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# Role of inward rectifier potassium channels in salivary gland function and sugar feeding of the fruit fly, *Drosophila melanogaster*

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ABSTRACT

The arthropod salivary gland is of critical importance for horizontal transmission of pathogens, yet a detailed understanding of the ion conductance pathways responsible for saliva production and excretion is lacking. A superfamily of potassium ion channels, known as inward rectifying potassium (Kir) channels, is overexpressed in the *Drosophila* salivary gland by 32-fold when compared to the whole body mRNA transcripts. Therefore, we aimed to test the hypothesis that pharmacological and genetic depletion of salivary gland specific Kir channels alters the efficiency of the gland and reduced feeding capabilities using the fruit fly *Drosophila melanogaster* as a model organism that could predict similar effects in arthropod disease vectors. Exposure to VU041, a selective Kir channel blocker, reduced the volume of sucrose consumption by up to 3.2-fold and was found to be concentration-dependent with an $EC_{50}$ of 68 μM. Importantly, the inactive analog, VU937, was shown to not influence feeding, suggesting the reduction in feeding observed with VU041 is due to Kir channel inhibition. Next, we performed a salivary gland specific knockdown of Kir1 to assess the role of these channels specifically in the salivary gland. The genetically depleted fruit flies had a reduction in total volume ingested and an increase in the time spent feeding, both suggestive of a reduction in salivary gland function. Furthermore, a compensatory mechanism appears to be present at day 1 of RNAi-treated fruit flies, and is likely to be the $Na^+$-$K^+$-$2Cl^-$ cotransporter and/or $Na^+$-$K^+$-ATPase pumps that serve to supplement the inward flow of $K^+$ ions, which highlights the functional redundancy in control of ion flux in the salivary glands. These findings suggest that Kir channels likely provide, at least in part, a principal potassium conductance pathway in the *Drosophila* salivary gland that is required for sucrose feeding.



## 1. Introduction

The molecular architecture of arthropod salivary glands have been analyzed in detail for a variety of model organisms, like *Drosophila melanogaster* [1,2], and hematophagous arthropods, including mosquitoes [3–5], ticks [6–8], fleas [9], and blackflies [10]. In the case of hematophagous arthropods, this work yielded a general understanding of saliva constituents that contribute to blood feeding through regulation of blood haemostasis by vasodilation, inhibitors of blood clotting, anesthetics, and anti-immune factors [11,12]. Yet, an understanding of the molecular machinery and physiological systems within arthropod insect salivary glands to enable salivation is limited.

Currently, tick salivary glands are the most commonly studied of all arthropods. Park and colleagues [13,14] have reported the presence of two dopamine receptors in the salivary glands of the blacklegged tick (*Ixodes scapularis*) that are expressed at two distinct locations of the gland and separately control the inward fluid transport and release of fluid to coordinate salivary secretion in ticks. Although considerable pharmacological evidence suggests the dopaminergic system is a major physiological pathway required for arthropod salivation, additional physiological pathways are present that likely contribute to salivary gland function as well. For example, mammalian salivary glands have been shown to rely on chloride ($Cl^-$)- and potassium ($K^+$)- ion gradients for proper salivary gland function [15], yet little to no literature exists on the $K^+$ or $Cl^-$ ion channels responsible for maintaining these gradients.

$K^+$ ion transport within the mammalian salivary glands is critical for generating saliva and inwardly rectifying potassium (Kir) channels have been shown to be essential to mammalian salivary gland function [16–19]. These channels function as biological diodes due to the unique biophysical property that facilitates the flow of potassium ions in the inward direction more easily than the outward direction [20]. All Kir channels share a similar molecular structure and are tetramers assembled around an aqueous membrane-spanning pore that are gated by

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polyvalent cations that occlude the pore at cell potentials more positive than the $K^+$ equilibrium potential ($E_k$) [21,22]. The numbers of genes encoding Kir channel constructs vary depending on species with humans possessing 16 Kir channel encoding genes [20], *Aedes aegypti* mosquito possessing 5 [23], and *D. melanogaster* with 3 [24]. The *Drosophila* Kir genes are termed *Ir*, *Irk*2, and *Irk*3 and encode Kir1, Kir2, and Kir3, respectively [24]. Tissue expression patterns of *Drosophila* Kir channels are highly variable and are described in Zhuo and Hong-Sheng [24] with the expression patterns in excretory systems summarized in Table 1.

Several recent lines of genetic and pharmacological evidence suggest Kir channels play important physiological roles in exocrine systems of dipteran insects as well. In *D. melanogaster*, embryonic depletion of Kir1 and Kir2 mRNA in Malpighian tubules significantly reduces transepithelial secretion of fluid and $K^+$ transport [25]. In *Aedes aegypti* and *Anopheles gambiae* mosquitoes, researchers have shown that pharmacological inhibition of *Ae*Kir1 with structurally distinct small-molecules (i.e. VU573, VU041) disrupts the secretion of fluid and $K^+$ in isolated Malpighian tubules, as well diuretic capacity, and $K^+$ homeostasis in adult females [26–29]. Furthermore, the *Ir* gene encoding *Drosophila* Kir1 is enriched in exocrine tissues and gene expression is increased by 37-fold in the salivary glands of larval and adult life stages (Table 1) [24,30]. The *Drosophila* salivary gland mainly consists of secretory cells that synthesize and secrete proteins required for feeding [31], and the high expression of Ir in the salivary gland may suggest a role in promoting secretion of salivary constituents.

