealing in the dsRNA site (Fig. 3A). However, no significant impact of the delivery method was observed for rehydration and soaking. Therefore, a part of the variation previously observed seems to be dependent on the delivery method. Indeed, the nanoparticles deliverance may affect gene relative expression. Finally, we also measured the impact of the mosquito larvae variation number per well on gene relative expression in the experiment. Indeed, although the experiment was designed for 15 larvae per well, the experiment showed little variations with 14 ± 2 larvae per well depending on conditions. However, the number of larvae per well did not impact significantly on the gene relative expression (**Figure S2**).

## 3.3. Gene copy number, GC content and transcript size correlate with RNAi efficiency

As our RNAi experiment showed very diverse knockdown efficiency related to target genes and delivery methods, after investigating the delivery method impact, we decided to look for molecular factors of target genes that could explain our results. We first considered the gene expression level as a potential

factor correlating with efficiency. The COL gene exhibited higher expression levels (3.7 ± 4 copies relative to housekeeping genes) than all the other genes, while the VEN gene was significantly less expressed (12.2 ± 6.2 copies relative to housekeeping genes) in most conditions. However, the expression level was not correlated with gene knockdown efficiency (**Table 3**). Secondly, we focused on the predicted off-target numbers for each gene. A total of three predicted off-target were identified for COL and one for KYN *in-silico*. Surprisingly, we did not observe any correlation between off-target numbers and knockdown efficiency (**Table 3**). We found out factors involved in gene knockdown efficiency. Firstly, a negative correlation was reported between the RNAi efficacy and the gene DNA copy number trough rehydration (Fig. 4A, **Table 3**). Secondly, we also evidenced a negative correlation between GC content and RNAi efficiency for both nanoparticle and soaking delivery (Fig. 4B, **Table 3**). Finally, we observed a positive correlation between the transcript size and RNAi efficiency for all tested approaches (Fig. 4C, **Table 3**). Therefore, both gene copy number, GC content and transcript size were correlated to silencing efficiency in our study. To exemplify those results, KYN (that responded well to dsRNA) appears as a very suitable candidate for RNAi knockdown as it is present in only one copy in *Ae. albopictus* genome, it holds a minor GC content (41%), and its transcript size is 1 835pb (which is the longest in this study).

# 4. Discussion

RNA interference is a powerful tool to explore gene functions and networks. While this method has been used for more than a decade, it still requires a meticulous plan of action to be optimally applied on a given model and target genes. In the present study, we aimed at investigating non-invasive dsRNA delivery methods efficiency, their potential side effects and the most suitable target genes to knockdown. Overall, this comparative study provides new insights for the optimization of RNA interference applications on the Asian tiger mosquito. It particularly shade light on the different levels of efficiency between delivery methods, the potential side effects of these methods, the main molecular characteristics involved in target gene knockdown and finally the importance of primer design to estimate gene silencing.

# 4.1. Efficiency variations between delivery methods

We showed that while being non-invasive, each of our selected methods can produce an efficient knock-down to at least one of the four targeted mosquito gene. Among those, soaking has been by far the most extensively used technique for treating mosquito larvae [45, 48, 49]. Previous studies have claimed that soaking efficiency reaches similar silencing levels than microinjection (*i.e.* the gold standard invasive approach) [50]. However, our results suggest that efficiency strongly varies among different targeted genes. Rehydration was the only method that consistently induced silencing for the two genes that could be silenced (*i.e. kynurenine 3-monooxygenase*-like and the *venom serine protease*-like genes), albeit silencing was higher for the *venom serine protease*-like gene with the soaking method. The exact mechanism involved in the efficiency of rehydration has not been elucidated yet, however it is highly probable that it relies on the rapid shift in transmembrane flux that may facilitate the dsRNA internalization due to rapid changes in the osmotic pressure [51]. Furthermore, dsRNA stability is

positively influenced by Na + concentrations [52] and residual presence of NaCl salts in the insect tissues and cavities may increase the molecule maintenance.

