The Role of *Gr5* in Regulating Sugar Feeding in the Yellow Fever Mosquito *Aedes aegypti*

– Comparative Analysis of Sugar Feeding Behaviour in Wildtype and *ΔGr5 Aedes aegypti* Using a Modified FlyPAD System

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Swedish University of Agricultural Sciences, SLU
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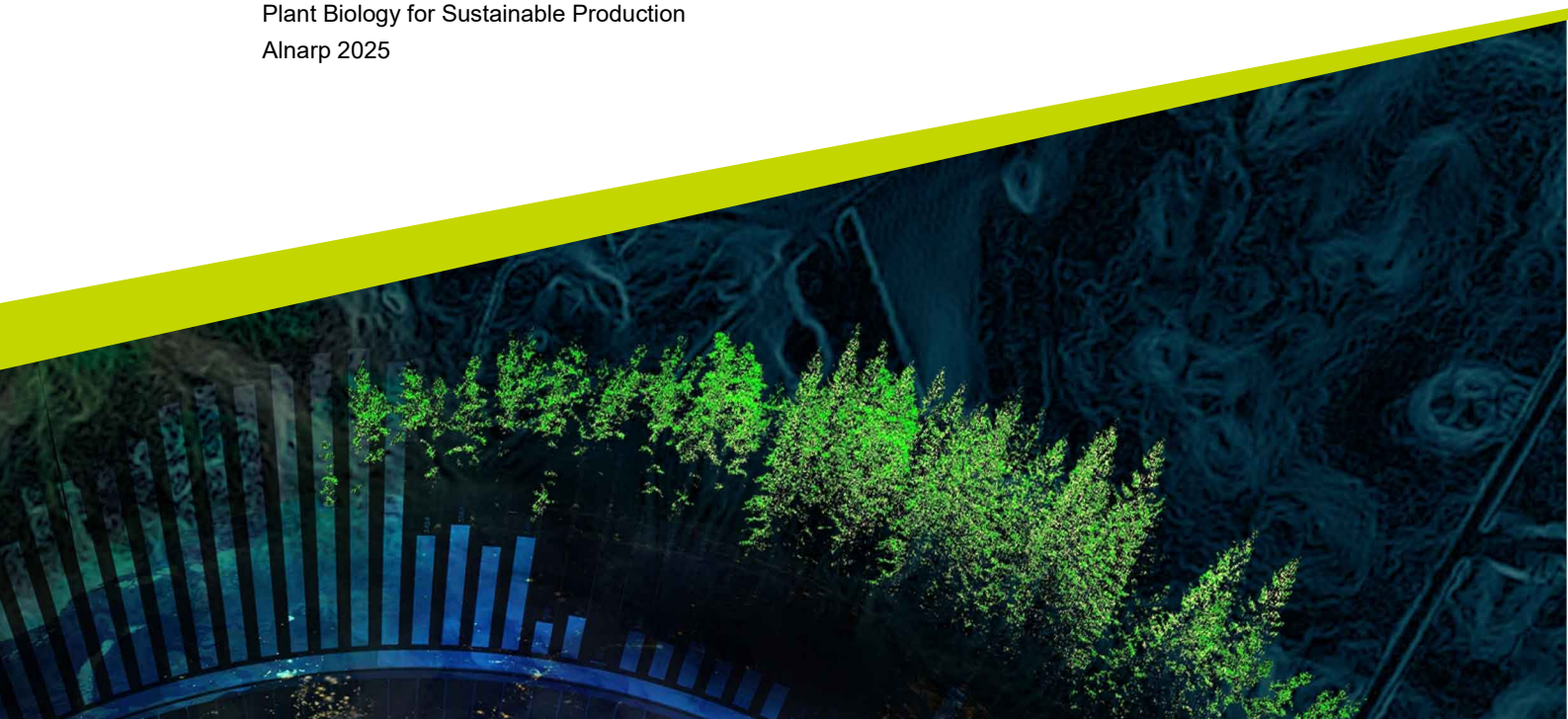

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Abstract

The sugar feeding behaviour of mosquitoes plays an essential role in their survival, reproduction, and vector competence. This project focuses on the role of the gustatory receptor gene *Gr5* of *Aedes aegypti* in sugar detection. Understanding how mosquitoes assess and respond to different sugars can provide insight into the evolution of sugar receptors in *Ae. aegypti* and improve vector control strategies. In this project, a modified FlyPAD system was applied to access the feeding behaviour of two wildtype *Aedes aegypti* strains, Liverpool and Rockefeller, and a *Gr5 gene* knockout mutant, *ΔGr5,* when provided with sucrose, glucose, fructose and trehalose at a series of concentrations containing dye. The feeding dynamics were recorded and defined as different feeding events. Food imbibed was quantified using a spectrophotometer and dye-based standard curves. Results showed that both wildtype strains exhibited similar sugar feeding dynamics, with maximum feeding around 100 mM. In contrast, the *ΔGr5* line demonstrated a marked reduction in feeding across all sugars, particularly sucrose and trehalose, and a lack of a significant dose response. The *ΔGr5* line displayed altered feeding dynamics for sucrose and trehalose, shifting from a sip-driven pattern to a "burst-pause" pattern. Statistical analyses revealed that *Gr5* knockout significantly disrupted the coordination of feeding parameters, especially for disaccharides.

The data suggest that *Ae. aegypti* Gr5 has a possible function of detecting disaccharides. And Gr5 might contribute to the formation of the *Ae. aegypti* gustatory receptor tetramer as a monomeric subunit. For a future step, crossing *Ae. aegypti ΔGr5* with different *Gr* knockout mutant lines, followed by comparative feeding assays of sucrose, glucose, fructose, and trehalose, is expected to reveal further details of the sugar-detection function of the Gr5 receptor in *Ae. aegypti*.

Keywords: Aedes aegypti, gustatory receptor, sugar feeding, *Gr5*, the FlyPAD system, behavioural assay.

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Abbreviations

Abbreviation	Description
ATSB	Attractive targeted sugar bait
ANOVA	Analysis of variance
DPE (dpe)	Days post-emergence
GPCR	G-protein coupled receptor
GR (Gr)	Gustatory receptor
PCR	Polymerase chain reaction
PER	Proboscis extension reflex
SR	Sugar receptor
VOC	Volatile organic compound

1. Introduction

Understanding how sugar-feeding behaviour is regulated may provide useful insights for vector control approaches, as well as for understanding the ecological and behavioural adaptations that shape *Aedes aegypti* as an efficient disease vector. Sugars serve as the primary energy source for mosquitoes, supporting activities such as flight, host seeking, and oviposition-site searching (Barredo & DeGennaro, 2020). Variations in the availability of floral or plant-derived sugars can influence mosquito survival, dispersal, and population dynamics, ultimately shaping their behavioural ecology and success as disease vectors (Swan et al., 2021; Tenywa et al., 2024). The impact of mosquito-borne disease highlights the need for improved mosquito control strategies, particularly those that can target mosquito behaviours critical to pathogen transmission, *e.g.*, adult female *Ae. aegypti* feed on both blood and sugar. Nulliparous female *Ae. aegypti* (1-5-day-post-emergence) show a greater preference for sugar meals than blood meals; meanwhile, older parous female *Ae. aegypti* prefer blood meals over sugar meals (Foster, 1995). Sugar feeding prior to blood meals can influence the vector competence of female *Ae. aegypti* adults for arboviruses by enhancing the antiviral immunity in the gut of mosquitoes (Almire et al., 2021). Thus, sugar-targeting vector control strategies can be applied at the early adult stage of *Ae. aegypti* that have not reached the infective stage, such as attractive targeted sugar baits (ATSBs), towards the mosquitoes (Tenywa et al., 2024).

1.1 Mosquito sugar meals

Adult *Aedes aegypti* of both sexes frequently consume a variety of natural sugar resources as a source of energy, although females can rely exclusively on blood for energy (Foster, 1995). The sugar meals originate from diverse natural sources, including floral and extrafloral nectars, fruits, as well as piercing plant tissue to get access to xylem and phloem (Foster, 1995; Müller & Schlein, 2005) and hemipteran (aphids and scale insects) honeydew (Peach et al., 2019). The types of sugars present in these sources vary: nectar primarily contains sucrose, glucose, and fructose (Liu et al., 2024), and sucrose is the primary form of sugar in xylem (Lemoine et al., 2013). In the absence of plant-based sugar sources, female *Ae. aegypti* have even been observed feeding on lepidopteran larval hemolymph under laboratory conditions (George et al., 2014). The sugar meals from hemipteran honeydew and lepidopteran larvae include components that are uncommon in plants, such as melezitose and trehalose, and the presence of these sugars in the diet of *Ae. aegypti* often signals a non-plant sugar source.

Glucose and fructose produce approximately 32 ATP upon metabolism (Dunn & Grider, 2025; Dholariya & Orrick, 2025). Sucrose (a glucose-fructose disaccharide with an α1→β2 bond) and trehalose (a disaccharide of two glucose units with an α 1→α1 bond) are first hydrolysed into their monosaccharide components before metabolism, with each molecule of sucrose or trehalose producing about 64 ATP (Shukla et al., 2015). Due to the different amount of energy per molecule that each type of sugar provides, *Ae. aegypti* exhibits varying preferences among different sugars. In *Ae. aegypti*, for the same concentration, the order of sugar acceptance is sucrose > trehalose > fructose > glucose. And sucrose cannot be distinguished from its monosaccharide components, the acceptance of 10 mM sucrose by mosquitoes is the same as that of a solution with 10 mM glucose and 10 mM fructose. For the same type of sugar at different concentrations, mosquitoes consistently show a preference for the higher concentration (Ignell et al., 2010).

Box 1 The public health threat of Aedes aegypti

Mosquitoes (*Diptera: Culicidae*) are widely recognised as one of the most dangerous animals to humans, due to their role in transmitting a wide range of infectious disease pathogens (Schmidt, 2005). Female mosquitoes can deliver pathogens with saliva injected into the vessels under the skin during blood feeding, which enables the transmission of arboviruses and protists (Lefteri et al., 2022). Mosquitoes are the primary vectors for five of the top ten vector-borne diseases ranked by global disability-adjusted life year (DALY) burden (Athni et al., 2021). One of the most significant examples is human malaria, in which five species of *Plasmodium spp.* parasites are transmitted by *Anopheles* mosquitoes and caused an estimated 263 million cases globally in 2023 (Singh & Daneshvar, 2013; WHO, 2024). Although only 2.5% of the 3,578 known mosquito species have been confirmed as vectors of human pathogens, an additional 6.8% have been identified as potential or likely vectors (Yee et al., 2022). Among disease vectors, *Aedes aegypti* is particularly important because it serves as the primary vector of the arboviruses that cause yellow fever, dengue, Zika, and chikungunya (Souza-Neto et al., 2019). The incidence of these diseases has increased in recent years, and their geographic ranges have expanded alongside the global spread of *Ae. aegypti*. As a result, it is estimated that 49.13% of the world's population is now at risk of exposure to one or more mosquito-borne viruses (Charrel et al., 2014; Kraemer et al., 2019).

1.2 Sugar feeding affects life history traits

Due to limited abdominal space, female mosquitoes face a trade-off between sugar feeding and blood feeding (Stone et al., 2011). On one hand, sugar provides a vital energy source that supports high-energy-consuming activities, such as flight. On the other hand, excessive sugar feeding can suppress blood feeding, which provides *Aedes aegypti* essential protein, lipids, iron, and other micronutrients for egg development (Foster, 1995). Female *Ae. aegypti* adults that rely exclusively on blood meals have shorter life expectancy compared to female adults that have

access to both sugar and blood meals, while at the same time, the presence of sugar meals does not affect the expected lifetime fecundity when the female adults have access to blood meals (Day et al., 1994). This trade-off of sugar feeding in mosquitoes makes sugar feeding behaviour essential for the reproduction of mosquitoes.