Considering 1) the overexpression of *Ir* in *Drosophila* salivary, 2) the critical role Kir channels serve in Malpighian tubules and 3) that salivary gland cells are also an exocrine tissue that rely on ionic gradients and water transport to generate saliva, we hypothesized that Kir channels are essential to proper salivary gland function and are critical in the highly intricate physiological processes of arthropod feeding. Therefore, the goals of the present study were to use pharmacological inhibition and salivary gland-specific genetic depletion of Kir channels in the model organism *D. melanogaster* to determine the physiological importance of Kir channels in fly salivary gland function as measured through sucrose feeding efficiency.

## 2. Methods

### 2.1. Drosophila stocks and rearing conditions

Four strains of *D. melanogaster* were used in this study. The wildtype Oregon-R (OR) strain was provided by Dr. Jeffrey Bloomquist at the University of Florida and was originally donated by Doug Knipple, Cornell University, Ithaca NY, USA. All GAL4-UAS fly strains were purchased from Bloomington Drosophila Stock Center (Bloomington, IN, USA). The GAL4-UAS strain 6870 expresses the promoter in the larval and adult salivary glands, the strain 42644 expresses dsRNA for RNAi of Kir1 (*Ir*) under UAS control, and the strain 41554 expresses hairpin RNA (hpRNA) under the control of UAS for RNAi of GFP and was used as a negative knockdown control. The genotypes of each strain are as follows: 6870, w[1118]; P{w[+mC] = Sgs3-GAL4.PD}TP1; 42644, y [1] sc[*] v [1]; P{y[+t7.7] v[+t1.8] = TRiP·HMS02480}attP2; 41554, y [1] sc[*] v [1]; P{y[+t7.7] v[+t1.8] = VALIUM20-EGFP.shRNA.2}attP2.

All fly strains have been maintained in culture at the Louisiana State University since April 2015. All fly strains were reared on standard medium in *Drosophila* tubes at 25 °C, 12·hour-12·hour photoperiod and 55% relative humidity. For dissection, flies were anaesthetized by chilling on ice and decapitated before dissecting out salivary glands in Schneider's medium (Invitrogen, Paisley, Scotland, UK).

#### 2.1.1. Chemicals

The Kir channel inhibitor VU041 and the inactive analog VU937 were originally discovered in a high-throughput screen against the *Anopheles gambiae* Kir1 channel [29]. Both compounds were synthesized by Dr. Corey Hopkins at the Vanderbilt Center for Neuroscience Drug Discovery using methods described in Swale et al. [29]. Dopamine and the $D_1/D_2$ antagonist fluphenazine dihydrochloride were purchased from Sigma-Aldrich. Structures of VU041 and VU937 are shown in Fig. 1.

### 2.2. Feeding assay

The capillary feeding assay (CAFE) was used to quantify the volume of sucrose solution consumed over a period of time and was performed essentially as described in Ja and colleagues [32]. A schematic representation and micrograph image of the assay design is shown in Fig. 2A and 2B, respectively. Briefly, both sexes were used in this assay due to the absence of any literature suggesting differential expression between the genders. One adult fly was placed into a 2 mL glass vial with a screw lid that was pierced with a glass microcapillary tube via a truncated 200-μL pipette tip. The microcapillary tubes contained 5% (wt/vol) sucrose solution that had a 5 μL mineral oil overlay to minimize evaporation during the time course of the experiment. Each experiment included an identical CAFE chamber without flies to determine evaporative losses (typically 5–10% of ingested volumes), which were subtracted from experimental readings. A concentration of 100 μM dopamine and 100 μM fluphenazine were used in Fig. 3A and 200 μM VU041 and 700 μM VU937 (solubility limits) was used in Fig. 3B to determine the influence pharmacological agents have on fly feeding. Exposure to VU041 yielded approximately 30% mortality whereas <10% mortality was observed in control and VU937 treated animals. All dead flies were excluded from all time points of the study regardless of the time point of death. Total consumption volume was calculated by measuring the change in meniscus changes from time zero with 1 cm = 1 μL. Mean ($n > 25$) values are shown as points for all figure panels that measure total consumption.

To determine the effect of increased potassium ions on the VU041-mediated reduction in sugar consumption, 500 μM potassium chloride was added to the 5% sucrose solution and ingested volume was

**Table 1**
Anatomical expression of Kir channels expressed in excretory systems of *Drosophila* [24].

| mRNA Signals | | | | | | | |
|---|---|---|---|---|---|---|---|
| Localizations | Ir | | | Irk2 | | Irk3 | |
| | Larval | Adult | Enrichment | Larval | Adult | Larval | Adult |
| Salivary gland | 725 | 7480 | 37.2 | 67 | 235 | 3 | 8 |
| Crop | – | 2871 | 1.4 | N/A | 1722 | – | 6 |
| Midgut | 782 | 506 | 2.5 | 36 | 117 | 7 | 4 |
| Hindgut | 302 | 83 | 0.4 | 3856 | 4564 | 4 | 48 |
| M. tubules | 844 | 1099 | 5.5 | N/A | 805 | 2898 | 4932 |

Note: Data was originally obtained from http://flyatlas.org and values shown were extracted from Luan and Li [24]. Values indicate intensities of RNA signal. N/A: no informative data. Enrichment is defined as the mRNA expression of the tissue/whole body mRNA expression.

