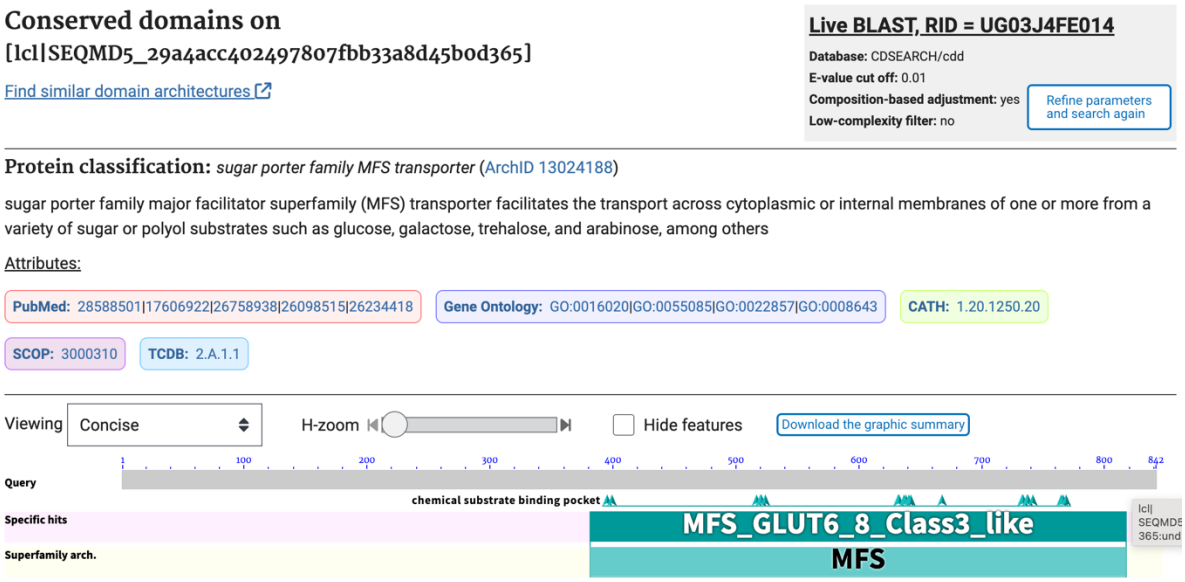
Trehalose Transport as a Male-Specific Axis of Mosquito Energy Metabolism and Reproductive Fitness

Nirmala Sankhala[1,2#] , Tanvi Singh [1,2#] , Pooja Yadav[1,2] , Vaishali Saini[1,2] , Pooja Rohilla[1,2] , Vartika Srivastava[1,2] , Gunjan Sharma[1,2] , Suchi Tyagi[1,2] , Sanjay Tevatiya[1] , Punita Sharma[1*], Rajnikant Dixit[1,2*]

[1]-Laboratory of Host-Parasite Interaction Studies, Department of Vector Genomics, ICMR-National Institute of Malaria Research, Dwarka, New Delhi, 110077, India.
[2]- Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, Uttar Pradesh-201002.
# Equally contributed

