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Original article

CRISPR/Cas9 mediated sex-ratio distortion by sex specific gene editing in *Aedes aegypti*

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ABSTRACT

Aedes aegypti is a principal vector for several viruses including dengue virus, chikungunya virus and zika virus. Economic burden of mosquito-borne diseases, relative failure of traditional control strategies and the resistance development against insecticides enforces towards genetic manipulation of *Ae. aegypti*. Hence, a key gene *doublesex* (*Aedsx*) which regulate sex differentiation and alternatively splices to form male and female specific transcripts ($Aedsx^M$ and $Aedsx^F$). CRISPR/Cas9 technique was employed to sex specifically disrupt the female-specific isoforms, $Aedsx^{F1}$ and $Aedsx^{F2}$, both of which were shown to be expressed only in female mosquitoes. Targeting of dsx^F at the developmental stage has resulted in various phenotypic anomalies of adult females. The rate of adult mutation phenotype was recorded between 29 and 37% along with anomalies of wing size, proboscis length and reduction in the sizes of pre-blood-meal and after blood-meal ovaries in dsx^{F1} and dsx^{F2} microinjected groups, respectively. These findings can be correlated with reduced fecundity rate of G_o female, where $Aedsx^{F1}$ and $Aedsx^{F2}$ groups showed reduction rate in range of 23–31%. Furthermore, hatching inhibition rate of 28 to 36% was also observed in G1 generation when compared to the wildtype. Overall, these results demonstrated that $Aedsx^F$ disruption has resulted in multiple female traits disruption including decreased fertility of the female that could directly or indirectly associated with reproduction and its disease transmitting abilities. All these findings suggesting that CRISPR works to alter the developmental pathways as predicted, and therefore this method potentially gives us the basis for the sex-ratio distortion system as genetic control approach for the management of this vector.

1. Introduction

The mosquito *Aedes aegypti* belongs to family *Culicidae*, and considered as a major vector of human infectious diseases i.e., dengue, dengue hemorrhagic fever, chikungunya, yellow fever and zika virus that increasingly infect millions of people every year (Adelman & Tu, 2016; Parra et al., 2016; Bhatt et al., 2013). The most prominent endemic disease caused by *Ae. aegypti* is dengue; a major health concern in tropical and subtropical areas. The causative agent of dengue is flavivirus dengue virus (DENV). The global cases of dengue fever (DF) and dengue hemorrhagic fever (DHF) have been intensely increased; about 50 million infections are reported annually in past years in more than 100 countries which are declared endemic by World Health organization (WHO) (Falcon et al., 2019; Zahoor et al., 2019).

By considering the medical and public health significance of *Ae. aegypti*, numerous control measures have been devised to manage this menace to combat diseases it caused. However, economic load of mosquito control programs, failure of many control strategies, environmental influence of chemical pesticides and importantly insecticidal resistance development (Ranian et al., 2021) has led the substantial efforts to develop most targeted and non-insecticidal technique using germline transformation based on genetic modification or the sex-ratio distortion system (Alphey et al., 2013).

Currently, genome editing techniques have exposed that genetic manipulation of biased sex-ratio in a population may contribute for *Ae. aegypti* control. It has been reported that the

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sex-determination pathway is triggered by specific sex-determining signals which is further regulated by a cascade of genes, either by activation or repression of the effector genes (Vicoso & Bachtrog 2013; Vicoso & Bachtrog 2015). In *Drosophila*, X-chromosome dosage regulates transcriptional activation of *Sex-lethal* (*Sxl*) gene. The functional *Sxl* protein regulates the splicing of *transformer* (*tra*) which together with the *transformer2* (*tra2*) and other splicing factors control the sex specific splicing of *doublesex* (*dsx*) and *fruitless* (*fru*). *dsx* and *fru* are also reported in *Ae. aegypti* as key regulators of sex-differentiation pathway but their sex-specific splicing is under the control of other upstream genes. *dsx* is a double switch gene works in coordination with the other regulatory genes, male and female specific *dsx* variant production is under the control of expression of TRA-2 proteins (Herpin & schartl 2015).

The results of RNAi-mediated knockout of the *dsx* gene in *Bombyx mori* revealed that two DSX proteins are essential for complete female-specific differentiation (Shukla et al., 2011). Further knockout experiment of *dsx* isoforms has also resulted in sex-specific sterility (Suzuki et al., 2005). In *D. melanogaster* and other Dipterans like *Musca domestica,* pre-mRNA of *doublsex* gene is alternatively spliced to produce both male and female specific isoforms. The double switch behavior of *dsx* gene to produce dsx^F and dsx^M isoforms was also reported in *Tribolium castaneum* (Shukla and Palli, 2012), *Anopheles gambie* (Scali et al., 2005), *Culex pipiens* (Price et al., 2015), *Apis mellifera* (Cho et al., 2007) showing thereby, highly conserved role of *dsx* gene for sex-determination across different insect species. Therefore, this gene can be targeted for genetic manipulation in order to control *Ae. Aegypti* (Salvemini et al., 2011). In the present study, initially female specific isoforms of $Aedsx^F$ were identified, characterized and then the CRISPR/Cas9 system was employed to disrupt the potent female isoforms $Aedsx^{F1}$ and $Aedsx^{F2}$. The knockout of dsx^F for sex-ratio distortion system could provide a genetic control approach for the management of this vector.