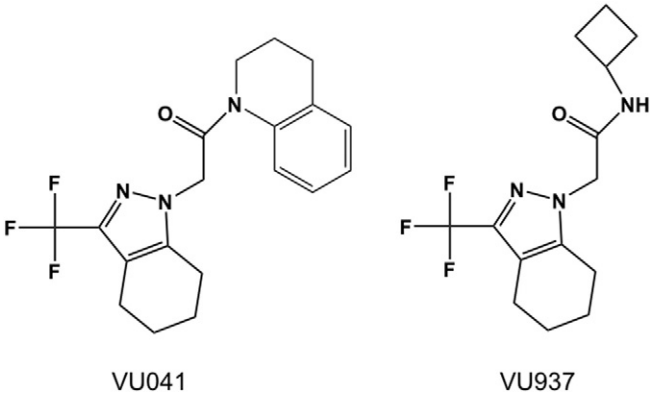


**Fig. 1.** Chemical structures of Kir channel inhibitors used in this study.

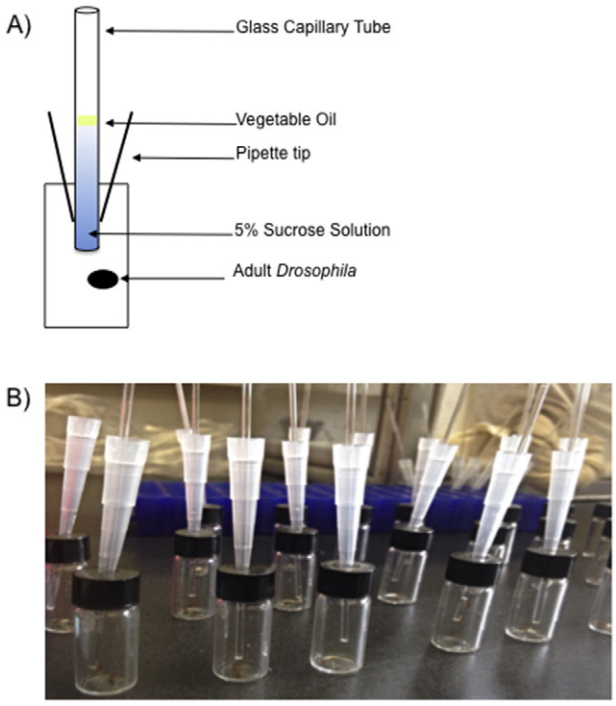


**Fig. 2.** *Drosophila* feeding assay used in this study modified from Ja et al. A) Schematic diagram showing the feeding assay that utilizes a glass capillary tube held by a pipette tip and one fly added to each chamber. B) Photograph of the feeding chambers.

determined as described above. Treatments included a negative control of 5% sucrose solution, a treatment control of 5% sucrose solution + 500 μM potassium chloride, the treatment of 5% sucrose solution + 500 μM potassium chloride + 200 μM VU041, and 5% sucrose solution + 200 μM VU041 (Fig. 3D).

The feeding time assay was used to determine how long individual flies spent on the open end of the capillary tube, presumably feeding on the sugar solution. Flies with a genetic depletion of salivary gland specific Kir channels were used in this experiment and were monitored 1-, 2-, and 3-days post emergence. A GoPro HERO 3 video camera was mounted in front of the CAFE assay to record the flies over a 24-period. Videos were uploaded to a computer and the time spent feeding was measured and recorded as a mean ($n > 10$) value.

### 2.3. Fluorescence microscopy

Methods were performed similar to those described in Mascari and Foil [33] with minor adjustments for species differences. Briefly, individual adult flies were fed 5% sucrose solution plus 500 ppm rhodamine B with the CAFE feeding (described above). Individual specimens were placed in the well of a glass concavity slide and covered with a glass coverslip to prevent air currents in the laboratory from moving specimens during observation. The slides were placed on the stage of a fluorescence stereomicroscope (SteREO Lumar.V12, Carl Zeiss, Gottingen, Germany) and observed using incandescent illumination. Digital images were captured with AxioVision version 4.6 (Carl Zeiss) by using an 800-ms exposure time. The specimens then were observed under fluorescence microscopy using a rhodamine filter cube (excitation wavelength, 540 nm; emission wavelength, 625 nm). All fluorescent images shown in Fig. 4 were captured at an exposure time of 300-ms. Minimal to no auto-fluorescence of the negative control negated the need to optimize the fluorescence exposure time.

### 2.4. Genetic knockdown of salivary gland specific Kir1

Advances in *Drosophila* genetics has enabled tissue specific knockdown of specific genes through the GAL4-UAS system. This technology has been used for the previous decade and is based on the properties of the yeast transcriptional activator Gal4 that activates transcription of its target genes by binding to upstream activating sequence (UAS). The GAL4-UAS construct binds next to the gene of interest, which in this case is hairpin RNA (hpRNA) for Kir1, to genetically enhance or decrease mRNA expression [34–36]. The two components, GAL4 and UAS are carried in separate *Drosophila* stocks that allow for hundreds of combinatorial possibilities after a simple parental cross. In this study, we utilized a strain of fly that expressed the GAL4-UAS promoter only in the salivary glands of all life stages, which enabled the salivary gland specific knockdown of Kir1.

