Improving mosquito (*Aedes aegypti*) sex-sorting methods for sterile insect technique using RNAi gene knockdowns

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Abstract

Doublesex (*dsx*) is an alternatively spliced mosquito (*Aedes aegypti*) gene that controls sex development by producing male (DSX^M) or female (DSX^F) transcription factors that regulate gene expression in a sex-specific manner. As both DSX^F and DSX^M bind the same DNA sequence, their ability to regulate differential gene expression is predicted to be modulated by other proteins that interact with DSX. A previously conducted protein-protein interaction study identified several proteins that bind to DSX^F and their role in altering female development was explored in this study by knocking down their corresponding transcripts using RNA interference during the larval stages of development.

Knockdowns of the genes *nop-14*, *wdr-48*, and *rnmt* was achieved by feeding mosquito larvae HT115 strain *E. coli* transformed with a pl4440 plasmid containing a dsRNA template specific to each of three target genes. The knockdowns were carried out at their normal rearing temperature, 28°C, and at 22°C to slow mosquito development. Knockdown of one of the genes, *wdr-48*, at either temperature, significantly increased the time to pupation of females compared to males. This delayed development of females could prove useful in the sex sorting of males and females during the pupal stage by providing a method to select only males for a sterile male insect (SIT) population control program for *Ae aegypti*. Of note, the 22°C growth conditions provided a larger difference in the pupation times between the sexes than the 28°C conditions did, and thus may provide even better sex-sorting for mosquito SIT.

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Introduction

Mosquitoes are one of the world's deadliest animals, capable of spreading a plethora of diseases to millions each year (Zabalou et al. 2004). The lack of effective vaccines, drugs, or treatments for these illnesses means that the most viable option for disease prevention is widespread mosquito population control (Lees et al. 2015). Due to growing public concern about the long-term environmental consequences of using pesticides, and increasing development of mosquito resistance to such measures, interest has grown in species-specific natural control methods (Nolan et al. 2011; Bonds 2012). One such method is the sterile insect technique (SIT), which involves the sterilization and release of male mosquitoes to facilitate infertile mating with wild females (Lees et al. 2015). This sterilization can be achieved in several different ways, the most promising of which being the use of RNA interference (RNAi) to knock down genes associated with mosquito fertility (Whyard et al. 2015; Faraji and Unlu 2016).

Sterilization of mosquitoes necessitates a stringent separation of male and female mosquitoes before release since female mosquitoes will facilitate spread of these blood-borne diseases by biting humans (Benedict and Robinson 2003). Furthermore, as release of female mosquitoes with sterile males will reduce the mating frequency among wild mosquitoes, it is best to eliminate female mosquitoes from being released at all (Benedict and Robinson 2003). The process by which this is done is called sex-sorting. This too, may be achieved in several different ways, one of which is using RNAi.

RNAi is a natural cell function that is initiated by introducing double-stranded RNA (dsRNA) into an organism (Whyard et al. 2015). The RNA is then unwound into two

strands of single-stranded RNA (ssRNA) called the “guide” and “passenger” strands (Tijsterman and Plasterk 2004; Lopez et al. 2019). The guide strand is induced to pair with a complementary mRNA sequence, which is then destroyed by a catalytic protein called Argonaute (Lopez et al. 2019). This prevents the mRNA from being translated into a protein, therefore preventing its expression, which can, in turn, alter the phenotype of the affected cell, tissue, or entire organism.

RNAi may be used to take advantage of sex-based dimorphisms present in the larval or pupal stages of several different species of mosquito. In the *Aedes* genus, females take on average a day longer than males to pupate, meaning that the pupae developing later may be sorted out from the earlier-pupating males (Gilles et al. 2014). This method of sex-sorting is currently imprecise, as some female larvae may pupate early, or some males pupate late (Papathanos et al. 2009). To improve the precision of the technique, RNAi may be leveraged to increase the time of pupation in females to a degree such that they may be easily sorted from males, if not killed altogether.

The sex-determination pathway of mosquitoes culminates in a single gene called *doublesex* (*dsx*) (Biedler and Tu 2016). This gene’s mRNA is differentially spliced into two isoforms, which are translated to the proteins DSX^F (the female isoform) and DSX^M (the male isoform) (Biedler and Tu 2016). These isoforms have identical DNA-binding domains, meaning that they themselves don’t determine mosquito sex but indirectly influence it by binding different proteins to sex-specific oligomerization domains (Whyard, unpublished). Determining the identity and function of the proteins that interact with these isoforms (particularly DSX^F) may reveal what influences the delay in

female pupation and result in an increase in pupation time for more accurate sex-sorting.

Proteins interacting with DSX^F were identified in a protein-interaction pulldown assay (Heschuk, unpublished data). In this assay, purified DSX^F was mixed with female pupal nuclear extracts, and the resulting proteins that had bound to DSX^F were subjected to mass spectrometry to determine their identity. Two of these pulldown assays were performed, and a total of 23 proteins that interact with DSX^F in *Aedes aegypti* were identified. In the current study, RNAi-mediated knockdowns were used to target three genes encoding three of the DSX^F-interacting proteins to determine if they have an effect on female mosquito larval and pupal development.

To identify proteins that have a higher likelihood of influencing growth and development of female larvae, the three genes chosen for this study were selected based on the following criteria: 1) the genes were confirmed to be expressed predominantly in females (>2 times greater than in males); 2) the genes are expressed predominantly in mosquito larvae; and 3) the gene is expressed in the mosquito gut; and 4) the gene has not been previously studied by this lab. These criteria were evaluated using transcriptomic data available in the public information database of Vectorbase (vectorbase.org).

It was hypothesized that the knockdown of one or more of these genes would extend pupation time in female larvae, which may indicate that these genes' encoded proteins influenced the differential pupation times seen in males and females. Identification of the genes that affect the timing of pupation could therefore permit

research into increasing the time of pupation, and therefore lead to development of a more accurate and efficient sex-sorting method for SIT.

Materials and Methods

Aedes aegypti Colony Maintenance

Aedes aegypti of the Liverpool strain were reared at 25°C in 50% humidity with a photoperiod of 16 hours light:8 hours dark (Whyard, unpublished). Adults were maintained using a constant supply of 10% sucrose solution and were given weekly feedings of rat's blood to encourage egg production in females (Whyard, unpublished). Eggs were collected on a moist paper towel and allowed to develop for at least a week. Eggs were then introduced to deoxygenated water to induce hatching. The larvae were then transferred to containers of tap water with rabbit food pellets and maintained at 28°C until the pupal development stage (Whyard, unpublished). Pupae were transferred to colony cages to prevent their escape upon eclosion and to maintain colony levels.