2. Materials & methods

2.1. Rearing of mosquitoes/ isogenic population

Two lines of *Ae. aegypti* were taken and all mosquitoes' life stages were reared under standard conditions as defined by Gantz et al., (2015) (*Supplementary material*).

2.2. Preparation of injection components

2.2.1. gRNA design

To specifically disrupt the female-isoforms dsx^{F1} and dsx^{F2}, the gene sequence of AAEL009114 ($Aedsx^F$) was retrieved, which have shown to be expressed only in female. The gene sequence was selected as a reference to study in-silico approach for the design of sgRNA. The candidate search for the genomic target included the 20-nucleotides gene sequence unique to the rest of the genome and target sequence just adjacent to the Protospacer Adjacent Motif (PAM). For knock-out experiment the *dsx-gRNAs* were designed targeting per target of the gene excluding the 5′ and 3′ UTR. The guide RNAs for each isoform were designed and assessed manually for off-target activity by genomic region available at genomic tools CHOPCHOP https://chopchop.cbu.uib.no/ and CRISPR direct https://crispr.dbcls.jp/ (Table S1). The only female-specific isoform2 & isoform3 of *Aedsx* spans over the region of 8 & 7 exons on the chromosome number 1. The gRNAs specifically designed to target the upstream boundary of exon5 at the position of the coding sequence.

2.2.2. Design of plasmid construct

Standard molecular biological protocol was used to construct the plasmids. The *PiggyBac* vector plasmid AAEL005635-Cas9 was designed containing the bacterial cas9 coding sequence (CDS) along with the fluorescent marker gene DsRed1. The effector plasmid gRNA[Dsx^F].1026A was used to make the construct, details are available in *Supplementary material* & Fig. 1.

2.3. Microinjection and screening

For the evaluation of CRISPR/Cas9 induced modification in *Ae. aegypti,* an optimal relative proportion of components were used by following the methodologies of Wang et al., (2019) and Dong et al., (2015). Fertilized eggs were collected within 30 min after the oviposition. The injection pressure and the level of desiccation were also adjusted. Microinjection manipulation was accomplished at about 2 h, eggs were placed under insectary condition at 26 ± 1 °C, 60 ± 10% RH for hatching. A total of 450 eggs of each group was injected with CRISPR/cas9 construct, each group was divided into three replicates of 150 eggs along with the control group which was injected with the double distilled water.

2.4. Phenotypic characterization and microdissections

After four days of incubation period hatching rate, live pupae and adult's mutation phenotype (F/M) rates% were observed. The phenotypes of pupae and adults were performed with stereo microscope. Adults were collected in the falcon tube and anesthetized on ice for 5 min before dissection. Wing length and proboscis lengths were assessed in wild type as well as in the mutant groups.

2.5. Reproductive assay and transgenic progeny screening

G_o females were allowed to back-cross with wild-type males. G1 eggs were collected and allowed to hatch by following the same procedure. Fecundity and hatching rates were assessed as a measure of fertility, blood feeding was visually observed and fecundity rate was evaluated by counting the number of eggs per lay by using wild type as a control. To confirm the possible effects of $Aedsx^F$ disruption on the ovarian development, ovary length was also assessed in Pre and post defibrinated blood meal. Ovaries were mounted and analyzed in the 4–5 days old adult females. Scanned images were subjected to the FIJI and adobe Photoshop software.

2.6. Qualitative and quantitaive Real-time PCR analysis

To confirm the mutagenesis at *Aedsx* locus, the genomic DNA was used for PCR template for the amplification of region flanking the target site. DNA was extracted from the mutant and wild type embryos of both male and female larvae and adults by following the protocol optimized by Zulhusnain et al., (2020).

2.7. Qualitative and quantitative real time PCR analysis

RT-qPCR was carried out to evaluate the relative gene expression for the knockout experiments and to amplify the amplicon surrounding the putative CRISPR/Cas9 cut sites. RT-qPCR was performed by using the primers listed in Table S2. (*Supplementary material*)

2.8. Statistical analysis

Experimental work was carried-out in replicates. All the data of the morphological measurements and reproductive assays were obtained by combining the results of three biological replicates.