A major factor involved in RNAi efficiency is the ability to translocate dsRNA within cells. Presence of RNAses and digestive enzymes degrading dsRNA in the insect gut lumen was often pointed out as a major bottleneck affecting the translocation efficiency (reviewed by [53]). Combining RNAse inhibitors with an optimal delivery method is a promising alternative to improve dsRNA delivery efficiency. However, RNAses are not likely to be the major limiting factor to knock down gene expression since, in comparison with other insects, mosquito RNAses display relatively low activities [54]. Our results confirm that soaking and rehydration can also be very efficient in gene silencing in the absence of RNAse inhibitors. Therefore, the use of RNAse inhibitors does not seem mandatory for *Ae. albopictus* gene silencing. Nevertheless, delivery of dsRNA in *Ae. aegypti* have been shown to be more stable when simultaneously combined with delivery of dsRNA targeting the insect dsRNAses [55]. To increase the dsRNA stability and uptake, vehicles such as chitosan nanoparticles was often considered as being more favorable [34–37]. However, in the experiment we have conducted, nanoparticles-coupled dsRNA only succeeded to silence KYN and with lower efficiency compared with other methods. Factors that still need to be deciphered (such as differences in dsRNA translocation pathway) may have led to this observable difference in dsRNA delivery efficiency.

Two main pathways have been described in insects for dsRNA uptake, either *via* transmembrane channel or *via* clathrin-mediated endocytose [4]. Recent results suggested that the latter is favored for naked dsRNA uptake in *Ae. aegypti* [56] as well as for chitosan nanoparticles coupled with dsRNA [57]. Therefore, the lack of efficiency in this study could be due either to a dysfunctional RNAi pathway activation (after nanoparticle internalization) or the importance alternative translocation pathways for dsRNA in *Ae. albopictus* that are less prompt to be activated by nanoparticles (such as transmembrane channels). For instance, *Drosophila* cells present SID-1 transmembrane proteins which have been identified as a passive channel specific for dsRNA [58]. Such system enables cell to directly intake dsRNA, thus facilitating activation of the RNAi machinery. *Ae. albopictus* possesses a *sid-1* orthologous gene, which could explain the better silencing efficiency when using naked dsRNA uptake in comparison with vehicle binding.

In our study, rehydration appears as the most efficient non-invasive delivery method, meanwhile, on top of its side effect on gene expression, chitosan-nanoparticles appear to be the less efficient delivery method. In a context of field applications, the rehydration is the most inconvenient approach that would need to induce an osmotic stress to larvae before delivering dsRNA into breeding sites. At the opposite, the two easier methods for field delivery of insecticide dsRNA seem less efficient to knockdown gene expression. More investigations are necessary to confirm those observations, for instance tests in mesocosms and with dsRNA targeting genes that influence the mosquito survival would help to dig further the influence of delivery method in a context of vector control. However, if those results were to be confirmed RNAi would not be very suitable for mosquito control in the field.

## 4.2. Provisioning method can collaterally impact gene expression

Ensuring the absence of any collateral effects due to both the dsRNA uptake itself and recruiting the RNAi machinery is an important part of the proper validation of RNAi-mediated knock-down. To that end, we assessed unspecific variations in the expression levels of the different genes tested in this study in response to the provisioning of dsRNA targeting an exogenic gene that is absent from the mosquito genome (*i.e. gfp*). No side effects were reported for the soaking and rehydration. However, when dsGFP were bound to chitosan nanoparticles, the expression of YEL and VEN genes was enhanced. The chitosan concentration used in this study had no impact on mosquito larva survival, but chitosan nanoparticle itself (with no dsRNA) was shown to be lethal for several insects [59] including mosquito larvae [60]. Moreover, chitosan presents antimicrobial, antitumoral and antioxidant activities [61] and has been shown to alter cells innate immunity when coupled with siRNA [62]. It could consequently alter the insect transcriptome and lead to unpredicted phenotypes. In our study, we showed differential gene expression between free nanoparticles and dsRNA-bound nanoparticles. Although dsGFP are supposed to be neutral to *Ae. albopictus*, presence of dsRNA can nonspecifically affect gene expression in the host organism as already observed for honeybees [63], *Drosophila* [64] or parasitoid wasps [65]. This effect could simply be related to the recruitment of RNAi pathways and their collateral influence on other interconnected pathways [66, 67]. Surprisingly, no differences were observed when considering the other delivery methods, that is to say this is the combination of nanoparticles and dsRNA that triggers this non-specific response. Use of nanoparticles should then be considered cautiously for gene function characterization. Although it was successfully used for dsRNA insecticide-based approaches in mosquitoes and other insects [68–71], their nonspecific impact on untargeted species should then be regarded when evaluating the efficiency of such method.