A schematic representation of the cross that enabled salivary gland specific knockdown of Kir1 is shown in Fig. 5A and was modified from Johnston [37]. Briefly, knockdown was achieved by crossing virgin females from the respective Kir1 RNAi strain (Bloomington stock 42644) with males from the salivary gland expressing GAL4-UAS strain (Bloomington stock 6870). The flies were given 96 h to mate and oviposit prior to removal from the *Drosophila* tube. $F_1$ offspring were allowed to emerge and adults were used in the study immediately upon emergence. The genotype expression of the Kir1 RNAi (Bloomington stock 42644) was on the X-chromosome and therefore, male GAL4-UAS flies (6870) were crossed with virgin females from strain 42644 or 41554. A schematic diagram outlining the GAL4-UAS system and cross performed is shown in Fig. 5A.

### 2.5. Statistical analyses

Concentration response curves and $IC_{50}$ values using VU041 and VU937 were generated by fitting the Hill equation using variable-slope, unconstrained, nonlinear regression analyses performed with GraphPad Prism (GraphPad Software, San Diego, CA). Mean cumulative consumption values for VU937 and VU041 were compared to control consumption values per day by a one-way ANOVA with a Dunn's multiple comparisons post-test. Time spent feeding in Kir1 and GFP genetic knockdown studies were compared to control values at each day using one-way ANOVA with a Dunn's multiple comparisons post-test. Statistical significance for all studies was denoted at $P < 0.05$.

### 2.6. RNA isolation, cDNA synthesis, and quantitative PCR

Total RNA was isolated and extracted from 30 pairs of *Drosophila* salivary glands using TRIzol® Reagent (Life Technologies, Carlsbad, CA) and purified using the RNeasy kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized from poly(A) RNA using the SuperScript® III First-Strand Synthesis System for real-time quantitative PCR (qRT-PCR) (Life Technologies) according to manufacturer instructions. qRT-PCR was then performed on an Qiagen Rotor Gene Q 2Plex Real-Time PCR System using the operating instructions. Relative quantification was carried out using the $2^{-DDCT}$ method [38], and beta-actin was used as the reference gene. Appropriate controls, such as DNAse and removal of reverse transcriptase, were performed to ensure the sample was not contaminated with genomic DNA. All primers used in this study were purchased from Life Technologies with primer reference numbers for the *irk1* and actin genes are Dm02143600_s1 and Dm02361909_s1, respectively. Five biological replicates were conducted and each was analyzed in triplicate. The graphed output displays the average fold-change in mRNA levels relative to the wild type Oregon-R control salivary glands.

## 3. Results

### 3.1. Effect of pharmacological inhibition of Kir channels to sugar feeding

Dopamine has been shown to stimulate salivation in arthropods [13, 14] and was therefore used as a positive control in this study to determine the utility of the CAFE assay for measuring alterations in feeding efficacy after exposure to small-molecules that target salivation pathways. Mean ($n > 10$) daily consumption volumes of sucrose for control flies were 1.25 μL 1 day post emergence (PE), 1.75 μL 2 days PE, 2 μL 3 days PE, and 1.35 μL 4 days PE (Fig. 3A). Dopamine was found to statistically increase the total volume of sucrose consumed when compared to control animals. We observed a 1.7-fold increase in consumption at 1 day PE ($P < 0.05$), 1.3-fold at 2 days PE ($P < 0.05$), 1.2-fold at 3 days PE ($P < 0.05$), and 1.4-fold at 4 days PE ($P < 0.05$) (Fig. 3A). Conversely, the dopamine receptor inhibitor fluphenazine was shown to significantly reduce the total volume of sucrose solution ingested by 3.6-fold at 1 day PE ($P < 0.01$), 3.1-fold at 2 days PE ($P < 0.001$), 2.4-fold at 3 days PE ($P < 0.001$), and 2.2-fold at 4 days PE ($P < 0.001$) (Fig. 3A). The ability to measure feeding differences using pharmacological probes of the dopamine receptor enabled us to explore the role of Kir channels in *Drosophila* sucrose feeding through small-molecules developed against the mosquito Kir1 channel. Mean ($n > 25$) daily consumption of sucrose for control flies during this experiment were 1.3 μL 1 day PE, 1.65 μL 2 days PE, 1.35 μL 3 days PE, and 1.45 μL 4 days PE, nearly identical values as observed during the dopamine studies. These data are compiled into Fig. 3B and are expressed as cumulative consumption. Pharmacological inhibition of Kir channels with the Kir channel inhibitor, VU041, was shown to significantly reduce the total volume of sucrose solution ingested by flies by 2.6-fold at 1 day PE (P: 0.01), 2.7-fold at 2 days PE ($P < 0.001$), 2.9-fold at 3 days PE ($P < 0.001$), and 3.2-fold at 4 days PE ($P < 0.001$) (Fig. 3B). Importantly, the volume of sucrose solution ingested by flies exposed to the inactive analog of VU041, termed VU937, was not significantly different than control treated flies (Fig. 3B). The influence of VU041 to feeding was found to be concentration dependent with an $EC_{50}$ of 68 μM (95% CI: 54 μM–79 μM), yet no difference in volume consumption was observed with VU937 at concentrations ranging up to 500 μM (Fig. 3C). To visualize the volume of ingestion of sucrose solution, we added the fluorophore Rhodamine B to the sucrose solution with and without VU041. Micrographs shown in Fig. 4 clearly illustrates that exposure to VU041 (Fig. 4C) yields a reduced intensity of fluorescence when compared to control treated flies (Fig. 4B), indicative of a reduced volume of sucrose solution ingested, reminiscent of the data described in Fig. 3B.

