# RNA Interference-Based Sterile Insect Technique in Mosquitoes: Overcoming Barriers to Implementation

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## ABSTRACT

The yellow fever mosquito (*Aedes aegypti*) is an important pathogen vector and model organism for mosquito researchers. Gene silencing by RNA interference (RNAi) has been used to determine genes' functions in the vinegar fly, *Drosophila melanogaster*, but comparable, genome-wide loss-of-function data are not available for mosquitoes. This is due to the difficulty of achieving transcript depletion by dsRNA feeding and to the complexity and size of the *Ae. aegypti* genome. To respond to these challenges, I set out to understand dsRNA degradation in the mosquito gut, analyze the transcriptome of male and female mosquito larvae and improve dsRNA delivery methods.

Several enzymes predicted to degrade dsRNA are found in the mosquito genome. I suspected that some of these enzymes could reduce RNAi efficiency of ingested dsRNAs in the larvae. I identified ten putative nuclease genes in the insect's genome, two of which are expressed in the gut of fourth instar larvae. By delivering dsRNA against these nuclease genes, RNAi efficiency against other transcripts was improved.

Sex determination in mosquitoes is controlled by a cascade of differentially-spliced transcripts that encode different transcription factors in the two sexes. To explore whether genes other than the few sex-determination genes are also spliced in a sex-specific manner, I used nanopore sequencing to sequence full-length transcripts from single reads in sex-sorted fourth instar larvae. This is the stage where development of sex organs begins, and I targeted some of these sex-biased transcripts using RNAi, inducing female-specific mortality. I also described several male-biased genes which may provide effective targets to produce sterile males. To enable low-cost RNAi assays, I developed and optimized dsRNA expression systems in bacteria and yeast.

An understanding of dsRNA degradation, together with identification of new sex-specific gene targets and improved dsRNA delivery methods will allow researchers to explore genes involved in sex differentiation and fertility in these important pests. Applying this knowledge may enable RNAi-based technologies for low-cost sterilization and sex-sorting for mosquito control.

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# Chapter 1: Introduction

## THE NEED TO CONTROL MOSQUITO POPULATIONS

Mosquitoes have plagued terrestrial vertebrates since the Jurassic period (Borkent and Grimaldi 2004), and the need to control mosquito populations is growing as global temperatures continue to increase, and the distributions of mosquito species are predicted to expand (Bradshaw et al. 2016). Because they are vectors of both human and animal disease, mosquitoes are of major medical and wildlife conservation importance. Human diseases transmitted by mosquitoes are substantial contributors to the global burden of communicable disease. The most important of these are two parasitic infections, malaria and lymphatic filariasis (LF), and two arboviruses, dengue and Japanese encephalitis (JE). Disability adjusted life years (DALYs) due to malaria accounted for nearly one tenth of DALYs from all communicable diseases worldwide in 2010, while DALYs due to LF were around one third of the communicable disease total (Kassebaum et al. 2016). Though dengue and JE's contribution to global burden of communicable disease is considerably less (both around 0.1%) (Kassebaum et al. 2016), the mosquito vectors of arboviruses are predicted to expand their range with increased global trade (Tatem, Hay, and Rogers 2006). Increases in suitable mosquito habitats due to global climate change will also increase the burden of mosquito borne disease (Ramasamy and Surendran 2012). This is especially true for the dengue vectors *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse), which have both spread throughout the tropics and now threaten new regions northward into temperate Europe, Asia and North America, and southward in Australia (Beebe et al. 2013; Nicholson et al. 2014; Beaumier, Garcia, and Murray 2014; Ogden et al. 2014). Recent outbreaks of dengue in Japan and Florida caused concern in these wealthy parts of the world, and the

discovery of *Ae. albopictus* populations as far north as southern Canada (Giordano et al. 2019) and Germany (Pluskota et al. 2016) brings a vector of a tropical disease near populations unaccustomed to this risk. Diseases carried by mosquitoes have not posed a significant threat to health in these countries for decades (Packard 2010), but the renewed possibility of outbreaks of mosquito-borne disease has led to an increase in public attention and funding for mosquito control. Even in countries where mosquito-borne diseases have long been major threats, changing climate and increased international trade is enabling the introduction of new species. For example, *Anopheles stephensi* has invaded North Eastern Africa (Amenu et al. 2020), resulting in the need to rethink how malaria is controlled in that region.

Mosquito transmitted diseases also adversely affect wildlife. In the same way that range expansion is bringing new arboviruses and parasites to naïve human populations, wildlife of conservation concern is under threat from new diseases as mosquitoes are introduced (reviewed in (Bradshaw et al. 2016; Benzarti et al. 2019). The impact of introduced mosquitoes on birds is the best documented example of how introduced mosquito-borne diseases impact native wildlife. In North America, West Nile Virus (WNV) has contributed to the decline of native bird populations (Kilpatrick et al. 2013; Levine et al. 2017), and the extinction of hundreds of Hawaiian bird species illustrates how an imported mosquito can permanently alter the avian diversity (Dahlin and Feng 2019) and soundscape (Paxton et al. 2019) of an ecosystem.

Novel biotechnologies, such as the release of genetically-sterilized mosquitoes (Alphey 2014), may provide environmentally safer alternatives than our current broad-spectrum chemical insecticides (McGraw and O'Neill 2013), though debate over production of genetically modified organisms (GMOs) will likely delay their implementation. The aim of the research presented in this thesis is to improve RNAi-based sterile insect technique (SIT) as a non GMO, area wide

control method for disease vectors.

## MOSQUITO CONTROL METHODS CURRENTLY AVAILABLE

Methods for control of vectors of malaria have remained nearly unchanged in the past 50 years, and rely heavily on delivery of insecticide to mosquitoes at vulnerable stages in their life cycle. Area-wide control methods are available, but they face concerns over public acceptance or technical problems that limit large-scale implementation. In this section the wide range of chemical insecticides and area-wide approaches are reviewed

### CHEMICAL INSECTICIDES

Indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) are routinely used to target adult anthropophilic females during their blood-feeding phases. In addition, larviciding and breeding source reduction target developing aquatic stages that are restricted to groundwater; in theory, this can be a vulnerable point in their lifecycle but sheer numbers of larvae and breeding sites makes epidemiologically relevant control prohibitively costly. Additional approaches such as odour-based traps, attractive ovitraps, zooprophylaxis and autodissemination have been proposed (Lwetoijera et al. 2014; Njiru et al. 2006), but none are widely used.

IRS exploits resting behaviour of *Anopheles* mosquitoes associated with indoor resting (endophily) and indoor feeding (endophagy) behaviours. The two primary vectors of malaria in Africa are both endophilic and endophagic; the humid sheltered environment of traditional huts provides protection during the day, and at night, sleeping occupants provide an easy bloodmeal. In the period immediately after feeding, the mosquito is likely to rest on a vertical surface in the hut to expel water from the bloodmeal (Vaughan, Noden, and Beier 1991). Coating the walls of a hut with insecticide delivers insecticide to these resting mosquitoes. Insecticides used for IRS have included highly stable active ingredients such as DDT, but in most countries, pyrethroids

were used until the recent WHO recommendation to switch to non-pyrethroids for most malaria-endemic countries (Sherrard-Smith et al. 2018). Evidence for the efficacy of IRS is insufficient for its widespread use (Pluess et al. 2010), and major donors have recently become reluctant to fund IRS campaigns, which by their nature are highly disruptive of the household and community, and require intensive logistics, monitoring and training of spray teams (Curtis and Mnzava 2000). Because IRS does not provide a physical barrier to mosquito entry, it has been suggested that mosquitoes could change their behaviour away from indoor resting and bite quickly without resting on indoor surfaces, or the mosquito could avoid the house altogether by biting before the occupants have retired or after they rise in the morning (Ranson et al. 2011). With the advent of pyrethroid resistance in much of sub-Saharan Africa, one method proposed to mitigate resistance has been to deliver non-pyrethroid IRS combined with LLINs, thereby delivering two insecticide classes in the same house (West et al. 2015).

The mosquito control method with the largest body of supporting evidence and the most funding is LLINs. LLINs provide a physical barrier that probing mosquitoes contact, and since they target adult females, LLINs reduce the probability of a mosquito host surviving long enough to become infected with malaria, support the parasite's sexual development, and become infective to another human. This reduction in old, multiparous females is likely the mechanism by which LLINs provide a community protective effect (Hawley et al. 2003). LLINs have proven to be effective in reducing malaria transmission, clinical disease, and childhood mortality in large controlled trials (Hawley et al. 2003; Eisele et al. 2005). Despite this evidence, some countries such as Liberia fail to attain high LLIN usage despite repeated distributions (Zinszer et al. 2017). Nets require proper hanging, repair of holes, and they dramatically change the appearance of rooms used for sleeping. In hot climates, they can make the sleeper uncomfortably hot and

claustrophobic. Although they are designed to last for 4 years with continuous delivery of insecticide, in practice, their effective lifespan may be much less (Allan et al. 2012). Finally, LLIN manufacturers are currently limited to pyrethroid active ingredients to which resistance has expanded and intensified across Africa (Toé et al. 2015). Two LLIN manufacturers have enhanced the effectiveness of their pyrethroid based LLINs with the addition of synergists, and one manufacturer is launching a net with a baffle treated with a second insecticide class (Murray et al. 2020). Beyond such insecticide combinations, the development of LLINs with alternative active ingredients appears to be several years away (Churcher et al. 2016).

Source reduction and larvicides against *Anopheles* mosquitoes have been one of the most effective means of interrupting malaria transmission over the past century, but targeting larvae (a comparatively expensive approach) has fallen out of favour in the current era of successes with LLIN distribution (Packard 2010). Recent models and experimental work have shown that targeting larvae can be effective in marginal transmission zones where entomological inoculation rates are low (Yohannes et al. 2005; Fillinger and Lindsay 2011). Insect growth inhibitors kill larvae at extremely low doses, such that autodissemination by gravid females is possible. For example, a single leg of a female that has touched a puddle treated with pyriproxifen acquires enough insecticide to kill the larvae in a small adjacent breeding site (Lwetoijera et al. 2014). *Anopheles* mosquitoes tend to lay eggs in several locations, thereby distributing the insecticide to multiple oviposition sites.

Several novel methods of delivering insecticides to mosquitoes have been proposed recently, including using mass administration of ivermectin, mimics of human odor to lure mosquitoes into traps (Njiru et al. 2006; Chaccour and Rabinovich 2019), and lethal *Anopheles* ovitraps (Herrera-Varela et al. 2014), which attract and kill females looking for an egg-laying site.

Ivermectin mass drug administration in human and animal populations could kill blood-feeding mosquitoes regardless of their resting behaviour or timing of their bites (Ouédraogo et al. 2015; Chaccour et al. 2013). If other interventions like IRS and LLINs result in a change in biting and resting behaviour, these approaches could be an effective complementary intervention.

Day-biting aedine dengue vectors are particularly challenging to control with insecticides. Biting occurs outside of the hours where humans could be protected by LLINs and there is no easily-defined resting behaviour to exploit with IRS. Currently, no treatment or acceptable dengue vaccine is available (Capeding et al. 2014; Normile 2014). Reducing breeding sites for aedine mosquitoes is often advocated, although communities must be effectively mobilized. Even in very authoritarian countries like Singapore, public compliance is incomplete, and dengue cases continue. Delivery of insecticides to daytime biting mosquitoes is challenging, though a limited effect was shown using insecticide-treated curtains (Lenhart et al. 2013). The limited efficacy of other interventions such as LLINs and screening suggests that additional tools are needed (Wilson et al. 2014). Due to a lack of such tools, governments typically rely on ultra-low volume insecticidal sprays, despite limited evidence for their efficacy (Wahid et al. 2019).

Insecticidal and mechanical ovitraps are another control method in development, but not yet widely deployed. Gravid females are an attractive target; killing these mosquitoes is theoretically an efficient means of selectively reducing the subset of mosquitoes old enough to have acquired and transmit a virus or parasite (Smith et al. 2012; 2014). Early lethal ovitraps did not reduce populations of *Ae. aegypti* enough to warrant widespread use (Thailand, Brazil), but recent studies have shown larger population reductions, notably an 80% drop in trapped gravid females in Puerto Rico following deployment of CDC developed ovitraps (Barrera et al. 2014). Increasing the attractiveness of these traps relative to natural breeding sites is key for their

success, and preliminary results from one group suggest bacterial blends could lure more gravid females to their death (Paz-Soldan et al. 2016).

Insecticides are our main defense against mosquitoes. The effect of insecticides is often incomplete, and without major advances in insecticide delivery, control agencies will continue to battle insecticide resistance. Non-insecticidal approaches need to be modernized and brought to cost-effective scale.

## *WOLBACHIA*

One such non-insecticidal approach leverages the use of a common endosymbiont. *Wolbachia* (Hertig and Wolbach 1924), the intracellular α-proteobacterial symbiont of many arthropods, is not found in most wild populations of *Ae. aegypti* (Zabalou et al. 2004). SIT using *Wolbachia* carrying *Ae. aegypti* males is in use in several countries around the world (Gilbert and Melton 2018), based on the phenomenon of cytoplasmic incompatibility between sperm carrying *Wolbachia* and eggs lacking *Wolbachia* (Zabalou et al. 2004). Population replacement of non-*Wolbachia* carrying *Ae. aegypti* with *Wolbachia* carriers produces populations that are refractory to dengue virus. This approach has dramatically reduced dengue infections in Brazilian cities where it is currently in use (Durovni et al. 2019). There are several concerns with applying this method however, including community opposition to release of females and the possibility that introduced populations could lose the symbiont with increasing temperatures (Ulrich et al. 2016; Ross et al. 2017).

## RIDL

Release of insects carrying a dominant lethal gene (RIDL) is a GMO approach that does not require insecticides and enables area-wide control. RIDL male release results in population collapse by activation of a lethal gene in progeny of RIDL males and wild females. The

technology was developed in the early 2000s but has faced community opposition due to uncertainties about the release of genetically modified mosquitoes. RIDL has been trialed in Brazil where the target population of *Ae. aegypti* was reduced near zero following release of lethal allele-carrying males (Garziera et al. 2017). The RIDL method is reviewed by Black, Alphey, and James (2011) and functions by suppressing the lethal gene to permit the insects to develop into reproductive adults. Following mating with wild females, the lethal gene is activated and prevents survival of developing embryos. While the technique is largely effective, a small number of progeny of RIDL males can survive following male release (Evans et al. 2019). Although there is no evidence that the offspring of these survivors have increased fitness as recently suggested following mass release in Brazil (Evans et al. 2019), the introduction of genetic material from laboratory strains into wild populations may cause concern in affected communities.

## GENE DRIVES

The concept of the selfish gene capable of driving through a population has been proposed as a means of mosquito elimination or fixation of disease refractoriness alleles in wild mosquito populations. The field has seen remarkable progress towards field-ready approaches to drive lethal or refractoriness traits into populations of pest insects (Palacios et al. 2020). Field-readiness is not the same as publicly acceptable however, and considerable debate has been generated over the ethics and safety of genetically modified mosquitoes (Alphey et al. 2002; Fleur 2015). Public consent may be possible if confinable gene drives can be implemented (Raban, Marshall, and Akbari 2020). It will likely be some time before genetically-mediated sterilization and genetic drive systems are approved for widespread use, making non-transgenic methods an attractive alternative to improve SIT with modern methods while avoiding regulatory

hurdles.

## STERILE INSECT TECHNIQUE

SIT overcomes many of the challenges described for chemical insecticides and for other methods of area-wide control of mosquitoes. Shortly after the development of irradiation techniques, a method of releasing sterile males to reduce pest insect populations was proposed (Knipling 1955), and since then many insects have been targeted by SIT. The appeal of SIT may lie in the fact that it avoids the off-target effects of insecticides, but also because its area-wide effect does not require regular disruption of communities or regular input by community members. The mass release of sterile insects (SIT) has been used to reduce, and in some cases, eliminate pest species. If sufficient numbers of sterile males are present, few fertile eggs will be laid and the population will collapse. Examples of successful SIT programs include the screwworm fly in North America (Vargas-Terán, Hofmann, and Tweddle 2005), tse-tse flies in Zanzibar (Vreysen et al. 2000), and codling moths in Canada (Dyck, Graham, and Bloem 1993).

In most cases, sterile insect technique requires a mechanism to sex-sort, in addition to a mechanism to sterilize. A recent review of sex-sorting mechanisms describes how knowledge of sex differentiation at the level of molecular signal or the level of anatomical differences may be exploited in an SIT system (Lutrat et al. 2019). Currently available techniques to remove females from laboratory reared populations of dipterans range from simple mechanical separation systems to more complex repressible female-specific lethal transgene approaches (Lutrat et al. 2019).

Mosquitoes have been targeted by SIT, but the success of these programs has been limited (Alphey et al. 2010; Ageep et al. 2014). Renewed interest in developing transgenic lines of sterile insects has led to trials using irradiated males in Sudan (Ageep et al. 2014).

SIT for mosquitoes requires two steps: 1) sex-sorting to remove biting females and 2) sterilization of males. Currently available methods for both steps are reviewed to highlight the complexity of producing sterile males for SIT.

## CURRENTLY AVAILABLE SEX-SORTING METHODS

Sex-sorting, or "sexing", refers to the separation of males from females, and more specifically the removal of females. Sexing can rely on mechanical separation of the sexes based on natural or engineered sexually dimorphic differences, or sexing can use more complex technologies to modify gene expression and conditionally masculinize or kill females during development. Overall, sex-separation strategies need to meet several criteria, summarized as 'the 7 Ses' by Papathanos et al. (2009): small, simple, switchable, stable, stringent, sexy and sellable.

The methods of sex-sorting flies has been reviewed numerous times (Alphey 2014; Gilles et al. 2014; Bernardini et al. 2018; Häcker and Schetelig 2018), focusing mostly on particular species or genera, as well as on the engineering methods employed. Comprehensive reviews of sex-sorting flies for SIT were conducted in 2002 (Robinson 2002) and by our group in 2019 (Lutrat et al. 2019).

A repressible female-specific lethal transgene is a conditionally activated transgene that can be repressed to allow for colony maintenance. A widely used conditional method involves the tetracycline transactivator (tTA) either controlling the expression of a lethal transgene or itself triggering lethality. A *Ceratitis capitata* Wiedemann, 1824) strain yielding full female lethality has been developed using sex-specific *transformer (tra)* intron splicing to control the expression of the lethality construct (Fu et al. 2007). The same system was used to produce a *Bactrocera oleae* (Rossi, 1790) RIDL strain carrying sex-specific fluorescence, genetic sterility and conditional female-lethality (Ant et al. 2012). In cage tests, this strain efficiently eliminated a

wild-type population using weekly releases of transgenic males. Female-specific splicing of *tra* was also used in *Lucilia cuprina (*Wiedemann, 1830) to distort sex ratios (Yan and Scott 2015) and a similar system was used in *Cochliomia hominivorax* (Coquerel, 1858) by Concha and colleagues (2016) in combination with proapoptotic genes. One of the *C. hominivorax* strains is currently under evaluation for a mass-rearing program (Scott et al. 2017).

In several mosquito species, the female-specific expression of *actin-4* was exploited to conditionally express lethal effectors. As *actin-4* is expressed in the indirect flight muscles of females, the obtained phenotype is flightless females, not death. Such a system was developed in *Ae. aegypti, Ae. albopictus* and *An. stephensi* with full penetrance of the flightless phenotype in absence of tetracycline (Fu et al. 2010; Labbé et al. 2012; Marinotti et al. 2013). Although this system seems effective in laboratory studies, it has been suggested that tetracycline affects gut microbiota and impairs *An. stephensi* fitness, and may render inadvertently released females more susceptible to *Plasmodium falciparum* infection (Sharma et al. 2013).

Dieldrin resistance has been exploited in the ANO IPCL1 strain, an *An. arabiensis* (Patton 1905) strain in which the dieldrin resistance allele was translocated to the Y chromosome so that males are resistant and females susceptible to dieldrin (Yamada et al. 2012). This strain produces few adults for release, with male recovery being 13% of the initial number of eggs (Ndo et al. 2014; Yamada et al. 2014; Yamada et al. 2015) with a risk that released males spread highly toxic dieldrin into the environment (Yamada, Jandric, et al. 2013). However, this cisgenic strain has been backcrossed into different *An. arabiensis* genetic backgrounds (Munhenga et al. 2016; Dandalo et al. 2018). A similar strain in *Ae. albopictus*, TiCoq, was developed with a sex-sorting efficiency of 98% (Lebon et al. 2018).

Given that only female mosquitoes blood-feed, insecticide-infused blood meals have also proven effective for sexing (Yamada, Soliban, et al. 2013). With ivermectin provided at 7.5 ppm in blood meals, all *An. arabiensis* females died after 4 days, but the insecticide did not affect the males.

Recently, Kandul and colleagues described a system producing 100% sterile males in *Drosophila melanogaster* (Fabricius, 1787), with females dying mainly at the late larval stage (Kandul et al. 2019). The strategy involves crossing a strain expressing *Cas9* enzyme with another expressing *β-tubulin* (*β-tub*) and *sex lethal* (*sxl*) CRISPR targets. This study demonstrates a method to achieve both sterilization and sex-sorting in the F1 generation.

Regulatory RNAs may also serve as targets for sex-sorting. Long non-coding RNAs (lncRNAs) were targeted recently (Duman-Scheel 2019) to produce male-biased populations. In the tephritid fruit fly *B. dorsalis* (Hendel, 1912) injection of a microRNA (miRNA) produced ~90% phenotypic males (Peng et al. 2020).

## CURRENTLY AVAILABLE STERILIZATION METHODS

SIT has proven effective for a variety of insects, but its implementation is slowed down by the necessity of removing females before release. In addition to minimizing the health and economic risks posed by released females, models and trials have also shown that releasing only males was much more cost-efficient than releasing both sexes (Knipling 1955; Rendón et al. 2004). These cost savings may arise from either reduced costs in mass-rearing the insects and/or in field performance, where released males will not expend energy mating with co-released females.

Other genetic control methods, including RIDL and the *Wolbachia*-based Incompatible Insect Technique (IIT), also require consistent sexing methods. Models show that the release of only a small proportion of *Wolbachia*-infected females could lead to population replacement instead of

elimination. In mosquitoes, whose females cause nuisance and transmit pathogens very little, female contamination can be tolerated in any genetic control strategy.

## RNAI BASED SIT

A non-GM method for the production of large numbers of sterile males was proposed by Whyard et al (2015), who demonstrated that *Ae. aegypti* larvae could be fed dsRNAs that silenced spermatogenesis genes to produce sterile males while maintaining mating vigor. They also demonstrated that a male-biased population could also be obtained by feeding the mosquito larvae dsRNA targeting the female isoform of the *doublesex* (*dsx*) gene. Since then, others have attempted to build on these results, though a population of 100% sterile population with no female contamination remains elusive. For example, Duman-Scheel (2019) produced ~80% female bias in *Ae. aegypti* by targeting long non-coding RNAs with short hairpin RNA (shRNA)-expressing yeast (Duman-Scheel 2019), and similar female-biased populations (~80%) of *An. gambiae* (Giles, 1902) were produced by feeding long dsRNA against *dsx* expressed in *E. coli* (Taracena et al. 2019). For SIT by RNAi to be deployed with community consent, sex-sorting efficiencies near 100% are needed (Lavery et al. 2010).

RNAi works in this application by reducing the abundance of targeted gene products when Dicer cleaves dsRNA fragments and one RNA strand is complexed with *Argonaute* proteins in the RNA interference silencing complex. *Argonaute* is the functional component of the complex that cleaves the complementary mRNA or represses translation (reviewed by Meister et al 2013). When dsRNA targeting essential sex-determination or testis-specific transcripts is introduced into the cell, female specific lethality or male sterility is induced.

Conceptually, RNAi based SIT is very attractive as it overcomes many of the challenges facing other mosquito control methods. Unlike insecticidal approaches, the method is species-specific,

and because the germ line is not edited, concerns over GMOs that accompany RIDL and gene drive approaches do not apply. When compared to other forms of SIT, RNAi-based SIT is favorable due to minimal off-target effects of sterilization. The gene targets of RNAi-based SIT can be chosen to only impact spermatogenesis while preserving mating vigor, unlike radiation that impacts flight ability and longevity (Yamada et al. 2014), and unlike chemosterilants that require stringent disposal methods (Barek et al. 1998) and may impact the physiology and fitness of released males (Gato et al. 2014a; 2014b). Radiation-based methods also face restrictions due to strict regulations around importation of radioactive materials.

SIT by RNAi requires successful removal of females, as well as sterilization of males before release. Choosing genes to target requires an understanding of how female sex is determined, how female tissues differentiate from males, and an understanding of how spermatogenesis is regulated in the male mosquito. Recent reviews outline sex determination in flies, and illustrate the lack of knowledge beyond the patterns described in a few model species and important pests (Lutrat et al. 2019; Papathanos et al. 2018). The conserved pattern (Figure 1) is a cascade from a male determining factor (or, exceptionally the XY dosage compensation found in *Drosophila*) which controls sex-specific alternative splicing of *transformer/transformer-2* (*tra/tra2*) genes, and finally the sex-specific alternative splicing of *doublesex (dsx)*.