## 4.3. Implication of gene copy number, GC content and transcript size on RNAi efficiency

In agreement with other studies, we showed that RNAi gene silencing efficiency was strongly gene-specific. Indeed, KYN and VEN were successfully silenced using several methods while the *collagenase*-like and *yellow*-like genes could not be silenced by any of the tested approaches. As we reported only few direct side effects of the delivery methods used in this study, we explored the potential gene features that could be correlated with RNAi efficiency. For instance, a negative correlation was previously reported in HeLa S3 cells between the amount of transcripts and RNAi silencing efficiency due to the dilution effect [72]. However, we failed to evidence any correlation between gene expression level and gene silencing for mosquitoes in this current study. The presence of off-target is supposed to interfere with RNAi efficiency. Indeed, siRNA resulting from dsRNA degradation can sometimes interact with untargeted transcripts and alter host transcriptome. To the extent of our knowledge, this has never been studied on mosquitoes while off-target effects have been documented for potential nuisance in RNAi experiments in other models [28, 73, 74]. In this study, we did not provide evidence supporting that the number of off-targets compete with

the efficiency of target genes knock-down. A panel involving supplemental genes could help us to catch more variability in gene off target and copy number to strengthen the preliminary results we have obtained.

The targeted genes also show differences in other factors that could be of importance to explain RNAi efficiency such as their copy number, GC content and transcript size. A strong negative correlation between RNAi efficiency and gene copy number was found for rehydration delivery. This result is particularly surprising since gene copy number has been shown to be positively correlated with an engineered RNAi machinery in yeast [75]. Indeed, one of the major roles of the RNAi machinery is to control virus infection but also transposable elements (TEs) that are selfish multicopy genetic elements that can spread within genomes. Therefore, we would expect that RNAi activation should have evolved to be higher for multicopy expressed sequences but our results contradict this prediction. Around 50% of the Asian tiger mosquito genome is composed of TEs while other mosquitoes such as *Culex* spp. contains less than 20% of TEs [76, 77]. We therefore suggest that a low RNAi control of multicopy sequences may have favored the permissiveness of *Ae. albopictus* towards TEs. However, further longitudinal investigation of RNAi – gene copy correlations along the mosquito phylogeny would help us to dig further this point. Although, the impact of TEs directly on insect traits remains very unclear [78], several examples of plant phenotypic alterations due to TEs have been reported [79, 80]. As we previously reported, the Asian tiger mosquito is one of the most invasive species. Its invasive success has been mainly attributed to its phenotypic plasticity that enables it to invade temperate areas [81]. We genuinely believe that it should be interesting to investigate further a potential link between TEs permissiveness and phenotypic plasticity in *Ae. albopictus*.

We showed that gene silencing was negatively correlated with the proportion of GC nucleotides in mRNA for two delivery approaches. High GC content of mRNAs was previously reported as a potential factor limiting RNAi efficiency due to potential limitation for siRNA accessibility and low rates of decay of mRNAs [82]. As an example in human cells, miRNA were shown to be more efficient to knock down low GC content mRNA [83].

Finally, the gene transcript size was shown to be positively correlated with gene silencing. Such correlation may be attributed to a snowball RNAi self-amplifying effect that are due to a higher synthesis of secondary siRNA that accentuate the gene silencing when longer mRNA are degraded [86–88]. In other terms, longer mRNA targeted fragments produce more siRNA that in turn increase the gene silencing. This was previously observed on plants and nematodes where an amplification of silencing was shown after mRNA degradation due to RNA dependent RNA Polymerases (RdRPs) generating new endogenous dsRNA matrix for siRNA pathway [89, 90]. For a long time, nematodes have been assumed to be the only animal that could trigger this mechanism [91]. Based on predicted proteomes, the authors showed that most insects are probably lacking RdRPs in their machinery except few Diptera. Although the existence of such mechanism in Diptera still have to be demonstrated, our results are currently giving some credit to this hypothesis.

## 4.4. Primers amplifying the targeted gene region underestimates gene silencing

To estimate gene silencing *via* RT-qPCR, we designed two types of primers: either within or outside the dsRNA hybridizing site. We showed that primers designed to hybridize within the dsRNA targeted site exhibited lower performance to quantify gene knockdown compared to primers hybridizing outside the targeted region. These findings are consistent with previous reports [92–94]. This phenomenon is probably due to remnant dsRNA fragments that can accidentally be quantified leading to silencing underestimation. Moreover, Onchuru et al. pointed out that knockdown is commonly underestimated in the case of lowly expressed genes. Here, the tested genes present different expression level as previously discussed. The primers within the dsRNA site detected gene silencing only for the gene with intermediate expression (KYN) but not for a gene with low expression level (VEN). Therefore, our results confirm that caution should be taken when designing primers to quantify RNAi efficiency in *Ae. albopictus*.