Target Genes Selection and Cloning

Nuclear proteins were previously extracted from *Ae. aegypti* using a pull-down approach that utilized His-tagged DSX^F proteins bound to magnetic beads (Heschuk, unpublished data). Pull-downs were performed twice after the first set did not yield genes that performed the desired functions upon testing. In addition, using transcriptomic data available on VectorBase, target genes were selected if they 1) showed greater than a two-fold increase in females over males; 2) they were

predominantly expressed in larvae relative to other developmental stages; 3) they were expressed in the mosquito gut; and 4) these genes were previously unstudied. From these pull-downs, 23 proteins were discovered to interact with DSX^F, as determined by mass spectrometry analysis of the protease-digested peptides obtained from these proteins (Heschuk, unpublished data). The twelve with the greatest binding affinity for DSX^F are shown in Table 1. Based on this data, three previously unstudied proteins were chosen to use as targets for RNAi knockdowns in this study due to their high affinity for DSX^F, as well as their involvement in transcription machinery (Table 1) (Heschuk, unpublished data). The three genes selected were: *nop-14*, *wdr-48* , and *rnmt*. Gene sequences for each protein were obtained from VectorBase, and primers for the knockdown were designed using IDT's online Primer Design tool, to amplify fragments of target genes.

Table 1. List of DSXF binding partners used as targets for feeding assays and their dsRNA primers.

Target	Accession	Description	Molecular weight (kDa)	dsRNA primers (5' to 3')	
				Fwd	Rev
1	A0A6I8U8Y2	CAF1C_H4-bd domain-containing protein	52.3	CGTTTATGGG GCTGGGACT	AAAATCCGGC TTCACTCCGT
2	Q17NL0	DNA-directed RNA polymerases I, II, and III subunit RPABC3	17.1	ACATGGATCC GGAAGGCAAG	GTAATCCGCC GAACGAAACG
3	Q16RV2	Zinc finger protein, AAEL010847-PA	82.5	CCGAAGCGAT ACAAACGCAG	GTAATCCGCC GAACGAAACG
4	A0A6I8TQ91	Heterogeneous nuclear ribonucleoprotein k	43.9	TTCCGGTGCA TCCAATCAGA	CCATCGTGGT GCTGATCACT
5	A0A6I8U220	Uncharacterized protein	101.3	CCCATCGAGT CCGATTCCAG	TCTTGGCGTT TGCGTTCAAG

6	Q16PG0	Asparagine--tRNA ligase	54.9	AGGAAGCCAT CCACGTTCTG	GTCGGGACCG ATCCAAACTT
7	Q17AI3	AAEL005307-PA	35	CTGCGAAAAC AGGTGGAAGC	CGACCAACGT CTATCGGAGG
nop-14	AAEL014051	Nucleolar protein 14 (NOP-14)	97.5	TGAGCTGCCT CGAAAGTACG	TGGAGTTACA ACGGGATGGC
wdr-48	AAEL021400	WD repeat-containing protein 48 homolog (WDR-48)	78.2	CATTAAGGGT GGAGCTGCGA	CGTCGCATCC GTTTGAATCC
rnmt	AAEL021810	mRNA cap guanine-N7 methyltransferase (RNMT)	47.9	AAAAAAGAAT TCTTCGCCGCT GACGCCACA	AAAAAAGAATT CTTACGAATAG ACAGGTTTTCCC
8	Q16P83	Succinate--CoA ligase [ADP-forming] subunit beta (mitochondrial)	48.5	CGGCAAGATG ATCAAGCAGC	CTCCAGCATC CTCGGCATAG
9	A0A1S4FI59	SEC63 protein (putative)	86.3	AGAATGTCAG TGCGAACCGT	GAGCAATGCC GAACGATGTC
10	A0A6I8TQA0	Glutamate synthase (NADH)	229.1	GAATTCGGAT TCAGCACGGC	TGGGCGAAGA TCCAAAGCAT
11	A0A1S4FPS6	Pupal cuticle protein 78E (putative)	15.9	ACAGCAGCGA AGAAACGGTA	TGATGACATG CTCCGGTACG
12	A0A6I8U5Z1	Larval cuticle protein A3A	23.4	CTCCCTGATC GAACCCGATG	ACACTCGGGA GTTGGTCCTA

Targets 1 to 7 as well as *nop-14*, *wdr-48*, and *rnmt* are genes encoding for proteins located in the nucleus that show some transcriptional and nucleic acid binding activity. Targets 8 to 12 are genes encoding for proteins that appeared in relatively large quantities when DSX^F was used to isolate proteins. Targets 1 to 12 are genes discovered in the first round of mass spectrometry, while *nop-14*, *wdr-48*, and *rnmt* are genes found in the second round. Primer sequences are listed without restriction sites. Data provided by Heschuk (unpublished).

Bacterial Transformations

To determine the impact of transcript knockdown on targets identified in the mass-spectrometry analyses, RNAi was employed using a bacterial expression system to produce dsRNA. Each dsRNA was designed to correspond to 350-450 base pairs of the target mRNA transcript. Constructs were PCR-amplified using primers listed in Table 1 and blunt-end ligated into pJET 1.2 using T4 DNA ligase (Thermofisher). Following transformation into competent DH5α strain *E. coli* cells, individual colonies containing

the correct plasmid inserts were grown overnight in a broth culture and subsequently used for plasmid purification using the E.Z.N.A.® Plasmid DNA Mini Kit (Omega). The dsRNA templates were exposed to a digestion temperature of 37°C for 1 hour to initiate digestion out of pJET 1.2 using the Xbal and Xhol restriction sites designed into the plasmid, and ligated into a similarly digested pL4440 plasmid using T4 DNA ligase. The resulting pL4440 plasmid including the dsRNA template was again transformed into competent DH5α *E. coli*, and plasmid isolation using the E.Z.N.A.® Plasmid DNA Mini Kit (Omega) was performed again. The isolated pL4440 plasmid was then transformed into the destination vector, HT115 *E. coli*, a tetracycline-resistant and nuclease-deficient *E. coli* strain.

dsRNA Production

HT115 *E. coli*, a bacteria strain that produces T7 RNA polymerase when exposed to the sugar analogue IPTG (Isopropyl β- d-1-thiogalactopyranoside), was used to initiate production of sense and antisense RNA strands; production was initiated by the T7 promotors and carried out by the RNA polymerase. The resulting strands were then annealed to form dsRNA, which was used to perform a knockdown. This dsRNA corresponding to the gene of interest was introduced to mosquitoes by feeding them the transformed *E. coli* embedded in agar. A negative control was established by feeding mosquitoes HT115 strain *E. coli* cells with an empty pl4440 plasmid.