*Dsx* is a gene typically comprised of 4-5 exons (Salvemini et al. 2011). The inclusion or exclusion of these exons, or alternative splicing, yields sex-specific transcripts. Reverse genetics screens have targeted female specific transcripts to induce female sterility or lethality (Clough et al. 2014; Whyard et al. 2015; Kyrou et al. 2018). *Dsx* is a transcription factor in the *doublesex/mab-3 related* gene family. Reviewed by Kopp (2012), this gene family appears to be conserved in arthropods, but the diversity of roles that *dsx* plays in other lineages (Verhulst and

van de Zande 2015) suggests that it has been frequently co-opted to new roles. Briefly, male or female *dsx* is expressed in tissues that require sexual identity. In *D. melanogaster*, *dsx* is regulated by alternative splicing of *transformer (tra)*, another conserved gene that has been successfully targeted for female lethality, which in turn is regulated by *sex-lethal (sxl)* (Lucchesi and Kuroda 2015). In the muscids studied to date, only *tra* is known to be an upstream regulator of *dsx* (Akash Sharma et al. 2017). In mosquito species studied so far, only the male determining factors *nix* (*Aedes aegypti*) (Gomulski et al. 2018), *yob (An. gambiae)* (Krzywinska et al. 2016) and *guy1* (*An. stephensi*) (Criscione, Qi, and Tu 2016), are known or presumed regulators of *dsx*, but there may be other upstream factors regulating *dsx*. A role for *tra-2* in mosquito sperm development has been shown, resulting in reduction of female offspring in the second generation after RNAi knockdown (Hoang et al. 2016). Intriguingly, putative *tra/tra2* orthologues appear to be highly conserved in mosquitoes (Hoang et al. 2016), but *tra/tra2* has not been implicated as a regulator of *dsx* in any mosquito, although *tra-2* is involved in ovarian development in *Ae. albopictus* (Li et al. 2019). The M-factor in tephritid flies has recently been described and shown to regulate *tra*'s auto-regulatory positive-feedback loop (Meccariello et al. 2019). Similarly, in two phlebotomine sandflies, *tra* has been recently identified and shown to also be self-regulating (Petrella et al. 2019).

Alternative splicing is also used downstream of the master regulator *dsx*. Sex differentiation is the process by which female and male-specific morphologies and behaviours are produced. *DSX* has been predicted to bind to several targets in the *D. melanogaster* genome (Luo, Shi, and Baker 2011; Luo and Baker 2015), and a few of these targets have been functionally validated (Luo and Baker 2015). In mosquitoes, however, very little is known regarding the regulation of transcript abundance and alternative splicing events that lead to sexually differentiated features. It can be

speculated that aedine sex differentiation pathways are complex, based on the diversity of *dsx* splice variants (n=11) (Matthews et al. 2018), but how these protein coding and non-protein encoding transcripts interact during development remains a mystery.

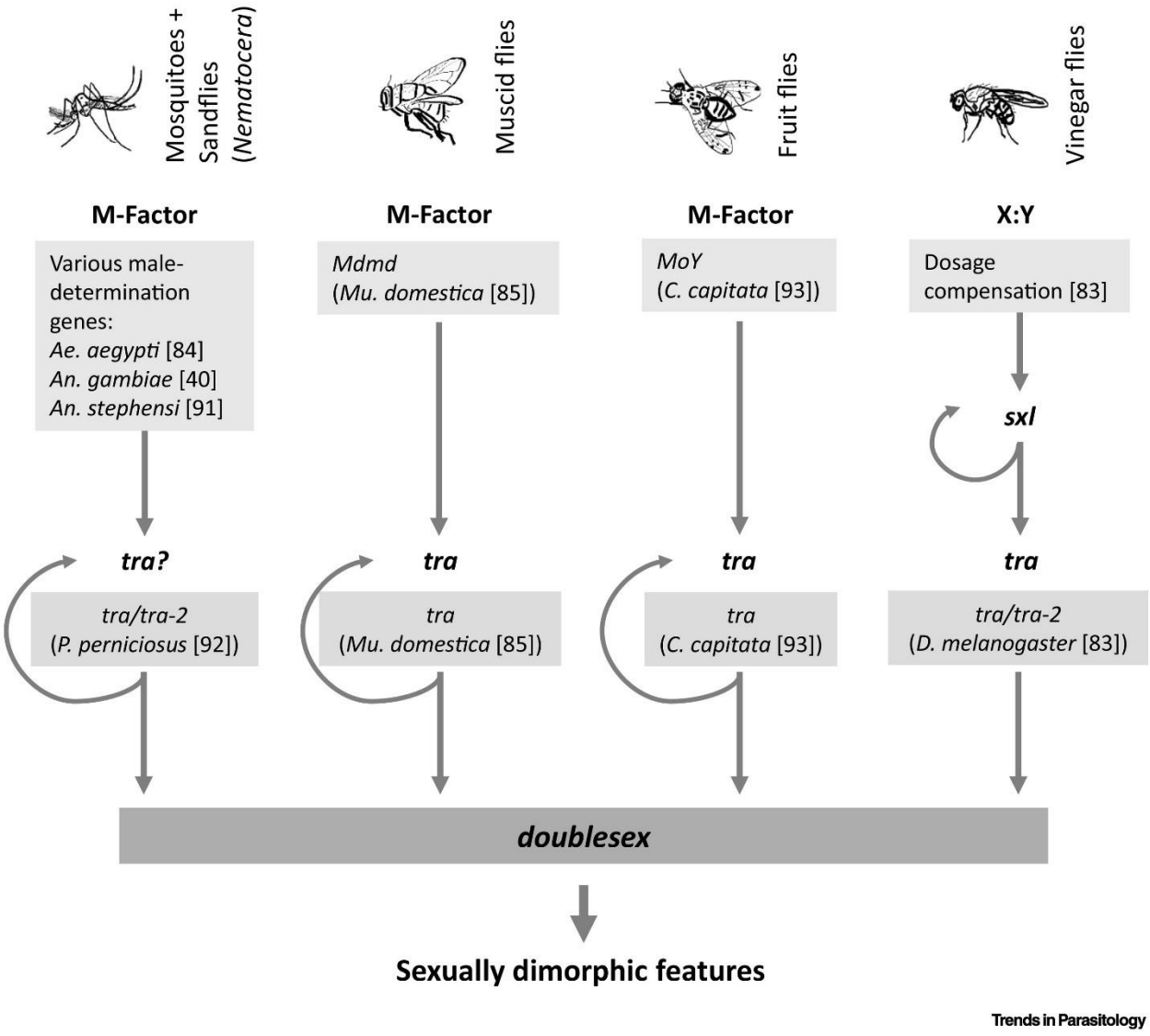


**Figure 1:** Dipteran sex-determination pathways reproduced from (Lutrat et al 2019), reference numbers refer to references in the original manuscript.

Successful design of SIT by RNAi depends on good quality genomic and transcriptomic data. *Ae. aegypti* has become a model organism in the past decade, with a well annotated genome.

This has come as a result of long-read sequencing and community annotation (Matthews et al. 2018). Following the success of making *Ae. aegypti* into a model organism (Matthews and Vosshall 2020) , recently conducted RNA-seq (Gamez et al. 2020) is bringing *Ae. albopictus* gene models closer to a quality needed to conduct rationally designed loss-of function screens. The highly repetitive genome of *Ae. albopictus* is not well assembled, however, and long-read sequencing is needed to accurately describe splice variants and assemble intronic and intergenic regions.

## RNAI BASED INSECTICIDES

RNAi by delivery of dsRNA was discovered in the 1990s (Fire et al. 1998) and has allowed for loss of function assays, revealing gene function in model organisms. Many of these phenotypes are lethal, raising the possibility of applying RNAi as an insecticide. The cost of producing dsRNA remains high, but there is growing interest in developing new insecticides against pest insects by inducing lethal phenotypes using RNAi (Huvenne and Smagghe 2010; Whyard, Singh, and Wong 2009; Bolognesi et al. 2012). Methods of delivery include soaking aquatic stages in dsRNA, topical application on leaves, and transformation of plants to express dsRNA against crop pests (Yu et al. 2013).

## BARRIERS TO SUCCESSFUL RNAI BY FEEDING

Despite the potential of dsRNA feeding to insects as a sex-sorting method, sterilant or insecticide, many barriers to achieving 100% effective RNAi by feeding remain to overcome. Each step towards RNAi by feeding dsRNA is subject to barriers that insects have evolved to defend against exogenous dsRNA. Insects deal with the threat of dsRNA by degrading it in the gut after ingestion. If dsRNA survives degradation, there are barriers to uptake by insect cells and finally there may be mechanisms to moderate mRNA depletion in the cytoplasm. In this

section the barriers to RNAi by feeding are discussed, preceded by a brief review of dsRNA processing in insects.

## UNDERSTANDING DSRNA PROCESSING IN CELLS

Small RNAs, including micro RNAs (miRNAs), small interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs) are molecules in eukaryotes that regulate the expression of endogenous genes or recognize and cleave exogenous viral RNA. piRNAs are specific to germ-line cells and are primarily involved in suppressing the activity of transposons by mechanisms that are not yet fully understood (Weick and Miska 2014). In contrast, miRNAs and siRNAs have been well-studied and they share some common features and functions. Both are distributed in the cytoplasm, where they bind to regulatory proteins in the argonaute family to silence gene expression either by cleavage of mRNAs (siRNAs) or translational repression (miRNAs). While both types of small RNAs can come from either exogenous or endogenous sources, in general, miRNAs are transcribed and processed in the nucleus and siRNAs come from outside the cell, typically derived from viruses or are exogenously applied to cells experimentally.

miRNAs were first discovered in the nematode *C. elegans* (Lee, Feinbaum, and Ambros 1993), but have since been found in every metazoan examined (Cloonan 2015). Over 1900 miRNAs are encoded in the human genome (‘MirBase’ 2020), and they are estimated to regulate the expression of approximately one third of the genes in humans. miRNAs regulate gene expression in eukaryotes by inhibition of translation or destabilization of mRNA (Bartel 2004; Yang et al. 2014). The complex miRNA biogenesis pathway can be summarized as follows: long (100nt) hairpin RNA is transcribed in the nucleus, cropped ~22nt away from the last junction before the loop of the hairpin and then exported to the cytoplasm (Ha and Kim 2014). This hairpin is processed by Dicer to form a duplex which is loaded onto an argonaute protein to form an

immature RNA-induced silencing complex (RISC). After unwinding of the duplex in RISC, the complex is mature and is able to bind with partial or full specificity to target regions on mRNA 3' UTRs. Though miRNAs are expressed endogenously to down-regulate gene expression, the introduction of exogenous miRNAs has been shown to increase levels of target mRNAs, possibly by competing with endogenous miRNAs (Cloonan 2015).

The gene regulatory effect of siRNAs is analogous to that of miRNAs, but their origin is normally from outside the cell. siRNAs may be derived from long dsRNAs or may be introduced as single siRNA units, though in insects, uptake of long dsRNA is more efficient than siRNAs (Yu et al. 2013). Transport of dsRNA across the cell membrane may be mediated by as yet unidentified receptors that are drawn into the cells by clathrin-mediated endocytosis (Xiao et al. 2015). Once in the cytoplasm, dsRNA is cleaved by Dicer into ~22nt siRNAs. siRNAs bind to an argonaute family protein, the basic components of RISC, along with other proteins. One strand of the duplex is unwound, producing an active RISC complex with a guide strand specific to mRNAs which are degraded by endonucleases of the RISC complex.

## INSECTS VARY IN THEIR RESPONSE TO DSRNA FEEDING

Not all insect clades respond to dsRNA feeding. For example, many Lepidoptera appear to be resistant (Terenius et al 2011; Kolliopoulou and Swevers 2014) to dsRNA feeding. There are several reasons that have been proposed (Cooper et al. 2019) to explain variation of RNAi efficiency between the insect orders, including degradation of dsRNA in the gut, lack of uptake by gut epithelial cells, deficient components of the RNAi pathway and lack of systemic transmission of the RNAi trigger. Within the transcriptome of an insect there may be further variability of RNAi efficiency due to variation in how target sequences are presented to the RNAi machinery and the regulatory network of the target gene. Although an enormous research

effort has been applied to solving this variation in RNAi response in important agricultural pests (Terenius et al. 2011; Wynant et al. 2014; Cooper et al. 2019), a unified understanding of the mechanisms of RNAi refractoriness in insects and means to circumvent it remain elusive.

## DEGRADATION IN THE GUT

In some insects, gut nucleases can degrade the ingested dsRNA within minutes, thereby preventing RNAi (Christiaens, Swevers, and Smagghe 2014; Singh et al. 2017; Cooper et al. 2019; Tayler et al. 2019). This phenomenon has been described in mosquitoes (Singh et al. 2017), although the enzymes involved in dsRNA degradation in the mosquito gut have not been functionally validated.

DsRNA nucleases have been described and implicated functionally in several agricultural pests, providing a template for similar studies in mosquitoes. In the Colorado potato beetle *Leptinotarsa decemlineata* (Say, 1824), for example, Spit and colleagues observed that direct injection of dsRNA into the hemocoel could knock down target transcripts, but ingested dsRNAs were rapidly degraded by gut nucleases, thereby preventing effective RNAi (Spit et al. 2017). This experiment was followed by functional validation whereby knockdown of two nucleases improved the RNAi response. In the locusts however, knockdown of four nucleases proved ineffective at restoring the RNAi response, which the authors suggested was either due to incomplete knockdown of all four nucleases, or other unidentified nucleases were still active (Spit et al 2017). In the pea aphid *Acyrthosiphon pisum* (Mordvilko, 1914), dsRNases have similarly been shown to reduce RNAi efficiency (Christiaens, Swevers, and Smagghe 2014). Chung et al. (2018), working with the same species, reported that an RNAi response was only observed when a nuclease was knocked down together with the genes they were investigating. Successful RNAi in lepidopteran insects has been only rarely achieved, with dsRNases

contributing to low RNAi efficiency in the silkworm moth *Bombyx mori* (Linnaeus, 1758) ( (Liu et al. 2012) and in the Oriental corn borer *Ostrinia furnicalis* (Guenée, 1854) (Guan et al. 2018). Nucleases have also been implicated in reducing RNAi efficiency in a dipteran pest, the Queensland fruit fly *B. tryoni*. Co-delivery of dsRNAs targeting two *B. tryoni* dsRNAses and one target gene dramatically improved knockdown of the target transcripts within three days of feeding on the dsRNA mixture (Tayler et al. 2019).

## UPTAKE AND TRANSPORT OF DSRNA

Escaping degradation by nucleases in the insect gut is not enough to trigger RNAi. DsRNA must also cross epithelial cell membranes and then be transported into cells where the target gene is expressed. After the remarkable success of RNAi in *C. elegans* (Fire et al. 1998), researchers were surprised to find many dipterans and lepidopterans to be resistant to RNAi (Huvenne and Smagghe 2010; Terenius et al. 2011). Some have argued that this resistance is due to the absence of homologs to *C. elegans* SID-1 transporter proteins, which serve as transporters of dsRNAs in nematodes, and may serve similar functions in other species (Terenius et al. 2011). Dipterans lack any homologs of SID-1, and yet, uptake of dsRNA into gut cells has been observed in a variety of dipteran insects (Walshe et al. 2009; Whyard, Singh and Wong 2009; Zhang, Zhang and Zhu 2010; Li, Zhang and Zhang 2011), and in cultured dipteran cells (Clemens et al. 2000; Saleh et al. 2006). Receptor-mediated endocytosis is believed to facilitate the dsRNA uptake in Diptera (Saleh et al. 2006; Ulvila et al. 2006), and quite possibly many other insects (Saleh et al. 2006; Xiao et al., 2015; Cappelle et al. 2016; Pinheiro et al. 2018), but it is unclear whether specific dsRNA receptors mediate uptake. Finally, other mechanisms may be involved in dsRNA transport, including caveolar endocytosis and micropinocytosis (Vélez and Fishilevich 2018).

## BARRIERS TO RNAI IN SIT APPLICATIONS

As described in the sections above, feeding dsRNA to insects is prone to many barriers. With current technology, RNAi-based SIT will only be economical if feeding of dsRNA is used. Therefore, RNAi-based SIT depends on dsRNA successfully entering the gut, avoiding degradation, being taken up by gut epithelial cells and finding its way to the target tissue. Sterilization of males depends on dsRNA reaching the testes, and female specific lethality can only occur if dsRNA reaches tissues where the targeted sex-differentiation gene products are expressed. Because reproductive organs are separated from the gut by hemolymph and the peritoneal sheath, many questions remain unexplored regarding how sterilization and sex-sorting of mosquitoes by RNAi takes place.

One such gap in our knowledge is the identity of genes involved in sperm development. Gene targets for sterilization of mosquitoes are typically inferred from high throughput *D. melanogaster* screens, meaning only conserved genes are tested. Promising results have been described for the spermatogenesis genes *zpg* and *fzo*, in *Ae. aegypti* (Whyard et al. 2015), but RNAi-mediated knockdown in these experiments fell short of 100% sterility in treated males. To preserve mating fitness, target genes involved in spermatogenesis must not serve other functions in the brain, which may mean that many highly conserved genes will not be suitable for large-scale SIT. Additional targets for sterilization identified in *D. melanogaster* include *Ipo9*, a gene recently shown to be necessary for the recruitment of factors necessary for late-stage gametogenesis (Palacios et al. 2020), and *lost boys* which is essential for retrograde swimming needed to enter the spermatheca (Yang et al. 2011).

Similarly, gene targets for sex-sorting in Diptera have been limited to a small set of highly conserved genes (Lutrat et al. 2019). The recent discovery of lncRNA targets for sex-sorting in

*Ae. aegypti* notwithstanding (Duman-Scheel 2019), complete removal of female mosquitoes by dsRNA feeding will require the identification of additional sex-differentiation targets.

With limited proven targets to optimize for SIT, and the relatively large evolutionary distance between vinegar flies and mosquitoes, more research to describe suitable mosquito sex-sorting and sterilization targets is needed.

## DSRNA DELIVERY METHODS

Variability in RNAi response has led to a wide range of approaches to overcome dsRNA degradation in the gut, and to enhance dsRNA uptake in the cells. These include modified dsRNA bases to a range of encapsulation techniques.

The simplest dsRNA protection method may be microorganisms: expression of dsRNA in transformed microorganisms provides natural encapsulation method. In *Ae. aegypti* larvae, it has been shown that synthesized siRNAs against essential genes yields moderate mortality, but when shRNAs against these targets were expressed in bacteria or yeast, mortality approached 100% (Hapairai et al. 2017). It is also possible to engineer symbionts to express shRNA against essential genes in pest insects. This has been accomplished in the horticultural pest *Frankliniella occidentalis* (Pergande, 1895) and the Chagas disease vector *Rhodnius prolixus* (Stål, 1859) (Whitten et al 2016). RNAi by symbiont expression is considered an inoculative approach as the engineered symbiont can spread through the population (Whitten et al 2016).

Widely used in cell culture to protect dsRNA and deliver it to cells, cationic liposomes encapsulate nucleotides within clusters or lipid bilayers and deliver them across cell membranes. A liposome encapsulation approach has been used in *D. melanogaster* to knock down transcripts and induce mortality (Whyard et al. 2009), whereas naked dsRNA did not perturb the target transcripts or induce mortality in that species. Similar work with *D. suzukii* (Matsumura, 1931)

confirmed that cationic liposomes were needed for dsRNA uptake, suggesting that epithelial cells in the gut of both species do not efficiently transport dsRNA into the cytoplasm (Taning et al. 2016) . Bacterial min icells protect dsRNA using a similar mechanism to the lipid bilayer formed by liposomes. This approach mimics the cell membrane and enhances uptake while protecting nucleotide cargo. Currently, the delivery of nucleic acids using bacterial minicells has been restricted to mammalian systems (Giacalone et al. 2006).

To overcome degradation in the gut, nucleotides may be modified such that nucleases are unable to cleave the dsRNA strand. The addition of 2' methoxyl bases to the ends of siRNAs increased RNAi efficiency in the lepidopteran pest *Plutella xylostella* (Linnaeus, 1758) (Gong et al 2011, 2013). Another example of this approach is in development for use against agricultural pests by Trillium Agriculture (Hauser 2015). This company uses dsRNA constructs with three self-complementary loops, with each end modified to protect against nucleases. Further stabilization and uptake optimization are achieved by complexing the dsRNA with proteins. Another approach used to mitigate the effects of nucleases in the gut is to exploit the metalloenzyme-blocking property of EDTA. EDTA enhanced RNAi to induce mortality has been demonstrated in the pentatomid pest *Euschistus heros* (Fabricius, 1798) (Castellanos et al. 2019).

Cationic polymers have been shown to improve dsRNA stability and uptake into cells, thereby improving RNAi efficiency. These include chitosan, which was shown to improve RNAi efficiency in *Ae. aegypti* (Zhang et al. 2010, Lopez et al 2019) and several agricultural pests (*Spodoptera exigua* (Hübner, 1808) (Christiaens et al. 2018), *O. furnacalis* (He et al. 2013) and *S. frugiperda* (Smith, 1797) (Parsons et al. 2018). A guanylated polymer used to deliver dsRNA to the lepidopteran *S. exigua* larvae improved the efficiency of RNAi (Christiaens et al. 2018), a pest that was previously thought to be minimally responsive to RNAi (Wynant et al. 2014).

DsRNA protection and gradual release was accomplished with a similar polymer marketed as BioClay (Mitter et al. 2017) though its efficacy in mosquitoes remains untested.

Viral-like particles (VLPs) may be an additional means of delivering dsRNA to insects, though their efficacy has only been tested in vertebrates. Production of VLPs is possible in commonly used microorganism expression platforms, yielding encapsulated dsRNA that is readily taken up by cells (Hoffmann et al. 2016). Such an approach overcomes the problems both dsRNA degradation in the gut and cellular uptake.

## UNDERSTANDING DSRNA STABILITY, DELIVERY METHODS AND SEX-DIFFERENTIATION WILL ENABLE IMPLEMENTATION OF RNAI-BASED SIT

The path to a fully scalable, field-ready RNAi-based SIT program faces many barriers ranging from poorly understood stability and uptake of dsRNA to the function of the genes targeted to sterilize and sex-sort. Establishing methods that overcome these barriers is needed for the deployment of RNAi-based SIT at low cost in resource-poor settings.

To achieve the goal of RNAi-based SIT, a rational approach to the design of RNAi delivery is needed. This must account for the following factors: A) dsRNA stability in feeding pellets and in the insect gut, B) the need for optimized delivery methods, and C) an understanding of gene targets tailored to mosquito reproductive biology.

The experiments described in the chapters that follow were designed to improve RNAi-based SIT in mosquitoes by testing how dsRNA survives the insect's gut and attempt to find ways of overcoming this natural defense mechanism against dsRNA. In addition to finding ways of cheaply producing dsRNA and protecting it from degradation in the gut, delivery methods that enhance uptake by epithelial cells were tested. And lastly, transcripts that regulate male and

female differentiation early in larval development were identified, with the goal of discovering new targets for sex-sorting and sterilizing mosquitoes for release.

To achieve these goals, the following objectives were defined, each falling under one of three themes:

**Theme 1: Nuclease activity in the mosquito gut.**

Variation in response to dsRNA feeding experiments has been described in several insect clades, and several approaches to overcome this barrier to RNAi efficacy have been proposed. In the current study, I set out to measure dsRNA degradation in the larval gut of mosquitoes. I then attempted to reduce nuclease activity to prevent the degradation of dsRNA in mosquitoes and thereby improve RNAi efficiency.

**Theme 2: Sex-specific splicing in mosquito development.**

The process of differentiation into male and female morphologies is not well understood in mosquitoes. Identifying the alternative splicing events that lead to differences in males and females offers the potential of identifying gene targets for SIT. To identify genes that are alternatively spliced in males and females, I devised a means of sex-sorting in the larval stage, and then conducted long-read transcriptomic analyses to identify novel alternatively-spliced RNAs in each sex.

**Theme 3: Microbial production methods and optimization of feeding parameters**

Cost effective dsRNA delivery to larval mosquitoes is likely to depend on the development of efficient microbial expression systems. To this end, I tested dsRNA production in bacteria and yeast, and optimized the parameters of feeding these microorganisms to mosquito larvae.

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# Chapter 2: RNA interference is enhanced by knockdown of nucleases in the yellow fever mosquito *Aedes aegypti*.

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## INTRODUCTION

RNA interference (RNAi) is a well-established reverse genetics method used to explore gene functions in insects. While being an indispensable research tool in many insect species with numerous reviews of their applications (Joga et al. 2016; Heigwer, Port, and Boutros 2018; Schuster et al. 2019), RNAi responses are highly variable, with some insects having only limited RNAi functionality (Terenius et al 2011; Li et al. 2015; Joga et al. 2016). The variability in RNAi responses in different insects has been attributed to a broad range of factors, including variations in the double-stranded RNA (dsRNA) stability within different insects, differences in cellular uptake of the dsRNA trigger molecules, differences in intracellular distribution and processing of the dsRNAs, and differences in systemic distribution of the effector molecules (Scott et al. 2013; Cooper et al. 2019). Of these factors, the most extensively studied is the stability of the dsRNAs when delivered to the insects. RNAi-mediated knockdown of gene transcripts is achievable in many insect species by injecting dsRNA directly into the hemocoel, but as this delivery method can be technically challenging and/or time-consuming, many researchers have sought to develop feeding formulations to administer dsRNA for higher throughput applications. While many successful dsRNA feeding experiments have been reported (Kunte et al. 2020; Scott et al. 2013), a growing number of research groups have observed that

dsRNases within the insects can greatly reduce the efficacy of RNAi (Joga et al. 2016; Cooper et al. 2019).

In some insects, gut dsRNases can degrade the ingested dsRNA within minutes, thereby preventing RNAi, despite the insect having the essential core RNAi machinery. In locusts, for example, direct injection of dsRNA into the hemocoel was used to knock down target transcripts, but ingested dsRNAs were rapidly degraded by gut dsRNases, thereby preventing effective RNAi (Spit et al. 2017). In the same study, the authors found that knockdown of two dsRNases improved the RNAi response in the Colorado potato beetle *Leptinotarsa decemlineata* (Spit et al. 2017). In the locusts, however, knockdown of four dsRNases proved ineffective at restoring the RNAi response, which the authors suggested was either due to incomplete knockdown of all four dsRNases, or other unidentified dsRNases were still active. In the pea aphid *Acyrthosiphon pisum*, dsRNases have similarly been shown to reduce RNAi efficiency (Christiaens, Swevers, and Smagghe 2014). Chung and colleagues (Chung et al. 2018), working with the same species, reported that an RNAi response was only observed when a dsRNase was knocked down together with the genes they were investigating. RNAi in lepidopteran species is generally poor, often only achieving modest knockdown of transcripts if high doses of dsRNA are used. Like other insects, dsRNases have been found to contribute to low RNAi efficiency in the silkworm moth *Bombyx mori* (Liu et al. 2012) and in the Oriental corn borer *Ostrinia furnicalis* (Guan et al. 2018). DsRNases have also been implicated in reducing RNAi efficiency in a dipteran pest, the Queensland fruit fly *Bactrocera tryoni*. Co-delivery of dsRNAs targeting two *B. tryoni* dsRNases and one target gene dramatically improved knockdown of the target transcripts within three days of feeding on the dsRNA mixture (Tayler et al. 2019).