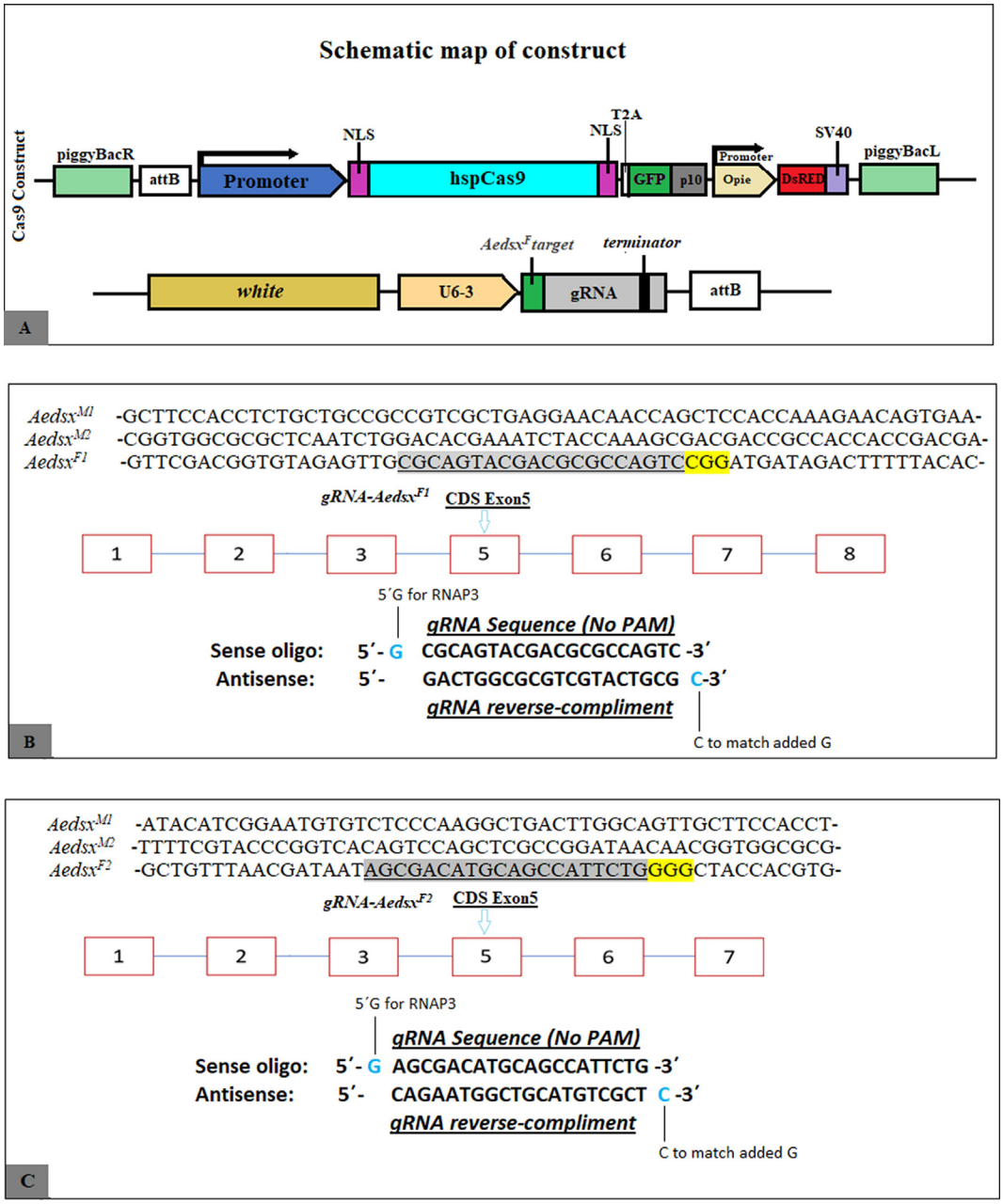

Fig. 1. Targeting the female-specific isoform of *Aedsx*. (A) Schematic representation of the HDR knockout piggybac-based and gRNA construct. (B, C) sequence alignment of the *dsx* exon-5 boundary in female ($Aedsx^{F}$) and male isoforms ($Aedsx^{M1}$ $Aedsx^{M2}$), and the gRNA sequences for both isoforms dsx^{F1} and dsx^{F2} (underlined and highlighted in grey) and reverse complementary sequences used to target the gene with the protospacer adjacent motif (PAM) are shaded in yellow while introns are not drawn to the scale. The isoforms 2 & 3 of $Aedsx^{F1}$ & $Aedsx^{F2}$ spans over a region 8 & 7 exons respectively.

The data was subjected to statistical analyzing software SPSS22.0 with an independent students *t*-test. Standard one-way ANOVA was used to evaluate the average phenotypical parameters. The data is expressed as means ± SEM and statistical significance was assumed for $p \leq 0.05$.

3. Results

3.1. The doublesex (dsx) gene in Aedes aegypti and CRISPR/Cas9 technique

Of sexual differentiation in *Aedes aegypti*, a primary signal triggers a central gene leading towards a cascade of molecular mechanism which then controls the alternative splicing of the bottom switch gene *doublesex (dsx)*. *Aedsx* gene comprises of nine exons distributed over the chromosome 1 and is alternatively spliced to produce male and female splicing variants $Aedsx^{M}$ and $Aedsx^{F}$. In the present study, two male and two females specific *dsx* transcripts were identified. The only female specific isoform-2 and isoform-3 of *Aedsx* spans over a region of 3631 bp and 3173 bp on the chromosome number 1 with 8 & 7 exons, respectively.

