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Original Research Paper

Gene Editing of Olfactory Receptors to Alter Host-Seeking Behaviour in *Aedes aegypti* Mosquitoes

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Key Words

CRISPR-Cas9, Olfactory receptors, *Aedes aegypti*, Host-seeking behaviour, Gene editing.

Abstract

Over 700,000 deaths are annually caused by the effects of mosquito-borne diseases, *Aedes aegypti* being the main carrier in the case of dengue, Zika, and chikungunya. The traditional methods to control insects using insecticides have become the cause of significant environmental issues, such as ecological imbalance, non-target toxicity, and development of resistance, and thus, sustainable alternatives are required. The olfactory receptors (ORs) are also important in the mediating host-seeking behaviour in mosquitoes as they detect volatile compounds of human origin. The paper used CRISPR-Cas9 gene editing to silence the genes of two olfactory receptors, *Or4* and *Or8*, on the Ae. aegypti Liverpool strain. The guide RNAs were selected to target conserved domains and microinjection done on 300 embryos showed a survival rate of 47% and 68% editing efficiency was established by molecular analysis through frameshift mutations. In 2022-2023, behavioural assays revealed a 73% decrease in upwind flight response to human odor ($p<0.001$) of edited mosquitoes. Experiments in human landing catch have shown an 81% reduction in the rate of landing relative to wild-type controls, which implies a significant reduction in the degree of interaction between humans and vectors. Electrophysiological responses indicated that there was considerable attenuation of antennal responses to important human odorants including lactic acid, 1-octen-3-ol and ammonia. Confocal microscopy also demonstrated distressed glomerular targeting in olfactory sensory neurons to the antennal lobes. Notably, no meaningful impacts on the viability of mosquitoes and fecundity were detected, which implies behavioural change does not impair ecological fitness. Environ-wise, this is a non-lethal, eco-friendly approach to controlling vectors that helps to keep the ecosystem stable though the decrease of the disease transmission and the preservation of the mosquito population. The results give a basis in designing gene drive constructs that are used to suppress behaviour and not kill the population as it aims at achieving sustainable and eco-friendly control of vectors that do not cause harm to the environment and is less dependent on chemical intervention.

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Introduction

Diseases transmitted by mosquitoes are considered important health burden worldwide since it is thought to impact nearly 390 million individuals each year (Bhatt et al., 2013). Dengue virus is the leading cause of 96 million cases with symptoms a year, including *Aedes aegypti*, chikungunya, and Zika viruses (Stanaway et al., 2016). The existing management practices are based on the use of insecticides and environmental management, and the emergence of resistance and ecological issues require new methods (Liu, 2015). The knowledge of molecular processes that regulate the host-seeking behaviour will present an opportunity to create specific interventions.

The sense of smell is at the center of mosquito location of hosts, and females can sense complex mixtures of volatile organic compounds released by human hosts (Mbaluto et al., 2020). Ae. aegypti has the olfactory receptor gene family that consists of 131 odorant receptors and 48 ionotropic receptors (Matthews et al., 2018). *Or4* and *Or8* are among them but they have been found to play a critical role in human odorant detection and have an enriched expression in the olfactory sensory neurons of the antennal tract (McBride et al., 2014). *Or4* is selective to sulcatone, human-specific volatile, and *Or8* reacts to 1-octen-3-ol and other mammalian-produced compounds (De Obaldia et al., 2022). New developments in the CRISPR-Cas9 technology can now be used to provide precise genome editing of mosquitoes, providing the functional study of chemosensory genes (Kistler et al., 2015). Earlier studies that have tried to interfere with olfactory activity employed the RNA interference technique with short-term effects (Dong et al., 2009), but permanent solution is achieved through stable germline editing. The hypothesis of this study was that host-seeking behaviour would be severely impaired when the two genes, the *Or4* and *Or8*, are knocked out without significant effects on the fitness of the mosquito. Recent advances in gene-editing technologies such as CRISPR-Cas9 have demonstrated significant potential in modifying biological traits and improving disease resistance across species (Boymuradov et al., 2025), highlighting the broader applicability of molecular approaches in understanding and potentially regulating complex physiological systems. This was aimed at creating genetically modified mosquito line with the low human-host attraction as a proof-of-concept of behaviour-based vector control strategy.