In mosquitoes, many researchers choose to deliver dsRNA by direct hemocoel injections, to ensure precise dsRNA doses and consistent knockdown of the genes under study (Pelletier et al. 2010; Wang et al. 2012; Vogel, Brown, and Strand 2015; Regna et al. 2016; Edgerton et al. 2020). DsRNA feeding in mosquitoes has proven effective in inducing RNAi, but, typically, only partial reductions in the target transcripts or limited knockdown phenotypes have been described (Mysore et al. 2015; Durant and Donini 2018; Lopez et al. 2019). Improvements in RNAi efficacy in mosquitoes have been observed when the dsRNA was fed to larvae either encapsulated (Zhang, Zhang, and Zhu 2010; Das et al. 2015) or expressed within microorganisms (Whyard et al. 2015; Hapairai et al. 2017; Taracena et al. 2019; Rocco et al. 2019). In these latter cases, the enhanced RNAi efficacy was attributed to protection of the ingested dsRNA from gut dsRNases.

Gut dsRNases in mosquitoes and their possible impacts on RNAi efficacy have not been described before. In this study, we used phylogenetic analyses to identify a diversity of nucleases in the mosquito *Aedes aegypti*. Two nucleases expressed in the larval gut were suspected of contributing to variable RNAi responses in this insect. Using bacterially produced short (21 to 26 nucleotide [nt]) hairpin dsRNAs (shRNAs), in a manner similar to that described by Hapairai et al. (2017) we examined whether RNAi efficiency can be enhanced by knocking down the activity of two of these dsRNases, thereby enabling other dsRNAs to reduce their target transcripts more effectively.

## MATERIALS AND METHODS

### DSRNASE EXPRESSION PROFILING AND PHYLOGENETIC ANALYSES

Ten *Ae. aegypti* genes were identified by homology with *Drosophila melanogaster* dsRNAse gene CG6839 and to dsRNases in several agricultural pest insects, including *Ostrinia furnicalis*

(Guan et al. 2018), *Acyrthosiphon pisum* (Christiaens, Swevers, and Smagghe 2014), *Leptinotarsa decemlineata* (Spit et al. 2017), and *Bactrocera tryoni* (Tayler et al. 2019). Mosquito homologs to gene AAEL008858 (hereafter referred to as 8858) were identified from current Vectorbase gene sets, and a neighbor-joining tree was created in MEGA X: Molecular Evolutionary Genetics Analysis Across Computing Platforms (Available from https://www.megasoftware.net/). Primers for each *Ae. aegypti* gene were designed using NCBI Primer-BLAST (Basic Local Alignment Search Tool). Wild-type mosquitoes used in this study were Liverpool strain *Ae. aegypti* obtained through BEI Resources (Atlanta, GA), NIAID (National Institute of Allergy and Infectious Diseases, National Institutes of Health): *Aedes aegypti*, strain LVP-IB12, eggs, MRA-735, contributed by David W. Severson. This colony was maintained at 12:12 light:dark photoperiod at 28°C with heparinized rat blood provided weekly and 10% sucrose provided *ad libitum*. Eggs were collected on paper towel and hatched into dd$H_2O$ bubbled with nitrogen to induce rapid hatching. Larvae were dissected immediately post-hatch (0 h) and at 6 time points until at 48 h. Isolated guts and remaining carcasses were dissected from pools of 10 mosquitoes, and RNA was extracted from the pooled tissues, or from individual intact insects, using the GeneJet RNA extraction kit (Thermo Fisher Scientific, Waltham, USA) according to manufacturer's specifications. RNA was treated with DNase-I (Thermo). The complementary DNA (cDNA) was prepared by reverse transcription with qScript (Quantabio, Beverly, USA) in 10 microlitre (uL) reactions, and 20 uL qPCR reactions were performed with SsoFast Evagreen supermix (BioRad, Hercules, USA) using the following thermocycling conditions: 3 min at 94 °C, 40 cycles of 94 °C for 20 s and 57 °C for 20 s, followed by melt-curve analysis on a CFX Connect PCR machine (BioRad). Transcript abundance relative to the ribosomal S7 gene (primer sequences are found in Table S1) was

calculated using the Livak method (Livak and Schmittgen 2001) for gut and carcass and L4 and adult females. Melt-curve analyses confirmed that only single amplicons were produced for all primer sets. As the PCR efficiencies for all primers ranged between 96 and 102% (Table S2), the single reference gene was considered appropriate for all transcript-level comparisons. Relative transcript abundances were calculated and expressed using a heat map generated in Microsoft Excel (Redmond, WA, USA).

## KNOCKDOWN OF DSRNASES BY FEEDING SHRNAS EXPRESSED IN BACTERIA

To reduce the activity of dsRNases *in vivo,* mosquito larvae were fed bacteria expressing shRNAs, targeting either a dsRNase gene or enhanced cyan fluorescent protein (*eCFP*). For treatments where two genes were targeted, mosquitoes were fed a mixture of two *Escherichia coli* strains. Target sequences were identified using Integrated DNA Technology's online Dicer substrate short interfering RNA (siRNA) design tool, and shRNAs were designed with a stem sequence of 21–26 base pair (bp) and a loop sequence of 9 bp (Figure S1). Annealed DNA oligos of the shRNA sequences were blunt-end ligated into the expression vector pJET1.2 (Thermo Fisher Scientific, Waltham, USA) downstream of a T7 promoter. A single shRNA construct was expressed by each *E. coli* strain. A non-specific control shRNA was synthesized containing a *Discosoma* Red DsRed sequence. *E. coli* (HT115 (DE3), available from University of Minnesota Caenorhabditis Genetics Center) cells were transformed with the plasmids, grown to mid-log phase (~16 h), and expression was induced with 0.4 M isopropyl β-d-1-thiogalactopyranoside (IPTG) for 4 h before harvesting. Harvested cells (40 mL) were pelleted by centrifugation at 5000 × g for 5 min and the pellets were then suspended in 4 mL 1% agar supplemented with 1 mL 10% sterile brewer's yeast (MP Biomedicals, Irvine, CA, USA) slurry. This mixture solidified in open-topped 5-mL syringes and sliced into 0.5-mL feeding pellets. Mosquito eggs

were hatched synchronously by submerging them in deoxygenated, previously boiled ddH2O into which nitrogen gas was bubbled for 2 min. In 100-mm Petri dishes, 40 larvae in 20 mL $ddH_20$ were provided with two pellets. These pellets were the exclusive food of the larvae from 4 h post hatch until the fifth day of development, when they were dissected to measure impact on dsRNase activity.

## EX VIVO DEGRADATION ASSAYS

Midguts were dissected in phosphate buffered saline (PBS) from bacterially fed fourth instar larvae, taking care to eliminate Malpighian tubules, crop, and gut contents sheathed within the peritrophic membrane. To standardize damage to the gut tissues, all guts were torn with forceps at five places. Pools of three guts were incubated overnight at 4 °C in 100 uL PBS before use in *ex vivo* degradation assays. To measure dsRNA degradation, 100 ng of dsRNA specific for the *β-glucuronidase* (Gus) gene (Whyard et al. 2015) was incubated with 7 uL of mosquito gut secretions in 1 × PBS pH 7.4 (Thermo Fisher Scientific, Waltham, USA). Aliquots of each sample were incubated at 28 °C for 0, 10, 30, or 60 min. Enzyme activity was halted by transferring the samples to ice. For negative controls, gut extracts were either heat-killed (20 min at 80 °C) before incubating with dsRNA for 60 min or contained only PBS with no gut extracts added. Samples for each of 4–8 biological replicates, with 2–4 technical replicates, were loaded onto 1.5% agarose gels and resolved for 30 min at 120 V in 1x Tris-acetate EDTA (TAE) buffer (Thermo Fisher Scientific, Waltham, MA). Gels were stained with ethidium bromide and imaged under UV illumination using a Gel Doc XR+ system (BioRad). The Band Analysis tools of Image Lab software, version 4.1, (BioRad) were used to calculate band intensities relative to heat-killed controls. For each time point, relative band intensity of RNAi treatments was compared to DsRed controls using an unpaired Wilcoxon rank sum test.

## CO-FEEDING OF DSRNASE AND REPORTER GENE SHRNA

To determine whether knockdown of dsRNases by shRNA improves RNAi when co-delivered with other RNAi triggers, we targeted the *eCFP* gene in the *Aedes aegypti* Orlando Gr3[*eCFP*] strain obtained from BEI Resources (Atlanta, GA) (McMeniman et al. 2014). The *eCFP* gene in this mosquito strain was expressed under the control of the ubiquitin promoter, and, hence, the fluorescent protein was expressed in all tissues throughout development, allowing for measurement of impact both in the gut and in the carcass. For co-feeding experiments 26 nt shRNAs expressed in *E. coli* were used with bacterial feeding pellets provided as the exclusive food from the day of hatching until fluorescent protein quantification. More stringent expression parameters were used compared to 22mer expression used for degradation assays. An overnight starter broth (2 ml LB ampicillin 50 ug/mL + tetracycline 10 ug/mL, [hereafter referred to as LB amp+tet] inoculated from glycerol stocks) was grown to mid-log phase and 1 mL of starter broth was used to inoculate 40 mL of LB amp+tet broth. This culture was grown to an optical density (OD) of 0.4 and induced with 0.4 mM Isopropyl β- d-1-thiogalactopyranoside (IPTG), then grown to an OD of 1.0. Bacteria expressing a scramble nt shRNA were used as controls. Bacteria from 40 mL of each dsRNase-gene shRNA expression strain was mixed 1:1 with double-stranded RNA against *eCFP* (ds*eCFP*) bacteria. An additional 2× ds*eCFP* control treatment was produced with cells from 80 mL of LB amp+tet broth, and feeding pellets were made as described above. Twenty mosquitoes were reared in 5 mL of water in deep-well, 100-mm Petri dishes. Following development on bacterial feeding pellets, fluorescence was measured in whole bodies of L4 mosquitoes, 5 days after hatching. RNAi efficiency against a fluorescent reporter was quantified using methods modified from previous studies in *Caenorhabditis elegans* (Timmons et al. 2003). To minimize size effects of background fluorescence, only larvae of uniform size were selected, and this size selection produced varying numbers of larvae analyzed

for each treatment. CFP fluorescence intensity was measured by placing a single live larva in 5 uL 1:1 1 × PBS:pure glycerol (to reduce their mobility) in each well of concave-bottomed 96-well plates and read using a Biotek Synergy H1 plate reader (Biotek, Winooski, VT) with excitation set to 435 nm and emission 505 nm. For these readings, a 7 × 7 grid with 600 nm spacing captured the entire bottom of each well, and the mean of the highest 10 of 49 values (indicative of the larva's position within the well) was expressed as a percentage of fluorescence relative to background autofluorescence levels observed in non-CFP mosquitoes treated in the same manner. The mean of each treatment was compared with a Wilcoxon sum-rank test. Survival of mosquitoes treated with shRNAs against the nuclease gene 8858 combined with *eCFP* shRNA treated was calculated by observing movement of larvae for a 30-s period on day five. Survival was expressed as a proportion alive of a starting number of 20 larvae per Petri dish. This was replicated four times for scramble nt controls and 8858 combined with *eCFP* shRNA. Adjusted mortality was calculated using Abbott's formula (Abbot 1987) and the means of survival per dish were compared with a Wilcoxon rank-sum test.

## MEASUREMENT OF DSRNA UPTAKE AND DEGRADATION IN THE MOSQUITO HEMOLYMPH

To quantify dsRNA in the hemolymph of mosquito larvae following soaking with long dsRNA, fourth instar *Ae. aegypti* larvae were soaked in 10 uL of 0.1 ug/uL ~350 bp dsRNA specific to the bacterial gene *β-glucuronidase* (Gus) (Whyard et al. 2015). Glass microcapillaries were used to withdraw approximately 0.5 uL of hemolymph from each larva. RNA from hemolymph of 10 pooled individuals was extracted and quantified relative to ribosomal S7 as described above.

# RESULTS

## MULTIPLE DSRNASES ARE FOUND IN MOSQUITOES

Sequences similar to other insect dsRNases (Spit et al. 2017; Christiaens, Swevers, and Smagghe 2014; Guan et al. 2018; Tayler et al. 2019) were identified using BLAST searches of the publicly available mosquito datasets (Vectorbase gene set AaegL5.2). Phylogenetic analyses revealed that aedine mosquitoes have a diversity of dsRNases, with the greatest number found in *Ae. aegypti* (n = 10) and *Ae. albopictus* (n = 10) (Figure 1). The 10 *Ae. aegypti* genes had a maximum E-value of 3e-27 at the amino acid level to the *D. melanogaster* gene CG6839 annotated a dsRNase. In addition, all 10 proteins were predicted by ProSite (Available at https://prosite.expasy.org/) to include the same functional domains as previously described dsRNases found in other insects (Guan et al. 2018; Christiaens, Swevers, and Smagghe 2014), with a signal peptide and a dsRNA cleavage domain. In all but one (AAEL006326) of the *Ae. aegypti* genes, the dsRNA cleavage domain included a conserved histidine residue that was functionally shown to be a proton acceptor in the well-described *Serratia marcescens* dsRNase homologue (Friedhoff, Gimadutdinow, and Pingoud 1994) (Figure S2).

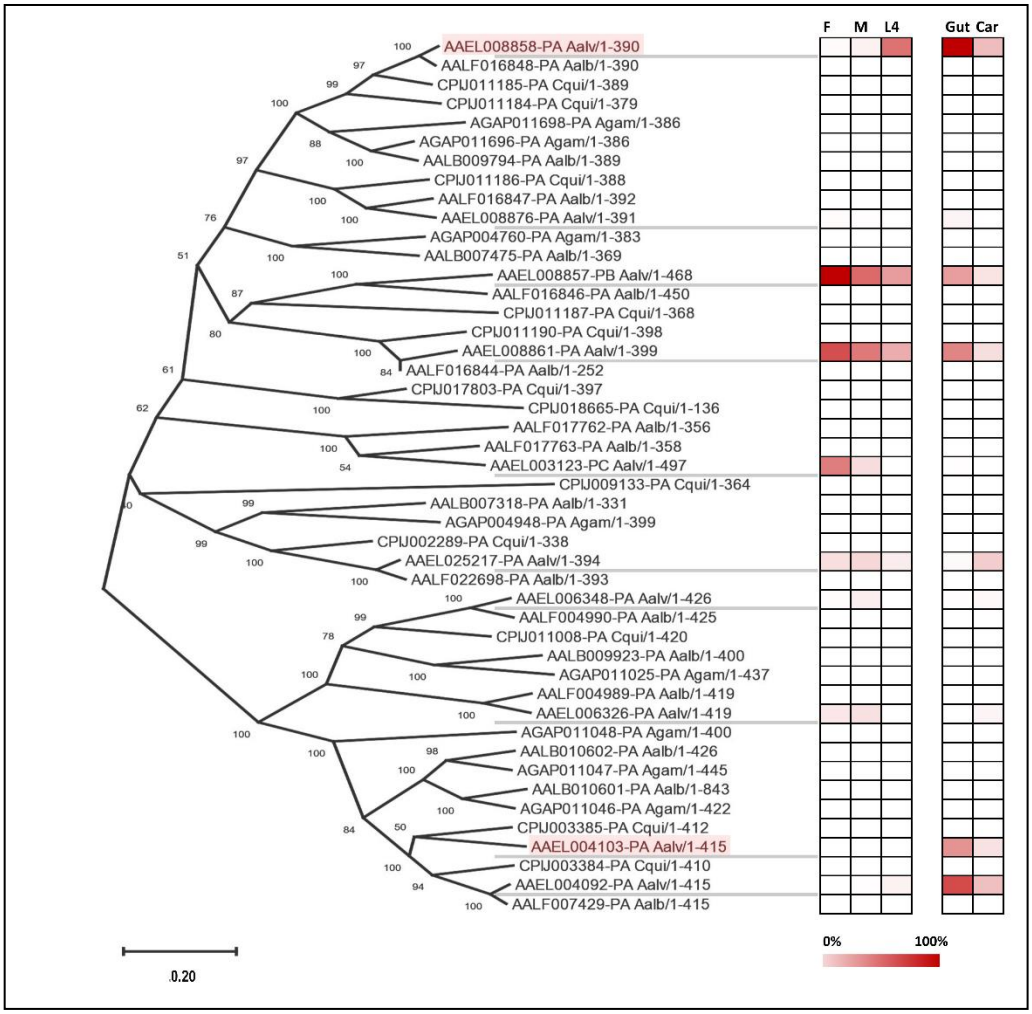
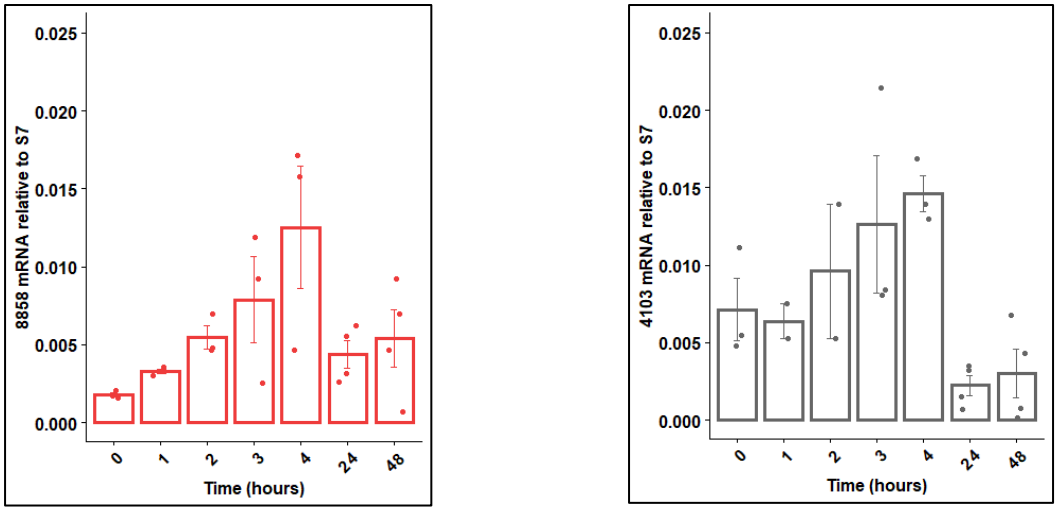



**Figure 1.** Phylogenetic tree of mosquito dsRNases and expression heat map of representative *Ae. aegypti* dsRNases. The neighbor-joining phylogenetic tree was produced in MEGA X from Clustal-Omega alignments' mosquito homologue proteins with scale bar indicating genetic distance. F, M, and L4 represent male, female, and fourth instar larvae, respectively. Gut and Car represent gut and carcass. Genes selected for further investigation are highlighted in pink. Expression is shown by a color scale where 100% is equal to the highest observed expression in each grouping: (female, male, larval stage 4) and (gut, carcass). 0% equals the limit of detection from four biological replicates per sample type.

## TWO DSRNASES ARE EXPRESSED IN THE GUT OF LARVAL *AE. AEGYPTI*

If RNAi technology is to be used to sex-sort or sterilize *Ae. aegypti* in a sterile insect technique (SIT) program, an ideal time to deliver the dsRNA would be during the larval feeding period, when dsRNA can be administered cheaply and efficiently to many insects simultaneously (Lutrat et al. 2019). Hence, we opted to focus our attention on dsRNases expressed within the guts of larvae, as these dsRNases would be the first to contact the administered dsRNAs. Based on qRT-PCR analyses, six of the 10 putative dsRNases were expressed mostly in the guts, relative to the rest of the body, and two of those dsRNase genes were more strongly expressed in larvae relative to adults (Figure 1). Expression of AAEL004103 and AAEL004092 is very low in the larval gut, but is gut-biased. In addition, the presence of a signal peptide sequence in AAEL004103 led me to investigate this gene further. Subsequent analyses focused on two larval gut-specific genes, 8858 and AAEL004103 (henceforward described as 4103). Both genes expressed throughout larval development, except during the initial h post-hatching, when very low transcription was observed for 8858 (Figure 2a, 2b). Expression of 8858 increased in some individuals between 3 and 4 h post-hatch (Figure 2b), corresponding to the inflation of the head capsule and initiation of feeding (Christophers 1960). Thereafter, expression of 8858 was variable.




(a) (b)

**Figure 2**: Expression profile of dsRNase genes 8858 (a) and 4103 (b) from pools of 10 *Ae. aegypti* larvae immediately post-hatch (0 h) to 48 h (early fourth instar). Expression was determined by qRT-PCR using ribosomal S7 as a control reference gene. Individual biological replicates (ranging between n = 2 and n = 4) and standard error are shown by dots and error bars, respectively.

## KNOCKDOWN OF DSRNASES REDUCES DSRNA DEGRADATION IN THE GUT

To assess whether the two dsRNases had a role in dsRNA degradation in the gut, larvae were fed *E. coli* expressing 22nt shRNAs targeting either 8858 or 4103. After five days of continuous feeding, degradation of dsRNA by midgut secretions was assessed in *ex vivo* assays. No dsRNA degradation was detected in dsRNA samples lacking gut extracts or in samples treated with heat-treated gut extracts. Gut extracts from negative control larvae that were fed bacteria expressing non-specific double-stranded RNA against DsRed (dsDsRed) degraded virtually all dsRNA within 30 min, indicating the presence of highly potent dsRNase activity in the gut tissues. In contrast, larvae fed ds4103 showed no significant degradation of dsRNA for the first 60 min.

Larvae fed ds8858 showed no significant degradation of dsRNA for the first 30 min, although by 60 min, most of the dsRNA was subsequently degraded (Figure 3).

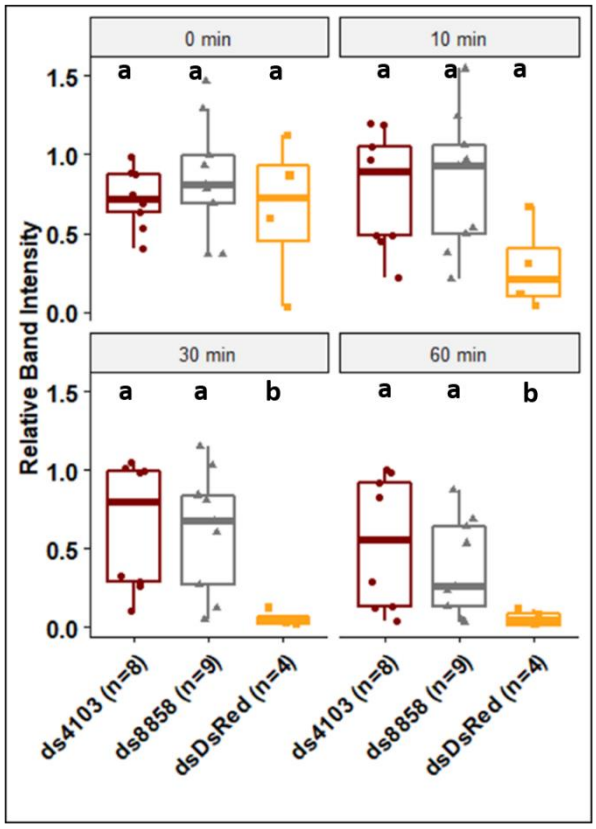



**Figure 3**: Degradation of long dsRNA after exposure to gut-derived enzymes. The shRNA were expressed in *E. coli* and fed to *Ae. aegypti* larvae in brewer's yeast-supplemented agar feeding pellets for five days at 28°C. Midguts were dissected and soaked in PBS overnight. The resulting enzyme mix was co-incubated with long dsRNA for 0, 10, 30, or 60 min and resolved by gel electrophoresis. Band intensity was calculated relative to heat-killed controls. Biological replicates are shown as jittered points (number of replicates ranged from four to eight per treatment). Comparisons by time and dsRNA treatment are denoted with different letters for Wilcoxon rank-sum *P*-values less than 0.05.

## CO-DELIVERY OF DSRNASE SHRNA WITH A TARGET SHRNA ENHANCES RNAI EFFICIENCY

To test whether co-feeding shRNAs against 8858 and 4103 could improve knockdown of a fluorescent transgene's expression, we co-delivered *E. coli* expressing shRNA against dsRNase genes and *eCFP* to an *Ae. aegypti* strain expressing *eCFP* under control of a ubiquitin promoter. Larvae fed bacteria expressing shRNAs targeting *eCFP* alone or together with a negative control "scrambled nt" shRNA failed to reduce *eCFP* fluorescence. Doubling the concentration of bacteria expressing *eCFP* shRNA resulted in only an 8% ($p = 0.127$) reduction in fluorescence compared to scrambled shRNA controls. However, when larvae were co-fed bacteria expressing shRNA targeting both *eCFP* and 4103, *eCFP* fluorescence was reduced by 32% ($p = 0.018$) relative to the negative shRNA controls (Figure 4). Co-feeding all three shRNAs (*eCFP*, 4103, and 8858) resulted in a non-significant reduction in fluorescence of 24% ($p = 0.09$). Treatments with the 26 nt shRNA targeting 8858 and *eCFP* suffered from adjusted mortality of 56% ($n = 4$, Wilcoxon $p = 0.029$) compared to "scrambled nt" shRNA controls (data shown in Table S5). No significant mortality was observed in any of the other treatments. Variation in number of larvae analyzed per treatment was due to size selection prior to fluorescence measurements.