Using CRISPR/Cas9 technique, a female-specific isoform of dsx^{F1} was targeted at the upstream boundary of exon 5 at the position of the coding sequence. This exon spans over a region of 458 bp. For the isoform dsx^{F2}, exon-exon boundary was targeted at the position of female specific exon5, the exon spans over the region of 465 bp in length. Both of the targeted exons were shown to be expressed only in female mosquitoes. To prevent the formation of functional

Aedsx^{F} while leaving the *Aedsx*^{M} transcript unaffected, the *dsx-sgRNAs* were designed targeting only coding regions of all female-specific isoforms of the gene excluding the 5′ and 3′ UTR most preferably targeting the exon-to-exon junction of the coding region with a source of Cas9. Two gRNAs designed, for each isoform and common regions of both isoforms and checked accordingly for their efficiency. Only female-specific and with higher efficiency gRNAs for each female-isoform are included in this knockout study (Fig. 1 & S1).

3.2. Microinjection of sgRNA-construct

For the analysis of traits developed in late pupal and adult stages, *Aedsx*^{F1} *or Aedsx*^{F2} targeting sgRNA-construct was delivered through microinjection into the eggs, the control group was injected with the double distilled water. A total of 450 eggs including three replicates, were injected with the *PiggyBac* vector plasmid containing the bacterial cas9 coding sequence (CDS) along with the fluorescent marker gene DsRed1 and designed sgRNA construct (Fig. 1A). The *PiggyBac* plasmid contains the promoter of AARL005635 drive expression of *Streptococcus pyogenes* Cas9 gene in *Aedes aegypti*, and gRNA effector plasmid gRNA(dsx^{F}) that expresses the gRNAs under the control of U6-promoter, were used to build the constructs. The mean hatching rates in the injected groups were 51.11% (230/450) and 54.00% (243/450) with the p-value = 0.00 (*Table S1*) in case of *Aedsx*^{F1} and *Aedsx*^{F2}, respectively, while in control group hatching rate was 74.44% (335/450). The rate of adult mutation phenotype was recorded as F/M (*Aedsx*^{F1} = 36.59% and *Aedsx*^{F2} = 29.36%), (p-value = 00) (Table 1).

3.3. Phenotypic characterization and blood feeding assay

Comparative analysis of morphological characteristics viz. wing span, body length and length of proboscis were made on the nectar feeding 10-days old female mosquitoes of mutant and control groups. A significant decrease in the wing length was observed in both *dsx*^{F1} and *dsx*^{F2} disrupted females. On average, mean wing length of the *dsx*^{F1} and *dsx*^{F2} groups was 16.40% and 18.09% (p = 0.001) smaller than the control group (Fig. 2A).

For blood feeding assay, females were given access to take the blood meal, but they showed anomalies in their proboscis and consequently in biting behavior. Morphological analysis revealed the reason behind this abnormal blood-feeding behavior was shortened proboscis in adult females. Comparative analysis with the control group showed 19.90% and 16.40% (p = 0.00) reduction in the proboscis length of the *dsx*^{F1} and *dsx*^{F2} mutant groups, respectively (Fig. 2B, Table S5).

3.4. Reproductive assay

Fecundity and fertility rates were investigated by assessing the number of larval progenies per lay by using wildtype as a control. Fecundity and hatching rates between the mutant and wildtype groups after the mating of mutant with wild-type males showed significant reduction in eggs production per female in *dsx*^{F1} and *dsx*^{F2} microinjected group. In G1 the *dsx*^{F1} mutant females laid 30.95% fewer eggs and *dsx*^{F2} mutant group laid 23.80% fewer eggs than the control group (p = 0.000) (Fig. 3A, Table S6). In addition to reduced fecundity, fertility rate was also assessed as a measure of

Table 1
Efficiency of CRIPR/Cas9 mediated mutagenesis.

Mean percentage of male and female pupae, mutant female pupae and adults							
Sr.	**Target Genes**	**sgRNA/Cas9 concentration (ng/μl)**	**Injected embryos**	**Female pupae/ Live pupae**	**Male pupae/ Live pupae**	**Mutation phenotype of female pupae/Total pupae**	**Mutation phenotype of female adult/Female pupae**
1	***Aedsx*^{F1}**	150/300	450	53.19 ± 3.54a	53.66 ± 2.18a	30.20 ± 2.11a	36.59 ± 2.18a
2	***Aedsx*^{F2}**	150/300	450	48.17 ± 0.91a	51.75 ± 0.84a	24.44 ± 1.70a	29.36 ± 3.02a
3	**DdH$_2$O**	–	450	54.11 ± 0.43a	4.23 ± 0.46b	–	–
4	**Statistical values**						
5	**P value**	–	–	0.18	0.000	0.10	0.12
6	**F value**	–	–	2.25	412.69	4.50	3.74
7	**Df value**	–	–	2	2	1	1

*Means sharing the same letter within each treatment is not statistically different.
Data represents three biological replicates and are expressed in mean ± SEM.