# 5. Conclusion

In this methodological study we showed that although, RNA interference *via* non-invasive delivery methods has been proposed as a promising technique to control gene expression in the Asian tiger mosquito, it may have some limitations. Rehydration delivery approach that provided the most promising results with simple soaking of larvae to a lesser extent. Conversely, nanoparticles coated with dsRNA are not a recommended delivery approach since they may modify non-target gene expression in larvae with a relatively poor gain in efficiency. Finally, RNAi efficiency seems to be strongly gene dependent and may be more efficient on large targeted transcripts with low GC content and limited copy numbers. Altogether these results argue the importance to test different parameters to optimize and further standardize this technique for future RNAi-based applications.

# Statements & Declarations

### Author Contribution Statement

MG designed the study under the supervision of AEH, CVM and GM. MG conducted the experiments with the help of VB, EM and LV as well as the advices of RR, AgV, AuV, AEH, CVM and GM. MG conducted the statistical analysis with the advice of GM. MG wrote the first draft of the manuscript and coauthors contributed to the redaction of the final version.

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### Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose.

### Data availability

The datasets generated during and/or analysed during the current study are available in the Zenodo repository, [A PERSISTENT DOI LINK WILL BE PROVIDED BEFORE THE PAPER WILL BE PUBLISHED]

### Ethics approval

Female mosquitoes were engorged on anesthetized mice to allow subsequent egg-laying. The protocol was reviewed by the Institutional Animal Care and Use Committee, acceptance reference number: Apafis #31807-2021052715018315.

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# Tables

**Table 1.** Primers used for qPCR in gene relative quantification estimations

| Target Gene | Primer Sequence | Amplicon size (bp) | dsRNA Site | $R^2$ Value | Efficiency (%) |
|---|---|---|---|---|---|
| RPS17 | FW: 5' GAACGACAGCAGCGAAACTT 3'<br>RV: 5' GTCACGAAACCAGCGATCTT 3' | 192 | - | 0.982 | 95.45% |
| RPL32 | FW: 5' TATGACAAGCTTGCCCCCAA 3'<br>RV: 5' AGGAACTTCTTGAATCCGTTGG 3' | 146 | - | 0.990 | 97.98% |
| YEL | FW: 5' GGGTGATCGTGGACCAAACT 3'<br>RV: 5' GCCTTCTGTGTCGACCTTCA 3' | 202 | In | 0.998 | 94.06% |
| | FW: 5' CACCTTTACTGCATCCGTACCC 3'<br>RV: 5' AGTTGCGTCACCAAGAATGTCC 3' | 200 | Out | 0.999 | 96.71% |
| KYN | FW: 5' CGCAGATCGTGTAACATGCG 3'<br>RV: 5' ACTCCACCTCGGCAAGAAAG 3' | 201 | In | 0.999 | 81.35% |
| | FW: 5' CCGTGCGAATGTCTTCTCTG 3'<br>RV: 5' TGAAGAGATGACGACCCAGT 3' | 170 | Out | 1 | 99.03% |
| COL | FW: 5' AAGCTCTTCCTCGCCGTTAT 3'<br>RV: 5' GGTTCGAGATGAGGGAACCT 3' | 223 | In | 0.983 | 81.03% |
| | FW: 5' GAACTGGGTTGGAATCCGTG 3'<br>RV: 5' GAGACGAACGACACAACACC 3' | 198 | Out | 0.999 | 95.12% |
| VEN | FW: 5' AGTGCCCAGGGAGAAGATTT 3'<br>RV: 5' GATGCCGAAACAAAATCCTAA 3' | 221 | In | 1 | 88.94% |
| | FW: 5' ATCCTACAGCACAACCACCA 3'<br>RV: 5' GTCCCACTTTGAGCAGAACG 3' | 208 | Out | 0.999 | 94.57% |