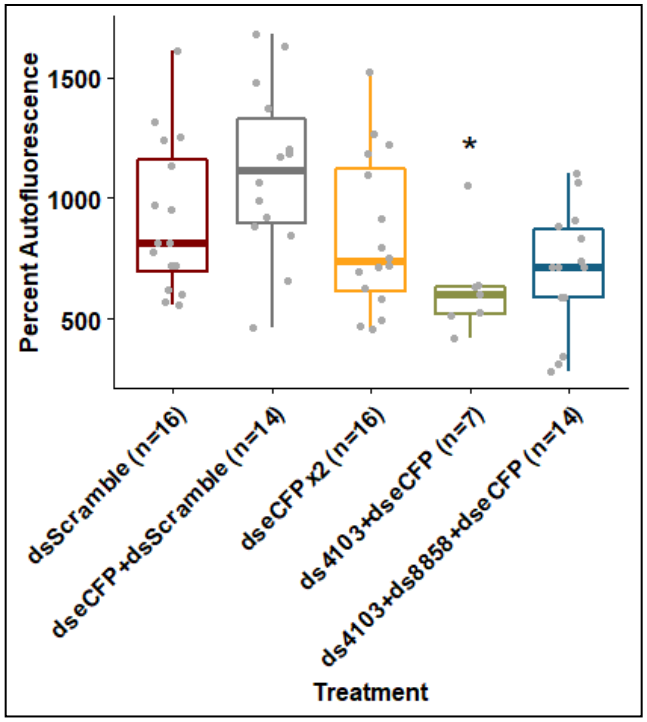



**Figure 4:** Co-feeding *eCFP* shRNA with dsRNase shRNA improved depletion of fluorescent protein by RNAi. The shRNAs were expressed in *E. coli* and fed to *Ae. aegypti* larvae in brewer's yeast-supplemented agar feeding pellets for five days at 28 °C. Whole larvae of uniform size were placed in conical-bottom 96-well plates and *eCFP* fluorescence was measured using a Biotek Synergy H1 plate reader. Box-plots show mean and upper and lower quartiles with range whiskers. Wilcoxon rank-sum *P*-value less than 0.05 relative to dsScramble controls are indicated by *. Ds8858+dseCFP treatments are not shown due to mortality (see Table S6).

## DSRNA ENTERS THE HEMOLYMPH WITHIN MINUTES OF DSRNA SOAKING

To explore dsRNA uptake by mosquitoes from the aquatic environment, mosquitoes were soaked in Gus dsRNA and the dsRNA present in the hemolymph was measured at various time points. DsRNA was detected in the hemolymph within the first 5 min of exposure, and levels steadily increased for the first 20 min. Thereafter, levels declined rapidly and dsRNA was not detectable at 60 min post exposure (Figure 5).

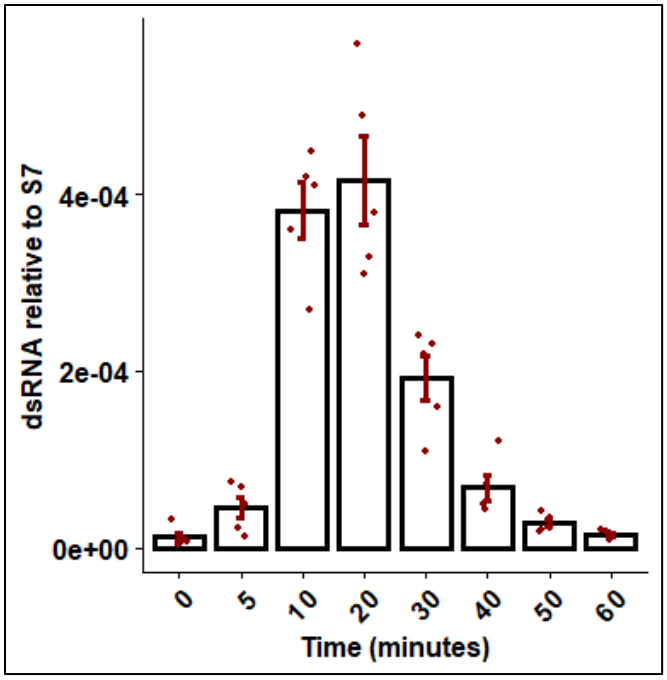



**Figure 5:** DsRNA recovered in hemolymph following soaking in dsGus for durations between 0 and 60 min. Fourth instar *Ae. aegypti* larvae were soaked in ~350 bp dsGus dsRNA 0.1 ng/uL and hemolymph was removed by microcapillary and dsRNA was measured by qRT-PCR, with ribosomal S7 used as a reference gene. Error bars represent standard error and bars represent mean of biological replicates (n = 5), shown as jittered points.

## DISCUSSION

RNAi efficiency in insects is variable and the factors contributing to this variability are still not well understood (Cooper et al. 2019). DsRNases have been shown to reduce RNAi efficiency by degrading RNAi trigger molecules in the gut, preventing uptake in target tissues (Singh et al. 2017). The impact of dsRNases on dsRNA degradation and reduced RNAi efficiency in mosquitoes was previously unknown. Here, we identified several putative dsRNases in the mosquito *Ae. aegypti*, and focused our attention on two dsRNases expressed in the larval gut.

Knockdown of one these dsRNases, 4103, proved effective in improving RNAi in the larval mosquitoes.

Functional predictions and phylogenetic analyses suggest that dsRNases, (Figure 1) with possible tissue and developmental specialization, are abundant in mosquitoes. From the publicly available data, aedine mosquitoes have the greatest diversity of dsRNases, with 10 predicted in *Ae. aegypti* and 10 in *Ae. albopictus*. In *Ae. aegypti*, the 10 dsRNases were found in close proximity on chromosomes 2 and 3, which suggests that the proliferation of these genes may have arisen through gene-duplication events. For example, genes AAEL008857, AAEL008858, AAEL008861, and AAEL008876 were found on a region of chromosome 3 spanning 24.3 kb (Table S4) and share highly similar amino acid sequences. Both of these aedine mosquitoes are relatively indiscriminate in their selection of freshwater breeding habitats, can develop in diverse aquatic environments, and outcompete local species in both man-made and naturally occurring aquatic habitats (Juliano and Lounibos 2005). We speculated that these dsRNase genes may have proliferated to permit exploitation of diverse habitats and food sources in both adult and larval stages. Gene duplication in *Ae. aegypti* in response to selective pressure has been documented previously, for example, in the GST epsilon genes (Matthews et al. 2018). Viral and eukaryotic dsRNA is encountered in the larval and adult diet, and dsRNases would undoubtedly assist in the degradation of these nucleic acids within the gut lumen. Interactions between viral pathogens and their mosquito hosts typically begin within the gut (Asgari 2018), and dsRNases in this tissue may provide a first line of defense by degrading viral dsRNA within the gut lumen. A variety of pathogens are known to use dsRNAs to facilitate the infection process, and, hence, degrading these nucleic acids within the gut could protect the insect host. Virally encoded double-stranded microRNAs (miRNAs), for example, have been implicated in modulating host gene expression,

and, more recently, eukaryotic protozoan parasites have similarly been observed to modulate immune responses in insect hosts (Asgari 2018; Bayer-Santos, Marini, and da Silveira 2017). Aside from offering possible protection from pathogens, dsRNases may also enable mosquito larvae to maximize nutrient acquisition from ingested microorganisms, as free nucleotides are a source of bioavailable phosphate in nutrient-poor aquatic habitats (Hossain, Koshio, and Kestemont 2019). The increased mortality rates of the larvae feeding on 8858 shRNA may indeed be a consequence of the larvae failing to digest free nucleotides in the environment. Mortality in 8858 shRNA feeding experiments is puzzling, in part due to the lack of mortality in experiments where 8858, 4103 and eCFP were targeted simultaneously. Future work to describe the role of 8858 in the gut lumen, as well in the development of other larval tissues is needed.

Our results suggest that dsRNA in the gut is degraded primarily by secreted dsRNases. Both *Ae. aegypti* RNase 4103 and 8858 were found to be necessary for dsRNA degradation *ex vivo*, as shown by shRNA knockdown. While it is possible that other enzymes, including bacterially-derived nucleases, may be contributing to dsRNA degradation in the larval gut, the improved durability of the dsRNA following the knockdown of these two dsRNases suggests that other dsRNases have only a small contribution to dsRNA degradation in the gut lumen. Previous reports of dsRNA degradation in *Ae. aegypti* assessed both processing of dsRNA by the RNAi machinery and degradation by dsRNases, suggesting that processing is a key limiting factor in dsRNA feeding experiments (Singh et al. 2017). Poor efficiency of dsRNA processing may be a factor limiting the success of RNAi experiments in *Ae. aegypti*, but our results suggest that a phenotypic response can be achieved by reducing dsRNase activity. Taken together, our results and other reports of a high degree of gene knockdown by dsRNA feeding (Hapairai et al. 2017) suggest that dsRNA processing in *Ae. aegypti* is sufficient to induce a strong RNAi response.

Based on our observation that dsRNAs were found within the hemolymph within minutes of ingestion, the durability of dsRNA in the early stages of feeding will be critical to improving the efficacy of the dsRNA at mediating knockdown in mosquito larvae, particularly if the dsRNAs must move systemically to reach targets beyond the gut. Co-feeding larvae with dsRNase-specific dsRNAs along with *eCFP* dsRNA proved effective at improving knockdown of that gene's proteins over several days. While the first ingestion of either ds1403 or ds8858 would not have immediate impact on their respective dsRNase levels in the gut, their activity was clearly reduced after five days, leading to improved knockdown of the reporter gene. Curiously, the dsRNA levels in the hemolymph decreased after 20 min, suggesting that further transfer of the dsRNA to the hemolymph had ceased. At this point in our research, it is not clear whether the insects had stopped feeding after this time point or if some mechanism of dsRNA sequestration was occurring. Future experiments are planned to assess whether dsRNA transfer rates are slowing after 20 min or if other dsRNases in the mosquitoes may be contributing to the rapid disappearance of dsRNA in the hemocoel.

The variation in RNAi efficiency in many insects may be the consequence of dsRNases rapidly degrading exogenously applied dsRNA. We speculated that a small, consistent dose of dsRNA is sufficient to induce RNAi, but in many insects, dsRNA was degraded before entering gut epithelial cells. Steady delivery of dsRNA (Hapairai et al. 2017) or daily dosing (Whyard et al. 2015; Taracena et al. 2019) in some insects can ensure that gene knockdown is initiated and sustained. However, for some insects, only highly concentrated doses of dsRNA were effective at knocking down targeted genes (Terenius et al. 2011). In many of these cases, despite most of the dsRNAs being depleted by dsRNase degradation, enough persisted to knock down the targeted genes. At lower dsRNA concentrations, or with only sporadic feedings of dsRNA, an

insufficient amount of dsRNA may survive dsRNase degradation within the gut to target the transcripts at the peak of their expression. Delivery of naked dsRNA also leaves the dsRNA vulnerable to degradation, whereas encapsulating the dsRNA can protect it from dsRNases. In previous studies where *Ae. aegypti* were soaked in siRNAs, RNAi-induced phenotypes were observed in less than 50% of individuals but approached 100% when larvae were continuously fed bacteria or yeast expressing shRNA (Hapairai et al. 2017). A combination of reduction in dsRNase activity, as reported here and in other insects (Christiaens, Swevers, and Smagghe 2014; Tayler et al. 2019), combined with protection inside the cells of a microorganism expression system (Whyard et al. 2015; Mysore et al. 2019) or liposomes (Tayler et al. 2019), may be an effective approach for many species. Such an approach may provide the sustained delivery of dsRNA needed for consistent transcript depletion and application of RNAi in pest management.

Successful induction of RNAi by feeding dsRNA is challenging, but overcoming these challenges will yield rewards to pest control. The possibility of co-delivery of dsRNase-specific dsRNA with their genes of interest may enable low-cost reverse genetics using RNAi, even against gene targets previously dismissed as unresponsive. This may include genes essential for male fertility and female-specific splice variants of sex determination genes. A promising extension of the current work is the use of RNAi enhanced by dsRNase knockdown to improve reliability of RNAi-based SIT. Coupled to low-cost dsRNA production systems in microorganisms, such a system may bring large scale SIT by RNAi within reach.

## CONCLUSIONS

Mosquito guts were found to have enzymes that degrade dsRNA. By knocking down two of these enzymes using RNAi, we were able to reduce dsRNA degradation and improve RNAi-

mediated knockdown of a fluorescent reporter. Our next steps will be to co-deliver dsRNA targeting transcripts of genes required for both gut dsRNases and sperm development, with the goal of achieving more reliable sterility by RNAi.

## SUPPLEMENTARY MATERIALS

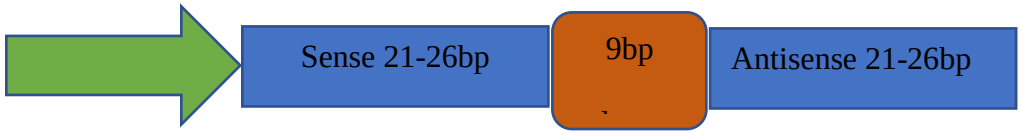



**Figure S1.** construct schematic of shRNA expression vectors in pJET1.2 cloning vector for all shRNAs used in this study.