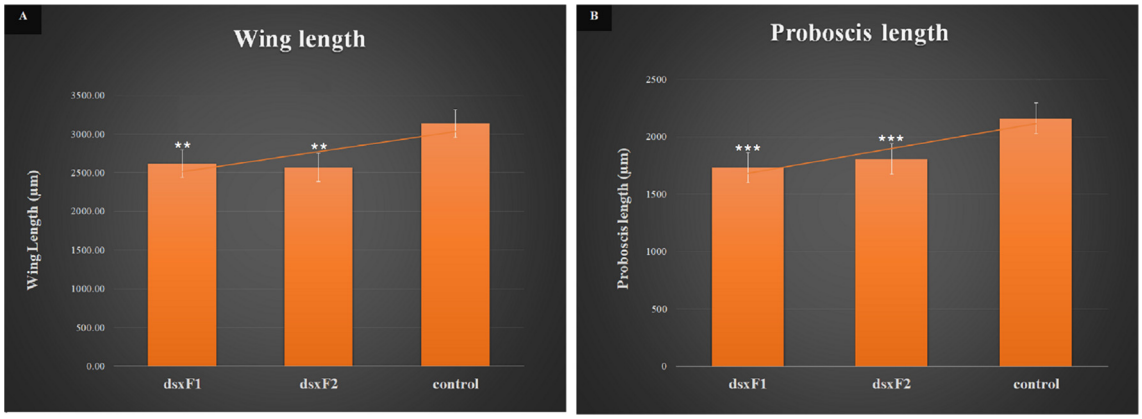

Fig. 2. Developmental disruption of *dsx* produced the adult female anomalies. (A) Mean adult wing length significantly decreased in *dsx*^{F1} and *dsx*^{F2}. (B) mean adult proboscis length also decreased in the muted groups.

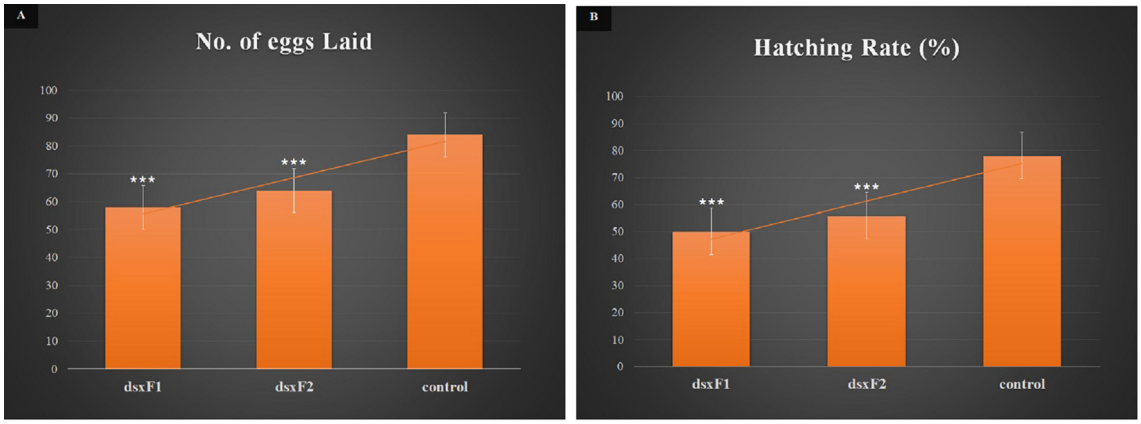

Fig. 3. Decreased female fertility and fecundity was observed by sex-specifically knock-out of female isoforms of *dsx*. (A) Mutant Females showed decreased fecundity rate in comparison to the control group. (B) Mean percentage hatching rate was also decreased in the mutant groups as compared to the control group. Error bars represent standard deviation.