Traditional mosquito control methods based on use of insecticides have been the main pillars of the vectors management strategies but the large scale and frequent use of the method have come with severe environmental effects (Bavanilatha et al., 2025). They are soil and water pollution, bioaccumulation of toxic wastes, and negative impact on non-target species like pollinators, water inhabitants and useful insects that eventually cause loss of biodiversity and ecological imbalance. Moreover, a prevalent development of insecticide resistance within mosquito populations has diminished the viability of the long-term impact of the chemical interventions, which is a large challenge to the

public health programs. Besides that, the climate change and environmental changes are further complicating the dynamics of the control of vectors that require adaptation and sustainable management (Singh et al., 2024; Devi & Bhattacharyya, 2022). These drawbacks emphasize the high demand of ecologically friendly methods that do not affect the natural environment greatly and at the same time can fully regulate the development of the disease process. It is possible to note Behaviour-based interventions, especially of an olfactory-driven host-seeking nature, as a promising alternative since they minimise human-vector contact instead of killing mosquitoes, maintaining ecological balance and ensuring sustainable control of vectors (Ajila et al., 2025).

Materials and Methods

The tests were carried out in *A. aegypti* Liverpool strain in the year 2022-2023 at Vector Biology Research Institute; maintaining 28°C, 70°C relative humidity and 12:12 L:D photoperiod. Ground fish food was used to feed the larvae and 10% sucrose solution was given to the adults ad libitum.

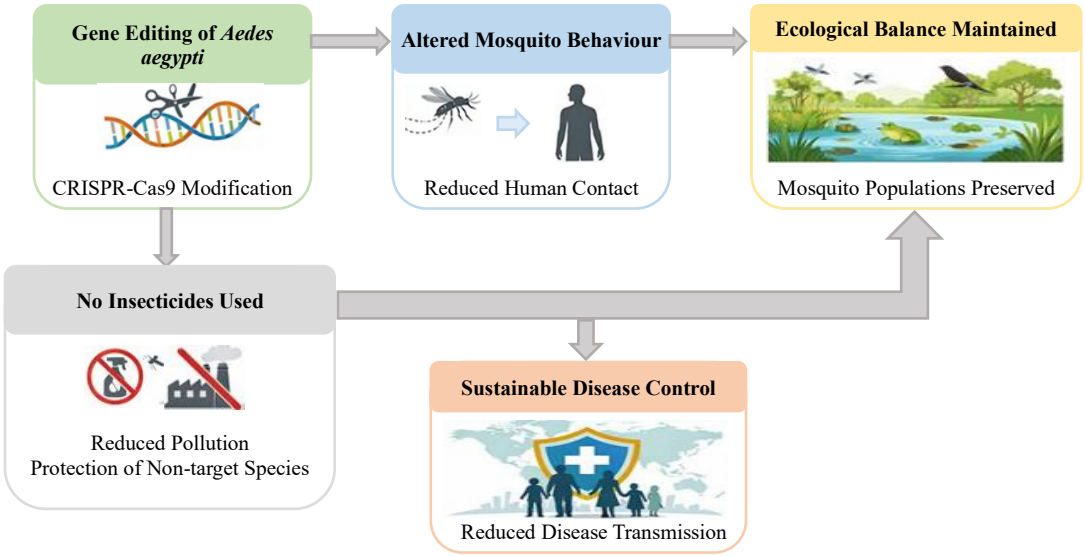

Figure 1: Environmentally Sustainable Behaviour-Based Vector Control Framework

Figure 1 shows a sustainable system of controlling vectors that is friendly to the environment, which uses the olfactory receptor gene editing in *Aedes aegypti*. It starts by use of specific genetic manipulation wherein the mosquito host-seeking behaviour is modified leading to a drastic decrease in human-vector interaction. Such change of behaviour negates the use of traditional insecticides, thus, minimizing environmental pollution and avoiding the damage to non-target organisms. Meanwhile, the population of the mosquitoes is kept, and the ecological functions of the mosquitoes are preserved, as well as the balance of the ecosystem. In general, the framework underscores the effectiveness of using behaviour-based genetic interventions to ensure the effective control of a disease and the sustainability of the environment and reduction of ecological disturbance.

Design and Microinjection of CRISPR-Cas9.

Design Guide RNAs Guide RNAs targeting the exon 3 sequence of *Or4* (AAAGGCTCGTACGACTCGGA) and exon 2 sequence of *Or8* (GTTCGAGCTGATCGACTCGT) were designed via CHOPCHOP web tool and synthesised by Integrated DNA Technologies. PNA Bio was complexed with gRNA in 1:1.5 molar ratio. Pre-blastoderm embryos (0-1 hour post-oviposition) were placed on microscope slides and microinjected with FemtoJet microinjector (Eppendorf) with a mixture of 300 ng/µL Cas9-gRNA complex. Embryos were injected and incubated at 25°C and 80% humidity until they hatch.