### 3.2. Effect of increased potassium ions to total consumption of sucrose

In mammals, the ion conductance pathways, particularly the outward flow of $K^+$ and $Cl^-$ ions, are critical for generation of saliva by arthropod salivary glands. Therefore, we hypothesized that Kir channels in *Drosophila* salivary glands are responsible for maintaining the high intracellular $K^+$ concentration that provides the $K^+$ ion gradient and enables the outward flow of potassium ions, presumably through $Ca^{2+}$-activated $K^+$-channels as is seen in mammals. To test this, we augmented the potassium ion concentration in the sucrose solution to increase the potassium equilibrium constant ($E_k$; based on the Nernst equation), which indirectly reduces the efficacy of intracellular $K^+$ channel inhibitors, such as VU041.

Supplementing the sucrose solution with 500 μM $K^+$ did not statistically alter the total volume of sucrose consumed when compared to

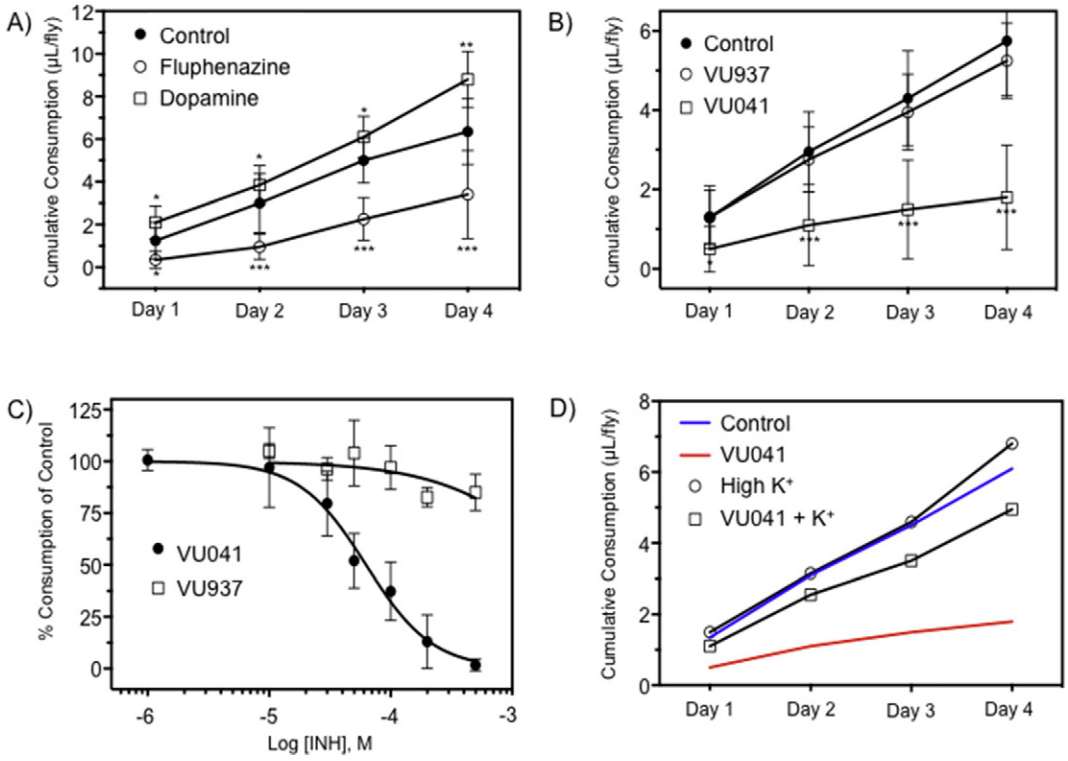


**Fig. 3.** Measurement of long-term sucrose consumption using the feeding assay. A) cumulative ingestion by individual flies ($n > 25$) over a 4-day period. Total daily consumption was compared between control flies (closed circles), dopamine hydrochloride (open square), and fluphenazine (closed circles). B) cumulative ingestion by individual flies ($n > 25$) over a 4-day period. Total daily consumption is compared between control flies (closed circles), Kir channel blocker VU041 (200 μM; open squares), and the inactive analog to VU937 (250 μM; open circles). C) Concentration-response curve comparing total consumption values collected on day 4 between VU041 (closed circles) and VU937 (open square). D) Effects of extracellular potassium addition to VU041-mediated reduction of consumption. Control (blue solid line) and VU041 (200 μM; red solid line) traces are shown in panel A but are duplicated for comparison to the consumption with 300 μM potassium alone (open circles) and 500 μM potassium + 300 μM VU041 (open square) traces. Asterisks represent statistical significance with * representing $P < 0.05$. **representing $P < 0.01$, and ***representing $P < 0.001$.