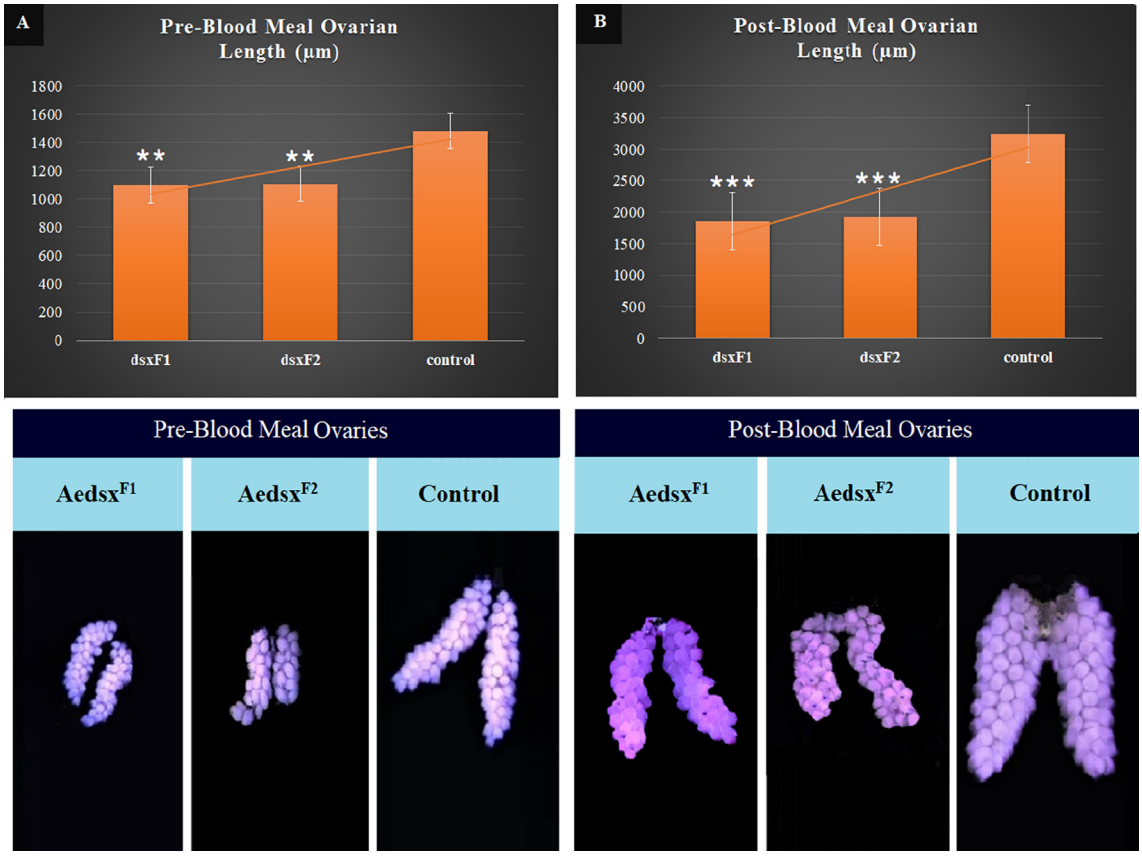

Fig. 4. CRISPR/Cas9 targets the *dsx* gene that controls the ovarian development. (A) Representative of Pre-blood meal ovaries of CRISPR/Cas9 based knock out of *Aedsx*^{F1}, *Aedsx*^{F2} isoforms and wild type 4–5 days old adult Ae. aegypti female. Mutant groups showed undeveloped ovaries. (B) representative of post-blood meal group, in comparison to the control group length of ovaries was significantly reduced in microinjected groups *Aedsx*^{F1} and *Aedsx*^{F2}. Data represents three biological replicates with 5–8 individuals in each replication and are shown in mean ± SEM (p = 0.000).

the percentage of hatched eggs. Likewise, hatching rate was also significantly reduced, on average the recorded rates for both *dsx*^{F1} and *dsx*^{F2} groups were 36.05% and 28.47% less than the control group (Fig. 3B, Table S7).

To investigate the possible cause of reduced fecundity rate, blood-feeding behavior and ovarian analysis was also performed and compared to the control group. Pre-blood meal ovaries were mounted and analyzed in the 4–5 days old females, in which phenotypic manifestations showed defects in ovarian development in the mutant group. In comparison to the wild-type on average 25.96% & 25.28% reduction in the ovarian size was observed in *dsx*^{F1} and *dsx*^{F2} micro-injected groups, respectively before they allow to take the blood meal (Fig. 4A, Table S8). Five days post to the blood meal 10–12 days old *dsx*^{F1} *and dsx*^{F2} injected female also showed smaller or undeveloped ovaries. On average, the length of *dsx*^{F1} post-blood meal ovaries was 42.60% smaller than the control