Conversely, insufficient sugar meals may leave the mosquito without the energy reserves needed for host-seeking flight (Foster, 2022). This trade-off is reflected in the balance between female mosquito lifespan and daily fecundity. For instance, in *Ae. aegypti* and *Aedes albopictus*, sugar feeding reduces both the frequency of blood feeding and daily fecundity. However, because sugar-fed females tend to live longer than those that rely solely on blood meals, the lifetime fecundity output remains unaffected by whether or not sugar is consumed (Braks et al., 2006). Although sugar feeding does not affect the reproduction of mosquitoes, it can influence the vector competence of female *Ae. aegypti*. Sucrose, glucose, and fructose can enhance the expression of antiviral genes in the gut, even though gut bacteria partially suppress sugar-induced activation of immune genes in the gut of females (Almire et al., 2021). Thus, sugar meals prior to an arbovirus-infected blood meal can protect female *Ae. aegypti* adults from infection by arboviruses.

Most imbibed sugar meals are stored in the ventral diverticulum (crop) and are transferred to the anterior midgut as needed to meet energy demands during flight or fill energy stores in the fat body and haemolymph (Clements, 1992). In contrast, blood meals are directed to the posterior midgut for rapid digestion (Trembley, 1952). When mosquitoes imbibe a large sugar meal within a short period, abdominal stretch receptors in the first three anterior abdominal segments mediate short-term host-seeking suppression through mechanosensation (Klowden, 1994). The host-seeking suppression is lifted after the sugar meal is digested, because the absence of proteins required for egg development prevents the activation of protein sensing or egg development signalling, which would otherwise induce sustained suppression of host-seeking and oviposition searching behaviours (Duvall, 2019).

The sugar-seeking behaviour can also influence the oviposition site selection of mosquitoes. Flowering plants that provide nectar can attract gravid females to oviposit in nearby areas. The energy obtained from sugar meals is used for both host-seeking flights and locating oviposition sites (Davis et al., 2016). This links mosquito sugar foraging behaviour with oviposition-site searching behaviour.

1.3 Sugar-foraging behaviour

Mosquitoes use both chemical and visual cues during sugar seeking at a distance (Foster, 2022). Since sugar solutions have a lower vapour pressure than water, sugar

themself are unlikely to act as volatile chemical cues. Instead, volatile organic compounds (VOCs) released by plants—including terpenes, aldehydes, and alcohols—serve as olfactory cues, which trigger the orientation of mosquitoes (Foster, 1995). Heat and carbon dioxide produced by flowers may serve as additional cues to VOCs to help mosquitoes locate host plants (Lomelí & Dahanukar, 2022). Visual cues also contribute at this stage, and at a shorter distance from the host plant, forming part of a multimodal floral signal complex together with carbon dioxide and chemical cues, thereby providing orientation guidance for nectar-foraging mosquitoes (Peach et al., 2019). Floral traits and the distribution of sugar sources among different plant species influence mosquito landing and feeding behaviour, highlighting the importance of sensory integration during the landing phase for successful sugar meal acquisition (Manda et al., 2007).

While chemical and visual cues can trigger and regulate sugar-seeking behaviour from a distance, gustatory cues are essential for assessing the palatability of food sources upon contact (Freeman & Dahanukar, 2015). When mosquitoes land on the surface of host plants, sugars stimulate the tarsi and trigger proboscis-oriented probing movements (Pappas & Larsen, 1976). If the tarsal sensilla on the legs of mosquitoes detect secondary metabolites of the host plant, such as quinine, it can repel the mosquitoes without imbibing food (Dennis et al., 2019).

When gustatory receptors on the proboscis and tarsi detect sugars, such as sucrose, glucose, and fructose, and assess the sugar source as suitable for feeding, the feeding phase will start (Baik et al., 2024). During this stage, the type of sugar and its concentration can affect the ingestion and the volume of ingestion, and the feeding can be inhibited by abdominal stretch receptors. (Foster, 1995). Given the broad influence of sugar feeding on mosquito life history traits, variations in sugar-feeding patterns could have implications for strategies aimed at controlling mosquito-borne diseases.

1.4 Detecting and discriminating sugars

Beyond serving as a critical energy source for adult mosquitoes, sugars also offer a valuable entry point into understanding the evolutionary shift from phytophagy to haematophagy in mosquitoes. Gustatory receptors (Grs) are proteins with seven transmembrane domains, with the N-terminus located intracellularly and the C-terminus located extracellularly. The Grs are ligand-gated ion channels, not G protein-coupled receptors (GPCRs), possess the opposite topology (Robertson & Kent, 2009). In *Aedes aegypti*, there are 79 Gr genes in the *Ae. aegypti* genome encoding 114 potential proteins. After removing 23 pseudogenic variants, there are 91 putatively functional Grs. Within these Gr genes in *Ae. aegytpi*, 41 Gr genes are expressed in the labellum and tarsi, and 12 of them have clear orthologs in

Anopheles gambiae, and 11 of those show conserved expression patterns in *Drosophila melanogaster* (Kent et al., 2008; Sparks et al., 2013). In *Ae. aegypti*, the expression of some Grs in the labella and tarsi is sex-specific. In males, genes such as *AaGr20e*, *AaGr27*, *AaGr36*, *AaGr39e*, and *AaGr43* are more abundantly expressed, whereas in females, *AaGr5*, *AaGr22*, *AaGr34*, *AaGr44*, and *AaGr56* are more highly enriched. The female-specific expressed Grs may be involved in the detection of cues from blood-feeding hosts (Sparks et al., 2013).

There are ten *Aedes aegypti* Gr genes that have been identified as sugar receptor (SR) genes based on sequence homology with the eight *Drosophila* SRs. Among these, seven are predicted to be functional SRs, while three are pseudogenes (Kent & Robertson, 2009; Sparks et al., 2013). However, these *Ae. aegypti* SRs do not follow a straightforward pattern of homology to the eight *Drosophila* Grs. Instead, they exhibit a complex evolutionary history involving gene duplication and loss events (Kent & Robertson, 2009). The *Drosophila* lineage, which is homologous to the *Ae. aegypti* female-specific expressing receptor *AaGr5* has been lost. Conversely, the *AaGr5* lineage corresponds to *AgGr16* in *Anopheles gambiae* and *CpGr5* in *Culex pipiens* (Kent & Robertson, 2009). Phylogenetic comparisons within the *Culicidae* family indicate that *Toxorhynchites amboinensis*, a strictly phytophagous sister taxon of *Ae. aegypti*—retains a relatively stable chemosensory gene repertoire, whereas *Ae. aegypti* possesses a greater number of Gr genes (Zhou et al., 2014). Notably, no *T. amboinensis* Grs show homology to *AaGr5*. The closest sugar-related receptors identified in *T. amboinensis* are *TaGr6*, *TaGr7*, *TaGr11.1*, and *TaGr11.2*, suggesting that an *AaGr5* homologue is absent in this phytophagous species. This divergence may be associated with the evolutionary specialisation of receptors involved in blood-feeding behaviour. However, the function of this putative female-specific sugar receptor, *AaGr5,* remains unknown. Its specific sugar ligands—whether it responds to a single type of sugar or to multiple sugars—as well as any other potential stimuli it may detect, have yet to be fully characterised.

1.5 The challenge

In order to improve mosquito control and understand vector evolution, we need to further investigate the regulation and evolution of the sugar-seeking behaviour of mosquitoes at the gene level, and link the behaviour with individual genes. The function of *Gr5* in *Aedes aegypti* is currently unknown, and it is also difficult to predict due to the absence of homology in *Drosophila* and in the phytophagous mosquito *T. amboinensis*. The function of *Gr5* in *Aedes aegypti* may provide further insight into the behavioural transition of mosquitoes from phytophagy to hematophagy. This project will address the challenge of linking the sugar detection in the sugar-feeding behaviour of *Ae. aegypti* to their genetic background, and

explore the impact of preference for different sugar diets on the amount of food imbibed.

1.6 The approach

In this project, behavioural assays were used to measure the feeding responses of wildtype *Aedes aegypti* and a *ΔGr5* mutant line to sucrose, glucose, fructose, and trehalose. Mosquito feeding behaviour can be evaluated qualitatively or quantitatively by using controlled variable experiments to investigate the factors that influence feeding. In the previous assessment of diet choice by *Ae. aegypti*, two-choice assays were applied with sugar diets containing yellow and blue dyes. After the assays, the colour of the mosquito midguts was scored, and a choice index of the diets was calculated. *Aedes aegypti* of the Rockefeller strain consistently showed a choice for higher concentrations of the same sugar, and for different sugars with the same concentration, the order of the choice by *Ae. aegypti* Rockefeller was sucrose > trehalose > fructose > glucose (Ignell et al., 2010). Adding dyes to mosquito food not only allows for qualitative assessment of feeding preferences but also enables quantitative measurement of food containing an inert dye imbibed through optical absorbance (Dawit et al., 2022). Although the current project used no-choice assays, in which mosquitoes were presented with a single food source at a time, the inclusion of dyes in the food facilitated the collection of additional data, such as the amount of food imbibed regarding feeding behaviour, for further analysis.

The FlyPAD quantitatively analyses *Aedes aegypti* feeding behaviour by measuring feeding timing and frequency, parameters correlated with food intake (Henriques-Santos et al., 2023) and sipping ability (Kim et al., 2013). To address the lack of cohesive dynamic and quantitative monitoring of mosquito feeding, the FlyPAD system, developed for *Drosophila* (Itskov et al. 2014), was modified to increase the chamber height and food well diameter to accommodate standing and walking female *Ae. aegypti* mosquitoes (Henriques-Santos et al., 2023). There are ten feeding factors that the FlyPAD system can collect from electrical signals generated by mosquitoes bridging a circuit between the electrodes on the platform and the bottom of the food well by imbibing the sugar diet. The factors include the number of, duration (s) of, and time (s) between sips, as well as feeding bursts and activity bouts. Feeding bursts by *Drosophila* were defined as three or more consecutive sips, and the activity bouts were defined as a set of two or more feeding bursts. These parameters simulate the process by which *Drosophila* evaluate food palatability and proceed to feeding once food is encountered. Whether these parameters will be informative in the mosquito remains to be observed.

1.7 Aims and objectives

The overall aim of this project was to investigate the feeding dynamics of a *Gr5* gene knockout line *ΔGr5* (*Gr5*$^{-/-}$) of *Aedes aegypti* on sucrose, glucose, fructose, and trehalose using the FlyPAD system.

The first objective was to design and optimise the method based on the FlyPAD system for measuring dietary imbibement in *Ae. aegypti*.

The second objective was to compare the feeding behaviours of the Gr5 knockout mutant line Δ*Gr5* with those of two wild-type *Ae. aegypti* strains Rockefeller and Liverpool across four sugars.

The third objective was to formulate hypotheses regarding the function of the *Gr5* gene based on the similarities and differences in sugar-feeding behaviour between the Δ*Gr5* line and the wildtype Liverpool strain.

Hypothesis: If the function of *Aedes aegypti Gr5* is to detect a specific sugar among sucrose, glucose, fructose, and trehalose, then *ΔGr5* (*Gr5*$^{-/-}$) mutant line would exhibit a loss of sensitivity to that particular sugar.