Supplementary material

1- In silico characterization

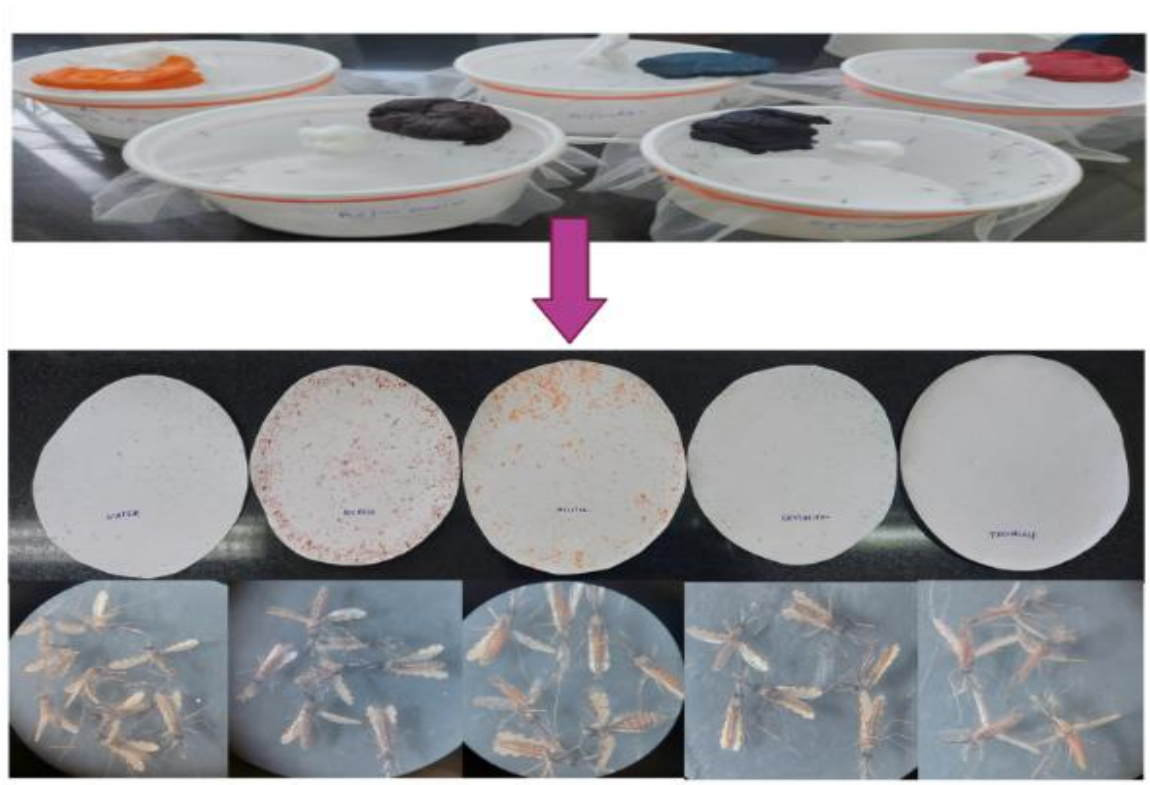


Name	Accession	Description	Interval	E-value
+ MFS_GLUT6_8_Class3_like	cd17358	Glucose transporter (GLUT) types 6 and 8, Class 3 GLUTs, and similar transporters of the Major Facilitator Superfamily	382-817	0

Supplementary Figure S1. Conserved domain analysis of the Anopheles stephensi trehalose transporter TRET1 (ASTE010234; 842 aa) using the NCBI Conserved Domain Database (CDD)

CD-Search tool (E-value cutoff: 0.01; composition-based adjustment: yes). The query sequence was classified as a sugar porter family MFS transporter (ArchID 13024188). A specific hit to the MFS_GLUT6_8_Class3_like domain (accession cd17358) was identified spanning residues 382–817 with an E-value of 0, corresponding to Class 3 glucose transporter (GLUT) types 6 and 8 and related transporters of the Major Facilitator Superfamily. The broader MFS superfamily architecture encompasses the same region. Predicted chemical substrate binding pocket residues are indicated along the query sequence.

2- Sugar survival assay



Supplementary Figure S2. Dye tracing confirmation of sugar feed uptake in adult Anopheles stephensi mosquitoes. (a) Experimental setup showing dye-supplemented sugar solution delivered via soaked cotton pads placed on cups housing adult mosquitoes within rearing cages. (b) Paper cutouts positioned beneath the cages displaying coloured spots from dye-containing excreta deposited following diuresis, confirming active ingestion and processing of the feed solution within 24 hours. (c) Adult male and female mosquitoes exhibiting visible dye accumulation through the translucent abdominal cuticle, providing direct evidence of feed uptake.

Treatment		**Sex of adult mosquito**	**Mean longevity (days)**	**Mantel cox test chi square value (Control 10% sucrose Vs test)**	**p-value (Control Vs test)**
Erythritol	2%	male	3	284.1	<0.0001

		female	3	292.4	<0.0001
	5%	male	3	293.8	<0.0001
		female	3	298.2	<0.0001
	10%	male	3	290.2	<0.0001
		female	3	294.9	<0.0001
Xylitol	2%	male	6	259.7	<0.0001
		female	7	286.8	<0.0001
	5%	male	9	179	<0.0001
		female	11	61.03	<0.0001
	10%	male	11	109.1	<0.0001
		female	14	9.818	<0.0017
Trehalose	2%	male	11	126.7	<0.0001
		female	13	0.4708	0.4926
	5%	male	11	136.4	<0.0001
		female	13	0.008	0.9284
	10%	male	9	185.9	<0.0001
		female	11	11.54	0.0007
Positive control	10% (Sucrose)	male	22,19,20= 20.33		
		female	14,17,10= 13.66		
Negative control	Water	male	3,3,4: Av. 3.33 days		
		female	3,5,5: Av. 4.33 days		