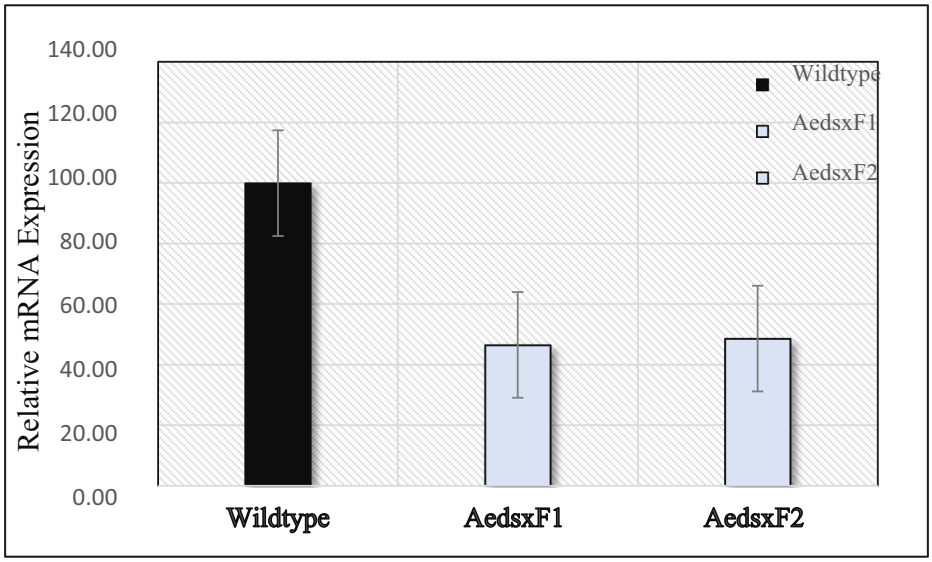

Fig. 5. The splicing pattern of *Aedsx*^{F} transcripts was observed by the RT-qPCR in wildtype and *dsx*^{F} mutants. qRT-PCR experiments demonstrated that the relative percent mean expression of *Aedsx*^{F1} and *Aedsx*^{F2} was significantly reduced in the mutant group as compared to the control group, The data shown are mean ± SE (F1-p = 0.05, F2-p = 0.00).

group, whereas *dsx*^{F2} group showed 40.00% reduction in ovary size (p = 0.00, Fig. 4B, Table S9).

3.5. PCR and qRT-PCR analyses

The mutation events or phenotypic defects induced by the sex-specific CRISPR/Cas9 mediated disruption of *Aedsx*^{F} were also confirmed through quantitative real time polymerase chain reaction (qRT-PCR). Reaction was performed for both adults selected on the basis of phenotypic abnormalities and wildtype. qRT-PCR was carried out based on SYBR green method and the relative gene expression was normalized against the housekeeping ribosomal protein S7 gene. Normalized delta C_t values in each experimental set were averaged and converted to percent expression. The qRT-PCR results demonstrated that average-percent *Adsx*^{F1} and *Adsx*^{F2} transcript's expression was significantly reduced relative to the wild-type (Fig. 5). The primers used were designed based on the female specific region that included the female specific region of Oligomerisation Domain (OD), OD2 domain. In *Aedsx*^{F} mutant group female-specific transcript was observed at lower level in female mosquitoes than in wild-type females. Although qPCR data supports the trends we observed in analysis of phenotypic assay, the level of fold changes tended to be dissimilar to the control group. Overall, the results confirmed the induced effective mutagenesis by means of highly efficient and flexible genome editing method CRISPR/Cas9 system of both female specific isoforms *Aedsx*^{F1} *Aedsx*^{F2} in adults with obvious defects.

4. Discussion

The *doublesex (dsx)* is a nexus that acts as a double switch gene in sex-determination cascade of insects and plays a vital role at the bottom of the pathway influencing the sexually dimorphic morphology, physiology and behavior of the mosquitoes (Wang et al., 2019). The *dsx* gene is highly conserved and has sex specific regions among different dipterans and other insect groups including Lepidoptera, Coleoptera and Hymenoptera. It has been revealed that *dsx* homologue of *Ae. aegypti, An. gambiae* and *Drosophila*, could be similar but *Aedsx* produces an additional novel female-specific splicing variant. Moreover, the partially conserved nature of the gene, exon organization and the extensive divergence in the intron lengths between *Aedes* and *Anopheles dsx* genes has been found. Comparative genomic analysis showed that *Aedsx* protein has two Oligomerization Domains (OD) (which serves as interface for protein and DNA interaction); OD1 (non-sex specific) and OD2 domain (consists of non-sex specific and females-specific part) (Salvemini et al., 2011).

It has been described that sex-specific silencing of *dsx* induced sterility in *D. melanogaster* adults (Chen et al., 2008). Molecular evolution of *Aedsx* splicing regulation mechanism concluded that *dsx* gene spliced sex-specifically to form two female and male specific isoforms, in *D. melanogaster* wherein knock-out of the female specific transcript of *Aedsx* has resulted in transformation of females into pseudo-males, while a male specific transcript was also found in *Aedsx*^{F} female mutants. Subsequent studies also showed that *dsx* sex-specifically splices to produce one female and male specific RNA variants in most of the insects, but it produces more than two isoforms in case of *Ae. aegypti, Musca domestica, Bombyx mori* and *Apis mellifera* (Hediger et al., 2004; Cho et al., 2007; Shukla et al., 2011; Shukla et al., 2012).

To selectively investigate whether *dsx* is suitable target for gene drive and to suppress the population reproductive capacity, we disrupted the female-specific isoforms of *dsx Aedsx*^{F1} and *Aedsx*^{F2} in *Ae. aegypti.* The upstream boundary of exon-5 which is female specific exon, was targeted for both *dsx*^{F1} and *dsx*^{F2} with the aim of blocking the formation of functional female-specific isoforms, which didn't affect the male development. CRISPR/cas9 mediated knockout of the female specific isoform of *Aedsx* had altered many morphological characteristics including wing length, proboscis length and other factors; hatchability rate, fecundity rate, ovarian size and shortened life span in females.