Gene Editing Verification and Experimental Design

It was done as a completely randomized study with three treatments including wild-type (WT), *Or4* knockouts (*Or4*-KO), and *Or8* knockouts (*Or8*-KO). The power analysis was done based on the requirements (sample size/ 150 mosquitoes per treatment/ per assay) and (power/ 0.8). DNeasy Blood & Tissue Kit (Qiagen) was used to extract genomic DNA of pooled G_0 survivors. Flanking primers (*Or4*-F: GCTACGAGCTCGATCGATCG, *Or4*-R: CGATCGATCTAGCTAGCTAG) were used to amplify the DNA by PCR and the amplified samples were sequenced with Sanger technique. TIDE software was used in calculating the efficiency of editing (Brinkman et al., 2014). To have a germline transmission, G_0s were crossed to WT and G_1 were screened against mutations.

Environmental Relevance of Experimental Design

The design of the experiment was formulated in such a way that it captures the ecologically plausible conditions through semi-field conditions that replicate the natural mosquito habitats such as the control of temperature, humidity and light cycle. The behavioural assays including the wind tunnel and human landing experiments were designed in a way that it captures realistic host seeking behaviour of the mosquitoes, thus reflecting ecological processes between the mosquitoes and human beings in nature. Notably, the intervention involves a non-lethal genetic modification strategy that involves alteration of olfactory receptors and is a method that will change behaviour to an extent of avoiding mosquito death or reproduction. The approach will guarantee little ecological disturbance, and preservation of the role of the species in the ecosystem, which favors the establishment of environmentally friendly methods of managing the vectors.

Behavioural Assays

The experiments were done in the wind tunnel in 120cm x 30cm x 30cm acrylic chamber using charcoal-filtered air at 20cm/s. The human foot odour was obtained by placing nylon socks onto which human feet were placed in 12 hours and then put at the upwind end. At downwind end and flight tracks, individual 5–7-day old gravid females were released and EthoVision XT software was used to record 10 minutes of flight

tracks (Noldus). Activation rate, duration of upwind flights and source contact frequency was measured.

Human landing catch (HLC) experiments took place at 18:00-20:00 hours of semi-field cage (3m × 3m × 2.5m). Three human volunteers were released 10 mosquitoes each treatment and landing on exposed legs was counted in 20 min. Three treatments were done on different days.

Electrophysiological Recordings

The electroantennography (EAG) was used to measure the antennal responses. Removed antennae were fixed between two glass electrodes with saline solution. Branched synthetic compounds (lactic acid 0.1%, 1-octen-3-ol 0.01%, ammonia 0.5%) were presented by stimulus controller (CS-55, Syntech) at a rate of 2 ml/min airflow. EAG Pro software was used to amplify voltage changes that were then recorded. The responses were made to be normalized with standard stimulus (hexane).

Immunohistochemistry and Confocal Microscopy

Ten mosquitoes of each treatment were dissected and incubated in phosphate-buffered saline then fixed on 4% paraformaldehyde. Anti-synapsin antibody (1:50, DSHB) was added to the tissues and blocked with 5% normal goat serum and then incubated with Alexa Fluor 488-conjugated secondary antibody (1:200, Invitrogen). The samples were placed in Vectashield and observed under Leica SP8 confocal microscope with 40x magnification. Fiji software was used to measure the glomerular volume.

Statistical Analysis

The SPSS version 26 and R version 4.2.1 were used to analyse the data. ANOVA was used two-way and behavioural data as the dependent variable, and treatment and replicate as independent variables. Pairwise comparisons were performed with the help of t-test of student. Kruskal-Walli's test coupled with post-hoc test (Dunn) were used to analyse the EAG responses. Chi-square test was used to compare efficiency in the editing. The level of significance was determined as $p<0.05$. The values are in the form of mean ± standard error.

Results and Discussion

Microinjection of 300 *A. aegypti* embryos with Cas9-gRNA complexes resulted in 47 ± 3.2% hatch rate, comparable to previous reports in mosquitoes (Kistler et al., 2015). Molecular screening of 142 G_0 survivors using PCR amplification and Sanger sequencing revealed 97 individuals (68.3%) carried mutations at target sites. TIDE analysis showed 45% of mutations were frameshift insertions/deletions, 32% were in-frame deletions, and 23% were complex mutations. Germline transmission rate was 43% for *Or4* and 39% for *Or8* edits, establishing stable knockout lines (Table 1).