2. Methods and materials

2.1 Mosquito rearing

Laboratory colonies of *Aedes aegypti* (Liverpool strain), *Ae. aegypti* (Rockefeller strain) and *Ae. aegypti* (*ΔGr5* (*Gr5*$^{+/-}$) line) were reared in the insectary under the conditions of 25 ± 2 °C, 65 ± 5% relative humidity with a 12 h:12 h light: dark light cycle. The larvae were reared in trays (20 cm × 18 cm × 7 cm) with 500 mL of distilled water and fed 10 mg of Tetramin® fish food (Tetra Werke, Germany) per larva per day. Pupae were collected into 30 mL cups (Nolato Hertila, Åstorp, Sweden) containing distilled water and transferred to Bugdorm cages (30 cm× 30 cm × 30 cm; MegaView Science, Taiwan) to emerge. Sucrose solution (10% w/v) was provided to *Ae. aegypti* adults *ad libitum* after emergence.

For oviposition, 4 days post-emergence (dpe) host-seeking females were starved without access to water or sucrose solution overnight. The following day, the females were provided with defibrinated sheep blood (Håtunalab, Bro, Sweden) using a membrane feeding system (Hemotek Discovery Workshops, Accrington, UK). The wildtype strains of *Ae. aegypti* were provided with blood to feed for 1 h, whereas the *Ae. aegypti ΔGr5* line, which is more reluctant to blood feed, was fed for 12 h. After blood feeding, mosquitoes were provided with 10% sucrose. At 24 h after the blood meal, 30 mL cups (Nolato Hertila, Åstorp, Sweden) filled with distilled water and lined with filter paper were provided for oviposition over the course of 24 h. The filter paper containing eggs was blotted dry with tissue paper and stored in trays (20 cm × 18 cm × 7 cm) for at least 7 days before being put into distilled water for hatching.

2.2 *Aedes aegypti ΔGr5* homozygous mutant line

2.2.1 Genotyping of *Aedes aegypti ΔGr5* mutant line

The *Ae. aegypti ΔGr5* mutant line was kept in rearing as heterozygote for the *Gr* allele (*Gr5*$^{+/-}$), due to the tendency to lose the mutation over generations, as well as to abnormal sugar and blood feeding behaviours in homozygous mutant lines. The *Gr5*$^{+/-}$ mosquitoes were separated based on their sex at 2 dpe. Adults were genotyped using the Phire Tissue Direct PCR Master Mix kit (ThermoFisher Scientific, USA). One meso- and one metathoracic leg from each virgin mosquito (2-3 dpe) were transferred into 1.5 mL microfuge tubes. A DNA extraction reaction, which contained 20 µL Dilution Buffer and 0.5 µL DNA Release Additive (Tissue Direct PCR Master Mix, ThermoFisher Scientific, USA), was added into each microfuge tube with legs. Pestles were used to grind the legs until the solution

became a suspension. The reactions were incubated for 10-15 min at room temperature, and then were placed into a preheated (98 °C) heating block for 2 min.

Gene-specific primers (Table 1) were designed to amplify genomic fragments of 355 bp indicating the wild type allele, and 261 bp indicating the knockout allele, for *Gr5*. The PCR reaction was carried out in a 20 µL volume containing 10 µL Direct PCR master mix (2X) (ThermoFisher Scientific, USA), 0.3 µL of each primer (10µL) and 9.4 µL of the DNA extracts, and performed according to the manufacturer's instructions in a thermal cycler (Bioer Technology, Hangzhou, China). The PCR products were amplified for 50 cycles using the following programme: 98 °C for 5 min; 98 °C for 5 s, 61.5 °C for 30 s, 72 °C for 90 s; and a final extension at 72 °C for 2 min. Products from the PCR were subjected to electrophoresis on TAE-agarose gels (50 V, 60 min), and visualised using the GelDoc Go Gel Imaging System (Bio-Rad, California, USA) to determine the genotype of individual mosquitoes.

Table 1. Primer sequences

Name	Primer sequences	Tm (°C)
AaGr5f	5′-GCTGAGCCAGAATTGACGC-3′	58.8
AaGr5r	5′-CATCATCGTGTACAGCATCCG-3′	59.8

Aa: Aedes aegypti*; f: forward; r: reverse,* Gr5*: gustatory receptor protein encoding gene, accession number for* Aedes aegypti Gr5 *gene is AAEL000043*

2.2.2 The verification of *Aedes aegypti ΔGr5* homozygous mutant line

On the agarose gel, homozygous mutants were identified by a band at 261 bp (Fig. 1A, blue arrow), whereas the homozygous wildtype displayed a band at 355 bp (Fig. 1A, yellow arrow). Individuals showing both bands were classified as heterozygotes (Fig. 1A, green arrow). Homozygous *ΔGr5* mutants, maintained for no more than five generations, were kept in cages and used for the feeding assays. After the assays, mosquitoes were collected and subjected to PCR analysis again to confirm that all individuals used in the feeding assays were homozygous (Fig. 1B). A difference in migration distance occurred between the bands on the two agarose gels, which resulted from different electrophoresis durations. During the establishment of the *ΔGr5* mutant line (Fig. 1A), electrophoresis was run for a shorter time (60 min), whereas genotyping conducted after the assays was run for a longer time (90 min) (Fig. 1B). The shorter duration was used during the build-up of the mutant line because a large number of mosquito samples were processed, allowing samples with unclear bands to be discarded. In contrast, the post-assay genotyping aimed to confirm that the tested mosquitoes remained within the mutant line, so a longer electrophoresis time was used to achieve clearer band separation.

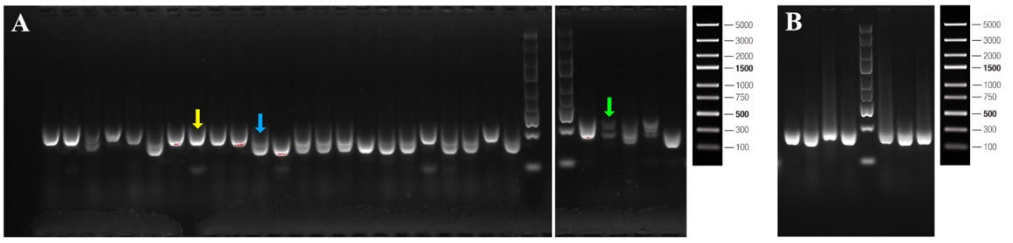


Figure 1. PCR amplification and product validation of Aedes aegypti ΔGr5 *mutant*

*(**A**) Agarose gel electrophoresis results showing PCR amplification of the* Gr5 *fragment in offspring from the* Aedes aegypti ΔGr5 *mutant heterozygous line. The blue arrow indicates the 261 bp fragment of the homozygous* Ae. aegypti ΔGr5 *mutant, the yellow arrow indicates the 355 bp fragment of the homozygous wild-type, and the green arrow indicates the PCR product of the heterozygote. (**B**) A PCR validation of the* Gr5 *fragment in* Ae. aegypti ΔGr5 *mutant mosquitoes after feeding assays, showing consistency of amplified fragments across different samples. DNA molecular weight markers (100-5000 bp) are shown on the right.*

2.3 Sugar diets for behavioural assays

The sugar diets of sucrose, glucose, and fructose (0.1 µM to 1 M in decadic steps in distilled water), as well as trehalose (0.01 µM to 100 M) contained blue dye (1 mg mL^{-1} Xylene cyanol FF; Sigma-Aldrich, Germany) to facilitate quantification of the amount of each sugar solution imbibed using a spectrophotometer-based microplate reader (Dawit et al., 2022).

2.4 Modification of the FlyPAD system

The FlyPAD system (HS902300, Pavel Itskov & Ekaterina Vinnik LDA, Cascais, Portugal) was designed as a method to monitor and quantitatively analyse feeding behaviour in *Drosophila* automatically (Itskov et al., 2014; Fig. 2A), and has been modified for use with mosquitoes (Henriques-Santos et al., 2023; Fig. 2B) with larger bottom electrodes (electrode 2, blue arrow) to carry the diet drop and additional plexiglass plates (yellow arrow) for heightening the arenae for mosquitoes to walk. To address the issue of false signals in the assays caused by mosquitoes accidentally triggering the electrodes while walking in the arena and touching the diet drops, a black O-ring (Biltema, Sweden) (Fig. 2C) was placed on the circular electrode (Electrode 1) of the arena (Fig. 2D).

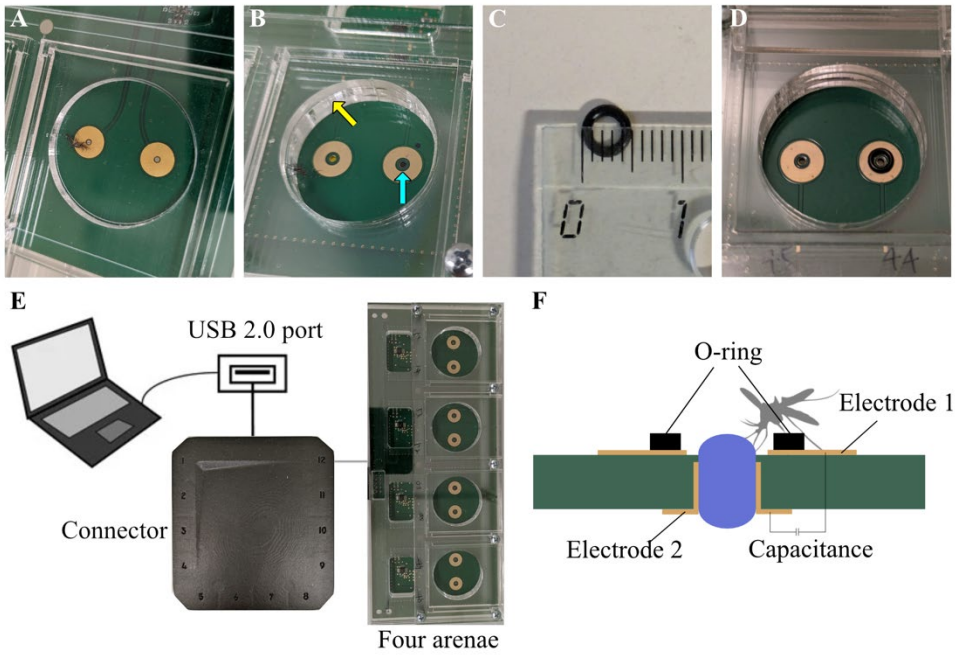


Figure 2. The FlyPAD system

*(**A**) The arenae of the FlyPAD system for* Drosophila*. (**B**) The arenae of the FlyPAD system for* Aedes aegypti*. The yellow arrow shows the additional plexiglass plate that was added to modify the height for* Ae. aegypti*, and the blue arrow shows the enlarged bottom electrodes (electrode 2) for carrying a larger drop of diet. (**C**) The O-ring (inner diameter: 3 × 1 mm, nitrile rubber). (**D**) The arena with the O-ring applied on the electrode 1. (**E**) Schematic of the complete FlyPAD system, modified from Itskov et al. (2014). (**F**) Concept for the use of capacitance measurement to monitor the interaction of the mosquito with the diet. When the mosquito landed on electrode 1 (golden ring) and imbibed the diet on electrode 2, the interaction was detected as a change in capacitance between the two electrodes.*

Briefly, on each multiplexing board, the FlyPAD system (Fig. 2E) has four behavioural chambers (arenae) consisting of two golden flat ring electrodes (channels) centred around, into which 2 µL of sugar diet was delivered into one of the holes in the centre of one of the ring electrodes, since this was a no-choice assay. When the mosquitoes were standing on electrode 1 and imbibing the diet drop, electrode 1 on where the mosquitoes were standing and electrode 2 containing the diet would form a circuit, and the feeding behaviour was recorded as an electrical signal (Fig. 2F). The electrical signals were defined as three types of feeding-related events, each characterised by number, duration, and interval. A capacitance signal exceeding the set threshold was defined as an activity bout. Based on processed capacitance traces, another absolute threshold was used to define the start (proboscis contact with food) and end (proboscis withdrawal) of a sip. When three or more consecutive sips occurred, and the intervals between these sips were shorter than twice the median inter-sip interval, they were defined as a feeding burst.