**Table 2.** Fixed effects impact on gene relative expression

| Conditions | Response variable | Fixed effect | F-value | *p-value* |
|---|---|---|---|---|
| dsGFP vs Control | Relative gene expression | Inoculation method | 33.0403 | **< 0.0001*** |
| | | Target gene | 11.6671 | **< 0.0001*** |
| | | dsRNA site | 4.7445 | **0.0299*** |
| | | Inoculation method : Target gene | 5.0719 | **0.00664**** |
| | | Inoculation method : dsRNA site | 0.5955 | 0.5517 |
| | | Target gene : dsRNA site | 13.4381 | **0.0003**** |
| | | Inoculation method : Target gene : dsRNA site | 9.9926 | **< 0.0001*** |
| dsRNA vs dsGFP | Relative gene expression | Inoculation method | 7.9865 | **0.0004**** |
| | | Target gene | 14.8707 | **< 0.0001*** |
| | | dsRNA site | 3.4190 | 0.0651 |
| | | Inoculation method : Target gene | 1.6450 | 0.1332 |
| | | Inoculation method : dsRNA site | 2.3361 | 0.0979 |
| | | Target gene : dsRNA site | 11.0656 | **< 0.0001*** |
| | | Inoculation method : Target gene : dsRNA site | 5.1874 | **< 0.0001*** |

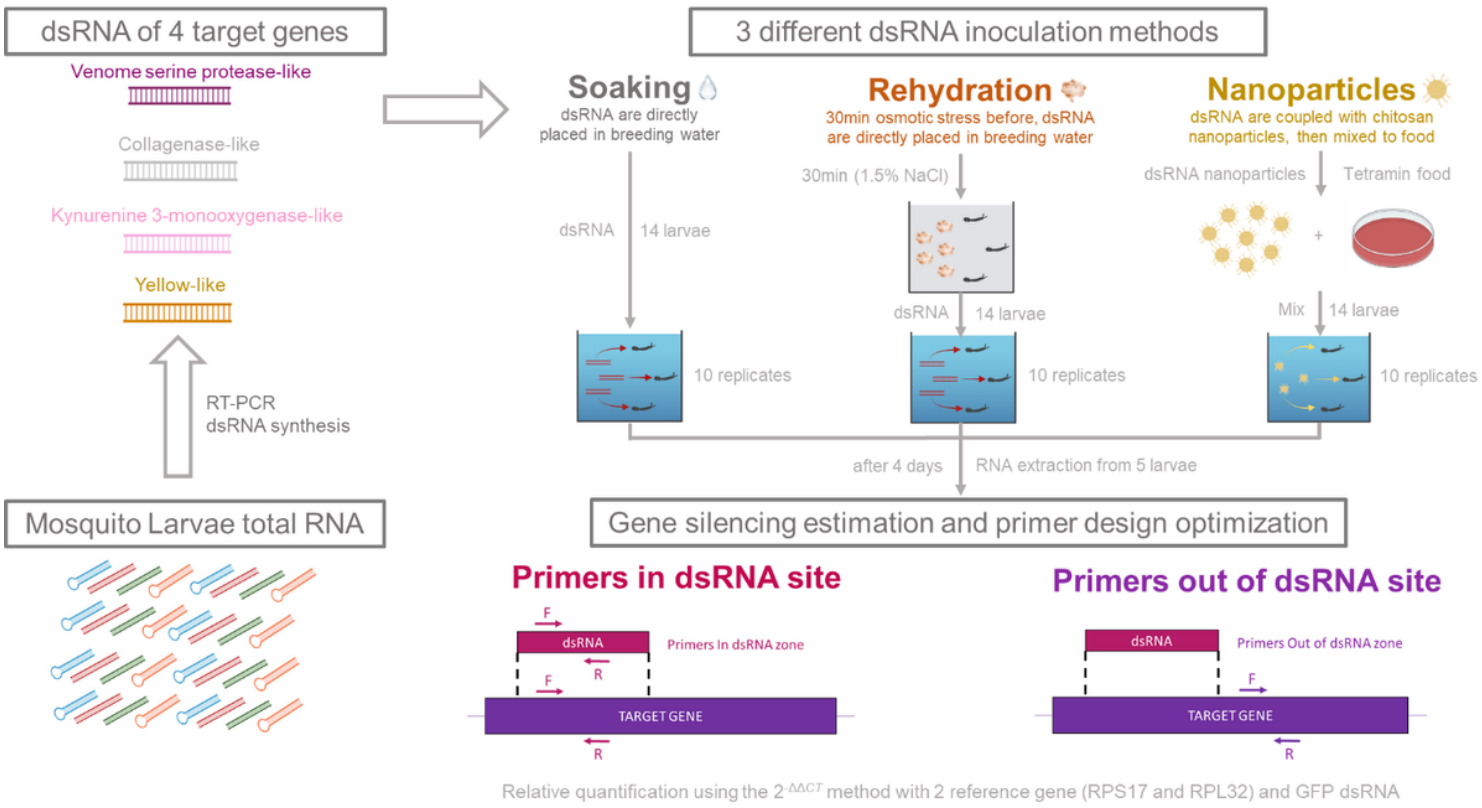
*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