The transformed *E. coli* was grown overnight at 37°C with shaking, in 4 mL LB broth cultures containing tetracycline and ampicillin. The ampicillin served to verify

whether the *E. coli* cells were transfected properly, as the plasmid also contains an ampR (ampicillin-resistance) gene that allows bacteria possessing it to grow in the presence of the antibiotic. Any *E. coli* cells that fail to grow must lack the ampR gene, and therefore, also lack the plasmid required for dsRNA production. The added ampicillin also prevented other bacteria from growing in these cultures and thus minimized possible contamination of the experiment.

Mosquito Larvae Feeding and RNAi Knockdown

The following morning, the 4mL broth cultures were used to inoculate a 36 mL mixture of LB, tetracycline, and ampicillin. The resulting bacteria was grown in this mixture at 37^{o}C with shaking until the culture reached an optical density of 0.4 at 600 nm. At this point, IPTG was added to the culture to induce dsRNA production, and after 4 hours, the cells were centrifuged at 4000xg for 5 minutes. The resulting pellets of bacteria were resuspended in a 1 mL slurry of brewer's yeast and deionized water, and subsequently heat killed via heating at 75^{o}C for 15 minutes. The dead bacteria and yeast mixtures were vortexed with 4 mL of molten 1% agar and poured into 5 mL syringes with the tips cut off. The syringes were left to solidify for 20 minutes, after which two 0.5 mL sections were cut from each solidified mixture and used to feed 20 larvae.

4 groups of 20 first instar larvae were grown at 22^{o}C and fed these agar plugs once every day, in petri dishes containing 20 mL of water. Three groups of 20 received dsRNA treatment for each gene studied (60 larvae each for targets *nop-14*, *wdr-48*, and *rnmt*) , and another three received a negative control treatment consisting of HT115 strain *E. coli* cells transformed with an empty pL4440 plasmid. Feeding assays were

repeated twice, so in total 120 larvae were treated with each target dsRNA, and 150 larvae were treated with the negative control as an extra 30 were examined for the control. The larvae were fed until pupation (which took between 9–19 days), at which time they were removed from the petri dishes to vials of deionized water. Pupae remained in these vials until adulthood, at which time the sex of the mosquito was visually determined. Male mosquitoes have bushy, hairy antennae, while females' antennae are not hairy. Female mosquitoes also have a long, needle-like proboscis they use for biting. These characteristics were used to distinguish between sexes.

Statistical Analysis

The time to pupation in each sex and the duration of pupation in each treatment of each treatment were averaged and the standard deviations were determined. The proportions of male and female adult mosquitoes were calculated and standard error was calculated from this data. A one-way ANOVA test was used to compare the average duration of pupation from each dsRNA treatment (*nop-14*, *wdr-48*, and *rnmt*) to the negative control treatment. A two-way ANOVA test was used to compare the proportion of males to females for each treatment, and the proportions of either sex to those of the negative control. A two-way ANOVA test was also used to compare average pupation times of males to females for each dsRNA treatment, and those pupation times to males and females of the negative control treatment. The number of male and female mosquitoes that pupated each day was recorded by removing pupae on the day of pupation and recording the sex of each pupae after eclosion. The proportion of males

and females pupating each day was calculated from the total number of either sex that pupated. The day by which 50% of mosquitoes of each sex had pupated was calculated to determine the ideal day for sex-sorting males from females.

Results

Sex Ratios

In the feeding assays, the sex of each mosquito that eclosed for each treatment was recorded, and the proportion of males and females was calculated. There was no significant difference ($p>0.05$) in the proportion of males (0.44 ± 0.19) and females (0.55 ± 0.19) found in the negative control, ds-*nop-14* (0.54 ± 0.06; 0.43 ± 0.08), ds-*wdr-48* (0.65 ± 0.28; 0.35 ± 0.28), or ds-*rnmt* (0.63 ± 0.09; 0.36 ± 0.08) experiments (Figure 1). Additionally, when compared to the proportions of sexes of the negative control population, there was no significant difference ($p>0.05$) in the proportions of each sex in any of the dsRNA treatment populations.

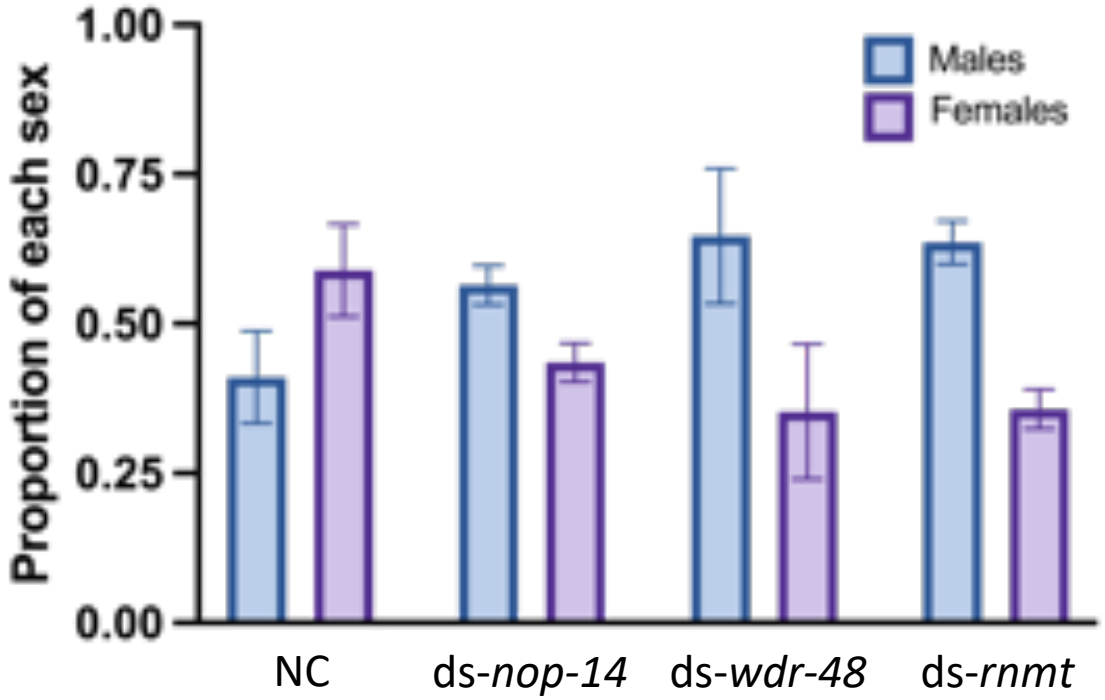

Figure 1. The male and female proportions of eclosed mosquitoes of three dsRNA (ds-*nop-14*, ds-*wdr-48*, and ds-*rnmt*) treatments compared to a negative control. Proportions were obtained from the mosquitoes that survived to adulthood from two separate feeding assays of 60 mosquitoes (120 total) for each dsRNA treatment and three feeding assays – two of 60 and one of 30 mosquitoes (150 total) – for the negative control treatment.