2.5 Behavioural assays using the FlyPAD system

2.5.1 Pilot behavioural assays

Pilot behavioural assays were conducted to establish the suitable conditions for the mosquitoes in the assays. Water was removed for the female *Aedes aegypti* Rockefeller mosquitoes before they became 2 dpe old to dehydrate for 0, 6, 12, and 24 h, and then provided with 10 mM sucrose solution containing 1 mg·mL^{-1} Xylene Cyanol for feeding behavioural assays using the FlyPAD system with 28 replicates. After the assays, the mosquitoes were placed in 1.5 mL centrifuge tubes individually and frozen at - 20 °C overnight, then 250 µL distilled water was added to the microfuge tubes and the samples were gently disrupted with a disposable pestle. The samples were centrifuged at 14534 rcf for 10 minutes (10000 rpm, AccuSpin Micro 17, Fisher Scientific, Waltham, Massachusetts, USA). The absorbance of the dye was measured (620 nm) using a spectrophotometric microplate reader (SPECTROStar® Nano, BMG Labtech, Ortenberg, DE). The results of the assays were analysed and ranked based on Spearman's correlation.

After 24 h of dehydration, the absorbance at 620 nm was most strongly correlated with total bout duration (Spearman's ρ = 0.7369) (Fig. 3A), followed by correlations observed after 12 h of dehydration between absorbance and both burst duration and total bout duration (Fig. 10, *Supplementary Material 1*). Across the dehydration series, feeding performance was higher in mosquitoes dehydrated for 12 and 24 h, with lower numbers of non-feeders (Fig. 3B).

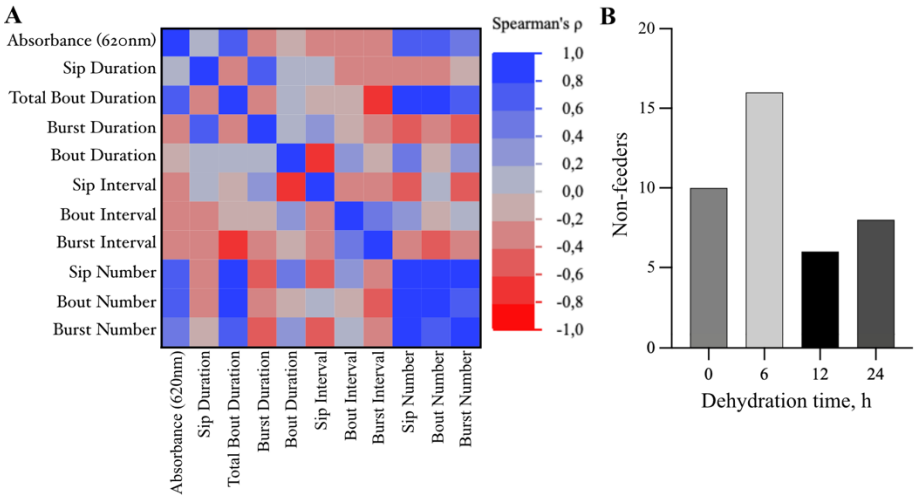


Figure 3. The results of the pilot behavioural assays

*(**A**) Spearman's correlation heatmap of the correlations between absorbance and ten feeding behavioural parameters of the assays in* Aedes aegypti *Rockefeller females after 24 h dehydration and given with 10 mM sucrose solution (n = 28). (**B**) The number of non-feeders in* Aedes aegypti *Rockefeller females that were exposed to different dehydration*

durations (0, 6, 12, 24 h) and given 10 mM sucrose solution, showing food acceptance under varying dehydration conditions.

2.5.2 Behavioural assays

Mosquitoes (2 dpe) used in behavioural assays emerged and were provided *ad libitum* access to water for 24 h, then the water was removed for 24 h. For the four arenae of each FlyPAD system, 2 µL of sucrose, glucose, fructose and trehalose solution in the same concentration containing 1 mg mL^{-1} blue dye (Xylene Cyanol FF) was placed on one of the electrode 2 of each arena (Fig. 2B) in order using a pipette. In each assay, ten FlyPAD systems were operated simultaneously, with every five systems at concentrations ranging from 0.1 mM to 1000 mM in decadic steps constituting one replicate. For each sugar at each concentration, the complete behavioural assay included 40 replicates. After initiating recording, individual mosquitoes (2 dpe) were placed in each arena, and data were recorded for 1 h. Mosquito feeding was assayed under red light (4-6 lux) at the environmental conditions stated above. Subsequently, mosquitoes were placed individually into microfuge tubes (1.5 mL) and stored at -20 °C until further analysis. After the behavioural assays, the remaining food in the arenae was removed using tissue, and then distilled water was gently applied to clean the electrodes. After the water was removed using tissue, 70% ethanol was applied to clean and then removed using tissue or Q-tips.

2.6 Diet quantification

2.6.1 Threshold finding for the spectrophotometer

The detection threshold of the spectrophotometer (Multiskan FC Microplate Photometer, Thermo Scientific, USA) was established to assess the sensitivity of the device to the lowest dye concentrations used for the behavioural experiments. Volumes ranging from 0 to 0.1375 µL of a 1 $mg \cdot mL^{-1}$ Xylene Cyanol FF solution were added to 250 µL of distilled water, and the absorbance at 620 nm was subsequently measured using the spectrophotometer. When the volume of 1 $mg \cdot mL^{-1}$ Xylene Cyanol FF solution was equal to or greater than 0.025 µL, the absorbance at 620 nm was found to increase linearly with feeding volume; however, 0.025 µL did not show a significant difference compared to distilled water without dye (Fig. 4). Therefore, after calculation, a food imbibed amount greater than 0.025 µL was considered valid.

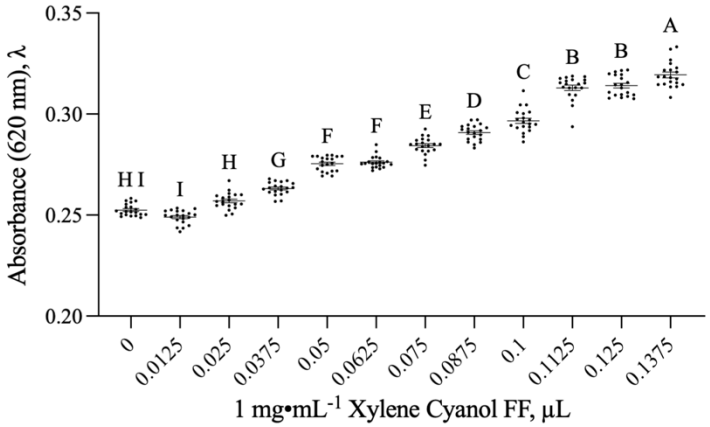


Figure 4. The relationship between the volume of 1 mg·mL⁻¹ Xylene Cyanol FF and 620 nm absorbance

The relationship between absorbance at 620 nm and different volumes of 1 mg·mL⁻¹ Xylene Cyanol FF. Different letters indicate significant differences among groups (One-way ANOVA, $p < 0.05$).

2.6.2 The standard curves

Primary standard curve

A primary stand curve has been made to establish a quantitative relationship between the dye concentration (1 mg·mL⁻¹ Xylene Cyanol FF) and absorbance in the mosquito homogenate. Unfed female mosquitoes were placed individually into 1.5 mL microfuge tubes and frozen at - 20 °C. Distilled water (250 µL) was added to the microfuge tubes, and the frozen mosquitoes were gently disrupted with disposable pestles. Volumes ranging from 0.125 to 2 µL of 1 mg·mL⁻¹ Xylene Cyanol solution were added into the tubes. The samples were centrifuged at 14534 rcf for 10 minutes (10000 rpm, AccuSpin Micro 17, Fisher Scientific, Waltham, Massachusetts, USA). After centrifuging, the supernatant (200 µL) of the samples was transferred to a 96-well microplate (Sigma-Aldrich) to generate the standard curves (Fig. 5A).

Secondary stand curve

Once the 620 nm absorbance of the supernatant of the mosquitoes (24 h dehydration, 2 dpe) had been measured, these were compared to the primary standard curves to calculate the volume imbibed by each mosquito. The total bout duration obtained after 24 h of dehydration in *Aedes aegypti* was selected and linearly fitted against the food imbibed amount calculated from the primary standard curve, and a secondary standard curve was produced (Fig. 5B). The food imbibed amount calculated based on the secondary standard curve was used in the

comparison of the feeding amount in *Ae. aegypti* wildtype Rockefeller and Liverpool strains and *ΔGr5* (*Gr5*$^{-/-}$) mutant line.

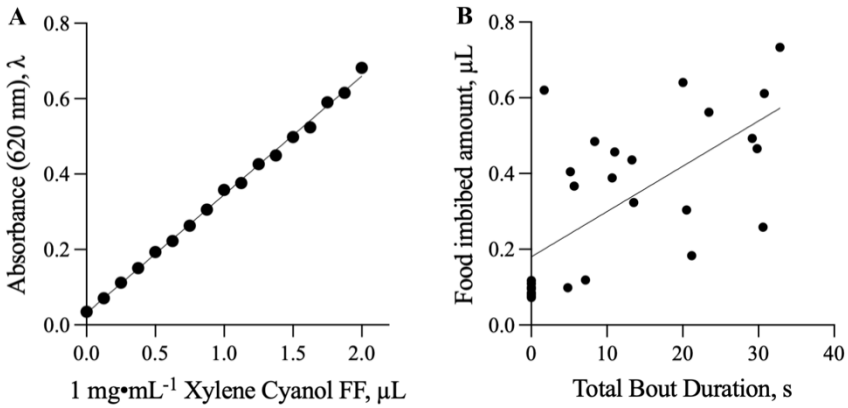


Figure 5. The standard curves

*(**A**) Primary standard curve: Linear regression of the volume of 1 mg·mL⁻¹ Xylene Cyanol FF (µL) against absorbance (620 nm), indicating high consistency in dye quantification (R^2 = 0.9977). (**B**) A positive correlation was observed between total bout duration (s) and food imbibed amount (µL), which formed the secondary standard curve (R^2 = 0.4354).*

2.6.3 Data analysis

The data collected during the behavioural assays using the FlyPAD system were analysed in Matlab (Mathworks, Natick, U.S.). The source code was from http://www.flypad.pt (Itskov et al., 2014). For the data collected during FlyPAD assays, the activity bouts are defined as time periods which surpassed a threshold of 500 ms, and the feeding bursts were defined as three or more consecutive sips with an inter-sip interval of less than two median inter-sip intervals of each mosquito (Itskov et al., 2014). The data collected were analysed by two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test using GraphPad Prism 10.6 (GraphPad Software, Boston, MA, USA) and JMP (JMP SAS Institute Inc., Cary, NC, USA).