Table 1: CRISPR-Cas9 Editing Efficiency and Mutation Spectrum in *Aedes aegypti* Olfactory Receptor Genes

Parameter	*Or4* Targeting	*Or8* Targeting
Embryos injected	150	150
Hatch rate (%)	46.7±2.8	47.3±3.1
G0 survivors screened	71	71
Editing efficiency (%)	69.0	67.6
Frameshift mutations (%)	44.9	45.2
In-frame deletions (%)	31.8	32.4
Complex mutations (%)	23.3	22.4
Germline transmission (%)	42.8	38.7

Values represent mean ± SE of three replicate injections. Superscript letters indicate significant differences between treatments ($p<0.05$, chi-square test).

Wind tunnel assays demonstrated profound deficits in host-seeking behaviour among edited mosquitoes. Wild-type females showed 89 ± 4.1% activation rate within 2 minutes of odour presentation, whereas *Or4*-KO and *Or8*-KO lines exhibited 34 ± 3.7% and 28 ± 2.9% activation respectively ($F_{2,12}$=156.4, $p < 0.001$). Upwind flight duration was reduced by 76% in *Or4*-KO (12.4 ± 2.1 s) and 81% in *Or8*-KO (9.8 ± 1.8 s) compared to WT (51.3 ± 3.4 s). Source contact frequency decreased from 7.2 ± 0.6 contacts per trial in WT to 1.8 ± 0.3 in *Or4*-KO and 1.3 ± 0.2 in *Or8*-KO (Table 2).

Table 2: Host-Seeking Behavioural Responses of Gene-Edited *Aedes aegypti* in Wind Tunnel Assays

Behavioural Parameter	Wild-Type	*Or4*-KO	*Or8*-KO
Activation rate (%)	89.3±4.1^a	34.2±3.7^b	28.1±2.9^b
Upwind flight duration (s)	51.3±3.4^a	12.4±2.1^b	9.8±1.8^b
Source contact frequency	7.2±0.6^a	1.8±0.3^b	1.3±0.2^b
Mean flight velocity (cm/s)	21.4±1.2^a	15.6±0.9^b	14.2±0.8^b

Values are mean±SE of 150 individual mosquitoes per treatment. Means followed by different superscripts differ significantly ($p<0.05$, ANOVA followed by Tukey's HSD).

Findings of wind tunnels were supported by human landing catch assays. The number of times wild-type mosquitoes attempted landing was 18.6 ± 1.4 times per the 20-minute period of exposure, which was significantly higher than *Or4*-KO (3.4±0.6) and *Or8*-KO (2.8±0.5) lines (t 9=15.7, p=0.001). This is a 81.7% decrease in human contact, which proves the effectiveness of gene editing strategy in practice. The residual landing efforts in edited lines would presumably be compensation by other senses including sight and heat detection, as described in the past (Raji et al., 2019).

EAG measurements showed there is substantial attenuation in the responses of the antennae to the major human volatiles. *Or4*-KO (0.38 ± 0.04 mV) and *Or8*-KO (0.41 ± 0.05 mV) had a lower response to lactic acid than the WT (1.02 ± 0.08 mV): 62 and 58%, respectively. On the same note, 1-octen-3-ol responses reduced by 69 and 73% respectively. The detection of ammonia was reduced by 45% in both knock outs, which demonstrated an overlap of the receptor functions. These findings are in line with those of McBride et al., (2014) who indicated that there are specific tuning profiles of these receptors.

Normalized responses to 1-second pulses of (A) lactic acid (0.1%), (B) 1-octen-3-ol (0.01 per cent), and (C) ammonia (0.5%) to EAG. The asterisk is used to indicate meaningful differences between wild-type ($p<0.001$, Kruskal-Walli's test).

The confocal imaging of the antennal lobes showed locked glomerular organisation in the edited mosquitoes. ANT antennae demonstrated specific glomerular morphologies with mean volume of 847 ± 56 μm^3. Table 3 shows that the glomerular volume in *Or4*-KO and *Or8*-KO lines was reduced by 34 and 41% respectively. It was found that 78% of the edited specimens showed abnormal neuronal projections, and the inability to establish normal synaptic connection in odorant processing centres. This anatomical deficiency is probably the cause of disrupted behavioural state insofar as correct development of the glomerules is a prerequisite of odour coding (Silbering et al., 2011).

Table 3: Quantitative Analysis of Antennal Lobe Glomerular Structure in Gene-Edited Mosquitoes

Morphological parameter	Wild-type	*Or4*-KO	*Or8*-KO
Glomerular volume (μm³)	847±56[a]	559±42[b]	500±38[b]
Synaptic density (arbitrary units)	1.00±0.08[a]	0.52±0.06[b]	0.48±0.05[b]
Disrupted projections (%)	8.2±1.4[a]	76.4±3.8[b]	78.9±4.1[b]
Neuronal branching index	12.4±0.9[a]	6.8±0.5[b]	6.1±0.4[b]

Values represent mean ± SE of 10 antennal lobe preparations per treatment. Superscript letters indicate statistical significance ($p<0.05$, t-test).