In *Ae. aegypti* body size is a sexually dimorphic, as female is larger than male. It was found that CRISPR/Cas9 mediated disruption of the female specific *Aedsx* isoforms had reduced wing development and reduced body size as well. The role of *dsx* in regulation of insect growth has been investigated by many other researchers; Wang et al., (2019) observed the phenotypic abnormalities induced by disruption of sex-specific splicing of *dsx in Plutella xylostella* wings. The correlation between reduced wing size and expression of *dsx* in *Nasonia vitripennis* was also reported by Leohlin et al.,

(2010). In agreement to these studies, the current results may suggest that *dsx* positively helps to regulate the growth of *Ae aegypti* too.

Recently, it was found that oral delivery of *Agdsx*^{F} dsRNA larvae has resulted in the reduction of female life span (Taracena et al., 2019); Gotoh et al., (2014) also confirmed the linkage of *dsx* gene with the other juvenile hormones signaling pathway in *Cyclommatus metallifer*. Hence, silencing of *dsx* gene may also contribute to disturb these pathways. Other research also exhibited deformation in sexual gonads and adult sterility as a result of *doublesex* disruption *B. mori*. Similarly, Shen et al., (2009) also described that genetic manipulation of sex determination genes have deep impact on the lifespan of *D. melanogaster*. In addition to this, overexpression of *dsx*^{M} male isoform dramatically contributed to decrease the lifespan of both male and female *Ae. aegypti*. Chen e al., 2019 also reported sex-specific morphological and sexually dimorphic defects of gonads and external genitals induced by the CRISPR mediated disruption of *dsx* in *Agrotis ipsilon*.

The *dsx* knockout also abridged the fecundity and overall fertility rate. The ovarian analysis showed the reduced size of pre-blood meal and post-blood meal ovaries that was correlated with the reduced fecundity and the fertility rate. Shukla et al. (2012) also reported the reduced fecundity and fertility rate in *T. casteneum dsx* mutant strain. Morphological defects of mutant strain helped to explore more possible causes for the reduced ovarian size and partial sterility. In addition to the reduced body size, anomalies in the proboscis were also observed; the individuals with malformed proboscis were unable to bite and to take enough blood meal thus, possibly the development of follicles was not completed; because no significant increase in the ovarian size was observed before and after taking the blood meal when compared to control group. Analysis of the sexual dimorphism in the olfactory system is mainly regulated by other gene *fruitless(fru)* in *Drosophila* and other insects but another interesting investigation proposed by Neville et al., (2014) who suggested the overlapping role of *dsx* and *fru*. In sex-specific neural development of *D. melanogaster*, small amount of the *dsx* and *fru* may form a complex or act as a co-regulator of other target genes. In support of this notion, Kyruo et al., (2018) also reported that the silencing of *dsx* has resulted in the abnormal development of proboscis of *A. gambiae* consequently individual couldn't bite or feed proficiently.

The current findings showed that CRISPR/Cas9 mediated *doublesex* disruption has resulted in decreased hatching rate, fecundity rate, reduced ovarian size and anomalies in the morphology at different developmental stages of *Ae. aegypti*. The qRT-PCR results confirmed CRISPR/Cas9 mediated mutagenesis of both female-specific isoforms *Aedsx*^{F1} and *Aedsx*^{F2} in adults and attributed to this, obvious respective defects and relatively reduced expression of sex-specific transcript in targeted group. The similar investigations were also retrieved by Wang et al. (2019) while working with *doublesex* in diamondback moth *Plutella xylostella*.

Therefore, our research findings provide the genetic basis for CRISPR/Cas9 gene drive targeting and role of *Aedsx* in sex determination in *Ae. aegypti*. The CRISPR/Cas9 gene drive targeting the *Aedsx*^{F} had altered the multiple female traits associated directly or indirectly to the reproduction and hence with the disease transmission abilities of the female *Ae. aegypti* mosquitoes. Owing to these features and potential to suppress the population, makes this suitable for future field-testing approach for the management of this menace.

5. Conclusions

In *Ades aegypti*, female differ from male in morphological, physiological and behavioral traits and the ability to transmit diseases. The *doublesex* acts as a double switch gene in the bottom of sex-determination pathway by regulating sexually dimorphic morphology, physiology and overall behavior of the mosquitoes. The present work was intended to develop a *Aedsx*^{F}-CRISPR-Cas9 line wherein the females were targeted. Males and females have specific splicing variant of this gene, CRISPR/Cas9 was employed to reduce the expression of female-specific variant of *Aedsx* that was identified, characterized and then employed into the system to disrupt the potent female specific isoform. Targeting of *dsx*^{F} had resulted in decreased body size, wing length, proboscis length and disruption to the fecundity and fertility rates. As a vector role of a female; these are critical aspects of pathogen transmission. Thus, targeting of *dsx* attributed to decreased fertility of the female and ultimately reduced ability to transmit pathogen makes this possible of potential control of transmission. This study not only demonstrated the basis for the elucidation of sex-determination pathway of *Ae. aegypti* but also suggested the more putative molecular target *dsx* for the application to the development of novel genetic control strategy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

All the date generated or analyzed during this study are included in this manuscript and supporting files.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2022.01.034.

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Further reading

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