3. Results

3.1 Sugar feeding is consistent across wildtype strains

The *Aedes aegypti* wildtype strains, Liverpool and Rockefeller, demonstrated similar sugar-feeding patterns to the disaccharides sucrose and trehalose, and the monosaccharides glucose and fructose using the FlyPAD system. Based on the average amount of food imbibed, no significant differences were found between the two wildtype lines for sucrose, glucose, fructose (Fig. 6A), and trehalose (Fig. 6B). In both lines, there was a trend that food imbibed amount increased with sugar concentration between 10 mM and 100 mM, but declined at 1000 mM, indicating that the imbibed peak for these three sugars occurred between 10 mM and 100 mM, but this trend was not significant. Trehalose was tested across a different concentration range (0.01-100 mM) for the Rockefeller strain, in this case, the food imbibed amount had an trend of increasing with concentration but still not significant (Fig. 6B). However the comparison of another parameter, sip number, which was also positively correlated to the 620 nm absorbance (Fig. 3A), showed a dose response of Rockefeller and Liverpool strain when the mosquitoes fed on sucrose, glucose and fructose (Fig. 7A). The feeding peak occurred on the concentration of 100 mM for sucrose, glucose and fructose, but for trehalose there was no dose response (Fig. 7B). These results suggest that there were no obvious differences in feeding behaviour between *Ae. aegypti* Rockefeller and Liverpool strains across the four sugars.

3.2 Gustatory receptor 5 regulates sugar sensitivity

Aedes aegypti ΔGr5 line imbibed significantly reduced on sucrose, glucose, fructose, and trehalose compared to the wildtype Liverpool strain during feeding assays using the FlyPAD system (Fig. 6C, 7C). And the dose response exhibited by the Liverpool strain when feeding on sucrose, glucose, fructose, and trehalose from the concentration range from 0.1 mM to 1000 mM was completely lost in the *ΔGr5* line (Fig. 7C).

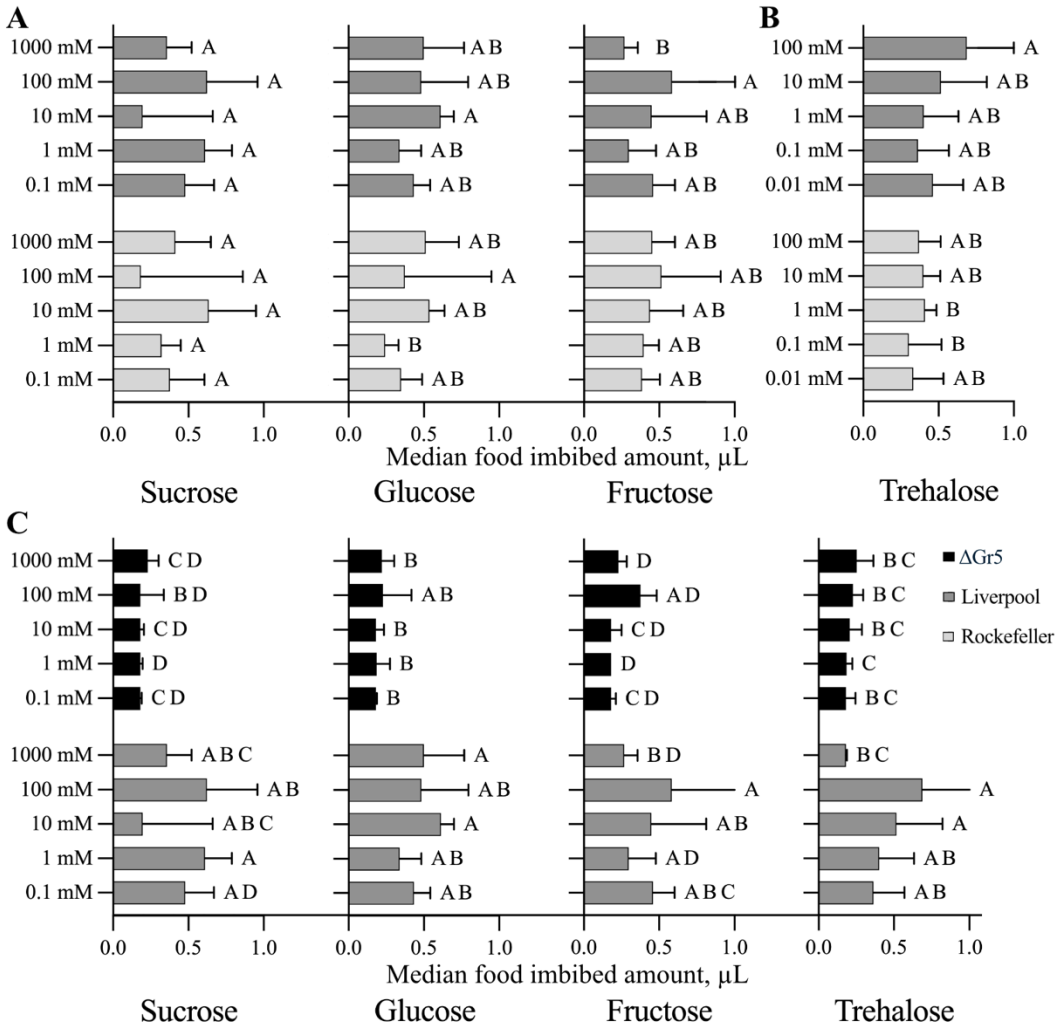


Figure 6. Comparison of median food imbibed amount across sugar concentrations and mosquito lines.

*(**A**) Median food imbibed amount (µL) of* Aedes aegypti *Rockefeller and Liverpool females on sucrose, glucose, and fructose at different concentrations (0.1-1000 mM). (**B**) Median food imbibed amount (µL) of* Ae. aegypti *Rockefeller and Liverpool females on trehalose at different concentrations (0.01-100 mM). (**C**) Comparison of the median food imbibed amount (µL) on four sugars between* Aedes aegypti *Liverpool and* ΔGr5 *lines at the same sugar concentrations (0.01-100 mM). Light grey bars represent Rockefeller, dark grey bars represent Liverpool, and black bars represent* ΔGr5. *Bars represent the median ± 95% confidence interval (CI). Within the same sugar treatment, different letters indicate significant differences among concentrations or mosquito lines (two-way ANOVA, $p < 0.05$, $n = 40$).*

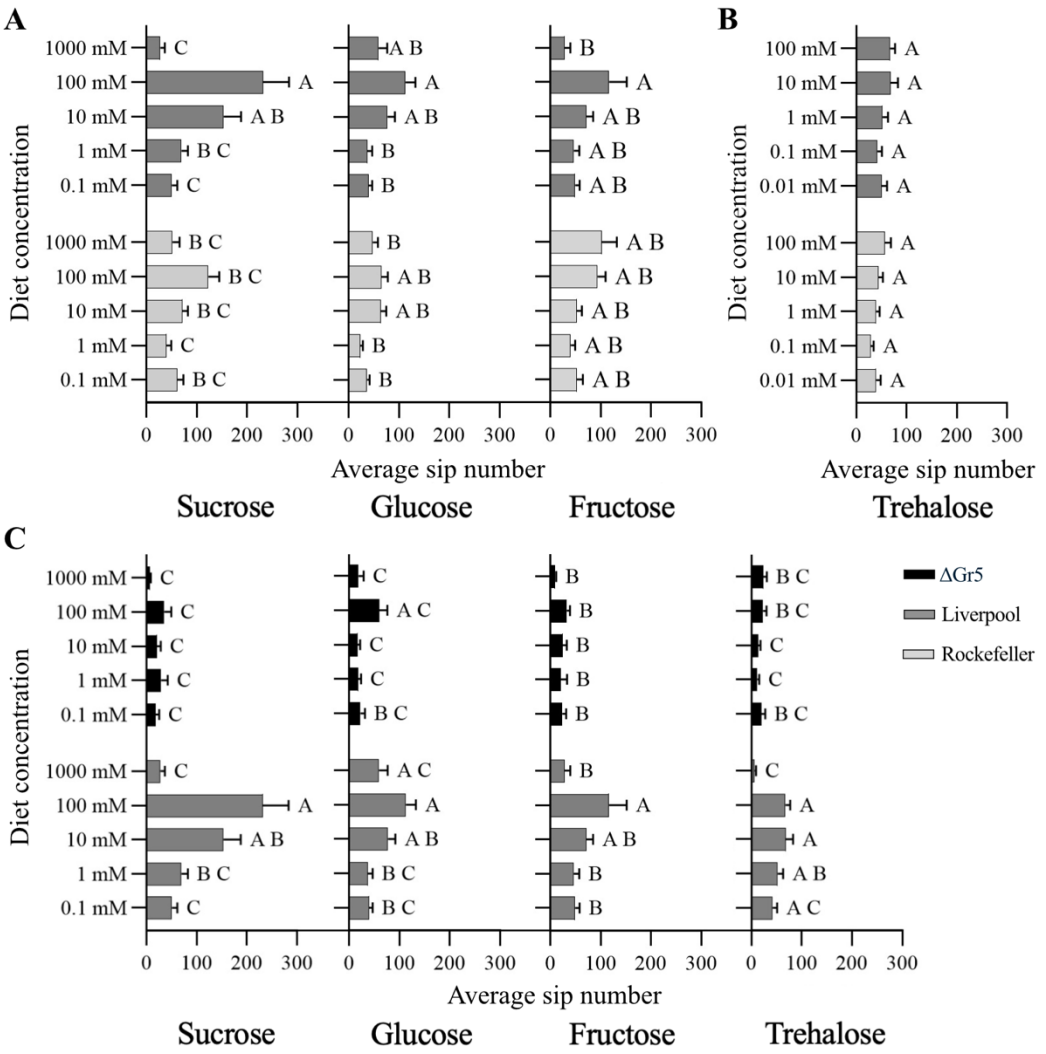


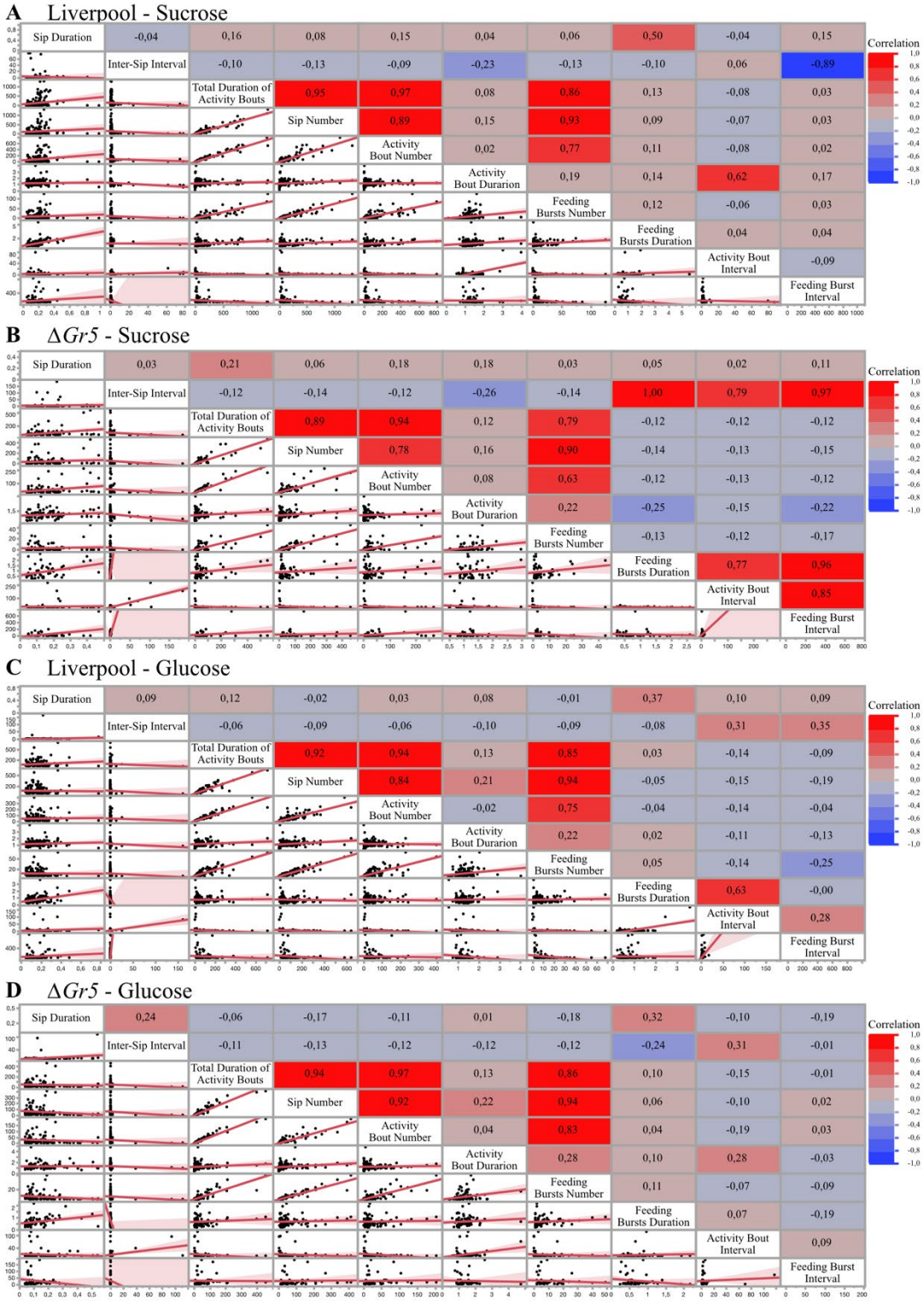
Figure 7. Comparison of average sip number across sugar concentrations and mosquito lines.