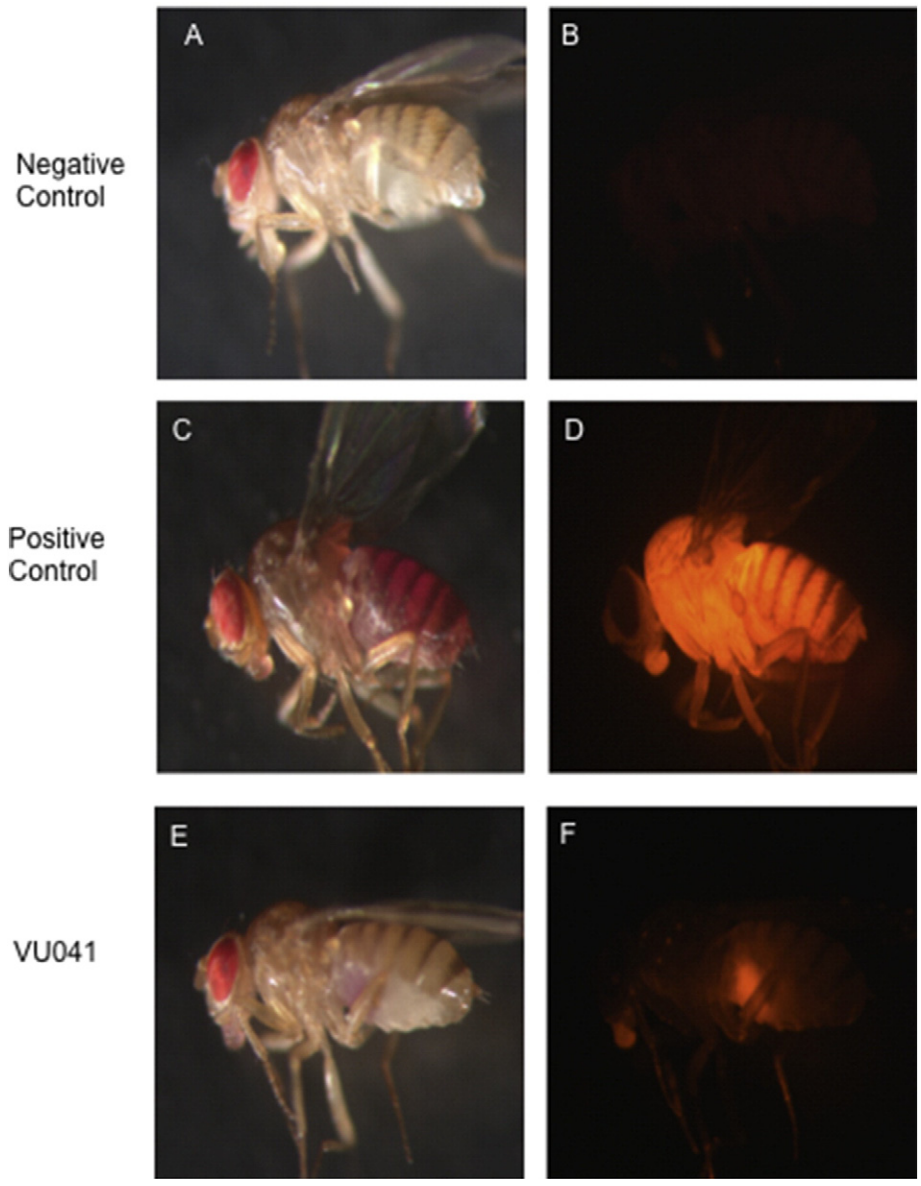


**Fig. 4.** Visualization of sucrose consumption through fluorescent imaging. Images of flies were taken under incandescent lighting (A,C,E) and by using fluorescence microscopy (B,D,F) with a Rhodamine filter. The negative control flies were not exposed to Rhodamine B whereas the positive control was exposed to 500 ppm mixed into the 5% sucrose solution and displayed a significant level of fluorescence. A representative of the VU041-treated animals is shown in E/F and highlights the marked reduction in sucrose consumption.

the control animals (Fig. 3D; open circles, blue line, respectively). However, the increased potassium ion concentration significantly reduced the efficacy of VU041 on all days studied. The mean consumption of flies that were exposed to 500 μM $K^+$ and VU041 were found to be 1.1 μL, 1.4 μL, 1 μL, and 1.45 μL at days 1-, 2-, 3-, and 4-PE, respectively, which were found to not differ significantly ($P = 0.7$) from control flies. Conversely, high significance ($P < 0.001$) was observed when we compared daily consumption of sucrose between flies only exposed to VU041 and those exposed to VU041 + $K^+$. Fig. 3D shows a 2-, 2.3-, 2.3-, and 2.75-fold increase in total consumption for days 1-, 2-, 3-, and 4 days-PE, respectively.

### 3.3. Knockdown efficiency of irk1 in salivary glands

Our data presented in Figs. 3 and 4 suggests a critical role of Kir channels in the feeding of adult *Drosophila*. But, the potential for small-molecule inhibitors to bind to additional proteins raised concerns that a combination of tissues could be responsible for reducing feeding efficacy. To address this concern, we utilized methods for knocking down Kir1 mRNA levels specifically in the salivary gland by RNA interference by using the GAL4-UAS system. Data show the salivary glands of the $F_1$ progeny of irk1 knockdown cross expressed 53% less *irk*1 mRNA relative to the wildtype (OR) and GFP dsRNA knockdown controls (Fig. 5B).