**Table 3.** Factors correlated to gene silecing efficacy based on inoculation methods

| Inoculation method | Factor | Spearman index | *p-value* |
|---|---|---|---|
| Nanoparticles | Gene relative expression | 0.31 | 0.0516 |
| | Off-target predicted number | 0.19 | 0.2380 |
| | Gene predicted copy number | -0.27 | 0.0888 |
| | GC content (%) | -0.35 | **0.0252*** |
| | Transcript size | 0.55 | **0.00024***** |
| Rehydration | Gene relative expression | 0.26 | 0.1058 |
| | Off-target predicted number | 0.11 | 0.4823 |
| | Gene predicted copy number | -0.77 | **>0.0001***** |
| | GC content (%) | 0.08 | 0.6346 |
| | Transcript size | 0.41 | **0.0092**** |
| Soaking | Gene relative expression | -0.22 | 0,169 |
| | Off-target predicted number | -0.24 | 0.1355 |
| | Gene predicted copy number | -0.14 | 0.3808 |
| | GC content (%) | -0.34 | **0.03344*** |
| | Transcript size | 0.42 | **0,0079***** |

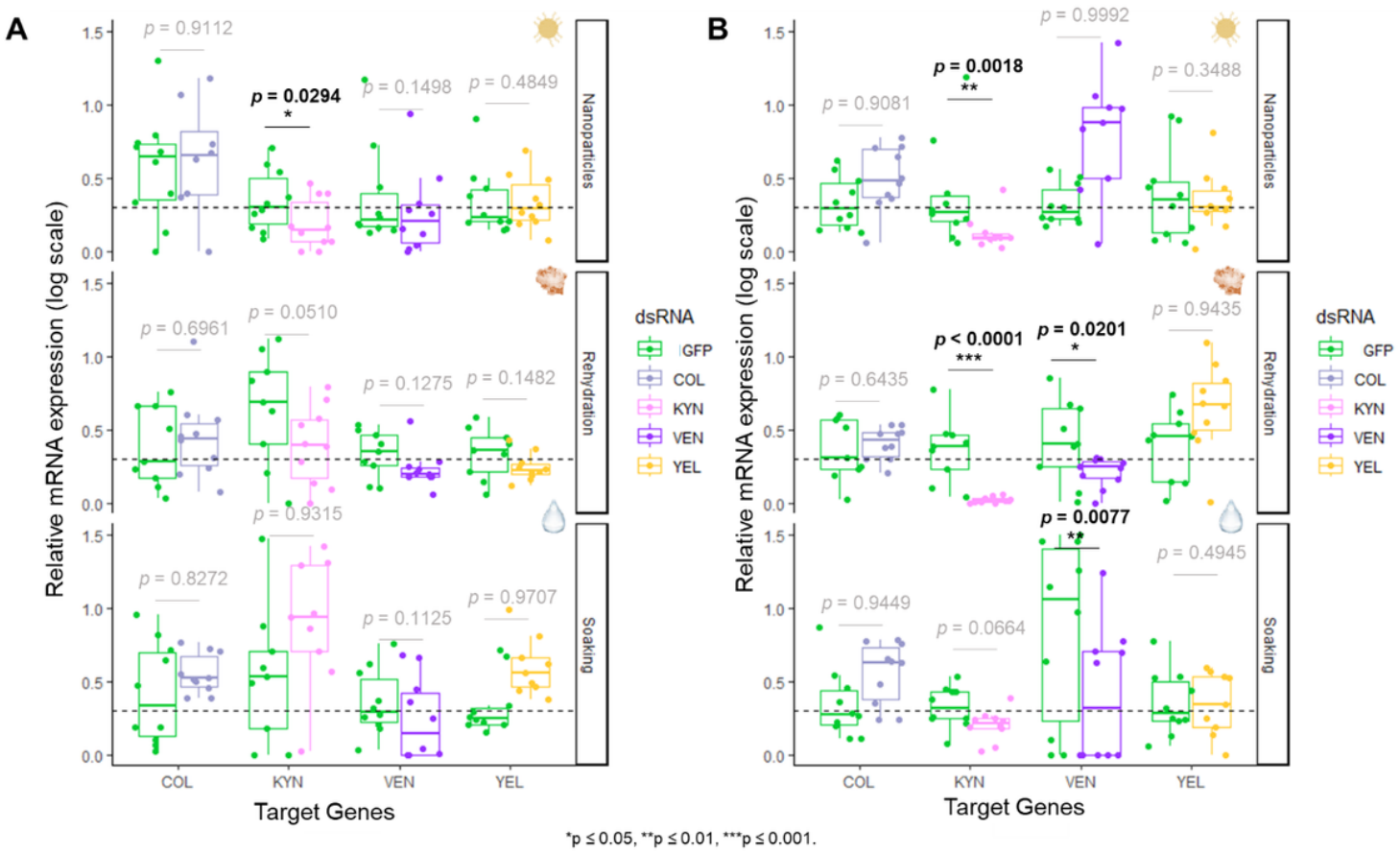
*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