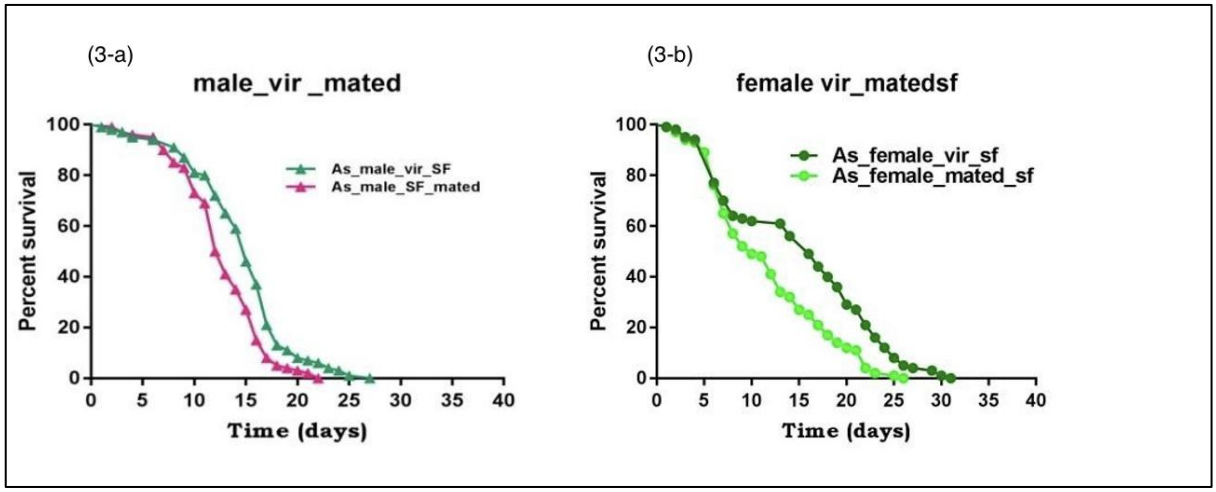
Supplementary Table S1. Mean longevity (days) and Mantel-Cox (log-rank) survival analysis of adult *Anopheles stephensi* ; mosquitoes fed on erythritol, xylitol, or trehalose at 2%, 5%, and 10% concentrations. Each treatment group comprised 50 mosquitoes across three biological replicates per sex. Survival of each treatment group was compared against the positive control (10% sucrose) using the Mantel-Cox chi-square test. Water-only fed mosquitoes served as the negative control. P-values less than 0.05 were considered statistically significant.

3- Mating status assay

Sex of adult mosquito	Treatment	Mating status	Mean longevity (days)	Mantel cox test chi square value (Control 10% sucrose Vs test)	p-value (Control Vs test)	Significance
female	Sucrose 10%	virgin	16	16.42 (Vir vs mated SF female)	<0.0001	****
		mated	10			
	Blood feeding	Virgin	9	4.6 (Vir vs mated BF female)	0.0320	ns
		mated	10			
male		virgin	15		0.0002	***

	Sucrose 10%	Mated(with SF female)	12.5	14.17 (Vir vs mated SF female)		
		Mated (with BF female)	12	24 (Vir vs mated BF female)	<0.0001	****

Supplementary Table S2. Mean longevity (days) and Mantel-Cox (log-rank) survival analysis of virgin and mated adult Anopheles stephensi mosquitoes maintained on 10% sucrose. Survival of each treatment group was compared against the respective control using the Mantel-Cox chi-square test in GraphPad Prism. P-values less than 0.05 were considered statistically significant.



Supplementary Figure S3- Kaplan-Meier survival curves of virgin and mated adult *Anopheles stephensi* mosquitoes maintained on 10% sucrose- (a) Male survival comparing virgin sugar-fed (As_male_vir_SF) and mated sugar-fed (As_male_SF_mated) groups. (b) Female survival comparing virgin sugar-fed (As_female_vir_sf) and mated sugar-fed (As_female_mated_sf) groups.

4- Mating sequence sample collection

Stage	Description of Condition	Time of Collection	Notes
Control	Before male–female mixing	12:00 PM	Baseline reference
Pre-swarm	After mixin g (3–4-day adults at 3 PM) but before visible swarm	6:00 PM	Same day as mixing
Active swarm	During visible swarming activity	7:00–8:00 PM	Peak swarm behavior

Stage	Description of Condition	Time of Collection	Notes
Coupled	During swarm; males and females observed in copula	7:00–8:00 PM	Collected in parallel with active swarm
Post-swarm	Next day, after swarm activity is complete	7:00 AM	Reflects recovery phase