### 3.4. Influence of Kir1 knockdown to feeding efficiency

Pharmacological or genetic depletion of Kir channels have been shown to inhibit Malpighian tubule function in flies [25] and mosquitoes [26,27], which may negatively influence feeding through an inability to osmoregulate at the level of the Malpighian tubules. Therefore, we performed genetic knockdown of salivary gland specific Kir1 channels to ensure the reduced ingestion of sugar water shown in Fig. 3 was indeed due to salivary gland failure and not reduced osmoregulatory capabilities stemming from tubule failure. The data in Fig. 6A show a significant reduction of total volume ingested for the Kir1 knockdown flies at post-emergence day 2 (P: 0.03), day 3 (P: 0.02), and day 4 (P: 0.005) when compared to control, but not at day 1 ($P > 0.05$). Importantly, the volume of sucrose ingested by the GFP knockdown flies did

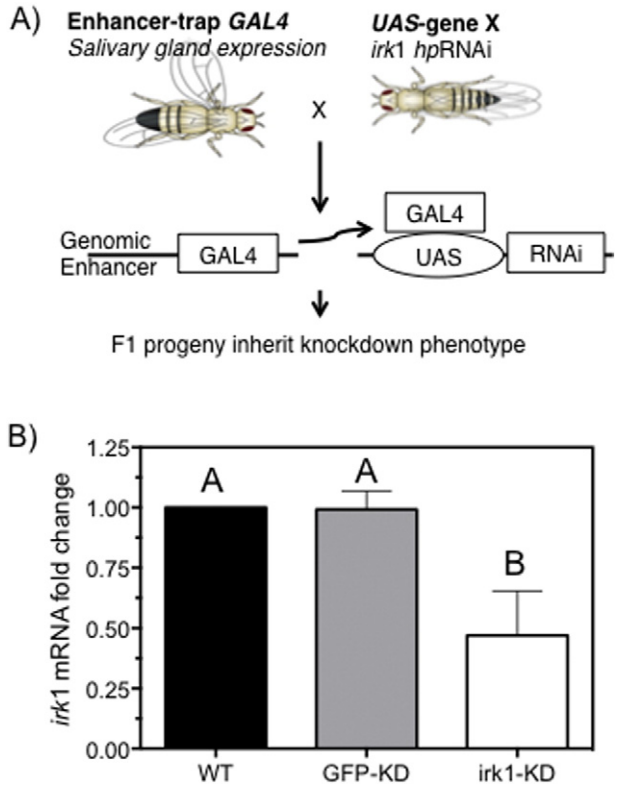


**Fig. 5.** Salivary gland specific RNAi-medated knockdown of irk1. A) Schematic diagram of the GAL4/UAS system for directed gene knockdown. B) Quantitative RT-PCR analysis showing *Dm*irk1 RNAi-based knockdown efficiency in the salivary glands of flies. Bars represent fold-difference of *irk*1 mRNA levels relative to beta-actin control group. The strains of flies used are as follows with Bloomington stock numbers in parentheses: WT (Oregon-R), GFP hpRNA (41554), *irk*1 hpRNA (42644), Salivary gland directed GAL4 (1824).

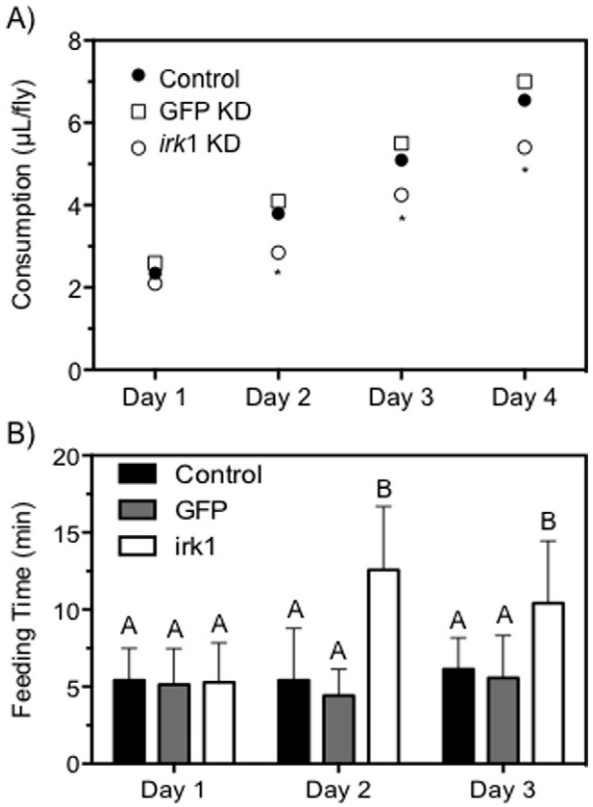


**Fig. 6.** Long-term sucrose consumption in salivary gland specific *irk*1 knockdown animals. A) Cumulative ingestion by individual flies ($n > 50$) over a 4-day period. Total daily consumption is compared between control flies (black circles), GFP knockdown (open squares), and salivary gland specific *irk*1 knockdown. Asterisks represent statistical significance at $P < 0.05$. B) Total time spent on capillary tube feeding on sucrose solution for 1-, 2-, 3-days post emergence. Bars represent mean (n:7–15) time spent feeding per treatment and error bars represent SEM. Bars not labeled by the same letter represent statistical significance at $P < 0.05$.