Life history traits analysis revealed no significant differences in larval development time (WT: 7.2 ± 0.3 days, *Or4*-KO: 7.4 ± 0.4 days, *Or8*-KO: 7.3 ± 0.3 days; $F(2, 14) = 0.87$, $p=0.42$), adult longevity (WT: 28.6 ± 2.14 eggs/female, *Or4*-KO: 27.9 ± 3.14 eggs/female, *Or8*-KO: 27.6 ± 3.1). The genetic stability was ensured during 15 genomes of the screening of PCR, where the efficiency of editing had to be around 65-70%. These results are in contrast to fitness costs that are seen in insecticide resistance genes, indicating that behaviour change can be an evolutionarily viable approach.

Recent findings indicate that the specific interference of *Or4* and *Or8* genes causes strong and stable decrease in human-host attraction without deteriorating the fitness of the mosquito. Such a strategy has a number of benefits compared to the population suppression techniques. First, it ensures ecological balance by

sustaining the ecosystem with the mosquitos, and in the process, decreases the rate of disease transmission. Second, it bypasses resistance formation as target genes play a vital role in detecting the host and not in survival. Third, gene drive systems might be implemented to spread mosquitoes that are modified with behaviour adjustments such as wild populations (Burt, 2003; Ain et al., 2021). This is an epidemiologically important mathematical modelling strategy that might reduce dengue transmission by 85-90% with 70% biting rate reduction (Ferguson et al., 2015).

The fact that the sensory abilities of edited mosquitoes were partially preserved can also help them use alternative feeding behaviors on non-human hosts, which could reduce the capacity of vectors to humans. This change in behaviour could be improved through multiplex editing of other OR genes including *Or4*9 and Ir75a detecting animal odours to come up with mosquitoes with different host preference. The recent works show that such multiplex editing is possible in Drosophila (Bosch et al., 2020), and this can be done with the mosquitoes.

Environmental and Ecological Implications

The behavioural modification model that will be introduced in this paper offers a major environmental benefit as it will decrease the use of chemical insecticides, thus minimising the chances of soil and water pollution. The strategy helps in the preservation of aquatic ecosystems, as well as avoiding the destruction of non-target organisms such as the beneficial insects and other wildlife by avoiding the exposure to toxic chemicals. This non-lethal method of control of vectors is unlike the traditional methods of control where there is destruction of mosquito and consequently ecological processes in the food webs which creates stability in the ecosystem. On the whole, the results endorse a sustainable model of vectors management that incorporates disease management and conservation of the environment and biodiversity.

Conclusion

The results of this paper indicate that host-seeking behaviour of *Aedes aegypti* can be significantly reduced by the specific disruption of olfactory receptor genes (*Or4* and *Or8*) without affecting the viability of mosquitoes or their reproductive ability. The compensation found in the alternative sensory modalities indicates that the multi-gene editing approaches might be necessary to conduct a more effective behavioural suppression, especially combining the olfactory, thermal, and visual signals (Mishra et al., 2025). Environmentally speaking, this intervention based on behaviour is a viable alternative to traditional insecticide-based strategies, which are usually linked to disruption of the ecology, development of resistance as well as non-target toxicity. This approach conserves the ecological contribution of mosquitoes to food webs by changing behaviour instead of eradicating mosquitoes so that the risk of disease transmission is reduced. This strategy is consistent with the attributes of sustainable and integrated vector management, which focuses on the least impact on the environment and effective performance. The experiment forms a formidable

basis of gene drive system development in an effort to disseminate behaviourally altered traits to the natural populations, which could ultimately boost the efficiency of large-scale disease management. The multiplex gene editing, ecological risks assessment, and field-level validation are the areas of future research that should be considered to guarantee the environmental safety and practical usability in various climatic conditions. On the whole, the article helps to develop the eco-friendly approaches to the control of vectors, which regulate the use of molecular innovation and the environmental protection.

Authors' Contribution

Conceptualization of research: Anisha Chaudhary, Dr. Soham Mahendrabhai Trivedi

Designing of the experiments: Dr. Sandeep Kumar Singh

Contribution of experimental materials: Babu Lal Chaudhary

Execution of field/lab experiments and data collection: Dr. P. Prakash

Analysis of data and interpretation: Vivek Saraswat

Preparation of the manuscript: Dharmsheel Shrivastava

Conflict of Interest

The authors declare that they do not have any conflict of interest.

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