# Figures



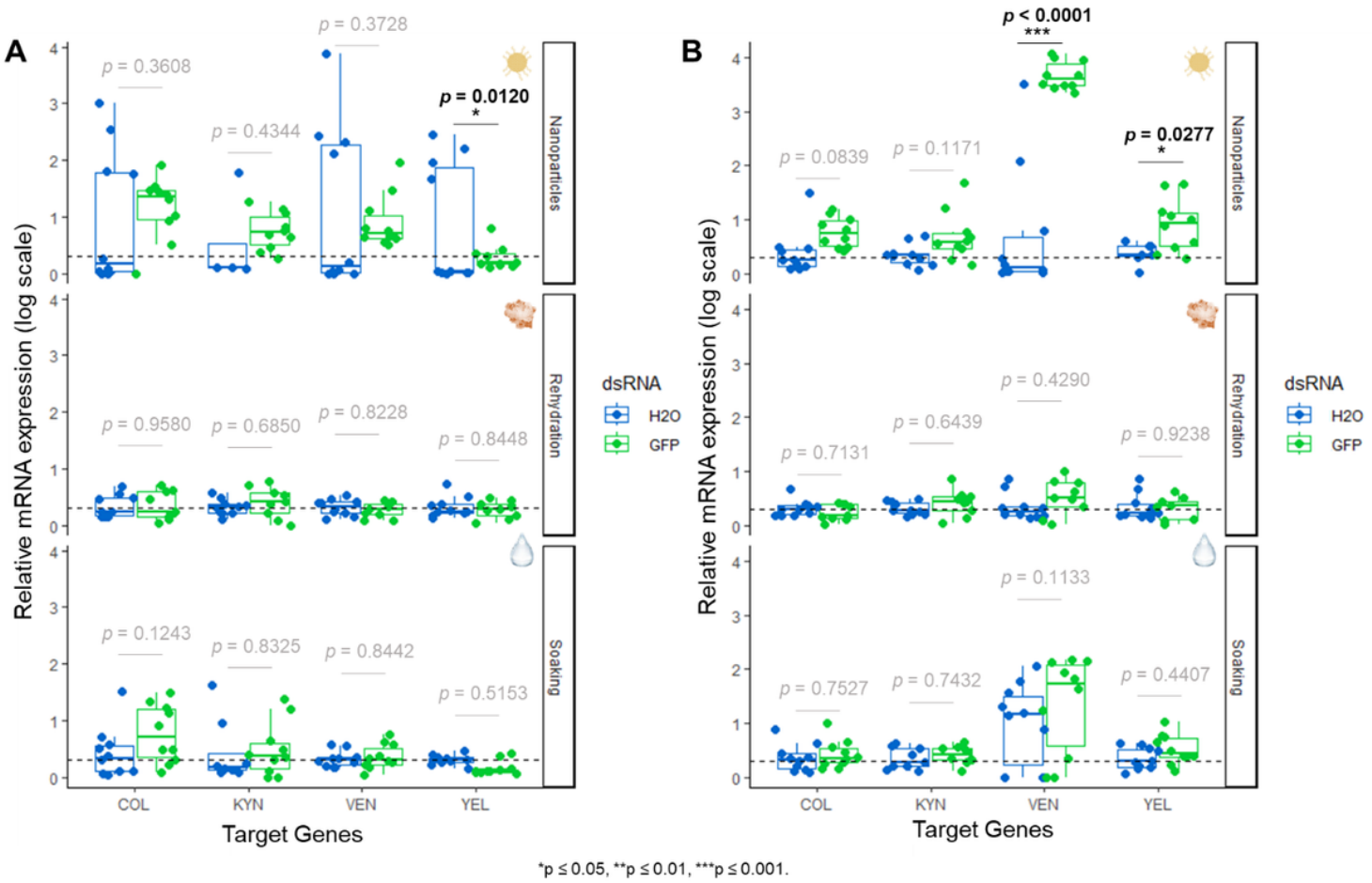

## Figure 1

**Experimental design for the comparison of RNAi delivery methods in *Aedes albopictus* larvae.** Using RNA total extraction, dsRNAs were amplified from 4 target genes (COL, KYN, VEN, YEL). Different non-invasive delivery methods (soaking, rehydration and nanoparticles ingestion) were tested separately with the same dsRNA concentration for each of the 4 genes. Finally, gene silencing efficiency was estimated with RT-qPCR using primers hybridizing either within or outside the gene-matching sequence of the dsRNA.




## Figure 2

**Differential expression of each target gene and delivery method in presence of non-specific and specific dsRNAs.** The relative gene expression has been represented for each gene (COL, KYN, VEN, YEL) either with a control involving non-specific dsRNA (dsGFP) or with dsRNA targeting each gene (*i.e.* dsCOL, dsKYN, dsVEN, dsYEL). Different dsRNAs were represented by a different color. (A) The gene expression has been estimated with primers matching within the specific dsRNA matching site and (B) outside of it. The black hatched line represents the mean for control condition (*i.e.* GFP).




## Figure 3

**Differential expression of each target gene and delivery method in presence or absence of non-specific dsRNA.