Male and Female Pupation Time

The time it took for male and female mosquitoes to pupate from the beginning of each feeding assay at 22^{o}C was recorded for each treatment. The average time to pupation (in days) for each sex in each dsRNA treatment was calculated, and the difference between male and female pupation times for each treatment were compared (Figure 2). The difference between pupation times of males and females of each dsRNA treatment were also compared to males and females of the negative control (Figure 2.)

There was no significant difference between the average pupation times of males (11.16±1.19 days) or females (12.73±1.55 days) following treatment with ds-*nop-14*, or between the average male (11.91±1.11 days) or female (13.18±1.27 days) negative control pupation times ($p>0.05$). Similarly, there was no significant difference between male (11.56±1.35 days) or female (12.34±1.39 days) pupation times of the ds-*rnmt* treatment and the average negative control times ($p>0.05$). In contrast, treatment ds-*wdr-48* did not exhibit significant differences in the average pupation time of male mosquitoes (12.06±0.99 days) when compared to that of negative control mosquitoes ($p>0.05$), but did exhibit a significant increase in average female pupation times (14.19±0.83 days) compared to the negative control times ($p<0.05$).

For the aforementioned assays, all mosquito larvae were reared at 22°C. The same assays were repeated at a higher temperature: 28°C, the normal rearing temperature for *Ae aegypti* in the lab (Whyard, unpublished). The average time to pupation of both sexes was compared, as well as the difference between pupation times of males and females of each dsRNA treatment; these were also compared to males and females of the negative control (Figure 3) (Heschuk, unpublished). There was no significant difference recorded between the average pupation times of females between the ds-*nop-14* treatment (7.42±0.63 days) and the negative control treatment (7.55±1.16 days) ($p>0.05$), but there was a significant increase in male pupation times following the ds-*nop-14* treatment (7.03±0.74 days) compared to the negative control (6.44±0.50 days) ($p<0.05$). Similarly, there was no significant difference between the average pupation times of females of the ds-*rnmt* treatment (7.68±1.05 days) and the

negative control treatment ($p>0.05$), but there was a significant increase in pupation times of males following the ds-*rnmt* treatment (7.10±0.64 days) compared to the negative control ($p<0.05$). However, there was a significant increase in both the male (7.20±0.62 days) and the female (8.71±1.22 days) average pupation times following treatment with ds-*wdr-48* when compared to the male and female pupation times of the negative control treatment ($p<0.05$).

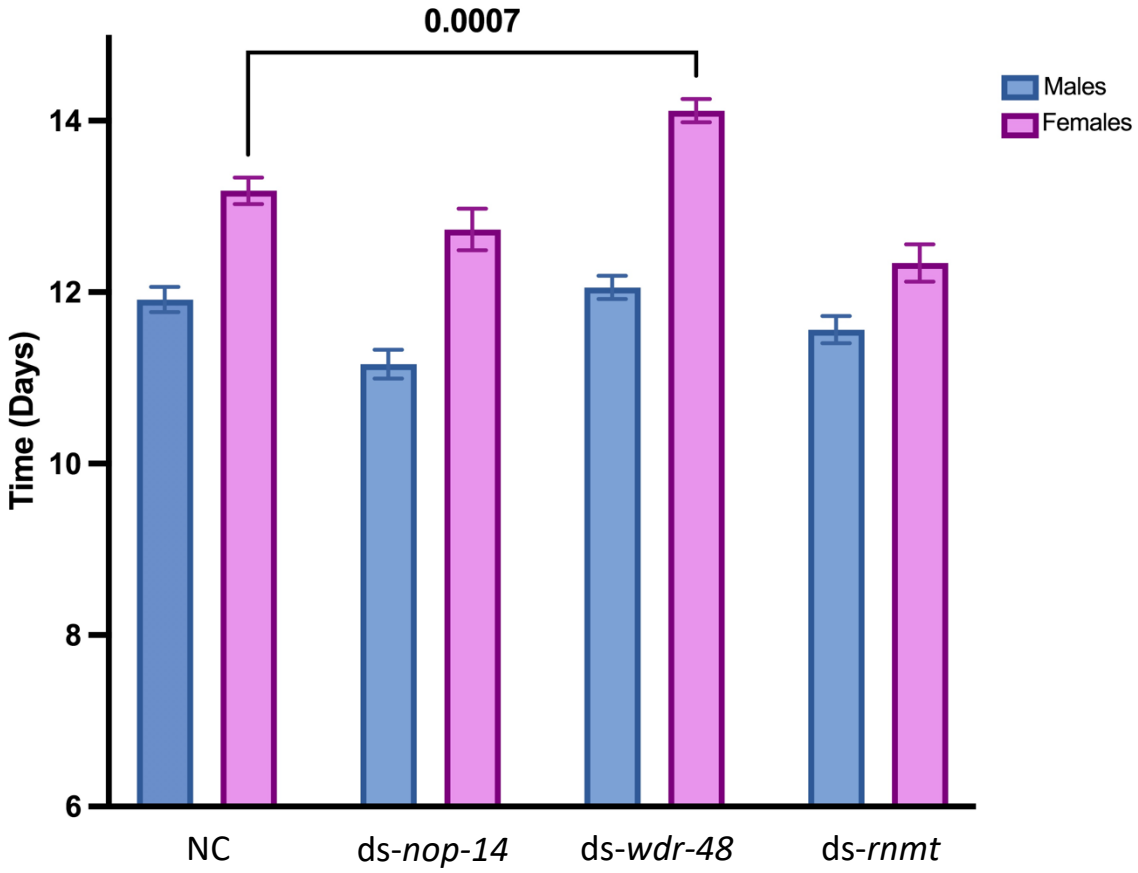

Figure 2. The average time to pupation of male and female mosquitoes when treated with either ds-*nop-14*, ds-*wdr-48*, ds-*rnmt*, or negative control treatments at 22°C. The time to pupation of

each knockdown treatment was compared to the negative control (NC) treatment, and the time to pupation of each sex was compared to the other sex within each treatment. The number above the bar graph indicates the p-value of the significant difference in pupation time between ds-*wdr-48* females and NC females (0.0007).