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sp|P13717|NUCA_SERMA      --------PALNP------ADTLAPADYTGANAALKVDRGHQAPLASLAGVSDWESLNYL 129
AAEL004103-PA             TNYLYTVNKQRQTIAQILQSQDLADDIVRDVNSGIYMARGHIAARVDFIYGTQQNATFWF 259
AAEL004092-PA             INTLYTVNRQRQTLATVLGSQAIADDLVRDASTGIFMARGHIAARADFIYGTQQNATFWF 259
AAEL006348-PA             MTKLYNIESQRKTFEKILGSSARADALLN-SKQDMFLARGHLAAKADFVFGAHQRATFWF 271
AAEL006326-PA             MAEIYSFDVQHATLGLILGSTNRANNLLN-RRKDIFIAKGRLAAQADFVYGSQQAATFRY 264
AAEL003123-PC             VSSAYNQESQLNRLVALFGADPNPWGSAEVYYNLSYLQRGHLVPDADQLFTTWQWSTYFY 346
AAEL025217-PA             LSTVYTQNSQRTRLANLLGSEELA--KQYISSS-SFFAKGHLTPDGDAVLNSWAGATYFY 245
AAEL008857-PB             ADALYTQTTQFERFEALLGSKAQA--EKYIEPGKTFLNRGHLTPRGDGIFQTWKHATFFY 290
AAEL008861-PA             PSTSYTQASQLERLTTLLGSAEQA--SRFVFTN-SFMARGHMSPDADGIYRSWQFTTYFF 246
AAEL008876-PA             PATSYTQQAQLTRLTELLGSEEQA--KKFISGGSYYMARGHLAPDADGIYRSWQWATFFY 241
AAEL008858-PA             PASSYTTASQATRLAVLLGSQAQA--DRFITTS-SYMSRGHLSPDADGIFRSWQWATYFY 240
                                                  :               . :*:     .    :   :
```

**Figure S2.** Clustal Omega alignment of 10 *Ae. aegypti* putative dsRNase genes aligned to *Serratia marcescens nucA* (P13717). The predicted proton acceptor histidine residue is highlighted in yellow.

**Table S1** Primer sequences used in qRT-PCR assays in this study.

| Accession # | GeneID | 5′-3′ forward primer sequence | 5′-3′ reverse primer sequence |
|---|---|---|---|
| **AAEL009496** | *S7rp* | AAATAAATTCGCTATGGTTTTC | CCTTCTTGCTGTTGAACTCG |
| **AAEL004092** | | CGAGTGGAATAAACTAGCTTCAG | CAAGCTCCAAGTATTCGCCT |
| **AAEL002042** | | ATGGTGACGCTGTGTTGAAC | GAGGGTCAGAACGTCGTAGA |
| **AAEL004103** | 4103 | AGATTCGCTCCATCCTGCTC | TGAACCCATTGCTGCAAACG |
| **AAEL006348** | | AGAACCGCTGTAGAATCGCA | GCCCAAAGAAATTTTACAGCCGT |
| **AAEL006326** | | GTACAACGGGGACTTCGACA | GCTCTACCTGTTCACCGCTT |
| **AAEL003123** | | AGCATACGTGGAATAAATCTGGT | TTGGACTCGAGCTGCATCTG |
| **AAEL008876** | | GGAGCTCACCTGGGAAGAAC | GACGCTGTATTGTTGTTTGCAT |
| **AAEL008858** | 8858 | CCGTTTTGTTGGGTTCGCAA | GCGTTGACAACCTGCCATTG |
| **AAEL008857** | | GATTCGACTCCAAGCTGCTC | ACACCGATGCACTGGGATAC |
| **AAEL008861** | | GGACGCGGATGGAATCTACC | GTTGACAACCTGCCACTTCG |

**Table S2.** Primer efficiency data for qRT-PCR assays used in this study. $R^2$ values and percent efficiency (E%) are shown for each primer set (Supplementary Table 2) and tissue type. PCR reactions with insufficient data are shown as not determined (ND).

| Gene | Female gut | | Female carcass | | Larva gut | | Larva carcass | |
|---|---|---|---|---|---|---|---|---|
| | **$R^2$** | **E%** | **$R^2$** | E% | $R^2$ | E% | $R^2$ | E% |
| **AAEL009496** | 0.995 | 97.9 | 0.992 | 101.1 | 0.991 | 97.2 | 0.989 | 102.1 |
| **AAEL002042** | 0.977 | 101.6 | 0.986 | 99.2 | 0.982 | 97.8 | 0.972 | 96.1 |
| **AAEL003123** | 0.995 | 101.7 | ND | ND | 0.998 | 100.9 | ND | ND |
| **AAEL004092** | 0.989 | 98.5 | ND | ND | 0.991 | 99.1 | ND | ND |
| **AAEL004103** | 0.991 | 98.5 | 0.987 | 99.9 | 0.996 | 101.1 | 0.984 | 101.8 |
| **AAEL006326** | 0.988 | 96.2 | ND | ND | 0.984 | 97.8 | ND | ND |
| **AAEL006348** | 0.99 | 98.9 | 0.989 | 96.3 | 0.997 | 96.7 | ND | ND |
| **AAEL008857** | 0.986 | 95.9 | 0.982 | 96.4 | 0.986 | 96.9 | 0.982 | 95.8 |
| **AAEL008858** | 0.994 | 96.8 | 0.991 | 97.4 | 0.992 | 96.9 | 0.989 | 97.8 |
| **AAEL008861** | 0.949 | 97.6 | ND | ND | 0.988 | 99.2 | ND | ND |
| **AAEL008876** | 0.987 | 96.8 | 0.982 | 96.2 | 0.991 | 98.8 | 0.995 | 97.4 |

**Table S3.** shRNA constructs used in this study.

| Strain Number | *E. coli* strain | Gene target and length | Target Sequence 5′-3′ |
|---|---|---|---|
| 1 | HT115 (DE3) | pJET-8858 22mer | ACAAGTACCTCTTGCGGTGGCG |
| 2 | HT115 (DE3) | pJET-4103 22mer | ATTGCTCGGAGCTAGGAGTGAA |

| | | | |
|---|---|---|---|
| 3 | HT115 (DE3) | pJET-8858 26mer | CCTGTTCTGGAATTCTGGCGAGACTA |
| 4 | HT115 (DE3) | pJET-4103 26mer | ATATTGCTCGGAGCTAGGAGTGAATC |
| 5 | HT115 (DE3) | pJET-*eCFP* 21mer | CCATGATATAGACGTTGTGGCTG |
| 6 | HT115 (DE3) | pJET-scramble 23mer | GTATAGTATAGTATACCGTATAA |
| 7 | HT115 (DE3) | pJET-DsRed 23mer | ACCGTGAAGCTGAAGGTGACCAA |

**Table S4.** Locations of ten *Ae. aegypti* dsRNase genes and current VectorBase (VB) community annotation status. Approximate locations of these genes on chromosome map are shown with arrows.

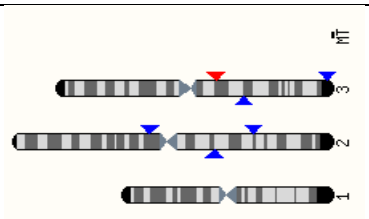
| Gene | Genome Location | Description |
|---|---|---|
| **AAEL025217** | Chromosome 2: 203659053 | No description |
| **AAEL008861** | Chromosome 3: 236574296 | deoxyribodsRNAse I, putative [Source:VB Community Annotation] |
| **AAEL008857** | Chromosome 3: 236610049 | deoxyribodsRNAse I, putative [Source:VB Community Annotation] |
| **AAEL008876** | Chromosome 3: 236632143 | deoxyribodsRNAse I, putative [Source:VB Community Annotation] |
| **AAEL008858** | Chromosome 3: 236643493 | deoxyribodsRNAse I, putative [Source:VB Community Annotation] |
| **AAEL003123** | Chromosome 3: 277923934 | deoxyribodsRNAse I, putative [Source:VB Community Annotation] |
| **AAEL006348** | Chromosome 3: 396923473 | deoxyribodsRNAse I, putative [Source:VB Community Annotation] |
| **AAEL006326** | Chromosome 3: 396927710 | deoxyribodsRNAse I, putative [Source:VB Community Annotation] |
| **AAEL004103** | Chromosome 3: 401525211 | deoxyribodsRNAse I, putative [Source:VB Community Annotation] |
| **AAEL004092** | Chromosome 3: 401536506 | deoxyribodsRNAse I, putative [Source:VB Community Annotation] |
| | |  |

**Table S5.** Mortality observed in ds8858 26mer shRNA feeding experiments compared to dsScramble controls. Survival was scored after five days of feeding on shRNA expressing bacteria in petri dishes with 20 larvae per dish.

| Treatment | Replicates | Mean Survival | Standard Error |
|---|---|---|---|
| **dsScramble** | 4 (n=80) | 0.88 | 0.037 |
| **ds8858+ds*eCFP*** | 4 (n=80) | 0.38 | 0.062 |
| **ds4103+ds*eCFP*** | 2 (n=40 | 0.83 | 0.053 |
| **2x ds*eCFP*** | 2 (n=40) | 0.90 | 0.071 |
| **ds4103+ds8858+ds*eCFP*** | 2 (n=40) | 0.70 | 0.035 |

**Table S6.** Mortality observed in ds8858 26mer shRNA feeding experiments compared to dsScramble controls, for each plate described in Table S5. Survival was scored after five days of feeding on shRNA expressing bacteria in petri dishes with 20 larvae per dish. Mean survival, standard error and Abbott's adjusted mortality are calculated for each treatment.

| Treatment | Biological Replicate | Survival Day 5 | Survival | Mean Survival | Standard Error | Abbott's adjusted mortality |
|---|---|---|---|---|---|---|
| **dsScramble** | 1 | 16 | 0.80 | 0.888 | 0.037 | 0.563698 |
| **dsScramble** | 2 | 17 | 0.85 | | | |
| **dsScramble** | 3 | 18 | 0.90 | | | |
| **dsScramble** | 4 | 20 | 1.00 | | | |
| **ds8858+dseCFP** | 1 | 7 | 0.35 | 0.388 | 0.062 | |
| **ds8858+dseCFP** | 2 | 6 | 0.30 | | | |
| **ds8858+dseCFP** | 3 | 6 | 0.30 | | | |
| **ds8858+dseCFP** | 4 | 12 | 0.60 | | | |

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# Chapter 3: methods for discovery of novel sex-specific splice variants

Chapter 3 includes contributions in implementation and preliminary analyses by Katerina Roznik

## INTRODUCTION

Current area-wide mosquito control strategies are focussed on population modifications, which will depend on high-fidelity sex-separation strategies and male sterility technologies. Because females transmit pathogens, when the sterile insect technique (SIT), *Wolbachia*-induced sterilization, or gene drive systems are implemented, females must be removed before males are released. Sex-separation is currently a major constraint on all three systems, since it increases the overall cost of rearing males, and incomplete sex-sorting adversely affects community acceptance (Mashatola et al. 2018; Lutrat et al. 2019). With few scalable, low-cost options for sex-separation available, new methods are needed.

Male sterilization methods for mosquitoes are similarly unrefined. Molecular approaches target testis-specific genes inferred from *Drosophila. melanogaster* (Whyard et al. 2015; Kandul, Liu, Wu, et al. 2019), or sterilization can be achieved by radiation (Bourtzis et al. 2016). Both approaches are prone to off-target effects in males that impede mating vigour. Adding to the repertoire of sterilization gene targets in mosquitoes is expected to increase the efficacy of RNAi-based sterilization approaches.

To sex-sort and sterilize, the biology of sex differentiation in mosquitoes must be understood. In the mosquitoes studied to date, males either lack a male-specific Y chromosome ( Hall et al. 2015; Gomulski et al. 2018), or the Y chromosome contributes very few transcripts (Papa et al. 2017). Sexual differentiation is therefore accomplished by post-transcriptional modifications,

primarily alternative splicing. In *Ae. aegypti,* sex-specific alternative splicing is initiated by the maleness factor *nix* that is necessary for male-specific splicing of *doublesex (dsx)* (Hall et al. 2015). Acting as central regulators of sexual identity in cells, sex-specific isoforms of DSX proteins regulate the transcription of male and female specific genes (Salvemini et al. 2011; 2013). In *Drosophila melanogaster,* a small number of direct targets of DSX have been identified (Luo, Shi, and Baker 2011; Luo and Baker 2015), but in mosquitoes little is known regarding the regulation of sexual dimorphism by the male and female isoforms of DSX. Additionally, the patterns and regulators of alternative splicing in males and females has not been described beyond the core sex-determination pathway genes.

Recent reviews by my group and others outlined the methods currently used to sex-sort mosquitoes (Mashatola et al. 2018; Lutrat et al. 2019). Briefly, these methods include exploiting size differences at the pupal stage (Carvalho et al. 2014), conditional female lethality of transgenic mosquitoes (Nolan et al. 2011; Facchinelli et al. 2019), and female-specific fluorescence and automated larval sorting (Catteruccia, Benton, and Crisanti 2005; Marois et al. 2012). Sterilization of pest insects is an area of very active research, but very few systems have progressed to commercialization (Bourtzis et al. 2016).

Sex-sorting and sterilization by induction of RNAi against female-specific splice variants has been demonstrated in *Aedes aegypti* (Whyard et al. 2015) and *Anopheles gambiae* (Taracena et al. 2019). In both species the central sex-determining factor *dsx* was targeted, but elimination of females was incomplete. Both studies exploit the sex-specific pattern of alternative splicing of *dsx* exons, a phenomenon that yields mRNA targets for RNAi that are sex-specific and essential for normal development. Genome-wide patterns of alternative splicing remain poorly understood in mosquitoes, although the recent assembly of *Ae. aegypti* uncovered thousands of previously

undescribed alternative splicing events (Matthews et al. 2018). Splicing is mediated by proteins in the spliceosome and their diversity is regulated in development. Descriptions of the spliceosome continually update our understanding of this complex regulatory mechanism (Papasaikas and Valcárcel 2016). Beyond the core subunits of this large ribonucleoprotein complex, the spliceosome includes subunits capable of mediating the inclusion of tissue and stage-specific exons in transcripts, thereby generating a diversity of proteins encoded by each gene. With more thorough genome sequencing efforts, considerably more alternatively-spliced transcripts will undoubtedly be identified. For example, there is a growing collection of what have been termed microexons, which are short 3-30 nt exons spliced primarily in the dipteran brain and gonads (Torres-Méndez et al. 2019), suggesting that microexon-containing splice variants may serve essential roles in specialized tissues. These gene products could be excellent targets for RNAi-based SIT, due to their tissue and temporal specificity.

Due to the lack of reverse genetics data available for mosquitoes, it is likely that there are undescribed sex-specific splicing events during larval development. In addition, it has been proposed that rare transcripts provide better targets for RNAi by feeding (Joga et al. 2016). I therefore set out to describe sex-specific splicing in female larvae and knock down these splice variants with shRNAs to assess which splice variants may be essential for female-specific development.

# METHODS

## SEX-SORTING BY EMBEDDING LARVAE IN AGAR AND VIEWING DEVELOPING TESTES

To generate pools of sex-sorted RNA for sequencing, a method of accurately separating male and female larvae was needed. In larval stages, sexual dimorphism of mosquitoes is subtle.

Primordial testes are developing in the fourth instar and provide a method of visually identifying males. Fourth instar larvae were sex-sorted by visualising the primordial testis in 4$^{th}$ instar larvae, as previously described (Christophers 1960). To facilitate visualization in live animals, we embedded live larvae in 0.7% molten agar at 42 °C, allowed the agar to solidify and viewed larvae under 20x magnification with a Zeiss Stereo Discovery v20 dissecting microscope.

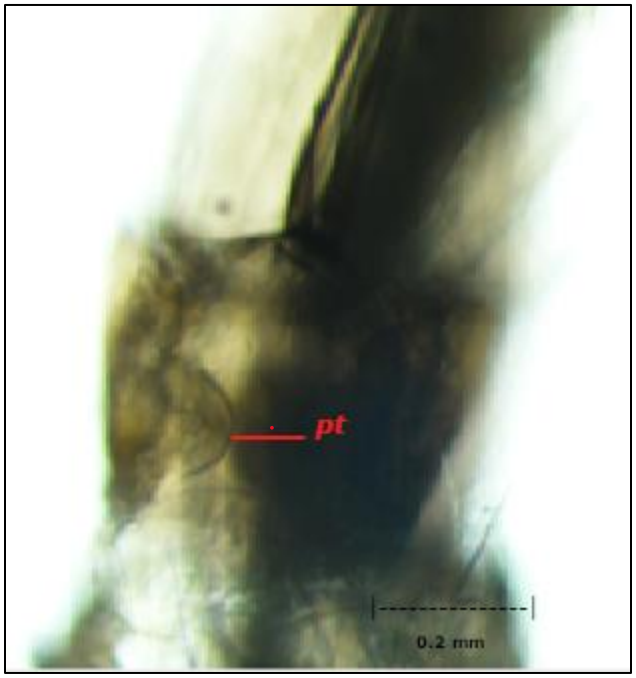



**Figure 1:** Male *Ae. aegypti* fourth instar larval terminal abdominal segments, showing primordial testis (pt). Fourth instar larvae were embedded in 0.7% agar at 42$^{o}$C to enable visualization of the developing gonad, the only sexually dimorphic feature visible at this developmental state.

For each sex, 3 pools of 10 individuals were homogenized in a Bullet Blender Tissue Homogenizer (Next Advance, Troy, NY) with 0.8mm glass beads followed by further tissue fragmentation with QiaShredder (Qiagen, Germantown, MD) and total RNA purification with

GeneJet RNA purification kit (ThermoFisher, Waltham, MA) according to manufacturer's specifications.

## NANOPORE SEQUENCING

To obtain sequences from full-length transcripts, I used Nanopore sequencing of cDNA from sex-sorted larvae. RNA concentration and quality were assessed using a 2100 Bioanalyzer (Agilent, Santa Clara, CA). Poly A RNA was enriched with the NEB Next Poly A module (NEB, Ipswich, MA), cDNA reverse transcribed with Superscript IV (ThermoFisher, Waltham, MA) and libraries were prepared using Oxford Nanopore Technologies (ONT) PCR cDNA kit SQK-PSC108 (Oxford Nanopore, Oxford, UK) according to the manufacturer's specifications

## LONG-READ TRANSCRIPTOMICS

The sequencing reads obtained from the RNA-seq were base-called using the base-calling program Guppy (ONT, Oxford UK). During this process, files with nucleotide sequences were obtained by converting voltage squiggles to called bases. Subsequently, these files were analyzed with ONT's Pinfish pipeline, available at https://github.com/nanoporetech/pinfish. Pinfish is a collection of tools that enable the processing of raw cDNA-derived .fastq files, alignment to genomic data, and prediction of novel mRNA isoforms using the Python computer programming language. This step generated gff (.gff) and bam (.bam) files. Each data file was collected for female (A, B, and C) and male (D, E, and F) pools of mosquitoes separately at two different stages of data cleaning, using polished and unpolished reads. Unpolished pinfish output includes all putative mRNA reads, whereas polished pinfish output removes artefacts that are predicted to be a result of 5' degradation.

Both polished and unpolished reads in .gff and raw .bam data files were examined using the read depth and splice prediction tracks displayed by IGV (Integrative Genomics Viewer) program

(Thorvaldsdóttir et al. 2013). The reference genome for IGV was obtained from NCBI (National Center for Biotechnology Information) (*Aedes aegypti* ID: 44 available from: https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Aedes_aegypti/101/). Female and male reads were compared manually in IGV, and relevant female-specific splice variants were identified. Manual examination of transcriptomic analysis of larval pools revealed some evidence of male contamination for female pool C, as many of the transcripts examined were identical with D, E, and F, rather than A and B. However, no transcripts of the male determining factor *nix* were found in pool C. Due to this ambiguity, female pool C was removed from further analysis.

To overcome the labour required to manually compare transcripts, we developed a pipeline using Bioconductor tools in the R programming language, available at https://www.bioconductor.org/. This pipeline compared locations of exons specific to the male and female genomes on the three chromosomes of *Ae. aegypti*. Once a sex-specific exon was identified in IGV, its location was compared to the *Ae. aegypti* genome database (vectorbase.org) to assign a putative gene name to the identified sequence.

## QUANTIFICATION OF GENE EXPRESSION IN INDIVIDUAL TISSUES

To validate the RNA-seq data and measure knockdown following RNAi experiments, we modified a direct qRT-PCR assay commonly used for analysis of cell cultures to examine transcript levels in dissected mosquito tissues. Midguts and gonads were dissected in 5ul droplets of PBS and then transferred to 5ul of PBS in PCR tubes. Samples were centrifuged briefly and incubated in 50 uL BioRad SingleShot (Hercules, CA) lysis buffer supplemented with 1ul proteinase K and 1 uL DNase. Samples were then incubated for 10 min at 22$^{o}$C, 20 min at 37$^{o}$C, 5 min at 75$^{o}$C and placed on ice. This RNA was used as template directly in 10 uL BioRad SingleShot SYBR qPCR reactions with each reaction containing 2 uL template RNA, 0.125 uL

reverse transcriptase 5 uL SYBR mix, 0.4 uL of each forward and reverse primer, and 2.1 uL molecular grade water. Thermocycling conditions for all primer sets were 10 min at 55°C to incubate the reverse transcription reaction, followed by 40 cycles of 2-step qPCR at 95°C and 57°C. This was followed by melt-curve analysis as recommended by the manufacturer. Each reaction was conducted in triplicate and analyzed using the ΔΔCt method relative to the ribosomal S7 gene (Livak and Schmittgen 2001).

## BACTERIAL SHRNA EXPRESSION SYSTEM TO KNOCK DOWN SPLICE VARIANTS

To test whether female-biased alternatively spliced genes are necessary for normal sex differentiation, I designed shRNAs to deplete three sex-specific transcripts identified by transcriptomic analyses. These genes (AAEL004441, AAEL005496, AAEL005884, referred to by the last four digits of their accession number hereafter) were chosen based on confirmed sex-bias observed in qPCR results and successful cloning into the expression vector. To rapidly produce shRNAs for larval feeding experiments, annealed oligos encoding 26mer shRNA fragments, with 9 nt loop sequences, were cloned into the pJET 1.2 blunt-end cloning vector downstream of a T7 promoter region. Resulting plasmids were transformed into HT115 (DE3) cells. As a negative control, a hairpin of a 26mer scrambled sequence (scramble nt) from an *Ae. aegypti* gene was produced in HT115 (DE3) cells. For each hairpin construct, LB starter broth was inoculated from glycerol stocks, incubated at 37°C overnight and 1 mL of starter culture was used to inoculate 80 mL 2YT media. This broth was grown in baffled flasks with shaking at 230 rpm to an optical density (OD) of 0.4, induced with IPTG of 0.4 mM, and grown to an OD of 1.0. Cells were harvested by centrifugation, heat-killed at 80°C for 15 min and suspended in agar with supplemental brewer's yeast. Open-topped syringes were used to measure 0.5 mL feeding pellets that were placed in deep well 90 mm Petri dishes with 20 newly hatched larvae and 50

mL of dd$H_20$. Feeding with shRNA expressing bacteria occurred once at the outset of the experiment, and when pellets were exhausted, liver powder was added sparingly to supplement nutrition. Larval growth and mortality were monitored daily, and for both experimental and control treatments, the proportion of males and females that emerged was recorded.

# RESULTS

## DIVERSE MALE AND FEMALE SPLICE VARIANTS ARE REVEALED BY LONG READ TRANSCRIPTOMICS

To understand alternative splicing in male and female *Ae. aegypti* larvae, I used long-read transcriptome analyses. Long-read Nanopore RNA sequencing identified 77 new female-specific splice variants, and 114 female-specific transcripts (Table 1). A total of 166 male specific genes were identified with the same methods, as well as 113 new male specific isoforms. For comparison, all predicted coding and long-non coding genes in the genome are listed in Table 1, as well as all genes found to be sex-biased by microrarray (Tomchaney et al. 2014).

**Table 1:** Predicted number of genes, and sex-specific genes predicted by splice variant analysis and transcript abundance. *Ae. aegypti*. Genes with sex-biased transcript abundance or splicing patterns were identified using long-read transcriptomics (LRT) and publicly available microarray data. From microarray data, a cutoff of 0.75 fold difference between male and female gene expression in pupae was used, which is an arbitrary value that is measurable by confirmatory qPCR.

| Gene Type | Number | Potential Targets for SIT |