** The relative gene expression has been represented for each target gene (COL, KYN, VEN, YEL) either with non-specific dsRNA (dsGFP) or in absence of dsRNA (H2O). Different colors represent the different treatments (*i.e.* H2O and dsGFP). (A) The gene expression has been estimated with primers matching within the specific dsRNA matching site and (B) outside of it. The black hatched line represents the mean for control condition (*i.e.* $H_2O$).

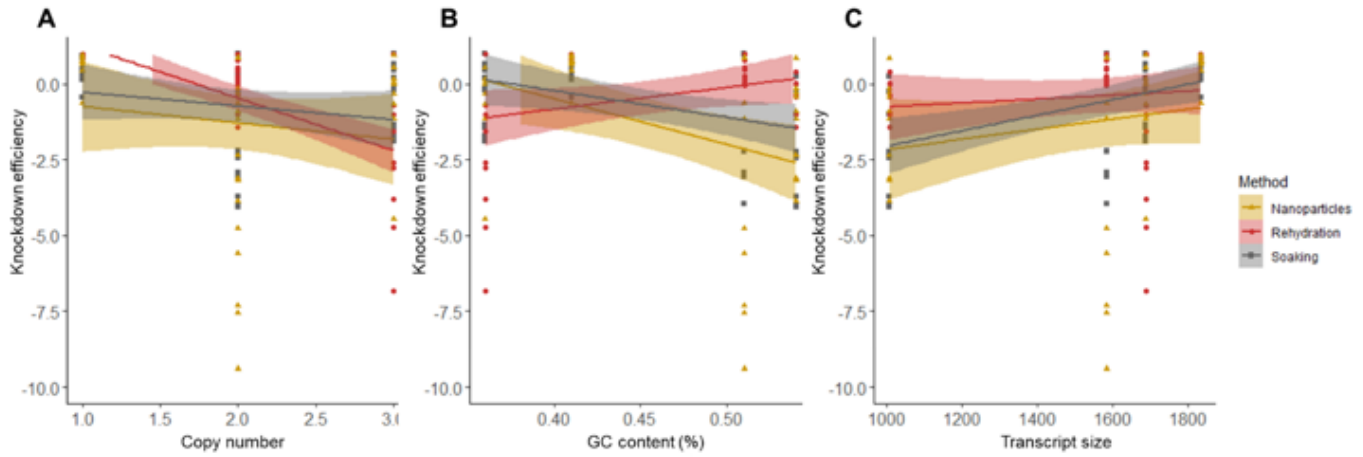



**Figure 4. Correlations between gene silencing efficiency and gene characteristics.** Correlations between gene silencing efficiency and different gene characteristics has been investigated. Different symbols (▲, ●, ■) and colors (yellow, red and grey) represent different delivery methods (nanoparticles, rehydration, soaking respectively). Non-parametric Spearman correlations were estimated between the silencing efficiency of target genes and (A) the copy number of each gene within the genome of *Ae. albopictus*, (B) the GC content (%) and (C) their transcript size and reported in Table 3.

### Figure 4

See image above for figure legend.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterial.pdf