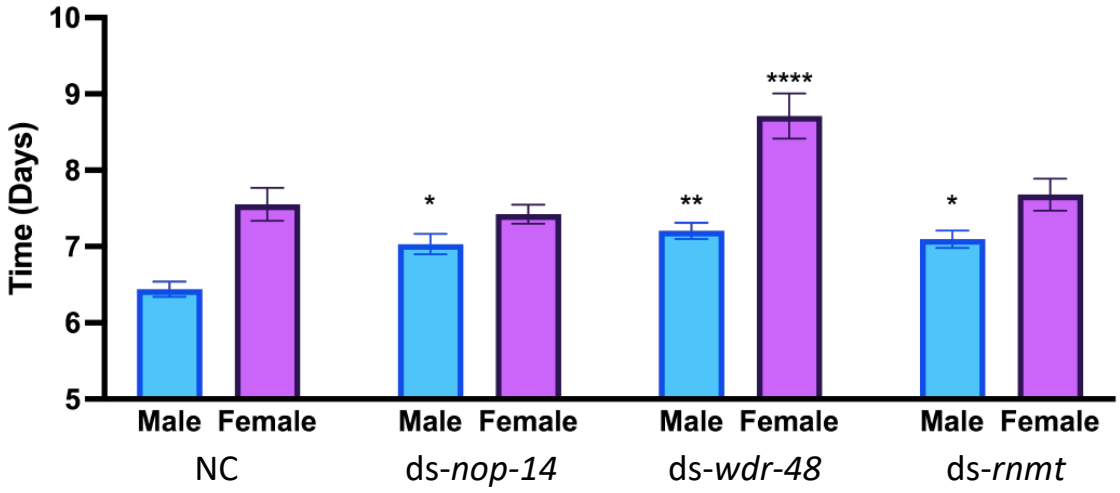

Figure 3. The average difference in pupation time between males and females in each dsRNA treatment at 28°C, as well as comparing male and female times to those of the negative control (NC) population. The asterisks indicate treatments that exhibited significant differences in mean pupation time when compared to the NC males or females. Data were provided by Heschuk (unpublished).

Male and Female Pupation Difference

The difference between the average male and average female pupation times was calculated for each treatment run (negative control, ds-*nop-14*, ds-*wdr-48*, and

rnmt). The average pupation difference of each dsRNA treatment was compared to the pupation difference of the negative control treatment (see Figure 4). Treatment ds-*nop-14* (1.57±0.29 days) did not exhibit any significant deviation of pupation time difference from the negative control pupation difference (1.27±0.21 days) ($p>0.05$; Figure 4). However, treatments ds-*wdr-48* and ds-*rnmt* both showed significant deviations. When compared to the negative control treatment, the ds-*rnmt* treatment reduced the pupation time difference between males and females (0.78±0.27) significantly ($p<0.05$), as opposed to ds-*wdr-48*, which increased the pupation time difference (2.13±0.19 days) significantly at 22°C ($p<0.05$).

The difference between average male and female pupation times was also calculated for the same treatments carried out at 28°C in an incubator by Heschuk (unpublished data). The average pupation difference of each dsRNA treatment was compared to the pupation difference of the negative control treatment (see Figure 5) (Heschuk, unpublished). All dsRNA treatments exhibited a significant deviation in pupation time difference of males and females from the pupation difference of the negative control treatment (1.11±0.24 days) ($p<0.05$; Figure 5). Treatments ds-*nop-14* (0.39±0.18 days) and ds-*rnmt* (0.58±0.24 days) showed a significant decrease in pupation time difference, while ds-*wdr-48* showed a significant increase in pupation time(1.51±0.31 days).

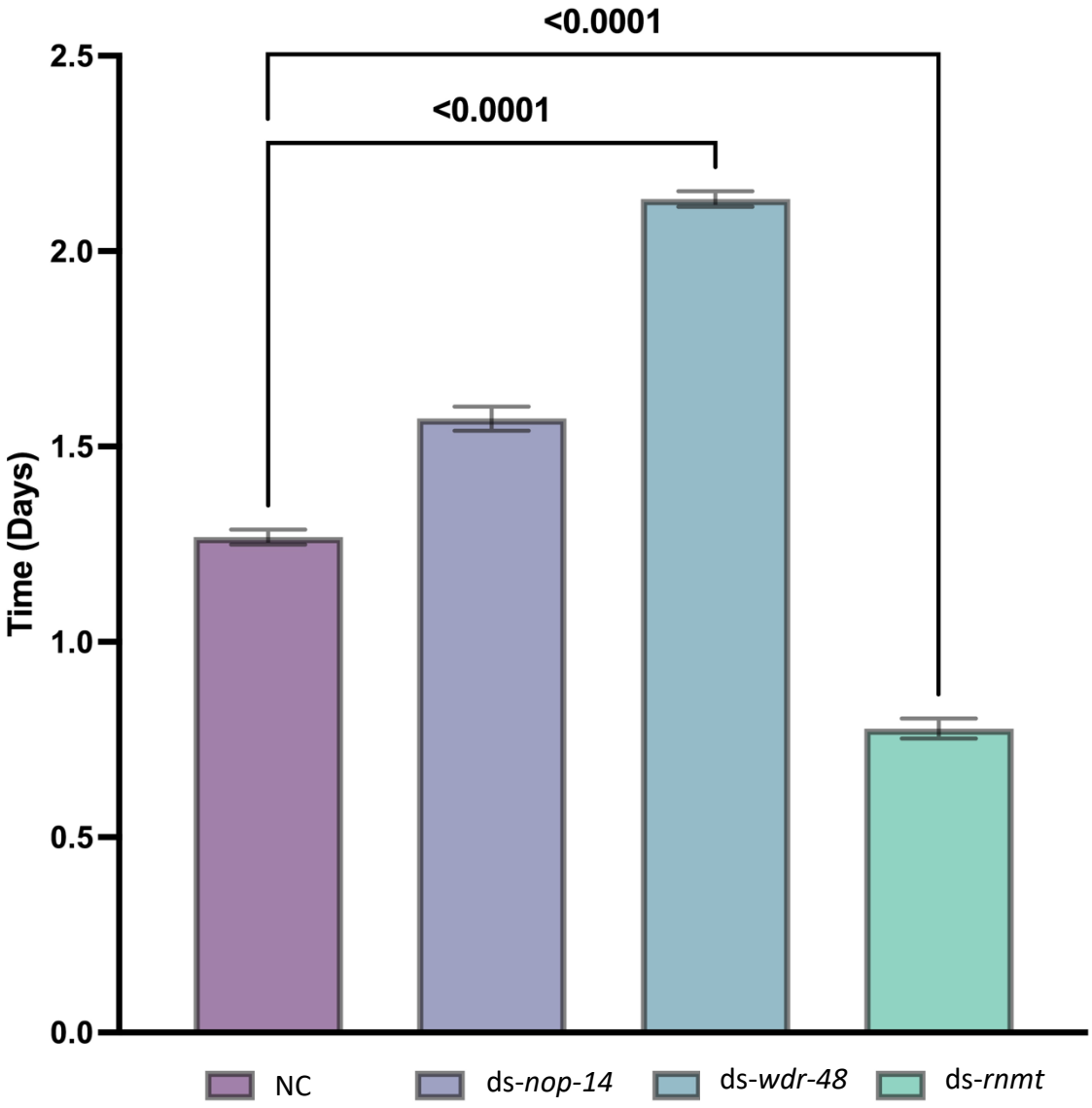

Figure 4. The average difference in the duration of pupation for mosquitoes treated with dsRNA knockdowns (ds-*nop-14*, ds-*wdr-48*, and ds-*rnmt*) at 28°C relative to a negative control (NC). The numbers above the graph indicate the p-value of the significant differences between ds-*rnmt* and the NC (<0.0001) and between ds-*wdr-48* and the NC (<0.0001).