*(**A**) Average sip number of* Aedes aegypti *Rockefeller and Liverpool females on sucrose, glucose, and fructose at different concentrations (0.1-1000 mM). (**B**) Average sip number of* Ae. aegypti *Rockefeller and Liverpool females on trehalose at different concentrations (0.01-100 mM). (**C**) Comparison of the average sip number on four sugars between* Aedes aegypti *Liverpool strain and* ΔGr5 *lines at the same sugar concentrations (0.01-100 mM). Light grey bars represent Rockefeller, dark grey bars represent Liverpool, and black bars represent* ΔGr5. *Bars represent the mean with SEM. Within the same sugar treatment, different letters indicate significant differences among concentrations or mosquito lines (two-way ANOVA, $p < 0.05$, $n = 40$).*

Analysis of additional feeding assay parameters revealed further differences between the *Aedes aegypti* Liverpool strain and *ΔGr5* line. Both lines exhibited a strong positive correlation between the total duration of activity bouts and the sip number across the four sugars (Fig. 8A-H). Overall, Liverpool showed similar responses to sucrose and fructose, with feeding bursts playing no obvious role (Fig. 8A, C), which suggested that the feeding behaviour was formed mostly as sips and

a few bursts. For glucose, feeding bursts contributed slightly more (Fig. 8B). Feeding behaviour on trehalose in Liverpool was more variable, with feeding bursts showing the weakest correlations with other parameters (Fig. 8D).

For sucrose, compared with Liverpool (Fig. 8A), the feeding bursts of *ΔGr5* (Fig. 8B) showed stronger correlations with other parameters, particularly between feeding burst duration and activity bout inter-bout intervals. For glucose, *ΔGr5* (Fig. 8C) displayed feeding parameters similar to Liverpool (Fig. 8D). For fructose, feeding bursts of *ΔGr5* (Fig. 8E) had completely different correlations with other parameters compared to Liverpool (Fig. 8F), which suggested that the bursts were sometimes very long and sometimes very short. In the feeding assays on trehalose, feeding bursts of *ΔGr5* (Fig. 8H) exhibited a similar modular pattern to it when *ΔGr5* were feeding on sucrose: inter-sip intervals, activity bout intervals and feeding burst intervals were strongly positively correlated. However, unlike sucrose, when *ΔGr5* were feeding on trehalose, the inter-sip intervals and feeding burst durations were negatively correlated.


A Liverpool - Sucrose
Sip Duration
-0,04
0,16
0,08
0,15
0,04
0,06
0,50
-0,04
0,15
Inter-Sip Interval
-0,10
-0,13
-0,09
-0,23
-0,13
-0,10
0,06
-0,89
Total Duration of Activity Bouts
0,95
0,97
0,08
0,86
0,13
-0,08
0,03
Sip Number
0,89
0,15
0,93
0,09
-0,07
0,03
Activity Bout Number
0,02
0,77
0,11
-0,08
0,02
Activity Bout Durarion
0,19
0,14
0,62
0,17
Feeding Bursts Number
0,12
-0,06
0,03
Feeding Bursts Duration
0,04
0,04
Activity Bout Interval
-0,09
Feeding Burst Interval
Correlation
B ΔGr5 - Sucrose
Sip Duration
0,03
0,21
0,06
0,18
0,18
0,03
0,05
0,02
0,11
Inter-Sip Interval
-0,12
-0,14
-0,12
-0,26
-0,14
1,00
0,79
0,97
Total Duration of Activity Bouts
0,89
0,94
0,12
0,79
-0,12
-0,12
-0,12
Sip Number
0,78
0,16
0,90
-0,14
-0,13
-0,15
Activity Bout Number
0,08
0,63
-0,12
-0,13
-0,12
Activity Bout Durarion
0,22
-0,25
-0,15
-0,22
Feeding Bursts Number
-0,13
-0,12
-0,17
Feeding Bursts Duration
0,77
0,96
Activity Bout Interval
0,85
Feeding Burst Interval
Correlation
C Liverpool - Glucose
Sip Duration
0,09
0,12
-0,02
0,03
0,08
-0,01
0,37
0,10
0,09
Inter-Sip Interval
-0,06
-0,09
-0,06
-0,10
-0,09
-0,08
0,31
0,35
Total Duration of Activity Bouts
0,92
0,94
0,13
0,85
0,03
-0,14
-0,09
Sip Number
0,84
0,21
0,94
-0,05
-0,15
-0,19
Activity Bout Number
-0,02
0,75
-0,04
-0,14
-0,04
Activity Bout Durarion
0,22
0,02
-0,11
-0,13
Feeding Bursts Number
0,05
-0,14
-0,25
Feeding Bursts Duration
0,63
-0,00
Activity Bout Interval
0,28
Feeding Burst Interval
Correlation
D ΔGr5 - Glucose
Sip Duration
0,24
-0,06
-0,17
-0,11
0,01
-0,18
0,32
-0,10
-0,19
Inter-Sip Interval
-0,11
-0,13
-0,12
-0,12
-0,12
-0,24
0,31
-0,01
Total Duration of Activity Bouts
0,94
0,97
0,13
0,86
0,10
-0,15
-0,01
Sip Number
0,92
0,22
0,94
0,06
-0,10
0,02
Activity Bout Number
0,04
0,83
0,04
-0,19
0,03
Activity Bout Durarion
0,28
0,10
0,28
-0,03
Feeding Bursts Number
0,11
-0,07
-0,09
Feeding Bursts Duration
0,07
-0,19
Activity Bout Interval
0,09
Feeding Burst Interval
Correlation

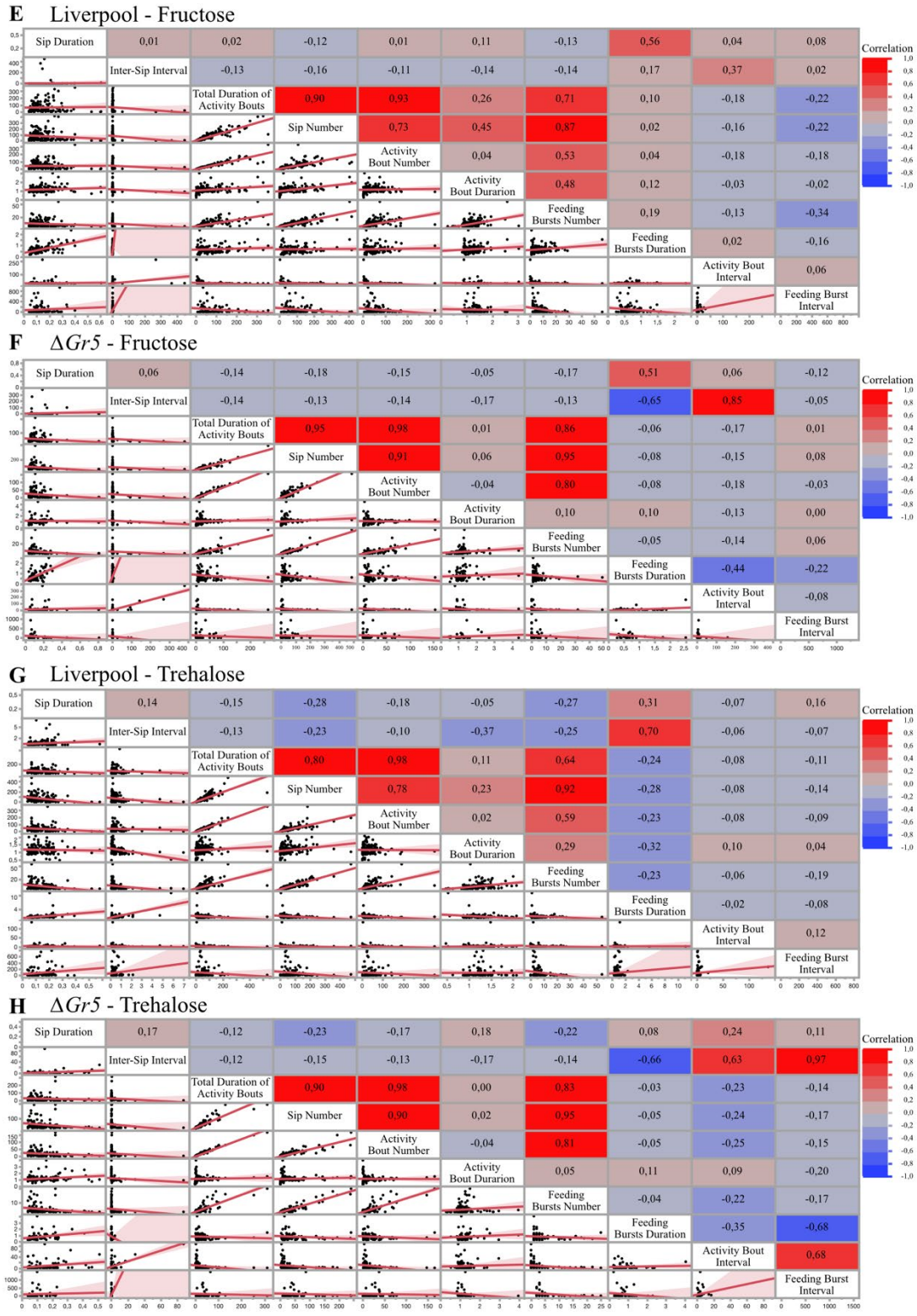


Figure 8. The correlation of all FlyPAD system parameters of Aedes aegypti *Liverpool and* ΔGr5 *feeding on sucrose, glucose, fructose and trehalose.*

Correlations among the FlyPAD system feeding behavioural parameters of Aedes aegypti *Liverpool and* ΔGr5 *females across four sugars. **(A-B)** Correlation analysis for sucrose in Liverpool (A) and* ΔGr5 *(B) females. **(C-D)** Correlation analysis for glucose in Liverpool*

(C) and ΔGr5 *(D) females. (**E-F**) Correlation analysis for fructose in Liverpool (E) and* ΔGr5 *(F) females. (**G-H**) Correlation analysis for trehalose in Liverpool (G) and* ΔGr5 *(H) females. The heatmaps show the correlation coefficients between sip duration, inter-sip interval, total duration of activity bouts, sip number, activity bout number, activity bout duration, feeding burst number, feeding burst duration, activity bout interval, and feeding burst interval. Scatterplots show individual data points with fitted regression lines. Red indicates positive correlations and blue indicates negative correlations. All behavioural assay data points were recorded from individual females (n = 40).*

3.3 Different feeding patterns of *ΔGr5* and Liverpool

Comparison of the correlations among ten parameters between *Aedes aegypti* Liverpool and *ΔGr5* revealed similarities and differences in feeding behaviour. Across the four sugars, two distinct feeding patterns could be observed based on the correlations among all parameters.