|---|---|---|
| Coding | 14677 | |
| Long non-coding | 3791 | 18,850 |
| Pseudogenes | 382 | |
| LRT Female Specific | 114 | |
| LRT Female Specific Novel Isoform | 77 | 470 |
| LRT Male Specific | 166 | |
| LRT Male Specific Novel Isoform | 113 | |
| Microarray Female Biased (75%) | 5512 | 14,644 |
| Microarray Male Biased (75%) | 9132 | |

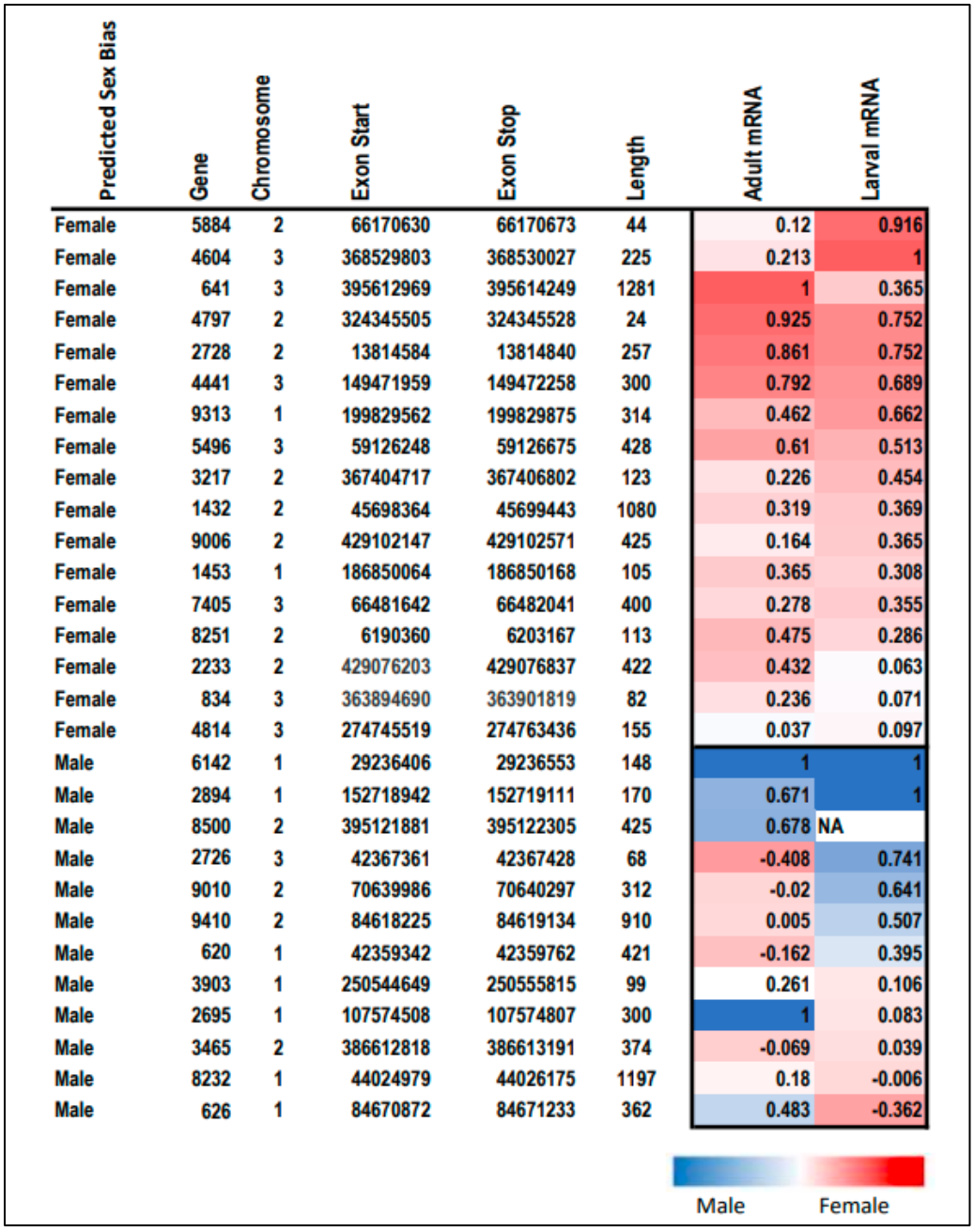
## SEX-SPECIFICITY SHOWN BY LONG-READ TRANSCRIPTOMICS IS VALIDATED BY QPCR

After identifying the alternatively spliced genes from long-read transcriptome analysis, I used qRT-PCR to validate their sex specificity. Transcripts for qRT-PCR analyses were selected based on their putative function in the developing larvae as annotated in Vectorbase.org databases. In selecting female genes for further examination, I used the Vectorbase gene expression reports to identify genes known to be expressed in the gut. The rationale for focusing on those genes was that cells within the digestive tract would be the first to be exposed to the ingested shRNAs and may therefore show a strong RNAi response and hopefully yield female lethality. Similarly, male specific transcripts identified by transcriptomic analyses were further examined for expression in the testis, with the aim of inducing male sterility.

The qRT-PCR analysis of the relative gene expression confirmed that for female-biased genes the RNAseq search algorithm was accurate, as 16 of 17 (94%) of predicted female-specific

transcripts were either exclusively expressed in females or were strongly (greater than 20%) female-biased (Figure 2). The cut-off value of 20% was chosen as a conservative threshold that can be accurately detected by qPCR. The genes that were found to be most conclusively female-specific were genes 4604 and 5884 in larvae, and 0641 and 4797 in adults (Figure 2).

qRT-PCR analyses of the male genes identified by the transcriptome did not confirm sex specificity as conclusively as was observed in females. In males, only 6 of 11 (54%) of the genes chosen for qRT-PCR analysis had a male bias greater than 20%. When I examined the expression pattern in gonads, 6 of 12 (50%) of the genes were highly male biased and one (2726) was found to be highly female biased (Figure 2).



| Predicted Sex Bias | Gene | Chromosome | Exon Start | Exon Stop | Length | Adult mRNA | Larval mRNA |
|---|---|---|---|---|---|---|---|
| Female | 5884 | 2 | 66170630 | 66170673 | 44 | 0.12 | 0.916 |
| Female | 4604 | 3 | 368529803 | 368530027 | 225 | 0.213 | 1 |
| Female | 641 | 3 | 395612969 | 395614249 | 1281 | 1 | 0.365 |
| Female | 4797 | 2 | 324345505 | 324345528 | 24 | 0.925 | 0.752 |
| Female | 2728 | 2 | 13814584 | 13814840 | 257 | 0.861 | 0.752 |
| Female | 4441 | 3 | 149471959 | 149472258 | 300 | 0.792 | 0.689 |
| Female | 9313 | 1 | 199829562 | 199829875 | 314 | 0.462 | 0.662 |
| Female | 5496 | 3 | 59126248 | 59126675 | 428 | 0.61 | 0.513 |
| Female | 3217 | 2 | 367404717 | 367406802 | 123 | 0.226 | 0.454 |
| Female | 1432 | 2 | 45698364 | 45699443 | 1080 | 0.319 | 0.369 |
| Female | 9006 | 2 | 429102147 | 429102571 | 425 | 0.164 | 0.365 |
| Female | 1453 | 1 | 186850064 | 186850168 | 105 | 0.365 | 0.308 |
| Female | 7405 | 3 | 66481642 | 66482041 | 400 | 0.278 | 0.355 |
| Female | 8251 | 2 | 6190360 | 6203167 | 113 | 0.475 | 0.286 |
| Female | 2233 | 2 | 429076203 | 429076837 | 422 | 0.432 | 0.063 |
| Female | 834 | 3 | 363894690 | 363901819 | 82 | 0.236 | 0.071 |
| Female | 4814 | 3 | 274745519 | 274763436 | 155 | 0.037 | 0.097 |
| Male | 6142 | 1 | 29236406 | 29236553 | 148 | 1 | 1 |
| Male | 2894 | 1 | 152718942 | 152719111 | 170 | 0.671 | 1 |
| Male | 8500 | 2 | 395121881 | 395122305 | 425 | 0.678 | NA |
| Male | 2726 | 3 | 42367361 | 42367428 | 68 | -0.408 | 0.741 |
| Male | 9010 | 2 | 70639986 | 70640297 | 312 | -0.02 | 0.641 |
| Male | 9410 | 2 | 84618225 | 84619134 | 910 | 0.005 | 0.507 |
| Male | 620 | 1 | 42359342 | 42359762 | 421 | -0.162 | 0.395 |
| Male | 3903 | 1 | 250544649 | 250555815 | 99 | 0.261 | 0.106 |
| Male | 2695 | 1 | 107574508 | 107574807 | 300 | 1 | 0.083 |
| Male | 3465 | 2 | 386612818 | 386613191 | 374 | -0.069 | 0.039 |
| Male | 8232 | 1 | 44024979 | 44026175 | 1197 | 0.18 | -0.006 |
| Male | 626 | 1 | 84670872 | 84671233 | 362 | 0.483 | -0.362 |



**Figure 2:** The genomic location, exon size and relative gene expression of 17 female and 12 male-biased alternatively-spliced exons in mosquito adult and larval tissues (female guts and male reproductive organs dissected from fourth instar larvae). The gene expression values (relative to a ribosomal protein gene's transcripts) were measured by qRT-PCR and analyzed using the Livak method (Livak and Schmittgen 2001), and then log-transformed to enable easy

identification of female-specific transcripts. A value of 1 signifies 100% sex-specificity, a value of 0 indicates no sex bias, and negative values indicate unexpected bias to the opposite sex. The scores for adults and larvae were determined based on the results of the most female-specific (highest $2^{-\Delta\Delta Ct}$ values) genes in female larval guts and adults. A score between 1 and 0 indicates that the gene is female-biased, as some transcripts may have been detected in males. Male larval transcripts were analyzed from isolated reproductive organs. qRT-PCR reactions that failed to amplify are shown as NA.

## LONG READ RNASEQ REVEALS A SUITE OF POTENTIAL RNAI TARGETS FOR SIT THAT ARE NOT IDENTIFIED BY MICROARRAYS.

Pipelines for the discovery of RNAi targets have historically relied upon short read RNAseq or microarrays, which typically fail to distinguish highly similar transcripts from one another. Long-read Nanopore RNA sequencing, in contrast, can provide accurate sequencing of long RNA transcript sequences, capturing reads up to 30 kb in length, and hence, this method was selected to identify sex-specific splice variants within the RNA samples derived from the sex sorted larvae. To illustrate the benefit of long-read transcriptomics, I analyzed the position of putative SIT RNAi targets discovered using Nanopore sequencing to a publicly available microarray dataset (Tomchaney et al. 2014). This dataset is the earliest developmental stage for which sex-differential transcript abundance was measured. This analysis revealed the abundance of sex-biased genes shown by microarrays (Table 1). My analysis also revealed variation in the density of sex-biased gene expression along chromosome 1. A preponderance of male-biased genes was found surrounding the so-called male determining region (Figure 4). In addition, a region with a preponderance of female-biased genes was found downstream of the *dsx* locus (Figure 3). Analysis of the chromosome position of long-read transcriptomics-identified genes did not identify any of the microarray-predicted male-biased genes surrounding the male-

determining region. The two methods did share the pattern of high density of female-biased genes downstream of *dsx* (Figure 3). Male-specific splice variants were discovered by our transcriptomic pipeline (n=166) with the greatest density within a 200 kb segment on chromosome 1, just outside the male determining region (Figure 3). Figure 4 shows the dense cluster of sex-biased genes flanking the male-determining region; beyond this region the density of sex-biased genes was very low (not shown). Analysis of single nucleotide polymorphism data revealed that the chromosome region flanking the sex-determining region is associated with reduced genetic variation (Figure 5). For SIT by RNAi, genes showing evidence of conservation and low variation may be expected to show a strong phenotypic response, and targeting regions with low variability will reduce the chances of mismatch to the dsRNA used deplete transcripts.

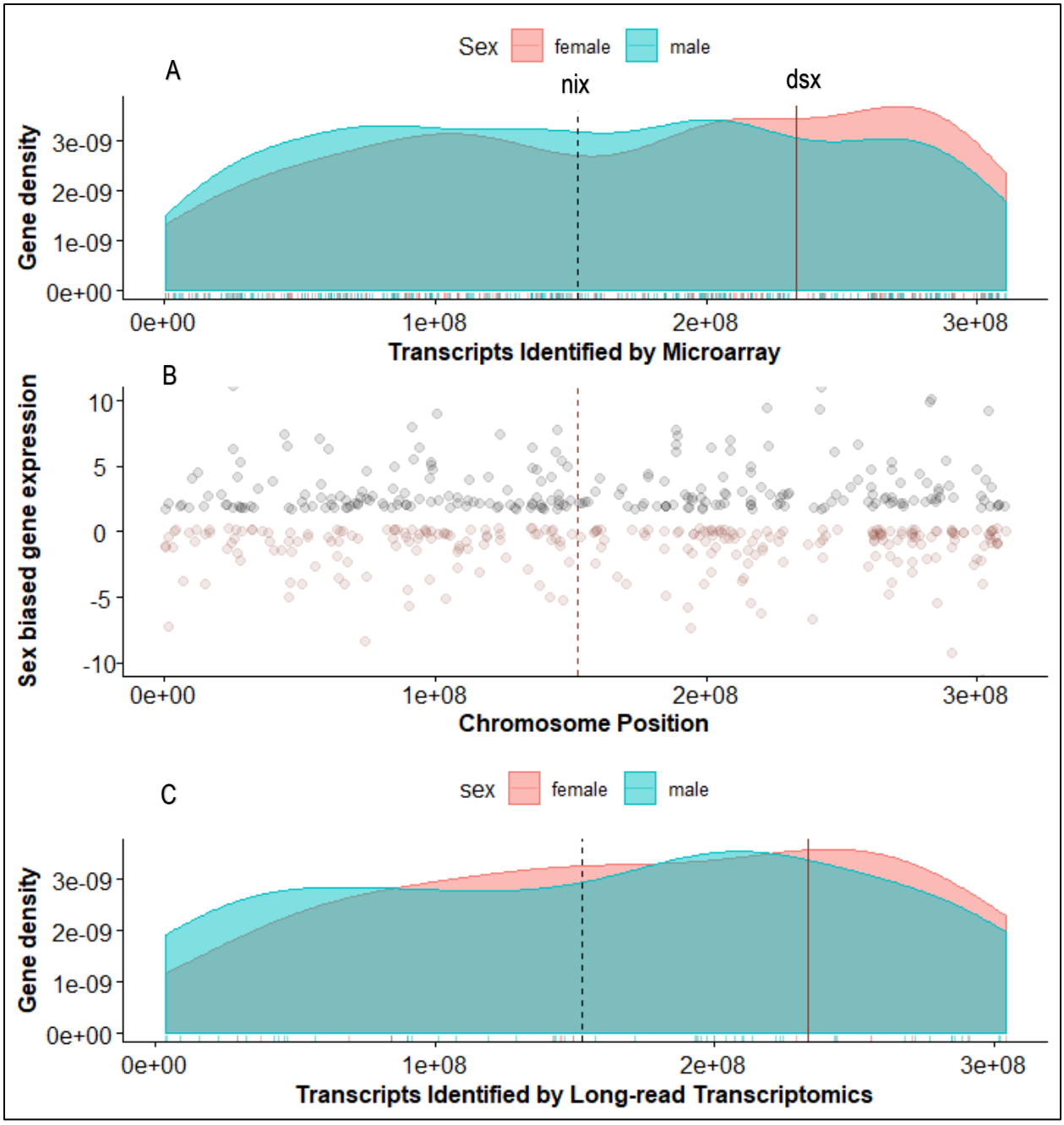



**Figure 3:** A comparison of the location of sex-biased gene expression measured by microarray eand long read transcriptomics (LRT) on chromosome 1 of *Ae. aegypti*. Sex-biased *Ae. aegypti* genes were identified by microarray and long-read transcriptomics. Pupal brain microarray data in top and middle panel is from publicly available data (Tomchaney et al. 2014). Panel A shows the locations of predicted male and female-biased genes as a density plot showing the number of

sex biased genes (greater than 75% $\log_2$ relative expression) as determined by microarray. Individual genes are shown as a rug plot below the density plot. The dashed vertical line indicates the location of *nix*, which is located centrally in the sex-determining region, while the red line shows the location of *dsx*. Panel B: *Ae. aegypti* chromosome 1 expression data was plotted along the entire chromosome. A dotplot is shown with relative expression of male biased (>15% $\log_2$ relative expression) (black dots) and female biased genes (pink dots). Male bias equal to one represents no difference between male and female samples. Panel C: *Ae. aegypti* LRT analysis predicted 114 female and 166 male sex-biased transcripts. Those found on chromosome 1 are shown as a density plot with a rug plot showing individual gene locations below the density plot generated using the ggpubr package in R (supplementary code is shown at the end of this chapter).

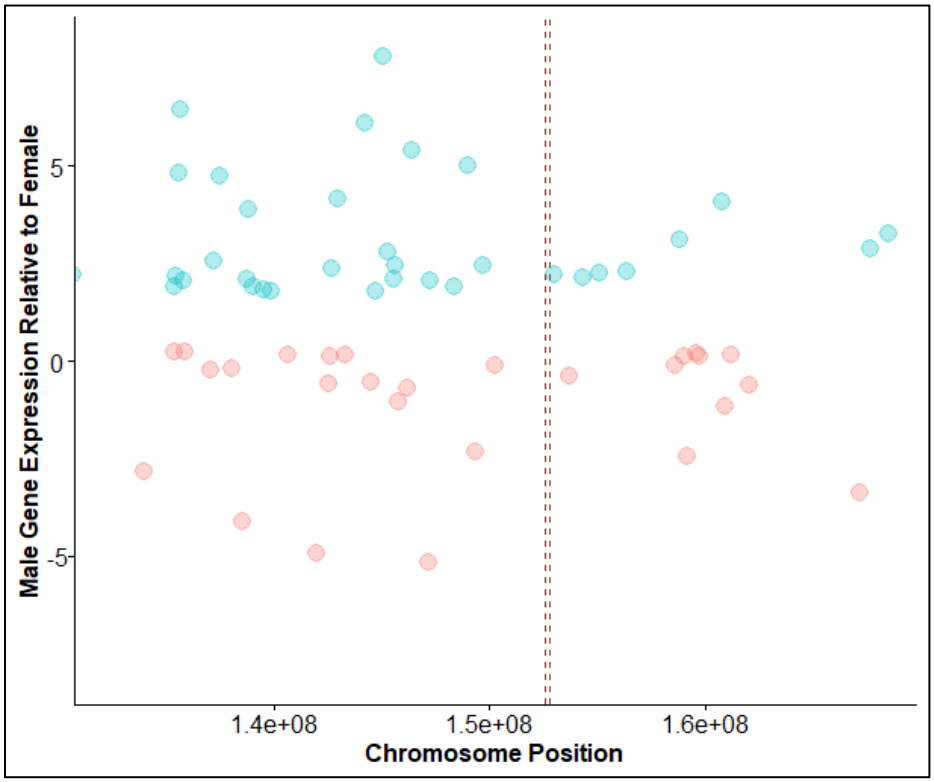


**Figure 4:** Two regions of sex biased gene expression flank the *Ae. aegypti* sex determining region (bounded by dashed lines). Dots represent genes with a ratio of $\log_2$ expression greater than +/- 0.75 in pupal heads with blue representing male biased genes and pink representing female biased genes. Male bias equal to one represents no difference between male and female samples. Plot is generated using the ggpubr package in R using publicly available data) (Tomchaney et al. 2014).

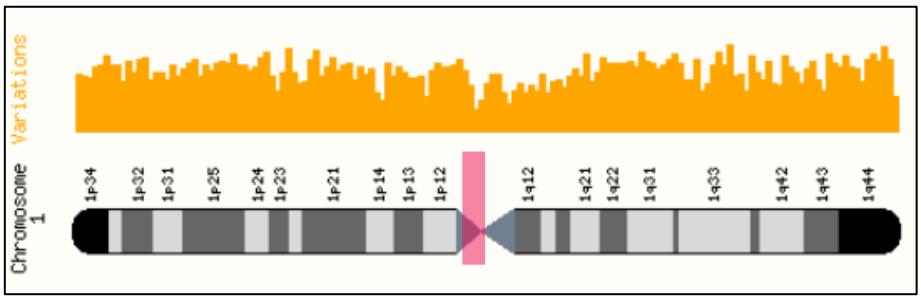



**Figure 5:** Genetic variation (plotted as a % of maximum reported variation) is reduced around the sex-determining region (highlighted in red) relative to most of the rest of the chromosome. Plot generated using Vectorbase genetic variation program.)

## A CLASS OF SMALL EXONS IS FOUND IN MALE SPECIFIC TRANSCRIPTS

The methods I used to discover sex-specific alternative splicing revealed numerous very short (6-90bp) exons that were not described in the publicly available gene sets (Table 2). These short exons are male specific and are the same length as exons described as neuronal microexons (Torres-Méndez et al. 2019). Splicing of microexons is enabled by a family of *srrm* splicing factor genes found throughout metazoans. Table 2 shows that 15 of 166 (9%) of male specific splice variants were formed by inclusion of exons less than 80bp, which are defined as microexons (Torres-Méndez et al. 2019). None of these male-specific splice variants containing microexons are described in publicly available gene models derived from short and long-read

RNAseq data. I then investigated published data regarding the tissue specificity of these microexons in *D. melanogaster* and found that "neuronal" microexons are also abundant in the gonads, a pattern not seen in vertebrates (Torres-Méndez et al. 2019). In mosquitoes the tissue specificity of these microexons has not been described.

An example of the potential utility of these microexons as potential targets for SIT is illustrated by the gene *Scp-2L3* (sterol carrier protein-2 like 3), encoding an intracellular lipid carrier expressed predominantly in larvae (Dyer et al. 2009; Li, Fan, and Papadopoulos 2016). My transcriptome analysis revealed that there is a 6bp microexon spliced in some transcripts of this gene (Figure 7).

**Table 2:** Microexons with male-specific splicing in *Ae. aegypti*, as identified by long-read transcriptomics.

| Genome address | Gene | Exon Length (bp) | Read Depth |
|---|---|---|---|
| 1:47,368,062-47,368,110 | | 25 | 34x |
| 1:91,700,989-91,701,062 | | 14 | 14x |
| 1:149,235,363-149,235,383 | | 20 | 15x |
| 1:203,005,983-203,006,081 | AeSCP-2L3 | 6 | 14x |
| 1:285,784,145-285,785,145 | | 79 | 64x |
| 1:304,361,715-304,361,720 | | 5 | 40x |
| 2: 37,426,654-37,429,103 | | 28 | 8x |
| 2: 45,706,427-45,711,327 | | 32 | 30x |
| 2: 64,834,195-64,839,095 | | 12 | 12x |
| 2: 76,244,745-76,249,645 | | 7 | 8x |

| | | |
|---|---|---|
| 2: 96,190,313-96,195,213 | 23 | 54x |
| 2: 291,313,541-291,315,990 | 90 | 30x |
| 2: 302,304,451-302,309,351 | 33 | 9x |
| 2: 375,479,549-375,484,449 | 31 | 12x |
| 3: 6,311,634-6,313,749 | 28 | 28x |
| 3: 304,091,142-304,095,374 | 14 | 22x |

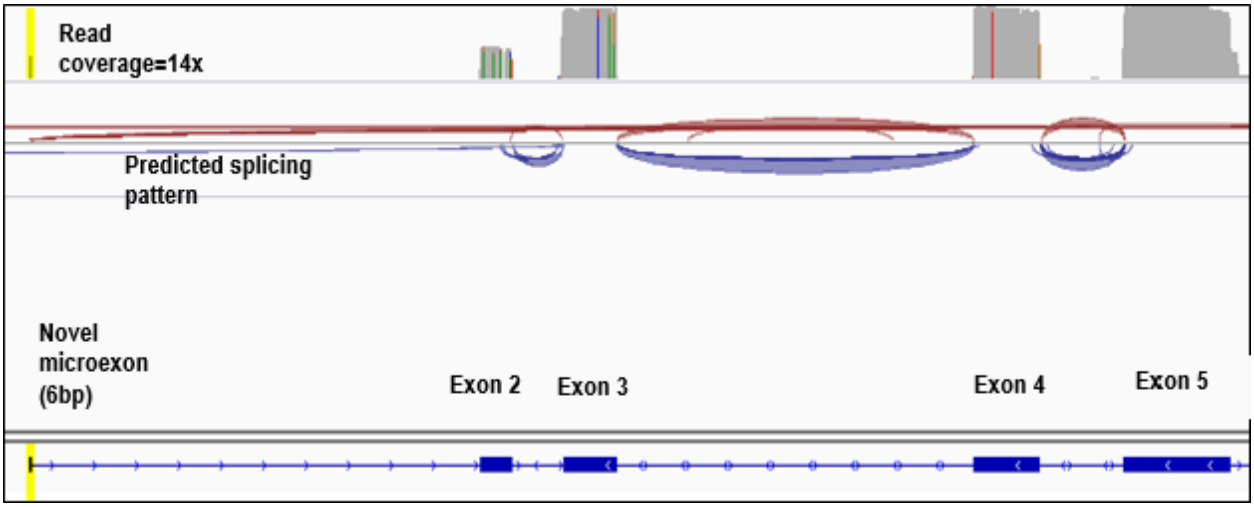



**Figure 6:** Microexon splicing pattern seen in *Ae. aegypti Scp-2L3*. Read coverage is shown in top panel. A 6bp microexon is highlighted in yellow (read coverage counts for this microexon were 14x, larger exons were sequence with 68x to 70x coverage). Curved, tapered pink bars represent evidence for introns. Variants are shown in four colours in the coverage plot, while consensus is shown in grey. Polished transcript models are shown in navy blue. Figure was produced using the publicly available IGV viewer software.

## KNOCKDOWN OF FEMALE-SPECIFIC TARGETS DID NOT PERTURB SEX RATIO

Depletion of female-specific transcripts was expected to induce a range of phenotypes with applicability to an SIT program or other mosquito control applications. These include delayed

development, reduced size, reduced fecundity or behavioural defects. As a preliminary set of experiments, I measured the development and mortality of mosquitoes fed bacteria expressing shRNAs against the exons identified in our transcriptomics pipeline (Figure 7). Three shRNA treatments (4441, 5496, 5884) had significantly more male adults emerge than scrambled nt treated controls (Fisher's exact test $P<0.05$, indicated by *).

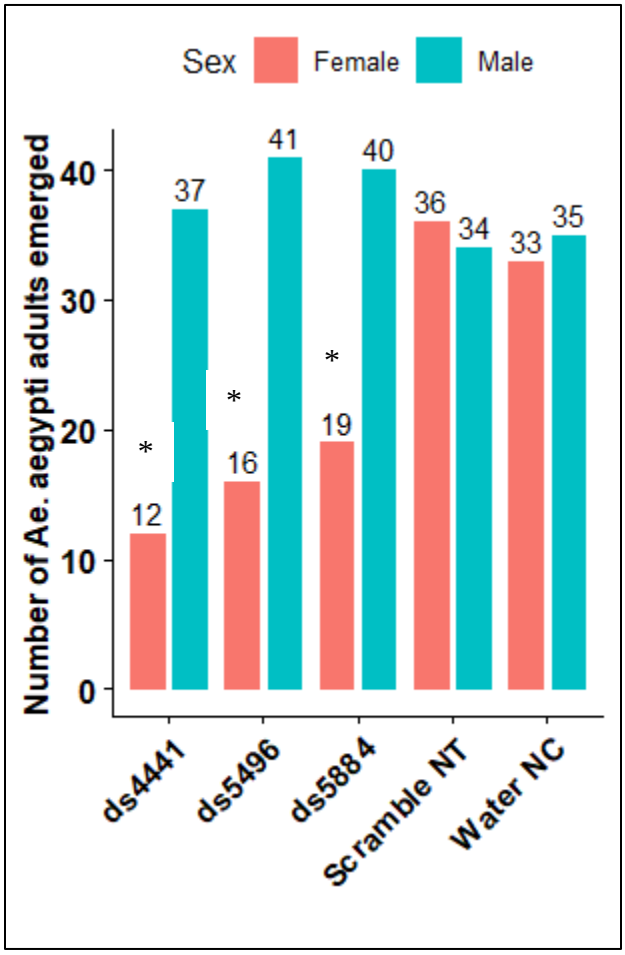



**Figure 7:** Knockdown of female splice variants by shRNA feeding. Bar graph shows proportions of treated *Ae. aegypti* larvae that reached adulthood, died or failed to develop (scored on day 13). Scramble NT is a scrambled nt shRNA control, water NC is water and brewer's yeast only. For each gene, 4 dishes with 20 larvae each were treated for a total of 80 larvae per gene.

# DISCUSSION

RNAi-based SIT depends on the successful depletion of gene products and resulting sterilization and female lethality. The current suite of gene targets known to be responsible for sex differentiation has not produced 100% sterility and female removal as required for mosquito control, preventing large-scale implementation. Proof-of principle experiments conducted to date have relied on targets identified based on homology with sex-determination and differentiation genes in *D. melanogaster*, meaning that many possible SIT mRNA targets remain undiscovered. With the goal of identifying novel splice variants in larval mosquitoes, we conducted long-read transcriptomic analysis from larvae sex-sorted at the 4$^{th}$ instar. This revealed 280 potential targets for sterilization and female lethality, of which 29 (10.3%) were tested by qRT-PCR. Additional gene targets for the optimization of dsRNA delivery, including novel dsRNase-encoding genes, were not described using the methodology used here. My methods produced low coverage at these loci and relied on alignment to the existing genome assembly.

Induction of RNAi by feeding allows for rapid loss-of-function screening, and can be scaled up to produce mosquitoes for area-wide control. The 3 female-specific splice variants tested by RNAi knockdown induced female lethality, though lethality was well below the threshold near 99% needed for an SIT application (Figure 7). Future work will refine the shRNA delivery methods and feeding parameters with the aim of revealing a clearer phenotypic response.

Existing methods for sex-separation require further refinement (Lutrat et al. 2019). Although Kandul and colleagues report that Verily's Debug project has the capacity for large scale separation of male *Ae. aegypti* (Kandul et al. 2019), detailed reports of their methods are not available. By adding to the repertoire of available targets for SIT applications, the current study may help move the field forward. Phenotypes of loss of function assays, either by Cas9-mediated

editing or RNAi transcript depletion, may include changes in rate of development, biting behaviour or mating behaviour. Perturbation of development or behaviour may directly apply to sex-sorting methods or male sterility, but may also aid in mechanical separation methods by delaying female growth. Alternatively, a phenotype that reduces blood-feeding behaviour may make some degree of female contamination in a male-release program acceptable.

Previous work in dipteran sex-differentiation has been largely restricted to *D. melanogaster*. In this species, the master regulator *dsx* controls differentiation genes by binding to their DSX binding sites and activating transcription (Luo, Shi, and Baker 2011; Luo and Baker 2015; Wagamitsu et al. 2017). To date, no reverse genetics screens have validated sex differentiation targets of DSX in mosquitoes, though the sex determination pathway is known to end with *dsx* (Salvemini et al. 2011). In the current study, we found that AAEL000834 is alternatively spliced and a female-specific variant was confirmed by qRT-PCR. This gene is a homolog to *D. melanogaster Fmo-2*, previously shown to be a target of $DSX^F$ (Luo and Baker 2015).

Female-specific transcripts of *dsx* are produced by alternative splicing controlled by the recruitment of splicing factors by Tra and Tra2 (Tian and Maniatis 1993). Alternative splicing as a means of producing diverse gene products is best understood in the fly brain, where diverse splicing events are enabled by the neuron-specific expression of splicing factors (Torres-Méndez et al. 2019). Intriguingly, compared to the vertebrate species analysed in this study, *D. melanogaster* had the highest relative abundance of microexons found in the gonads and viscera, suggesting that dipterans may have evolved a less tissue-specific repertoire of alternative splicing events to control gonad and gut development. The current study uncovers many alternative splicing events in the gut, and future work may reveal gut and gonad-specific splicing factors

that enable this transcript diversity in mosquitoes. Microexons are expected to provide ideal targets for transcript-specific RNAi constructs.

An example of the potential of microexons as new targets for SIT is the gene SCP-2L3. This protein has been described in detail (Dyer et al. 2009). While the role of this lipid carrier in mosquitoes is not well described, mammalian homologues are essential for production of testosterone in the testis (Li, Fan, and Papadopoulos 2016), and expression of this gene has been shown to be highly male biased (Tomchaney et al. 2014). The size of microexons is convenient for design of RNAi effectors. ShRNAs with 21-26mer transcript homology are potent RNAi effectors in mosquitoes (Hapairai et al. 2017) and partial homology of lengths less than 18nt is not likely to induce off-target effects. This means that microexons with lengths greater than 4bp that can be theoretically knocked down by a 22mer shRNA without depletion of transcripts that include adjacent exons.

Knockdown of gene splice variants identified in this study by RNAi will require further optimisation of dsRNA delivery to achieve high efficiency. As demonstrated in chapter 4 of this thesis, fine-tuning the microbial expression of shRNAs is required for optimal RNAi efficacy. While optimized shRNA expression parameters for female splice variant shRNA feeding experiments are still required, other methods to reduce abundance of non-transformed bacteria in the rearing water such as bleach and ampicillin treatment were not used in this study.

Recent developments in production of dsRNA in yeast (Mysore et al. 2019) and methods to mitigate effects of nucleases in the gut (Tayler et al. 2019) may provide a path towards mass rearing mosquitoes while knocking down the targets identified in this study. Another approach that could be used to target these genes includes the pgSIT method, in which guide RNA expressing and Cas9 expressing strains are crossed to edit the genome (Kandul, Liu, Wu, et al.

2019). Transgenic approaches are expected to face community opposition in some countries, making RNAi-based SIT the most likely method to achieve widespread adoption.

Implementation of RNAi-based SIT on a large scale will depend on identification and validation of a range of gene targets for sterilization and female lethality. The sex-differentiation genes identified in this study will provide dozens of novel targets to test at scale. In addition, the long-read transcriptomics approach and rapid shRNA feeding protocols described here will be useful as a template for future screening efforts.

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## SUPPLEMENTARY CODE