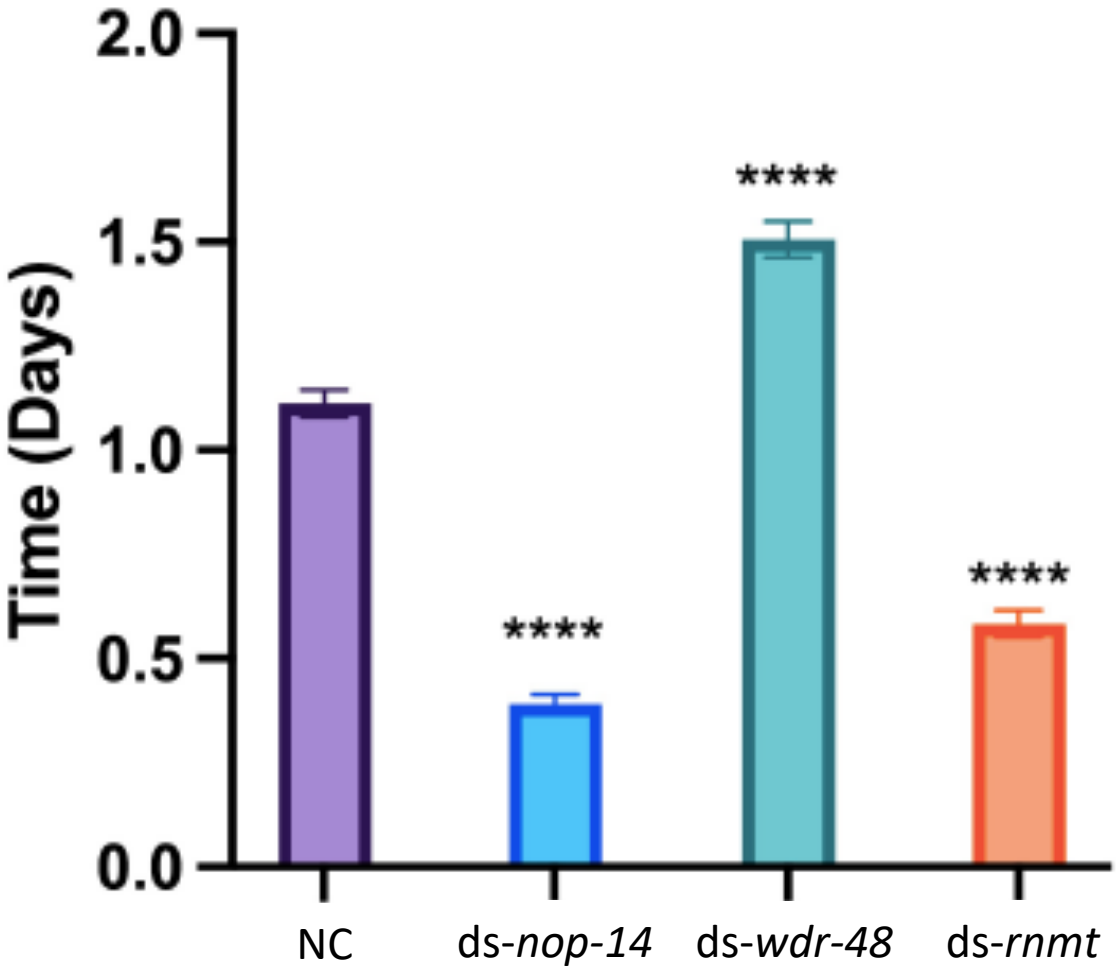

Figure 5. The average difference in time to pupation for mosquitoes treated with ds-*nop-14*, ds-*wdr-48*, and ds-*rnmt* dsRNA treatments, relative to a negative control (NC). The asterisks denote treatments that exhibit a significant difference in mean pupation time difference when compared to the NC. Data was provided by Heschuk (unpublished).

Sex-Sorting Timing Comparisons

The proportion of male and female mosquitoes pupating each day was recorded and the day at which median pupation of either sex was calculated (Figure 6). The median of male pupation (28 pupae) for the negative control treatment occurred at day 11.5 (between days 11 and 12), while the female median (33.5 pupae) occurred on day 13. The median of male pupation (25 pupae) of the ds-*nop-14* treatment occurred on day 11

post-hatching, and the female median (20.5 pupae) on day 12.5 (between days 12 and 13). The ds-*wdr-48* treatment's male median (24 pupae) pupation date was day 12 post-hatching, while the female median (18.5 pupae) occurred at day 14, with two full days between median pupation proportions. The male median (35.5 pupae) date of pupation for treatment ds-*rnmt* was day 11, while the female median (20.5 pupae) occurred on day 12 post-hatching.