The first pattern, which could be referred to as the sip-driven feeding pattern (Fig. 9A), was present when the Liverpool strain fed on sucrose, glucose, fructose, and trehalose, and when the *ΔGr5* line fed on glucose. In this pattern, there was a strong positive correlation between the total duration of activity bouts and the number of sips. The total duration of activity bouts and the corresponding amount of food imbibed were primarily driven by the number of sips and the inter-sip intervals. Since feeding bursts are defined on the basis of sip events, and sips occur within activity bouts, the number of sips was consistently strongly positively correlated with both the number of feeding bursts and the number of activity bouts. The amount of food imbibed scaled linearly with the number of sips, while sip duration showed only weak correlations with other parameters, indicating that in both lines sip duration was not substantially influenced by sugar type.

The second feeding pattern, which could be referred to as the burst-pause feeding pattern (Fig. 9B), was observed when the ΔGr5 line fed on the two disaccharides, sucrose and trehalose. In this pattern, the total duration of activity bouts, number of sips, number of activity bouts, and number of feeding bursts remained strongly positively correlated with one another. However, in contrast to the sip-driven pattern, inter-sip intervals, activity bout intervals, and feeding burst intervals were highly positively correlated. This suggests that in this feeding pattern most sip events occurred collectively as feeding bursts, whereas isolated single sips were greatly reduced.

The *ΔGr5* line also exhibited a different feeding pattern when feeding on fructose compared with the Liverpool strain, but it was not the same as the burst-pause pattern which *ΔGr5* fed on sucrose and trehalose. When *ΔGr5* fed on fructose, only the correlations between inter-sip intervals and activity bout intervals increased significantly, whereas the correlation between inter-sip intervals and feeding burst intervals showed minimal change.

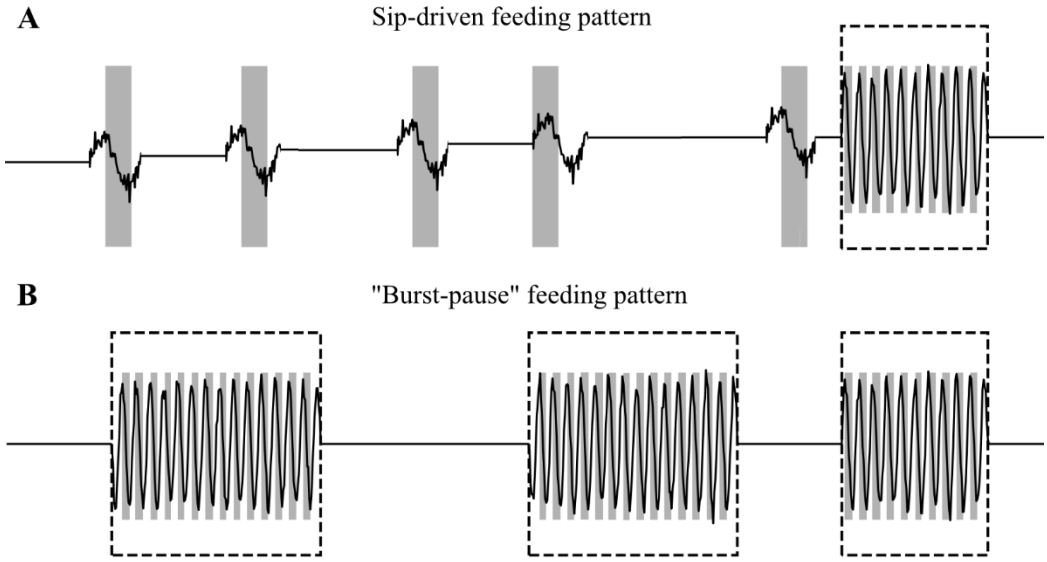


Figure 9. Two feeding patterns exhibited by the Aedes aegypti Liverpool strain and ΔGr5 mutant line in response to sugars, modified from Itskov et al. (2014).

*(**A**) Sip-driven feeding pattern. In the Liverpool line across all four sugars, and in the* ΔGr5 *mutant line when feeding on glucose, the feeding pattern consisted of both individual sips and bursts. However, this model was characterised by a higher number of sips and relatively fewer bursts. (**B**) "Burst-pause" feeding pattern. In the* ΔGr5 *mutant line when feeding on sucrose and trehalose, sips were fewer and scattered, while feeding occurred primarily in bursts composed of multiple consecutive sips. Grey bars mark the duration of individual sips, and dashed boxes highlight bursts.*

4. Discussion

4.1 The *Aedes aegypti* Liverpool strain and Rockefeller strain had similar feeding dynamics

The sugar feeding behaviours in response to the four sugars were consistent between the two wildtype *Aedes aegypti* strains, Liverpool and Rockefeller. Both strains exhibited a preference for higher sugar concentrations; however, unlike the results reported by Ignell et al. (2010) in mosquito sugar diet choice assays which the mosquitoes always preferred higher concentrations, the feeding peak when mosquitoes fed on sucrose, glucose and fructose occurred at 100 mM, which was particularly pronounced in the Liverpool strain. This difference may be attributed to the variation in the volume of sugar solution provided in the two experiments. In Ignell et al. (2010), approximately 300 µL of sugar solution was provided for the assays, whereas in this project, only 2 µL was provided. During the FlyPAD assays, crystallisation of the 1000 mM sugar solution was occasionally observed, which may have suppressed mosquito feeding. The similar feeding dynamics observed between the two wildtype strains indicate that long-term laboratory colonisation has not altered the Liverpool strain's feeding responses to the four sugars. This supports the reliability of the Liverpool-*ΔGr5* comparison and excludes potential bias caused by genetic divergence between laboratory-maintained mosquito strains.

4.2 Potential role of *Aedes aegypti* Gr5 in disaccharide detection

The loss of a functional Gr5 strongly suppresses the feeding behaviour of sucrose, glucose, fructose and trehalose in *Aedes aegypti*, suggesting a role for Gr5 in sugar detection. Both wildtype strains of *Ae. aegypti* exhibits strong and stable feeding responses to sucrose, glucose, and fructose, but a lower motivation to feed on trehalose, consistent with the typical distribution of sugar sources in *Ae. aegypti* natural diets-in which sucrose, fructose, and glucose are abundant in plant nectar, while trehalose is found mainly in insect-derived sources, such as honeydew and haemolymph (Liu et al., 2024; Thompson, 2003; van Neerbos et al., 2020). In contrast, the *ΔGr5* line, generated by the CRISPR/Cas9 knockout of the *Ae. aegypti Gr5* from the Liverpool strain genetic background showed altered correlations involving feeding burst parameters. The functional knockout of the *Gr5* gene shifted *Ae. aegypti* feeding behaviour on sucrose and trehalose toward a feeding burst-dominated "burst-pause" pattern, in which the structure of feeding bursts rather than continuous sips became the defining feature of sugar-feeding behaviour. The comparison of feeding behaviours between the *Ae. aegypti* Liverpool strain and *ΔGr5* line suggest that knockout of the *Gr5* gene impairs the sugar detection

pathways of two disaccharides, sucrose and trehalose, partially impairs fructose perception, and has little to no effect on glucose detection. The feeding behavioural dynamic change on sucrose and trehalose suggested that Gr5 in *Ae. aegypti* might have the function of detecting disaccharides.

Other than the feeding dynamic change of *Aedes aegypti ΔGr5* line feeding on disaccharides, the overall reduction of feeding of *Ae. aegypti ΔGr5* line on the four sugars might have provided behavioural evidence of how the Gr complexes assemble. Gr5 likely participates in the assembly of sucrose, glucose, fructose and trehalose receptor complexes in *Ae. aegypti*. The structure of insect Grs has been found to be tetrameric (Frank et al., 2024; Ma et al., 2024). Several studies have reported that the loss of a single Gr can result in the loss of sensitivity to multiple sugars. For example, deletion of *Gr64a* in *Drosophila* leads to reduced sensitivity to a range of sugars, including sucrose and maltose (Isono & Morita, 2010). In addition, the detection of certain sugars requires the involvement of multiple Grs; for example, *Gr64f* need to act in combination with *Gr64a* to enable *Drosophila* to detect sucrose, maltose, and glucose (Jiao et al., 2008). Research on insect gustatory receptor neurons has also shown that multiple Gr genes are co-expressed within sugar-responsive neurons (Dahanukar et al., 2007). Based on these previous findings and the observed feeding dynamic change of *Ae. aegypti ΔGr5* line across the four sugars compared with the wildtype strain in this project, it is possible that the sugar-detecting function of *Ae. aegypti* Gr5 involves co-expression with other Grs, and that Gr5 acts as a monomeric subunit contributing to the formation of a Gr tetramer.

4.3 The limitation within this project

The limitation within this project occurred with the secondary standard curve (Fig. 5B) that was used to quantitatively analyse the food imbibed amount through the total duration of activity bout, because the squared R value was very low (R^2 = 0.4354). Spectrophotometric analysis for each sample appears to be a better method for estimating volume imbibed instead of calculating using a parameter from the assays based on the standard curve. If the equipment allows, a method capable of precisely measuring the change in the body weight of mosquitoes before and after the assays could provide a more accurate way of quantifying food imbibe. This approach would eliminate the interference caused by the mosquitoes' own pigments when using the spectrophotometer.

Another limitation concerns the concentration ranges tested for trehalose. In the Rockefeller-Liverpool comparison, trehalose concentrations were limited to 0.01-100 mM, whereas other sugars were tested across 0.1-1000 mM. Although this range was corrected in the Liverpool-*ΔGr5* comparison to align all four sugars at

0.1-1000 mM, including a 1000 mM trehalose condition for the Rockefeller-Liverpool comparison would have provided a more complete picture of their dose responses. The initial restriction arose because trehalose has relatively low solubility in distilled water, and high concentrations easily crystallised on the FlyPAD electrodes during the assays that would last for one hour. Nevertheless, to improve analytical precision, the 1000 mM trehalose was subsequently included in the assays for Liverpool strain and *ΔGr5* line.