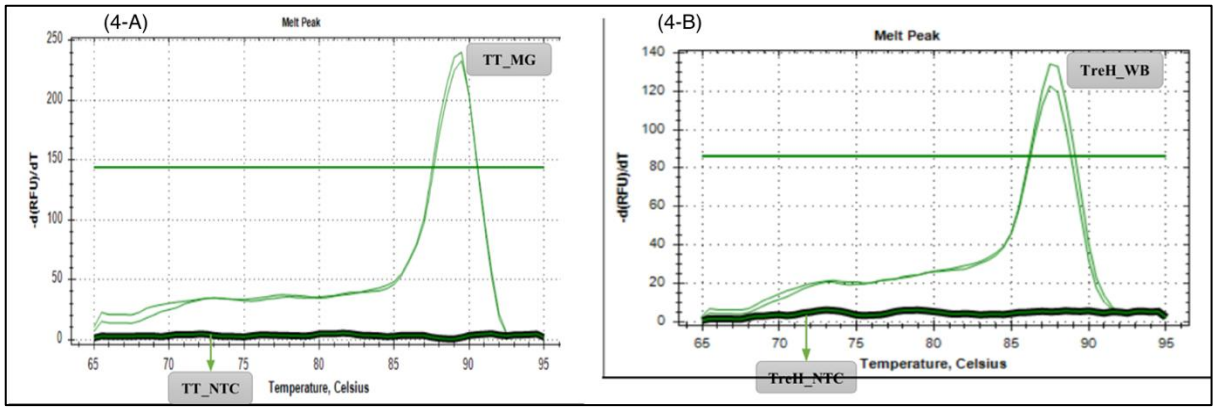
Supplementary Table S3. Description of mating stage conditions and corresponding sample collection time points used for tissue-specific gene expression profiling of adult Anopheles stephensi males. Mosquitoes were sampled across five stages of the swarming and mating sequence: control (pre-mixing baseline), pre-swarm, active swarm, coupled (in copula), and post-swarm (following day recovery).

5 – **Quantitative PCR**

Supplementary table – 4 – Primer sequences

Gene	**Sequence**
Trehalase	Fw: 5' ACGTTTAACAAAACGAGAGC 3’ Rev: 5' GCCGGTATTGATGGAGTAT 3’
Trehalose Transporter	Fw GGTGTACTGTATCGGGTTTG Rev TAAGTCCCTTGGTTTCCATA
S7	Fw 5'- ATG GTG TTC GGT TCC AAG GTG -3' Rev 5’- CTT CTT GTT GTT GAA CTC GAC CTC -3 ‘
Actin	Fw: 5’ TGCGTGACATCAAGGAGAAG 3’ Rev: 5’GATTCCATACCCAGGAACGA 3’

Supplementary Table S4. Forward (Fw) and reverse (Rev) primer sequences used for RT-qPCR analysis of trehalase (TreH), trehalose transporter (TreT), and the reference genes ribosomal protein S7 and Actin in *Anopheles stephensi*.



Supplementary Figure S4. Melt curve analysis confirming primer specificity for RT-qPCR assays. (A) Melt peak profile of the trehalose transporter (TT) primer pair amplified from midgut (MG) cDNA, showing a single sharp peak indicative of a specific amplification product, with no amplification detected in the no-template control (TT_NTC). (B) Melt peak profile of the trehalase (TreH) primer pair amplified from whole-body (WB) cDNA, displaying a single specific peak with no amplification in the corresponding no-template control (TreH_NTC). The presence of single melt peaks and flat NTC traces confirm the absence of primer-dimers and non-specific amplification.

5- RNAi – silencing

TT_DSR	Fw: TAATACGACTCACTATAGGGGATGAGGATGAGAACAGCTT Rev:TAATACGACTCACTATAGGGCATCGAGACTCTTGTGTTTG
TH_DSR	Fw: 5' TAATACGACTCACTATAGGGGGATTCGTACTGGATCGTAA 3' Rev: 5' TAATACGACTCACTATAGGGCATCGAGACTCTTGTGTTTG
GFP	dsGFP_F: TAATACGACTCACTATAGACGACGGCAACTACAAGACC dsGFP_R: TAATACGACTCACTATAGGAACTCCAGCAGGACCATGT

Supplementary Table S5. Forward (Fw) and reverse (Rev) primer sequences carrying T7 promoter overhangs (underlined: TAATACGACTCACTATAGG) used for the synthesis of double-stranded RNA (dsRNA) targeting the trehalose transporter (TT_DSR), trehalase (TH_DSR), and green fluorescent protein (dsGFP; non-target injection control) in *Anopheles stephensi* RNAi knockdown experiments.

6- Additional information about data and analysis

Item	File Name/Type	Description
1	RMarkdown reports (.Rmd exported as PDF)	Reproducible analysis scripts with embedded data for all experiments except the trehalose assay, including RT-qPCR expression profiling, survival analysis, fecundity analysis, and RNAi knockdown validation

Item	File Name/Type	Description
2	t_data.csv and s_data.csv	t_data.csv -Raw trehalose assay data used for hemolymph trehalose and glucose concentration calculations, s_data.csv for standard data
3	Copulation video (.mp4)	Video documentation depicting mating behaviour and in copula sample collection procedure during swarming
4	FASTA file (.fasta)	protein sequences of trehalose transporter orthologs used for phylogenetic analysis
5	Newick tree file (.nwk)	Maximum-likelihood phylogenetic tree output used to generate Figure 1c

Supplementary Table S6. Description of data and materials deposited in Figshare - 10.6084/m9.figshare.31648699

Private link - https://figshare.com/s/53c8781ae50abcaaab28