```
## load required libraries
library(dplyr)
library(ggplot2)
library(ggpubr)
library(ape)
library(sushi)
library(cowplot)
library(ggExtra)
#read in tab delimited file from vectorbase
AaeEx <- read.delim("D:/vbo_archive_20200227_1/Aedes-aegypti_EXPR-STATS_VB-2019-06.txt.gz",
                    as.is="gene_id")
#read in base features gff file. Does not have a simple accession number variable
#Don't use read.delim for gff3
AaeBase <- read.gff("D:/Aedes-aegypti-LVP_AGWG_BASEFEATURES_AaegL5.2.gff3.gz")
glimpse(AaeEx)
glimpse(AaeBase)
#subset tomchaney pupal heads data only
picktom <- c("gene_id",
             "X.MEAN..Male.vs.female.Aedes.aegypti.pupal.heads..Tomchaney.et.al...2014..male",
             "X.MEAN..Male.vs.female.Aedes.aegypti.pupal.heads..Tomchaney.et.al...2014..female")
tomchaney <- AaeEx[picktom]
tomchaney2 <-rename(tomchaney, male =
X.MEAN..Male.vs.female.Aedes.aegypti.pupal.heads..Tomchaney.et.al...2014..male,

female=X.MEAN..Male.vs.female.Aedes.aegypti.pupal.heads..Tomchaney.et.al...2014..female,
             accession="gene_id")
glimpse(tomchaney2)
tomchaney2$value <- tomchaney2$male / tomchaney2$female
```

```
twoormore <- filter(tomchaney2, value > 40)
#plot
plot(twoormore$value,twoormore$gene_id)
#
ggbarplot(twoormore, x = "gene_id",
          y = "ratio",
          color = "coral4",
          fill= "coral4",
          ylab="Male bias (relative log2 expression)",
          xlab="",
          size=0.9,
          palette= "uchicago",
          legend="NULL",
          font.tickslab = c(12, "bold"),
          font.x = c(12, "bold"),
          font.y = c(12, "bold"))+
  rotate_x_text(angle = 45)
#code to merge our analyses with vectorbase gene model data and tomchaney
#subset AaeBase to only include gene
AaeGene <- filter(AaeBase, type == "gene")
#rename
AaeGene2 <- rename(AaeGene,
                   chrom=seqid,
                   chromStart=start,
                   chromEnd=end)
#create a vectorbase accession number variable
AaeGene2$accession <- substr(AaeGene2$attributes, 4, 13)
#Sort by accession
arrange(AaeGene2, accession)
glimpse(AaeGene2)
glimpse(tomchaney)
#use inner-join to filtering join AaeGene to tomchaney2
AaeGenEx <- inner_join(AaeGene2, tomchaney2, by="accession")
glimpse(AaeGenEx)
#calculate male bias (non-normalised ratio to female
AaeGenEx$malebias <- AaeGenEx$male/AaeGenEx$female
#create a variable called sexbias to color code plot below
AaeGenEx$Sex <- if_else(AaeGenEx$malebias > 1, "male", "female")
```

```
#select only chromosome 1 and bias greater than `25%
ch1male <- filter(AaeGenEx, chrom==1, malebias>1.75 | malebias<0.25)
glimpse(ch1male)
#plot scatter
malescatter <- ggscatter(ch1male, x="chromStart", y="malebias",
       xlab="Chromosome Position",
       ylab="Sex biased gene expression",
       ylim=c(-10,10),
       color="Sex",
       palette=c("coral4","black"),
       legend="NULL",
       alpha=0.12,
       size=2,
       tickslab=FALSE,
       font.x = c(12, "bold"),
       font.y = c(12, "bold"))+
  geom_vline(xintercept = 152554860, linetype = 2, color = "coral4")
#
#zoom in on sex-determining region with male data only
ggscatter(ch1male, x="chromStart", y="malebias",
              xlab="Chromosome Position",
                     ylab="Male Gene Expression Relative to Female",
                     ticks="FALSE",
                     ylim=c(-8,8),
                     xlim=c(132558583,168005350),
                     color="Sex",
                    palette= c("coral4,black"),
                     legend="NULL",
                     alpha=0.3,
                     size=4,
                     tickslab="FALSE",
                     font.x = c(12, "bold"),
                     font.y = c(12, "bold"))+
    geom_vline(xintercept = 152554860, linetype = 2, color = "coral4")+
     geom_vline(xintercept = 152761862, linetype = 2, color = "coral4")

#expression plot density plot with rug
malexplot <- ggdensity(ch1male, "chromStart", fill = "Sex",
```