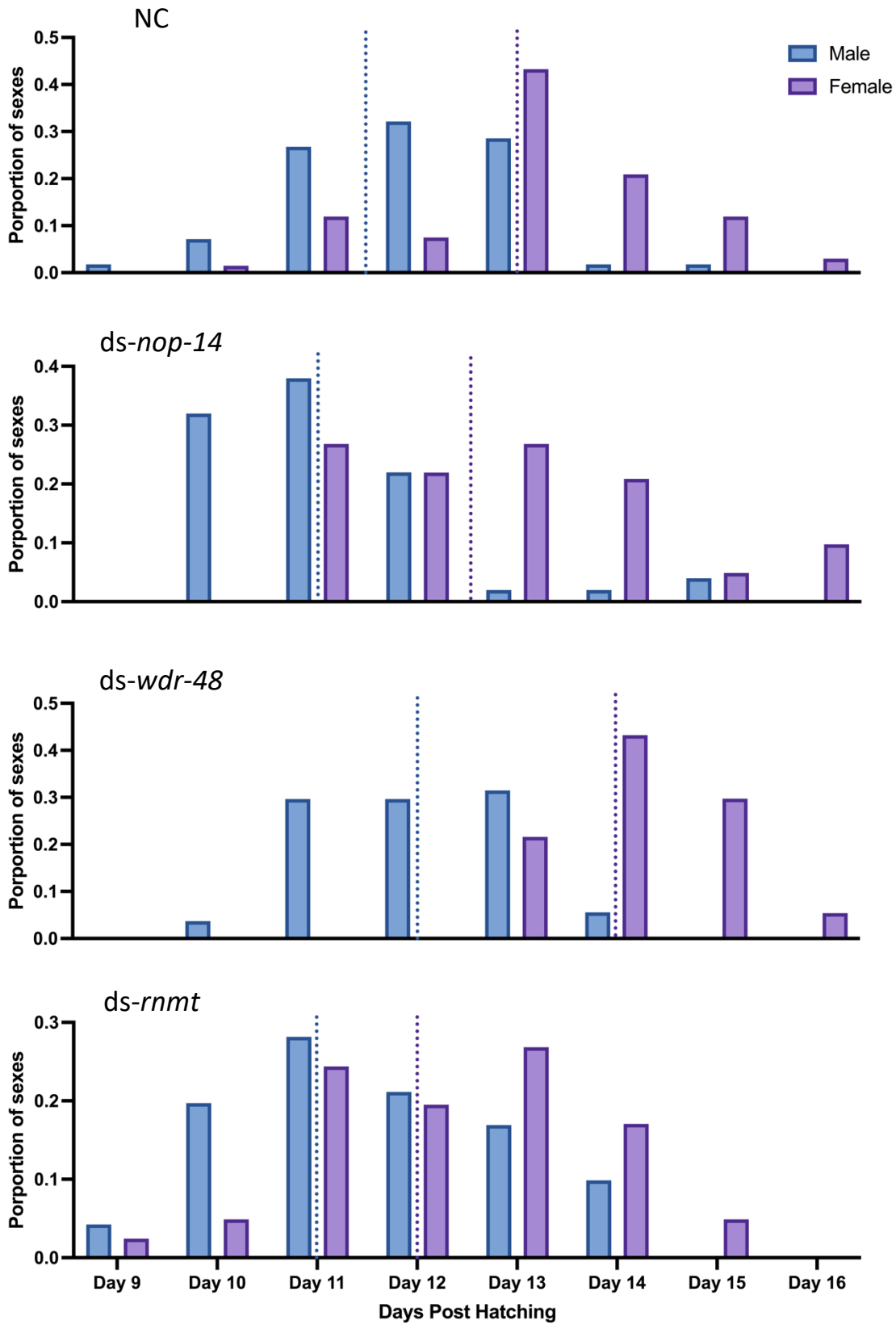

Figure 6. The proportion of male and female mosquitoes pupating per day in negative control, ds-*nop-14*, ds-*wdr-48*, and ds-*rnmt* treatments. Proportions were calculated from the total

amount of mosquitoes that eclosed for each treatment. The dotted lines indicate the number of days post-hatching by which the median number of male or female mosquitoes had pupated (blue indicates the median number of males; purple indicates the median number of females).

Discussion

Insect population control through the use of SIT has been a topic of great interest in recent years as it is species-specific and precludes the need for broad-spectrum pesticides that harm or kill other species. Mosquito SIT involves the release of sterilized mosquitoes; however, the co-release of female mosquitoes with the males should be avoided as they may still seek blood meals following mating, and may thus exacerbate the spread of mosquito-borne disease (Whyard et al. 2015). Additionally, if released alongside males, they may distract the males and prevent them from mating with wild females as desired (Papathanos et al. 2018). It is therefore necessary to sex-sort mosquitoes and remove females from the population prior to release.

Male *Ae. aegypti* mosquitoes, when raised at 28°C, typically pupate about one day sooner than females, but this timing is inconsistent. Females may pupate at the same time as males, making it difficult to sex-sort mosquito pupae based on developmental timing. In this study, mosquitoes were fed *E. coli* expressing three different dsRNAs selected to target genes encoding proteins that interact with DSX^F. Two of these dsRNA treatments (ds-*nop-14* and ds-*rnmt*) exhibited no significant impact on the developmental timing of female mosquitoes, but another, ds-*wdr-48*, caused females to pupate considerably later than the negative controls. This is emphasized by

the significant pupation gap between males and females seen in the ds-*wdr-48* treatment (Figure 4, Figure 5).

This delay in female pupation resulting from the ds-*wdr-48* treatment made it possible to collect pupae at day 12 and ensure all were male (figure 6). Using this delay, 50% of the total males were collected on day 12 from those produced in a batch of treated larvae. In contrast, pupae of the negative control treatment would need to be removed on day 9 post-hatching to prevent any female collection, and only 1% of the total males would be salvaged; any collection past this date would risk female contamination. The pupae of the ds-*nop-14* treatment would need to be collected by day 10, and only 32% of the total males would be removed at that point. The ds-*rnmt* treatment exhibited no day during which male mosquitoes alone pupated; there was female contamination in the pupae removed during each day of pupation.

According to the VectorBase annotation, the *wdr-48* gene is predicted to code for the tryptophan [W]-aspartic acid[D] repeat-containing protein 48, also known as USP-associated factor 1 (UAF1). This highly conserved protein serves as a deubiquitinating (DUB) enzyme in multiple species such as fruit flies, the nematode *Caenorhabditis elegans*, mice, and humans (Hodul et al. 2020; Snyder and Silva 2021). *wdr-48* binds and initiates catalytic activity of the DUB ubiquitin-specific protease 46 (USP-46), which is a protein that removes ubiquitin from the glutamate receptor GLR-1 and regulates its activity (Hodul et al. 2020).

Ubiquitination is a post-translational modification that regulates synapse function and other neuronal processes; DUBs are proteases that remove ubiquitin from specific

target proteins to help regulate these processes primarily by signalling the destruction of proteins it is bound to (Snyder and Silva 2021). In *C. elegans*, the activity of USP-46 controls glutamatergic behaviour by regulating the amount of glutamate receptor levels in neurons (Hodul et al. 2020). Glutamate receptor levels are controlled by ubiquitination and de-ubiquitination by USP-46, which protects the receptors from degradation in the lysosome (Hodul et al. 2020). USP-46 also affects GABA signalling and depression-like behaviours, regulates cell proliferation and tumorigenesis, and mediates the Fanconi anemia and DNA translesion synthesis DNA damage response pathways in many different species (Hodul et al. 2020; Li et al. 2022). *wdr-48* function has not currently been validated in mosquitoes yet, however as its function is conserved, its knockdown may be expected to affect female development. This gene was two-fold more expressed in females than males, meaning the knockdown could have disproportionately affected females and resulted in their increased pupation time.