4.4 Future perspective

The feeding behavioural assays in this project were conducted as non-choice assays, in which mosquitoes were offered only one concentration of a single sugar per assay, which provides evidence of diet imbibing dynamics. Previous studies of *Aedes aegypti* feeding behaviour often used two-choice assays, where two diet solutions labelled with different dyes were presented simultaneously, allowing feeding preference to be inferred from dye ingestion. Applying a two-choice assay within the FlyPAD system would not only preserve the detailed information about the feeding behaviour, but would also provide direct evidence of diet preference. Moreover, presenting mosquitoes with multiple sugar concentrations or diets simultaneously would better mimic natural foraging scenarios. However, in the pre-experiments, it was revealed that some dyes themselves could affect feeding dynamics. For instance, when *Ae. aegypti* Rockefeller mosquitoes were provided with 10 mM sucrose solutions containing two dyes Acid Yellow 17 and Xylene Cyanol, the activity bout duration differed significantly between the dye treatments, even under red-light illumination to minimise visual bias (Fig. 11, *Supplementary material 2*). Since the source of this effect remains unclear, non-choice assays were chosen to adopt in this project. Once dyes that do not interfere with feeding dynamics are identified, future work can reintroduce two-choice assays to more rigorously evaluate sugar feeding preferences. In addition, crossing *Ae. aegypti ΔGr5* with other Gr gene knockout lines may allow further exploration of the potential synergistic roles of Gr genes in sugar detection in *Ae. aegypti.*

5. Conclusion

In this project, the FlyPAD system was applied to assess the feeding responses of two *Aedes aegypti* wildtype strains (Rockefeller and Liverpool) and a *Gr5* knockout homozygous mutant line (*ΔGr5*) across different concentrations of sucrose, glucose, fructose, and trehalose. The meaning of this project lies in extending the FlyPAD system, which was previously developed primarily in *Drosophila*, to mosquitoes, thereby uncovering fine-scale details of sugar-feeding behaviour. Traditional feeding behavioural assays often relied on imbibed food amounts to infer feeding preferences. By contrast, the FlyPAD system allowed to capture more detailed dynamics of the feeding process, enabling the description of distinct behavioural patterns associated with different diets. Two *Ae. aegypti* wildtype lines exhibited similar dose response to the sugar diets, with the feeding peaks generally occurring between 10 and 100 mM. In contrast, the *ΔGr5* line showed markedly reduced food imbibed amounts for all four sugars compared to its background strain Liverpool, and displayed altered feeding dynamics for sucrose, fructose, and trehalose, but not glucose. Based on the comparison of the FlyPAD system parameter correlations, *Ae. aegypti ΔGr5* mutants exhibited a characteristic "burst-pause" feeding pattern on sucrose and trehalose, whereas Liverpool mosquitoes for all four sugars and *ΔGr5* for glucose displayed a sip-driven feeding pattern.

The hypothesis regarding the function of *Aedes aegypti* Gr5 that it detects a specific sugar among sucrose, glucose, fructose, and trehalose has not been confirmed. The Gr5 in *Ae. aegypti* appears to have the function in detecting disaccharides, and *Ae. aegypti* Gr5 might contribute to the construction of Gr complexes for multiple sugars, including disaccharides and monosaccharides.

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Popular science summary

A key to the sweet side of the mosquitoes: *Gr5* Gene Behind Sugar Sensing

When we think of mosquitoes, the image that often comes to mind is that of a buzzing flying vampire greedy for blood. But beyond their notorious crave for blood, these insects also rely heavily on sugar for energy - to fly, mate, and locate their hosts. Both male and female yellow fever mosquitoes (*Aedes aegypti*) feed on flower nectar and other sugar sources, with only the females feeding on blood for reproduction. Understanding how mosquitoes sense sugar could reveal new ways to stop them from spreading viruses like dengue and Zika.

In our study, we focused on a taste gene *Gr5* of the yellow fever mosquitoes. This gene helps mosquitoes detect sweetness through sugar receptors on their mouthparts and legs. By knocking out this gene, we aimed to find how crucial Gr5 is for sugar detection and the feeding behaviour of the mosquitoes.

To do this, we reared two types of the yellow fever mosquitoes in the lab — one normal and one genetically modified to lack the *Gr5* gene. Using a device called the FlyPAD system which was originally developed for fruit flies, we offered the mosquitoes four types of sugar solutions at different concentrations: sucrose, glucose, fructose, and trehalose. The device precisely recorded each sip, allowing us to measure not only how much they fed but also how often and how long each feeding bout lasted.

Normal mosquitoes showed a clear preference for a medium concentration of the sugar meals, especially sucrose (commonly found in nectar) and trehalose (present in the body fluid of other insects). But mosquitoes missing the *Gr5* gene consumed far less sugar overall, with unstable feeding patterns — frequent pauses with only brief sips. Interestingly, their behaviour toward glucose didn't change much, even though they consumed smaller amounts than normal. This suggests that the *Gr5* gene plays a key role in sensing certain types of sugars and is essential for the sugar feeding behaviour.

Why is this important? Because sugar doesn't only serve as a role of fuel for mosquitoes, it also affects the lifespan of mosquitoes and how effectively they can transmit viruses. By uncovering the function of the gene in sensing the sugars, researchers could design better solutions to stop the mosquitoes from transmitting diseases during their sugar seeking phase and before they bite humans.

In short, by studying a single taste gene of the yellow fever mosquito, we tried to find more molecular details for the sugar feeding behaviour. Future research on genes like *Gr5* could even help explain how these once plant feeding insects evolved into the infamous “flying vampires” we know today.

Appendix

Supplementary Material 1

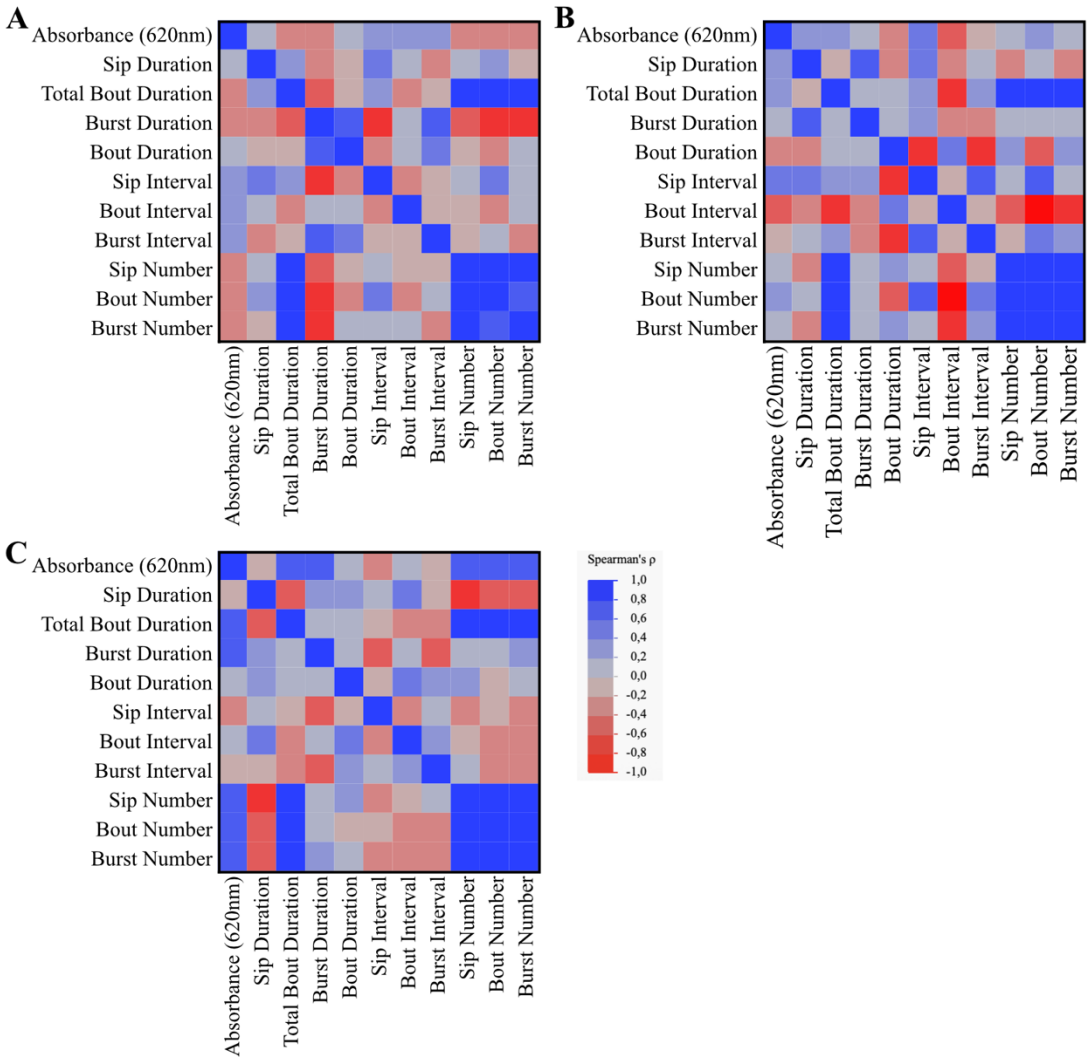

Figure 10. The Spearman's correlation heatmap of parameters when mosquitoes were dehydrated for different time lengths before the mosquitoes reached to 2 dpe.

Spearman's correlation heatmap of the correlations between absorbance and ten feeding behavioural parameters of the assays in Aedes aegypti *Rockefeller females after 0 h* **(A)**, *6 h* **(B)** *and 12 h* **(C)** *dehydration and given with 10 mM sucrose solution (n = 28).*

***Supplementary material 2**:*

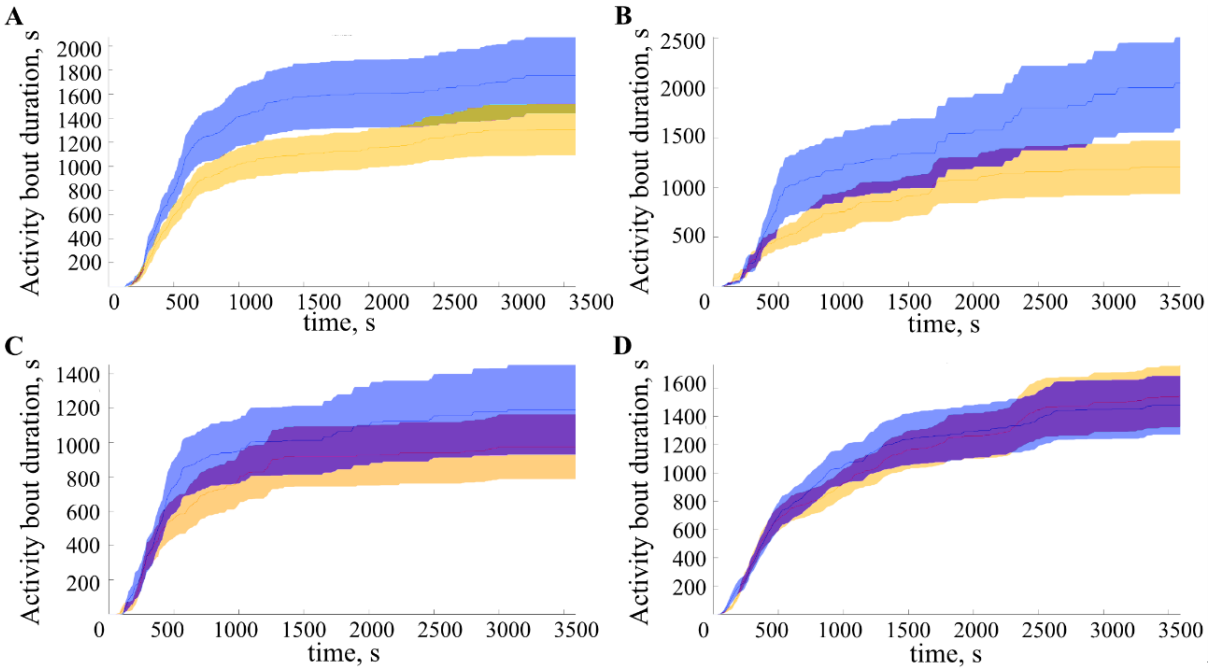

Figure 11. Factors involved in generating artefacts obscuring the dose-dependent sugar feeding of Aedes aegypti *Rockefeller females.*

*Activity bout duration of feeding on 10 mM sucrose containing Xylene Cyanol (1 mg mL^{-1}, blue) vs. Acid Yellow 17 (6 mg mL^{-1}, yellow) dyes under white light (**A**) and red light (**B**). **C**. Activity bout duration under low-level red light for 10 mM sucrose containing 1 mg mL^{-1} blue and yellow dyes as well as **D**. containing no dyes.*

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