```
    xlab="Transcripts Identified by Microarray", ylab="Gene density", rug=TRUE,
    color="Sex",
    axis.text.x = "",
    font.x = c(12, "bold"),
    font.y = c(12, "bold"))+
  geom_vline(xintercept = 152616641, linetype = 2, color = "black")+
  geom_vline(xintercept = 233124572, linetype = 1, color = "coral4")
# Arranging the plot using cowplot
plot_grid(malexplot, malescatter, ncol = 1, nrow=2, align = "hv")
#
#comparing to our data (katerina's results)
AaeMaleKat <- read.csv("D:/AaeMaleKat.csv",
                 as.is="accession")
AaeMFKat <- read.csv("D:/SpliceKat.csv",
                   as.is="accession")
#join male and female with Base data file from vectorbase
AaeGenExMFKat <- inner_join(AaeGene2, AaeMFKat, by="accession")
glimpse(AaeGenExMFKat)
#
#select only chromosome 1 for male and female data
ch1MFKat <- filter(AaeGenExMFKat, chrom==1)
#
katdensity <- ggdensity(ch1MFKat, "chromStart", fill = "sex",
                    xlab="Transcripts Identified by Long-read Transcriptomics", ylab="Gene density",
rug=TRUE,
                    axis.text.x = "",
                   color="sex",
                    font.x = c(12, "bold"),
                    font.y = c(12, "bold"))+
  geom_vline(xintercept = 152616641, linetype = 2, color = "black")+
  geom_vline(xintercept = 233124572, linetype = 1, color = "coral4")
# Arranging the plot using cowplot
plot_grid(malexplot, malescatter, katdensity, ncol = 1, nrow=3, align = "hv")
```

# Chapter 4: improved microorganism dRNA expression systems

## INTRODUCTION

Production of dsRNA in transgenic microorganisms is a scalable technology that overcomes the high cost of alternative means of dsRNA synthesis. Expression systems include the commonly used strains of *Escherichia coli* (Timmons et al. 2003) and *Saccharomyces cerevisiae* (Hapairai et al. 2017) as well as highly specialized insect symbionts (Asgari et al. 2020). Each approach has advantages in terms of ease of development, biocontainment, and efficacy of dsRNA production and delivery. As discussed in chapters 1 and 2 of this thesis, there are many barriers to RNAi by feeding, including degradation by nucleases in the gut and restricted dsRNA uptake by epithelial cells. Microorganism expression systems may assist in overcoming barriers to dsRNA uptake by protecting dsRNA within the cell membrane of the microorganism. In insects such as mosquitoes that normally feed on bacteria, algae and yeast as larvae, microorganism delivery systems may be a highly efficient means of rearing and inducing the phenotypes necessary for SIT, namely, sterility and female lethality.

The first dsRNA expression platform was the expression vector pL4440 in RNase III-deficient *E. coli*, developed for use in *Caenorhabditis elegans* by Andrew Fire and colleagues (Fire et al. 1998). The pL4440 plasmid used in this study contains dual promoters to drive transcription of both the sense and antisense RNA strands. The two strands can anneal to produce the dsRNA within the bacterial cell, and are not degraded as long as they are produced in the RNase III mutant strain. In the production protocol, bacteria are grown to mid-log phase, induced with IPTG for four h and then fed to *C. elegans* in agar plates. Due to the high degree of RNAi

efficiency in *C. elegans*, the expression platform and the protocol for growth and induction required no further refinements as *C. elegans* can subsist entirely on a simple diet of *E. coli*. Feeding dsRNA-expressing *E. coli* to other organisms has been attempted, and while some successes have been reported (Orii, Mochii, and Watanabe 2003; Zhao et al. 2013; Taning et al. 2016), RNAi efficiency seldom matches that seen in nematodes, and it is clear that further refinements to the *E. coli* expression system are needed to yield highly concentrated dietary dsRNA. While some insect larvae and adults can be fed concentrated *E. coli* in food pellets (Whyard et al. 2015; Tayler et al. 2019), *E. coli* is unattractive to many insect pests and cannot be delivered in sufficient quantities. Alternative microorganisms are needed to deliver dsRNA to these insects.

Since the early days of RNAi testing in worms, bacterial expression systems have been used as a cost-effective means of delivering dsRNA to a range of organisms. Yeast may provide a more attractive dsRNA expression platform for insects. *Saccharomyces cerevisiae* is a commensal found in the guts of many insects, and has been isolated from mosquitoes, wasps, bees and beetles (Stefanini et al. 2016; Suh et al. 2005; Souza et al. 2016). Volatiles produced by *S. cerevisiae* attract ovipositing *D. suzukii* and *Ae. aegypti* (Hapairai et al. 2017), and insecticidal RNAi has been developed in which *S. cerevisiae* expressing dsRNA was fed to *D. suzukii* (Taning et al. 2016) and mosquitoes (Mysore et al. 2019). Yeast is a commercial product that can easily be produced in large scale, making it an attractive platform for both SIT and insecticidal RNAi applications. Despite the potential of yeast and bacterial dsRNA production systems, reports of their use in mosquito RNAi applications remain limited to a small number of research groups (Whyard et al. 2015; Hapairai et al. 2017; Taracena et al. 2019). The lack of widespread reports of dsRNA feeding in mosquitoes suggests that reproducibility across species and rearing

conditions is a challenge, and efforts to standardize insect rearing together with dsRNA production parameters are needed.

Delivering dsRNA to aquatic insects provides additional challenges compared to terrestrial insects that can be fed on solid food spiked with dsRNA (Tayler et al. 2019). One of these challenges is the overgrowth of bacteria and fungi in the rearing water. Recent reports of successful rearing of *Ae. aegypti* in axenic conditions (Vogel et al. 2017; Correa et al. 2018) suggest that the microbiome and microbial community of the mosquito's larval environment are not required for development. Hence, delivery of only the dsRNA-expressing microbes, without competing species present, may permit more effective dsRNA delivery methods to mosquitoes for use in SIT control programs.

With the goal of optimizing dsRNA production parameters in both bacterial and yeast expression systems, I designed experiments in which I knocked down essential genes previously shown to induce lethal phenotypes (Hapairai et al. 2017). By optimizing dsRNA delivery for lethality, the limitations of RNAi by dsRNA feeding can be understood and then applied to reverse genetics studies for basic biological research, and then applied to the highest impact application, RNAi-based SIT.

## METHODS

### DSRNA EXPRESSION IN BACTERIA

*Ae. aegypti* Orlando Gr3[*ECFP*] mosquito larvae (McMeniman et al. 2014) were fed bacteria expressing shRNAs targeting fasciculation and elongation protein zeta-2 (*fez2)* transcripts. This gene was chosen because its knockdown phenotype is reported to be lethal when reared at 26 $^{o}$C (Hapairai et al. 2017), and mortality is easily quantified in plate-based assays. The shRNAs were

designed with a stem sequence of 26 bp and a loop sequence of 9 bp. Annealed DNA oligos of the shRNA sequences were blunt-end ligated into the expression vector pJET1.2 downstream of a T7 promoter. *E. coli* (HT115 DE3) cells were transformed with the plasmids, grown to mid-log phase (~16 h) and expression was induced with 0.4M IPTG for 2 h before harvesting. Harvested cells (40 mL) were pelleted by centrifugation at 5000 xg, heat killed at 80$^{o}$C for 15 min, and the pellets were then suspended in 4 mL 1% agar supplemented with 1 mL 10% sterile brewer's yeast slurry. This mixture solidified in open-topped 5 mL syringes and sliced into 0.5 mL feeding pellets. Mosquito eggs were hatched synchronously by submerging them in deoxygenated previously-boiled dd$H_2O$ into which nitrogen gas was bubbled for 2 min. In 100 mm Petri dishes, 40 larvae in 20 mL dd$H_20$ were provided with two pellets. These pellets were the exclusive food of the larvae from 4 h post hatch until the end of the experiment.

## ASSESSING DSRNA PRODUCTION IN THE BACTERIA

To determine optimum growth parameters for bacterially expressed dsRNA production, a starter broth of *E. coli* was grown overnight with shaking in 2 mL LB ampicillin (50 µg/µL) tetracycline (12.5 µg/µl)) broth after inoculation from glycerol stocks. 50 ml of ampicillin tetracycline LB broth was inoculated with the resulting starter broth and grown to an OD of 0.4 when it was induced with 0.4 mM IPTG. Samples were taken every hour for an additional 4 h post induction. To quantify dsRNA, 1 mL of broth was harvested and direct qRT-PCR was conducted using the SingleShot qRT-PCR kit (BioRad, Hercules, CA) according to manufacturer's instructions using a BioRad CFX 1000 thermocycler. DsRNA abundance was calculated relative to the stably expressed ribosomal reference gene *rrsA* (forward: CTCTTGCCATCGGATGTGCCCA reverse: CCAGTGTGGCTGGTCATCCTCTCA) (Livak and Schmittgen 2001).

## YEAST DSRNA FEEDING

Transgenic *S. cerevisiae* containing single copy shRNA constructs were provided by Renaissance Biosciences (Vancouver, BC). The company was provided the shRNA sequences of the target mosquito genes, and they integrated the shRNA construct into the chromosomes of an auxotrophic (rrp6Δ::His  ski3Δ::Leu) strain. The shRNA was expressed under the control of the strong constitutive promoter (TEF1). The wild-type BY4742 with an empty plasmid (strain 36) was used a negative control strain for all feeding experiments. A list of the strains provided by RB are listed in Table 1.

Yeast expressing shRNA were grown by inoculating a 4 ml starter broth containing URA3 dropout media (ThermoFisher, Walton, MA), and grown overnight at 30$^{o}$C with shaking at 270 rpm. The resulting broth was used to inoculate 150 mL of URA3 dropout media grown for 48h in baffled flasks shaken at 270 rpm. Yeast was harvested by centrifugation at 4000 xg for 5 min. Media was removed and 190 µl of wet yeast slurry was provided to each Petri dish containing 20 newly hatched *Ae. aegypti* larvae (Liverpool strain) in 50 mL deionized water. Larvae were grown at either 28$^{o}$C or 22$^{o}$C for 6 to 10 days.

To produce rearing conditions with minimal non-transformed bacteria present, mosquito egg papers were soaked in a 10% bleach solution for 10 min and then dipped twice in 70% ethanol.

**Table 1:** Yeast strains used in *Ae. aegypti* yeast feeding experiments. Yeast was grown to late log-phase in liquid media and *Ae. aegypti* Liverpool strain mosquito larvae were fed 190 µl of wet yeast slurry.  Yeast strain, plasmid construct and selection marker (if any) are described.

| Strain # | Yeast strain | Construct | Selection marker |
|---|---|---|---|
| **1** | BY4742 | | |
| **2** | BY4742 rrp6Δ::His ski3Δ::Leu | | |
| **3** | BY4742 | TRP1::TEF1_boule | |
| **4** | BY4742 | TRP1::TEF1_gas8 | |
| **5** | BY4742 | TRP1::TEF1_fez2 | |
| **9** | BY4742 rrp6Δ::His ski3Δ::Leu | TRP1::TEF1_boule | |
| **10** | BY4742 rrp6Δ::His ski3Δ::Leu | TRP1::TEF1_gas8 | |
| **11** | BY4742 rrp6Δ::His ski3Δ::Leu | TRP1::TEF1_fez2 | |
| **15** | BY4742 | pRS343-Kan90-TEF1_boule | KanMX (G418) |
| **16** | BY4742 | pRS343-Kan90-TEF1_gas8 | KanMX (G418) |
| **17** | BY4742 | pRS343-Kan90-TEF1_fez2 | KanMX (G418) |
| **18** | BY4742 | pRS343-Kan90-TEF1_modsp | KanMX (G418) |
| **19** | BY4742 | pRS343-Kan90-TEF1_gnbpa1 | KanMX (G418) |
| **20** | BY4742 | pRS343-kan90-TEF1_gnbpa3 | KanMX (G418) |
| **21** | BY4742 | pRS343-Kan90-TEF1_eGFP | KanMX (G418) |
| **22** | BY4742 rrp6Δ::His ski3Δ::Leu | pRS343-Kan90-TEF1_boule | KanMX (G418) |
| **23** | BY4742 rrp6Δ::His ski3Δ::Leu | pRS343-Kan90-TEF1_gas8 | KanMX (G418) |
| **24** | BY4742 rrp6Δ::His ski3Δ::Leu | pRS343-Kan90-TEF1_fez2 | KanMX (G418) |
| **28** | BY4742 rrp6Δ::His ski3Δ::Leu | pRS343-Kan90-TEF1_eGFP | KanMX (G418) |
| **29** | BY4742 | pRS343-Kan90- | KanMX (G418) |
| **30** | BY4742 rrp6Δ::His ski3Δ::Leu | pRS343-Kan90- | KanMX (G418) |
| **31** | BY4742 | pRS343-Kan90_empty | KanMX (G418) |
| **32** | BY4742 rrp6Δ::His ski3Δ::Leu | pRS343-Kan90_empty | KanMX (G418) |
| **33** | BY4742 | TRP1::TEF1_eGFP | |
| **34** | BY4742 rrp6Δ::His ski3Δ::Leu | TRP1::TEF1_eGFP | |
| **35** | BY4742 | pRS343-ura20_empty | Ura dropout (YNB-Ura) |
| **36** | BY4742 rrp6Δ::His ski3Δ::Leu | pRS343-ura20_empty | Ura dropout (YNB-Ura) |
| **37** | BY4742 | pRS343-ura20_fez2 | Ura dropout (YNB-Ura) |
| **38** | BY4742 rrp6Δ::His ski3Δ::Leu | pRS343-ura20_fez2 | Ura dropout (YNB-Ura) |
| **39** | BY4742 | pRS343-ura20_eGFP | Ura dropout (YNB-Ura) |
| **40** | BY4742 rrp6Δ::His ski3Δ::Leu | pRS343-ura20_eGFP | Ura dropout (YNB-Ura) |
| **41** | BY4742 | pRS343-ura20_fez2_f30 | Ura dropout (YNB-Ura) |
| **42** | BY4742 rrp6Δ::His ski3Δ::Leu | pRS343-ura20_fez2_f30 | Ura dropout (YNB-Ura) |
| **43** | BY4742 | pRS343-ura20_eGFP_f30 | Ura dropout (YNB-Ura) |
| **44** | BY4742 rrp6Δ::His ski3Δ::Leu | pRS343-ura20_eGFP_f30 | Ura dropout (YNB-Ura) |
| **45** | BY4742 | pRS343-ura20_4103 | Ura dropout (YNB-Ura) |
| **46** | BY4742 rrp6Δ::His ski3Δ::Leu | pRS343-ura20_4103 | Ura dropout (YNB-Ura) |
| **47** | BY4742 | pRS343-ura20_8858 | Ura dropout (YNB-Ura) |
| **48** | BY4742 rrp6Δ::His ski3Δ::Leu | pRS343-ura20_8858 | Ura dropout (YNB-Ura) |
| **49** | BY4742 | pRS343_ura20_syt427 | Ura dropout (YNB-Ura) |
| **50** | BY4742 rrp6Δ::His ski3Δ::Leu | pRS343_ura20_syt427 | Ura dropout (YNB-Ura) |
| **51** | BY4742 | pRS343_ura20_fez2_#52 | Ura dropout (YNB-Ura) |
| **52** | BY4742 rrp6Δ::His ski3Δ::Leu | pRS343_ura20_fez2_#52 | Ura dropout (YNB-Ura) |

# RESULTS

## INSECTICIDAL RNAI BY *E. COLI* FEEDING IS NOT ROBUST IN *AE. AEGYPTI*

I set out to test RNAi efficiency using bacterial and yeast expression systems. Because it was previously reported that mosquito larvae treated with shRNAs targeting the neuronal gene *fez2* resulted in high mortalities (Hapairai et al. 2017), I selected this gene to target for an initial comparison of RNA efficiency using bacteria and yeast delivery systems. Bacterial feeding assays were conducted using the expression vector pJET 1.2 to produce shRNAs in HT115 (DE3) cells. As shown in Figure 1, feeding on *fez2* shRNA expressed by *E. coli* did not induce mortality relative to controls under these conditions.

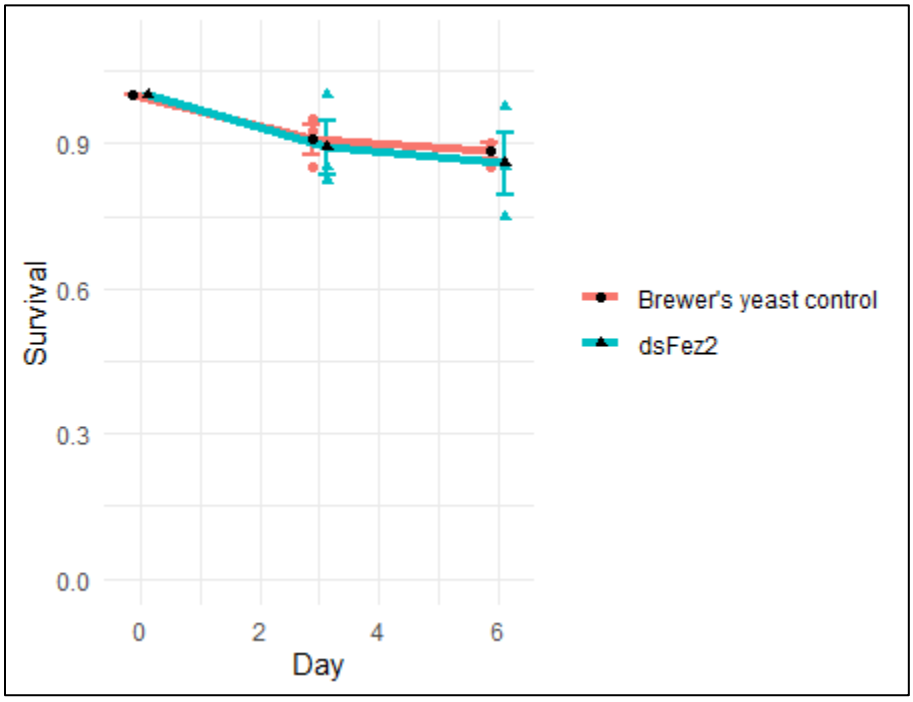


**Figure 1:** Survival plot of *Ae. aegypti* fed *E. coli* expressing shRNAs against *fez2* (dsFez2) compared to negative controls (brewer's yeast pellet with no *E. coli*). Larvae were reared at a density of 20 larvae in 50 ml water in 90 mm Petri dishes at 28$^{o}$C. Black points represent the

mean of three independent biological replicates, coloured dots represent biological replicates and error bars represent the standard error.

## OPTIMIZED PARAMETERS FOR BACTERIAL DSRNA PRODUCTION

After failing to achieve a high degree of mortality reported previously (Hapairai et al. 2017), I investigated why *E. coli fez2* shRNA feeding experiments did not induce mortality. One question that remained after reviewing protocols for RNAi in *C. elegans* was the timing of induction and harvesting of cells. To determine the optimal time to harvest *E. coli,* I conducted a time-course experiment and quantified dsRNA expression during log-phase growth, both before and after induction.

Optical density (OD) readings are an estimate of cell numbers, but require validation by CFU assays. To determine the relationship between OD readings and CFU, I plated diluted samples on LB-Amp plates and counted the number of colonies formed after 20h. The reference gene *rrsA* is a highly expressed gene with approximately 2.8 mRNA copies/cell (Neidhardt and Curtiss 1996). Figure 2 shows that the number of transcripts of long antiparallel dsRNA generated from the plasmid pL4440 peaks at 4 h post inoculation, or 2 h post induction with IPTG. Table 2 shows that this is also the time of maximum number of copies of dsRNA/cell, however the number of copies of dsRNA/cell is also high at the time of induction.

**Table 2:** dsRNA expression profile of *E. coli* cells

| **Time post inoculation (h)** | **Extracted Total RNA (ng/µL)** | **Expression relative to RRSA** | **dsGFP copies/cell** |
|---|---|---|---|
| 0 | 708 | 7.98 | 3.79E+04 |
| 1.5 | 1026 | 278.1 | 3.96E+06 |
| 2.5 (time of induction) | 1050 | 21.3 | 4.04E+06 |
| 4 | 1293 | 12.6 | 4.19E+06 |
| 5 | 1260 | 5.9 | 1.69E+06 |
| 6 | 1441 | 5.1 | 1.95E+06 |

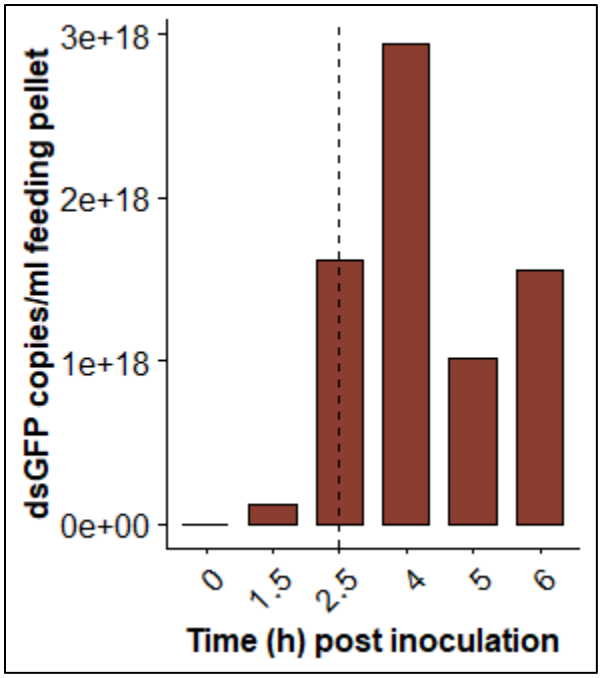


**Figure 2:** dsGFP strands per 1 ml *E. coli* mosquito feeding pellet measured by qRT-PCR. Dashed line indicates time of induction with IPTG.

## INDUCTION OF RNAI BY FEEDING TRANSFORMED YEAST IS NOT ROBUST

I then attempted to replicate the reported successful knockdown of two essential genes, *fez2* and *syt* using a yeast expression system, as described previously (Hapairai et al. 2017; Mysore et al. 2019). BY4742 rrp6Δ::His  ski3Δ::Leu yeast strains were transformed with pRS343_ura20_fez2_#52 and pRS343_ura20_syt4, plasmids that expresses *fez2* and *syt*-specific shRNA. No larval mortality, relative to negative control larvae, was observed after 6 continuous days of feeding on these transformed yeast (Figure 3).

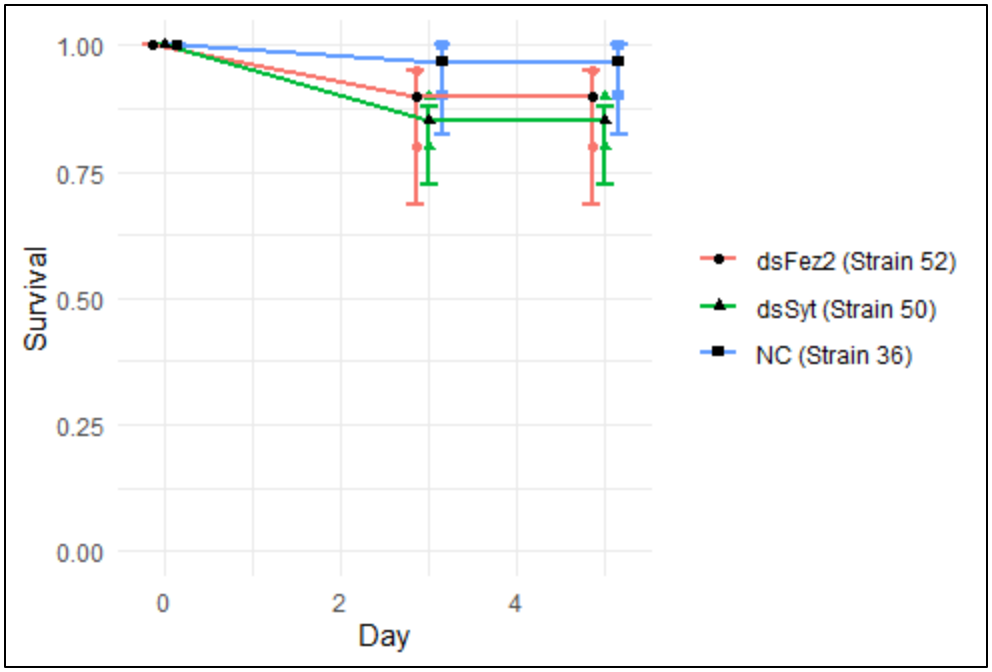


**Figure 3:** Survival plot of *Ae. aegypti* fed *S. cerevisiae* expressing shRNAs against *fez2* (dsFez2) and syt (dsSyt) compared to negative control (untransformed yeast). Larvae were reared at a density of 20 larvae in 50 ml water in 90 mm Petri dishes at 28$^{o}$C. Black points represent the mean of three independent biological replicates, coloured dots represent biological replicates each with 20 larvae and error bars represent the standard error.

In addition to *fez2* and *syt* mortality experiments, several other yeast expression strains were tested with the intent of using a yeast platform to replicate previously reported dsRNA injection and feeding experiments (Whyard et al. 2015; Hapairai et al. 2017; Mysore et al. 2019). The results of these are summarized with a qualitative description of the experimental result in Table 3. Notably, larvae suffered from high mortality in all experiments where G418 was used as a selection marker in the transformed yeast.

**Table 3:** Summary of yeast dsRNA feeding experiments with strain number, construct plasmid and a qualitative summary of the outcome of the feeding experiment.

| Strain # | Construct | Summary of RNAi Efficacy |
|---|---|---|
| **1** | | Control |
| **2** | | Control |
| **3** | TRP1::TEF1_boule | Did not induce measurable knockdown |
| **4** | TRP1::TEF1_gas8 | Did not induce measurable knockdown |
| **5** | TRP1::TEF1_fez2 | No mortality induced |
| **9** | TRP1::TEF1_boule | Did not induce measurable knockdown |
| **10** | TRP1::TEF1_gas8 | Did not induce measurable knockdown |
| **11** | TRP1::TEF1_fez2 | No mortality induced |
| **15** | pRS343-Kan90- | |
| **16** | pRS343-Kan90- | |
| **17** | pRS343-Kan90- | |
| **18** | pRS343-Kan90- | |
| **19** | pRS343-Kan90- | |
| **20** | pRS343-kan90- | Mortality observed, attributed to G418* |
| **21** | pRS343-Kan90- | |
| **22** | pRS343-Kan90- | |
| **23** | pRS343-Kan90- | |
| **24** | pRS343-Kan90- | |
| **28** | pRS343-Kan90- | |
| **29** | pRS343-Kan90- | |
| **30** | pRS343-Kan90- | |
| **31** | pRS343-Kan90_empty | |
| **32** | pRS343-Kan90_empty | |
| **33** | TRP1::TEF1_eGFP | Control |
| **34** | TRP1::TEF1_eGFP | Control |
| **35** | pRS343-ura20_empty | Control |
| **36** | pRS343-ura20_empty | Control |
| **37** | pRS343-ura20_fez2 | No Mortality Induced |
| **38** | pRS343-ura20_fez2 | No Mortality Induced |
| **39** | pRS343-ura20_eGFP | Control |
| **40** | pRS343-ura20_eGFP | Control |
| **41** | pRS343-ura20_fez2_f30 | No Mortality Induced |
| **42** | pRS343-ura20_fez2_f30 | No Mortality Induced |
| **43** | pRS343- | Control |
| **44** | pRS343- | Control |
| **45** | pRS343-ura20_4103 | Did not induce measurable knockdown |
| **46** | pRS343-ura20_4103 | Improved *eCFP* knockdown when combined with strain 40 |
| **47** | pRS343-ura20_8858 | Did not induce measurable knockdown |
| **48** | pRS343-ura20_8858 | Improved *eCFP* knockdown when combined with strain 40 |
| **49** | pRS343_ura20_syt427 | No Mortality Induced |
| **50** | pRS343_ura20_syt427 | No Mortality Induced |
| **51** | pRS343_ura20_fez2_#52 | Mortality induced at 22ºC |
| **52** | pRS343_ura20_fez2_#52 | Mortality induced at 22ºC |

*G418 mortality due to translational read-through has been previously described (Prokhorova et al. 2017)

## LETHAL RNAI IS IMPROVED WHEN DEVELOPMENT IS SLOWED

Due to the lack of any observable effect in transformed yeast feeding experiments, I attempted to prolong the exposure of the larvae to dsRNA by decreasing the rearing temperature to slow larval development, and thereby provide additional time for the RNAi machinery to deplete transcripts. The density of larvae was also reduced as a means of increasing the dose of shRNA-expressing yeast each mosquito consumed. These experiments were conducted at both 28°C and 22°C. There was a decrease in per-dish survival compared to empty plasmid controls (Student's t-test $P$=0.038), however at 22°C, larvae exhibited lower survival in all treatments (Figure 4).

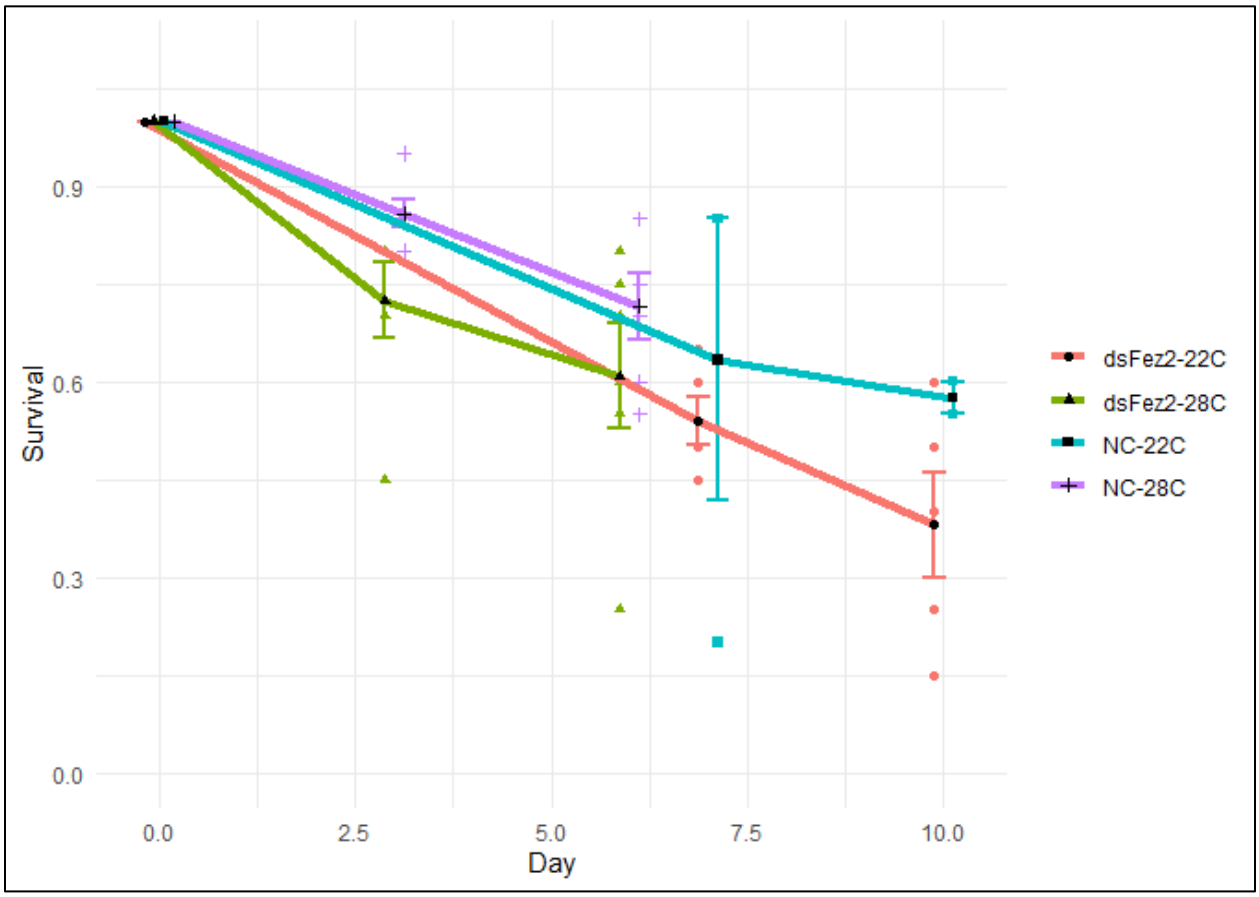

**Figure 4:** Survival plot of *Ae. aegypti* fed *S. cerevisiae* expressing shRNAs against *fez2* (dsFez2, strain 52) compared to negative control (untransformed yeast). Larvae were reared at a density of 20 larvae in 50 ml water in 90 mm Petri dishes at 28$^{o}$C. Yeast was delivered directly to the water as a wet paste. Black points represent the mean of three independent biological replicates, coloured dots represent biological replicates each with 20 larvae and error bars represent the standard error.

## LIMITING BACTERIAL GROWTH ENHANCES LETHAL RNAI WITH YEAST

In the yeast-feeding experiments described previously in this chapter, I observed variable mortality in *fez2* dsRNA treatments and unexpected mortality was observed in the negative control treatments. During optimization of yeast feeding, it was observed that although *fez2*-dsRNA induced slightly greater mortality than the negative controls, at the end of the feeding period, control-treatments also showed a high degree of mortality (Figure 1). Bacterial growth in the rearing water was evident visually and by smell. A potential explanation for this phenomenon is that the proliferation of bacteria in the rearing water was disrupting normal feeding and obscured any effects of RNAi-mediated mortality. I therefore attempted to rear larvae in near axenic conditions. Bleach-treating eggs before hatching and rearing larvae in water with 100 mg/ml ampicillin resulted in a qualitative reduction of bacterial growth and improved survival in control treatments (Figure 5), resulting in increased mortality due to *fez2*-dsRNA consumption.

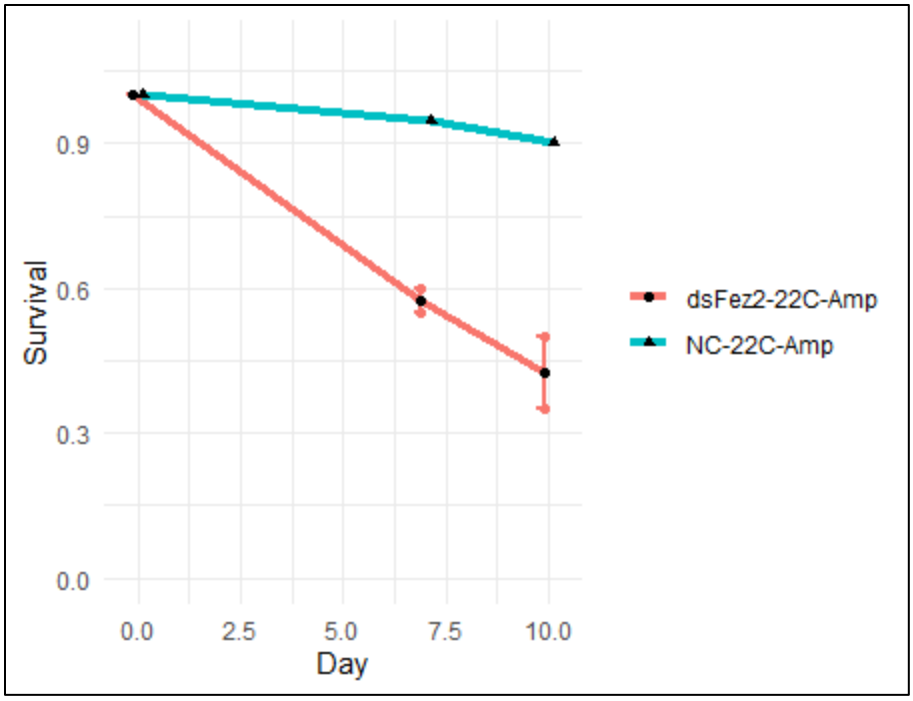


**Figure 5.** Survival plot of *Ae. aegypti* fed *S. cerevisiae* expressing shRNAs against *fez2* (dsFez2, strain 52) compared to negative control (untransformed yeast). Larvae were reared at a density of 20 larvae in 50 ml water in 90 mm Petri dishes at 28°C. Yeast was delivered directly to the water as a wet paste. Ampicillin (Amp) was added to rearing water and eggs were treated by soaking in 5% bleach solution for 5 min before hatching. Black points represent the mean of independent biological replicates (NC=1, dsFez2 =2), coloured dots represent biological replicates each with 20 larvae and error bars represent the standard error.

## DISCUSSION

Laboratory-scale dsRNA production typically relies on *in vitro* synthesis, which is too costly to deliver dsRNA to millions of mosquito larvae. Microorganisms are an attractive alternative dsRNA delivery system because they are easily transformed to express dsRNA and their production can easily be scaled up to feed large numbers of mosquitoes for use in SIT. While

progress has been made towards rearing transformed yeast and bacteria for dsRNA delivery (Whyard et al. 2015; Mysore et al. 2019), parameters for production and feeding are still relatively crude and this prevents the broad application of these methods for mosquito control.

Methods for mass producing dsRNA are needed to make reverse genetics studies by dsRNA feeding possible, and to make RNAi-based SIT cost-effective. Microorganisms are an ideal platform for dsRNA production, but microorganism growth and delivery methods must be optimized to ensure dsRNA is transcribed and biologically active upon consumption by mosquitoes.

My attempts to replicate published results where *fez2* knockdown yielded near complete mortality (Hapairai et al. 2017) failed using both bacterial and yeast expression systems. While attempting to replicate these previously published results, I identified two parameters in yeast feeding experiments that are essential for success. First, RNAi-induced mortality by feeding yeast shows temperature dependency. The development rate in larvae is temperature-dependent (Christophers 1960), as is the reproduction of naturally-occurring microorganisms on which the larvae feed. The observed increase in mortality in *fez2* yeast feeding experiments at 22$^{o}$C compared to 28$^{o}$C may be explained by the reduced rate of development, which in turn allows more time for the RNAi machinery to initiate mRNA depletion. Increased time between larval moults may also contribute to enhanced RNAi, although little is known regarding how RNAi pathways respond during the moulting process.

The second parameter described in this study is microbial overgrowth in the rearing media limiting the bacteria derived from eggs by bleach-treating them before hatching, as well as decreasing bacterial growth with ampicillin increased survival in control shRNA feeding experiments. This suggests that members of the microbial community in larval rearing water may

be responsible for some of the mortality observed in previously reported insecticidal RNAi studies (Whyard, Singh, and Wong 2009; Hapairai et al. 2017). In addition, by eliminating most of the bacteria in the rearing media, there may have been a shift in the nutritional source in fourth instar larvae. Larvae in experiments without ampicillin are expected to have initially fed on yeast, but then gradually fed on whatever bacteria began to grow in the rearing media. Ampicillin-spiked rearing conditions enabled a larger initial dose of dsRNA-expressing yeast, meaning the larvae fed on dsRNA right up to the end of the experiment.

The efforts to identify and knock out genes involved in RNA degradation in *S. cerevisiae* did not yield an increase in RNAi efficiency as measured in these assays. Yeast strains expressing shRNA against *fez2* performed similarly to feeding experiments using transformed *E. coli.* After optimizing rearing conditions to minimize growth of unwanted bacteria and yeast, and delivering a larger dose of transformed yeast, we showed an increase in survival of controls relative to *fez2* expressing yeast, but performance of these strains is still well below the near 100% mortality reported by others (Hapairai et al. 2017). This suggests that there are undescribed factors that inhibit RNAi in mosquitoes. These may be undescribed enzymes in the gut, resistance to dsRNA uptake and processing, or members of the microbial community in the aquatic rearing environment that produce dsRNA degrading enzymes. In addition, yeast expression of dsRNA and preparation of feeding pellets may require further optimization. As shown with bacterial expression experiments, dsRNA production parameters require strict control that is often not well described in the literature. My results suggest that several sources of reduced RNAi efficiency are important barriers to successful RNAi by feeding transformed microorganisms and these parameters deserve further investigation in the future.

For the broad application of microorganism-delivered dsRNA for SIT and reverse genetics studies in mosquitoes, a robust system of production and feeding is needed. This implies that the system can be applied to a range of gene targets with transcripts abundant at different developmental stages, and that the system is effective regardless of the variation in the gut and aquatic microbiome. As eloquently described in a recent discussion of reproducibility (Nosek and Errington 2020), the ability to repeat an experiment must extend beyond the narrow parameters set in proof-of-principle experiments. The experiments described in this chapter have identified several parameters worth closer inspection, highlighting the challenges of reproducing results in dsRNA feeding experiments, and suggesting a path forward to overcoming these challenges.

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# Chapter 5: Discussion

## RNAI-BASED SIT COMPARED TO OTHER MOSQUITO CONTROL METHODS

SIT has been used for decades in insect control but remains underutilized in mosquito control. The cost of sex-separation (Lutrat et al. 2019), as well as challenges in achieving sterilization while preserving mating vigor of males (Alphey et al. 2010), have resulted in slow progress towards cost-effective SIT. The use of RNAi to achieve sex-sorting and sterilization has been demonstrated in principle, and although challenges remain in optimizing this approach, RNAi-based SIT has great potential as a cost-effective approach.

RNAi-based SIT offers refinement compared with GMO SIT methods. If a low-cost method of producing dsRNA is optimized, it promises to be a low-cost method for mosquito control. RNAi technology could also face fewer regulatory and public perception barriers relative to GM technologies, thereby reducing the time from a lab-optimized methodology to wide usage. In addition, RNAi-based sterilization has been shown to produce fewer off target effects (Whyard et al. 2015) due to the tissue specificity of the targeted genes.

## OVERCOMING BARRIERS TO DSRNA STABILITY AND UPTAKE

Feeding dsRNA is expected to be the path forward if large scale RNAi-based SIT is to be realized. This is because it is currently not possible to inject dsRNA into the hemocoel of large numbers of mosquitoes. Despite the promise of dsRNA feeding for mosquito control, variability in RNAi responses during feeding experiments suggests that there are undescribed factors limiting dsRNA stability and uptake. Instability of dsRNA in the gut of mosquitoes has been described (Singh et al. 2017), but the enzymes responsible for degradation are not known.

DsRNA uptake is similarly poorly understood in mosquitoes. The work presented in this thesis answers several important elements related to dsRNA delivery to mosquitoes, which could have an impact on the development of RNAi technologies for mosquito control.

Protecting dsRNA from degradation within the mosquito's gut has been attempted using sophisticated encapsulation approaches, but my results suggests that microorganisms may provide protection of dsRNA. Several forms of dsRNA packaging to enhance RNAi in feeding experiments to insects have been tested. These include liposomes (Castellanos et al. 2019), nanoparticle complexes (Das et al. 2015) and protein complexes (Hauser and Olivier 2019). While these systems can provide protection of dsRNA in the gut and enhance RNAi, their cost may prohibit their application in SIT. In contrast, microorganisms provide a cost-effective means of encapsulating dsRNA, while also providing a scalable means of production.

In the same way, co-feeding target shRNAs with shRNAs directed against mosquito nucleases in the gut is a cost-effective way of overcoming dsRNA degradation. I showed that the nucleases 8858 and 4103 are involved in dsRNA degradation, and that co-feeding with nuclease 4103 enabled higher RNAi efficiency. I also conducted phylogenetic analyses that will enable future investigations into why there is such a diversity of nuclease genes in mosquitoes, and what their role is in development and nutrition.

Improved gene knockdown by dsRNA feeding can offer an array of benefits, both in terms of basic research and applied science. On the basic science side, RNAi is a highly useful reverse genetics tool, and improved knockdown of targeted genes will enhance RNAi's ability to validate the function of newly described genes. I showed that feeding shRNAs expressed in bacteria enabled validation of the role of putative nucleases in the mosquito gut and then applied these methods to sex-sorting and sterilization targets. Currently, most RNAi knockdown studies

in mosquitoes rely on hemocoel injections to deplete gene products. For example, a recent study into the role of a small number of immunity genes in mosquitoes was made possible by injecting hundreds of adult mosquitoes (Werling et al. 2019). Investigating gene function earlier in development, and with higher throughput, is possible using shRNAs expressed in microorganisms, which are readily consumed by mosquito larvae. In the same way that high throughput *D. melanogaster* RNAi screens revolutionized our understanding of fly development (Heigwer, Port, and Boutros 2018), an optimized shRNA cloning and expression system with streamlined rearing methods, such as those used in this dissertation, could make mosquitoes a model organism with robust loss-of-function phenotype data. Such a reverse genetics approach would speed up the discovery of genes involved in olfaction and oviposition choice, enabling the rational design of mosquito repellants and traps. Similarly, the role of regulatory RNAs such as lncRNAs could be investigated in a high-throughput pipeline.

Some may argue that CRISPR-Cas systems already provide a reliable reverse genetics system, but there are advantages to using RNAi for high-throughput screens. First, partial depletion of transcripts by RNAi enables the investigation of phenotypes that would be lethal if eliminated entirely by CRISPR-Cas editing. Second, despite improvements in CRISPR-Cas editing by targeting ovarian cells (Chaverra-Rodriguez et al. 2018), the low editing efficiency of CRISPR-Cas requires the screening of hundreds of mosquitoes and several generations of crosses to achieve a homozygous strain. CRISPR-Cas systems are a useful, but blunt tool for understanding gene function and regulatory networks. Therefore, pursuing RNAi-based methods for reverse genetics screens is worthwhile due to reduced timeline to confirmed phenotype, and the ability to detect phenotypes of genes that are lethal when knocked out.

Applying the microorganism-based protection of dsRNA to SIT against mosquitoes could take several forms. My work with yeast and *E. coli* showed that yeast and bacterial feeding pellets are stable in storage at 4 $^{o}$C, and the degree of depletion of *eCFP* seen in my experiments suggests that feeding pellets and yeast continue to provide dsRNA to mosquitoes in an aquatic environment at 28 $^{o}$C over several days. In yeast, there has been some work done towards optimizing production and shipment of feeding pellets (Mysore et al. 2019), but dsRNA production methods are still at a small scale for *E. coli* expression systems. My findings refine the production parameters for *E. coli* production of dsRNA. Future studies are expected to focus on bioreactor scale production and methods for storage techniques that preserve dsRNA in the cells.

## NEW TARGETS FOR SEX-SORTING AND STERILIZATION

Adding to the repertoire of genes that can be targeted for sex-sorting and sterilization is the next hurdle in achieving successful RNAi-based SIT. Understanding dsRNA stability is not enough to achieve a fully sterile population of mosquitoes. This is because some gene targets are not easily perturbed by RNAi.

The design of dsRNA against sex-determination and sterilization genes is only as good as our understanding of these biological processes. Sex-sorting and sterilization targets to date have largely focused on conserved pathways of sex determination and sex differentiation, assuming that sex determination pathways are shared with *D. melanogaster*. A recent study in tephritid fruit flies illustrates how far off the mark this assumption can be (Peng et al. 2020). This study found that a miRNA is the early embryonic male determining factor, a pattern not described in other flies. Dipteran sex-determination and differentiation pathways continue to surprise

developmental biologists, and I sought to focus on patterns of sex-specific alternative splicing in larval mosquitoes.

Sex-specific splicing was found to be a relatively common feature of the fourth instar larval transcriptome of *Ae. aegypti*. It was not, however, so common as to be overwhelming. Previously employed methods of gene discovery have used short read sequencing (Gamez et al. 2020) or microarrays (Tomchaney et al. 2014) to quantify gene abundance. Those approaches yield thousands of potential targets, mask alternative exon usage, and commonly result in researchers selecting a subset of targets based on gene ontologies or conservation with model organism. In addition, short-read transcriptomics and microarrays are biased towards differential expression of highly expressed genes, many of which are expected to be challenging targets for RNAi (Joga et al. 2016).

My methods provide a more streamlined approach of identification of targets. I identified ~150 sex-biased targets in each sex, most of which had low or moderate levels of expression. This number of genes is manageable, and the discovery pipeline is based in the biology of the organism under investigation with minimal bias introduced by the researcher.

Long-read transcriptomics also revealed splice variants with very small exons that were not found in the publicly available databases. Microexon-containing splice variants are likely to follow tightly regulated tissue and developmental-stage specific expression patterns seen in other organisms (Torres-Méndez et al. 2019). Beyond basic biology studies of their role in development of mosquitoes, microexons may provide ideal targets for sterilization and sex-sorting by RNAi. Their length of approximately 25 bp closely matches the length of commonly used shRNAs, and their tissue specificity is likely to minimize off-target effects.

An additional benefit of adding to the repertoire of gene targets for sex-sorting and sterilization is the possibility for improved tissue specificity. The regulatory networks controlling the expression and splicing of sex-differentiation genes are finely tuned, enabling the targeting of a transcript expressed exclusively in the testis. Although some success has been reported using homology with *D. melanogaster* to identify targets (Whyard et al. 2015), the expression of these genes may not follow the pattern seen in vinegar flies. The *de novo* approach I used identified sex-specific genes in the testis at the developmental stage in which dsRNA delivery is occurring. It is hoped that these will provide many targets for future screening of sterilization with minimal off-target effects.

## IMPROVED METHODS FOR MASS-PRODUCTION OF DSRNA

Finally, to implement SIT cheaply, we need a mass production system. As discussed in the context of dsRNA stability, microorganisms provide many advantages over the production of dsRNA *in vitro*.

I have described optimized methods of dsRNA production in *E. coli*. Although these systems have been in use for many years, the preponderance of studies have been in *C. elegans*, a species with a highly efficient RNAi system. This has allowed worm researchers some degree of latitude in achieving knockdown by feeding transformed bacteria. In more challenging organisms such as *Ae. aegypti*, the methods developed here will be of use for researchers in the SIT fields and anyone seeking to perform reverse genetics screens.

In yeast, I have also advanced the methods used for dsRNA production. Mutant yeast strains (BY4742 rrp6Δ::His  ski3Δ::Leu) were found to be effective dsRNA production strains. My results suggest that whatever improvement was derived from producing dsRNA in this knockout strain was masked by underlying barriers to dsRNA stability and uptake in the mosquito larvae.

The improved rearing conditions I developed may be the highest impact initially for this research, but once rearing conditions are further refined, the benefits of improved dsRNA production by mutant yeast strains may become apparent.

I also extensively tested yeast strains that used G418 (geneticin) selection and found that mosquito growth was impaired. Gentamycin perturbs protein synthesis in bacteria by inducing incorrect incorporation of tRNAs by the ribosomal 30S subunit. In eukaryotes similar translation defects due to gentamycin binding to the 80S subunit can occur (Prokhorova et al. 2017) with widespread impacts on development. In mosquitoes, similar impacts have not been described, but this may explain developmental delays and mortality in these assays. My recommendation is to avoid similar expression systems for mosquito rearing. This knowledge will be useful to researchers undertaking yeast dsRNA production studies.

While conducting dsRNA production studies I was unable to replicate published reports of complete larval mortality when *fez2* was knocked down (Hapairai et al. 2017). This led me to investigate the effects of rearing temperature on RNAi efficacy. Larval rearing at 22$^{o}$C resulted in mortality in all treatments, including controls. While lower temperature is known to reduce developmental rate and may thereby provide more time for the RNAi machinery to deplete target transcripts, it also reduces feeding rate and gives time for unwanted yeast and bacteria to flourish.

This finding led to another useful insight that can be derived from my research. Control of bacterial overgrowth during yeast feeding assays eliminates some of the variation in the microbial communities found in the mosquito gut and rearing environment. I found that bleach treatment of eggs before hatching and rearing in ampicillin-spiked water greatly decreased mortality in yeast feeding experiments. This insight will be of interest to anyone attempting to

conduct RNAi experiments in aquatic organisms. The provision of nutrient-rich foods is always prone to bacterial growth that confounds mortality estimates and impairs normal development. The protocol developed in this dissertation is likely to be adaptable to a range of aquatic arthropods.

Taken broadly, these results point to a problem of reproducibility in dsRNA feeding experiments. While it may be tempting to discard RNAi in light of the complexity of delivery in aquatic environments, these struggles may actually highlight fundamental biological questions and lead to future discoveries. A recent perspective (Nosek and Errington 2020) outlines the struggle biology faces in repeating experiments. Rather than abandoning lines of research due to apparent lack of robustness in the methodology, the methodology should be probed to reveal the underlying complexity in the system. It is hoped that my investigation into microbial production and rearing conditions provides a template for future work investigating the biology of dsRNA feeding in mosquitoes.

## APPLICATIONS OF RNAI-BASED SIT FOR HUMAN HEALTH AND CONSERVATION

The technologies developed in this dissertation could be applied widely to pest mosquitoes. SIT has been adapted to several anopheline and culicine species in the past, and RNAi-based SIT can be expected to perform well in many species.

For human health applications, the species with the greatest impact are *An. gambiae* and *Ae. aegypti*. Both of these species are well researched and deployment of SIT protocols will be able to follow established methods of rearing and release of sterile males (Andreasen and Curtis 2005; Zheng et al. 2015). Secondary African malaria vectors such as *An. arabiensis* and *An. stephensi* have established rearing protocols, making the development of RNAi-based SIT for these species

an attractive next step. One African malaria vector may not be amenable to an RNAi-based SIT approach, and this is *An. funestus*. Colonies have not been successfully maintained for this species, so a factory rearing approach will depend on the establishment of colony-maintenance protocols that enable mating in cages and normal oviposition behaviour. Aedine pest mosquitoes are perhaps the easiest species to which this technology can be applied. For example, *Ae. albopictus* shares many life history traits with *Ae. aegypti* and is easily reared in the laboratory. The insights into nuclease reduction described here may be applied to *Ae. albopictus* to reduce variability in RNAi response.

Mosquito pests of wildlife may provide additional target species to control with RNAi-based SIT. For example, avian malaria vectors such as *Culex quinquefasciatus (Say 1823)* are a major pest of Hawaiian birds (Dahlin and Feng 2019). An SIT program against these mosquitoes could have a significant conservation impact, and the low cost of RNAi-based methods may speed its adoption.

RNAi-based SIT is a species specific, scalable mosquito control platform that can be used to control multiple diseases simultaneously. The concept of integrating vector control methods across diseases is reviewed by Golding et al. (2015) who describe how effective mosquito control can be deployed to combat malaria, arboviruses and mosquito-borne nematodes. Applying this principle to RNAi-based SIT, a multi-species SIT can be envisioned. Such a program would require mass rearing, sex-sorting and sterilization using a range of dsRNAs that are active against important culicine and anopheline mosquitoes. Colonies of each pest could be produced from locally trapped adults and mass reared on microorganisms expressing dsRNA. Male release would be tailored to the mating behaviour and flight range of each species.

The potential of RNAi-based SIT is great. This dissertation describes several obstacles to achieving successful SIT by RNAi, and it provides solutions to overcome them. A better understanding of dsRNA stability, streamlined methods for identification of gene targets, and mass dsRNA production methods will be essential for the goal of broadly deployed SIT programs being realized.

## CONCLUSIONS

Mosquitoes that transmit pathogens are among the most devastating pests. They are responsible for more deaths than any other animal and the pathogens they carry impede the development of tropical countries. I have identified a path forward to bringing RNAi-based SIT for aedine mosquitoes to a form that can be commercialized. Understanding dsRNA degradation, gene targets and dsRNA production is likely to be critical to bring this from the lab bench to field applications.

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