The *nop-14* gene codes for the nucleolar protein 14 according to the VectorBase annotation. This protein is highly conserved in eukaryotes, and regulates the activity of the methyltransferase EMG1, which is required for DNA repair and cell replication (Lu et al. 2022). This protein is a stress-response nucleolar protein that is required for the maturation of 18S rRNA and production of the 40S ribosome (Li et al. 2019). NOP-14 inhibits the Wnt/β-catenin signalling pathway, which has been found to reduce proliferation of melanoma and breast cancer in humans, and acts as a tumor suppressor gene (Li et al. 2019; Lu et al. 2022). However, it has also been found to promote the growth and proliferation of tumor cells in human pancreatic cancers, and

therefore may also act as an oncogene (Lu et al. 2022). In short, due to its role in regulating cell proliferation, it is possible that its downregulation by the dsRNA treatment could affect the development of female pupae. Due to time constraints, it was not possible to assess the transcript knockdowns of these genes examined in this study, but it would be informative in future studies to use RT-qPCR to quantify the knockdown and its correlation to the phenotypes shown in these altered mosquitoes.

The knockdown of ds-*nop-14* did not have a significant effect on female pupation as the pupation time gap between sexes was not significant when compared to the negative control (Figure 4, Figure 5). The latest pupae could be collected without risking female contamination was day 10 of development, and only 32% of total males would be collected at this point (Figure 6). 50% of total males could be collected by the following day, but these males would be mixed with a significant number of females (27% of the total amount of females) and therefore the efficacy of the sex-sorting would be reduced. While it seems that the knockdown did have some effect on female development, which is likely given the function of the gene, it did not affect it to the same degree seen in dswdr-48 knockdowns.

It is possible that the gene is highly expressed in pupae (which is feasible given its importance to mosquito cell replication) and the dosage of dsRNA used was not enough to achieve a significant knockdown. It is important to note that too much bacteria in a feeding pellet may deter larvae and pupae from eating it, and the dosage therefore cannot be increased in mosquito food without affecting feeding habits (Whyard et al. 2009). Alternatively, the knockdown of this gene may up-regulate activity of others that

produce the 40S ribosome and they could compensate for the loss of this gene to ultimately restore function and produce and maintain the 40S ribosome and 18S rRNA. As with the other genes knocked down in the study, the use of RT-qPCR to quantify this knockdown would aid in explaining the phenotypes seen in these mosquitoes.

The last gene of interest, *rnmt* , encodes a mRNA cap guanine-N7 methyltransferase as mentioned in VectorBase. This enzyme catalyzes synthesis of a highly conserved eukaryotic 5'-terminal cap by methylation of the N-7 guanine position of the mRNA (Yokoska et al. 2000). This methyl cap targets the 5' end of RNA polymerase transcripts and regulates gene expression by affecting the processes of splicing, mRNA export, and initiation of translation; it also protects the mRNA from degradation (Gonatopoulos-Pournatzis et al. 2011). As seen with the *nop-14* gene, this gene is seemingly essential for growth and development of mosquitoes, yet it has no effect on the developmental timing of female mosquitoes (Figure 6). As with *nop-14*, this may be due to increased expression of the gene in pupae, which is certainly possible as cap methylation was found to be upregulated in mammals (Gonatopoulos-Pournatzis et al. 2011). Upregulation of the gene by other genes may mean that the amount of dsRNA delivered to the mosquitoes was insufficient to properly knock down the gene, and thus there was no effect. Alternatively, it is possible that other genes that perform a similar function are upregulated in the absence of this one's activity, and therefore ensure this essential step of transcription is carried out.

The dose of dsRNA delivered to each mosquito by the *E. coli* is unknown, and variable (Balakrishna Pillai et al. 2017). Ingested dsRNA is not limited to the gut, but can

spread to other cells of the body as well (Balakrishna Pillai et al. 2017). The mechanisms by which RNAi spreads from cell to cell from the gut is as yet unknown, but regardless of the means, RNAi does still occur (Singh et al. 2013). Previous studies of dsRNA ingestion by mosquitoes found that increasing the concentration of dsRNA in food past 0.5 μg/μl did not increase the spread of the RNAi further (Whyard et al. 2009).

The efficiency of the dsRNA in causing silencing is also variable, which may be due to the presence of dsRNAses in the gut epithelial cells (Giesbrecht et al. 2020). These destroy ingested dsRNA, meaning that oral doses of low concentrations of dsRNA may not survive in the gut long enough to cause gene silencing (Giesbrecht et al. 2020). Additionally, sporadic ingestion of dsRNA instead of constant ingestion may allow dsRNAses to destroy dsRNA before it completes its purpose; as it is impossible to control when mosquitoes eat, some may get irregular doses and the knockdown will fail (Giesbrecht et al. 2020). This has made it difficult to measure the dose of dsRNA each mosquito receives, and impossible to determine the optimal dosage of dsRNA a mosquito may need.

The use of SIT for mosquito population control is not a new technique, and has been studied for many years in many different trials. One such trial in Brazil was carried out using radiation to sterilize mosquitoes prior to sex-sorting and release (Carvalho et al. 2015). While exposure to radiation did successfully sterilize mosquitoes, it also substantially decreased male competitiveness in finding mates (Carvalho et al. 2015). Since sterile males didn't seek out wild females to the same degree as their wild counterparts, more sterile mosquito release was necessary to achieve the same degree

of population suppression achieved using RNAi and other methods (Carvalho et al. 2015).

In Mexico, a pilot trial of SIT using radiation the “incompatible insect” method was performed to determine the success of this technique (Martín-Park et al. 2022). It involved infecting irradiated mosquitoes with *Wolbachia* bacteria, which is vertically transmitted through maternal inheritance (Martín-Park et al. 2022). *Wolbachia* infections induced cytoplasmic incompatibility and thus mating between lab-grown males and wild females resulted in infertile eggs (Martín-Park et al. 2022). Here, radiation was used to sterilize any females that were accidentally released with the males to prevent population growth, but its indiscriminate use resulted in a decrease in male competitiveness (Carvalho et al. 2015; Martín-Park et al. 2022). Additionally, sterilization of females does not prevent them from biting, and their release could distract males with an already low mating drive from wild females. In short, the possible release of female mosquitoes into the wild as part of SIT is an obstacle that must be minimized for the safe implementation of the technique. To this end, the use of the dswdr-48 RNAi knockdown treatment for sex-sorting outlined in this study could be helpful when used in conjunction with different population control methods, particularly insect incompatibility as it precludes the need for sterilization of females by radiation and eliminates the complication of decreased male mating drive.

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