not differ from control. Although statistical significance was observed, a smaller than expected volume difference (c.a. 1 µL) between control and Kir1 knockdown flies was observed that may be due to the absence of an external stimuli preventing continuous feeding within the CAFE assay. Therefore, we assessed the time each individual fly rested on the bottom of the capillary tube, presumably feeding, at post emergence days 1, 2, and 3. Similar to the total consumption values, no significant difference in time spent feeding was observed for day 1, but a significant increase ($P < 0.001$) increase in time spent feeding was observed for the Kir1 knockdown flies over control flies for days 2 and 3 with a 2.3- and 1.9-fold increase, respectively (Fig. 6B).

## 4. Discussion

Despite the critical role the arthropod salivary gland serves in horizontal transmission of pathogens, an understanding of the machinery required for proper gland function is limited. Although rather limited in scope, pharmacological studies against the isolated tick salivary gland have implicated several components involved in the process of salivary secretion: dopaminergic pathway [13], $Na^+$-$K^+$-ATPase [39], GABA [40], and the muscarinic acetylcholine receptor [41]. Although these pathways are clearly important for saliva production and excretion, the complex nature of the gland suggests other pathways are likely critical for proper function of the salivary gland. Indeed, the results of the present study provide compelling data that a superfamily of potassium ion channels, known as inward rectifier potassium channels, is an essential conductance pathway in the salivary gland that mediates proper feeding in the model organism 4.1 *Drosophila melanogaster*.

Recent work on insect Kir channels have yielded insightful data suggesting these channels serve a critical role in Malpighian tubule function and fluid secretion [23,26,42]. The Malpighian tubules and salivary glands are physiologically related tissues as both are a polarized epithelial tissue [43,44], require water and ion transport for function, and are considered, at least in part, to be an exocrine tissue. Furthermore, the Kir1 channel has been shown to constitute the primary inward $K^+$ conductance in the mosquito Malpighian tubule and the analogous gene that encodes Kir1 in *Drosophila* is highly upregulated in the salivary glands of larval and adult flies [24]. Therefore, we hypothesized Kir channels also serve a critical role in salivary gland function and aimed to elucidate the role of these channels through pharmacological and genetic manipulations of the Kir1 channel measured through feeding efficiency.

The recent identification of selective and potent small-molecules designed to target insect Kir channels [29,45–47] have enabled researchers to begin to characterize the physiological role of these channels in various tissue systems. In this study, we used the recently discovered insect Kir channel modulator (VU041) and its inactive analog (VU937) [29] to characterize the influence these molecules have in the feeding cascade. We found that exposure to VU041 during feeding significantly reduced the volume of sucrose ingested, whereas VU937 had no influence to feeding efficiency, suggesting the observed phenotype is through Kir inhibition. However, due to the capability that small-molecules can inhibit unintended target sites and the fact Kir channels are highly expressed in the Malpighian tubules (Table 1) [29], it was impossible to ensure the observed effect to feeding was directly due to salivary gland failure. Therefore, we performed salivary gland specific RNAi-mediated knockdown of the Kir1 encoding gene. Results from this genetic depletion of Kir1 show a significantly less

efficient salivary gland (Fig. 6) and, when combined with the VU041-mediated reduction in sucrose consumption, strongly suggests the *Drosophila* salivary gland relies on the inward conductance of $K^+$ ions through Kir channels.

The data presented in this study raises the question as to what the physiological role Kir channels have in salivary gland function at the cellular level. Consideration of knowledge and hypotheses based on the role of Kir channels in mammalian salivary gland function and saliva production can be applied to expand our understanding of salivary gland physiology in arthropods. First, it has been shown that electrolyte secretion in the mammalian salivary glands is based on the secondary active transport of anions, principally $Cl^-$ (and/or $HCO_3^-$) ions [15]. In this model, $K^+$ channels in the basolateral membrane of acinar cells maintain the membrane potential of the apical cell membrane to be more negative than the Nernst potential for anions, thereby providing a driving force for the sustained electrogenic anion efflux across the apical membrane. The second model for a role of Kir channels in the mammalian salivary gland was described through cell-attached patch and whole-cell patch-clamp studies. Here, researchers demonstrated the presence of four primary $K^+$ channels, two of which are the outward mediated $Ca^{2+}$-activated $K^+$ channel and a Kir channel [18,19,48]. The inwardly rectifying property of the Kir channel was hypothesized to perform fast uptake of accumulated $K^+$ ions, in concert with $Na^+$-$K^+$-ATPase, into acinar cells with the $K^+$ influx depending on the relation between the membrane potential and the concentration gradient of $K^+$ across the basolateral membrane. This buffering action likely provides an ion gradient enabling the outward flow of $K^+$ ions through $Ca^{2+}$-activated $K^+$ channels. Such $K^+$ buffering action of Kir channels has been proposed in brain astrocytes [49–51], in retinal Müller cells [52] and also in retinal pigmented epithelial cells [53].

To begin elucidating the role of Kir channels in the insect salivary gland based on the mammalian hypotheses, we augmented the potassium ion concentration in the sucrose solution to increase the potassium equilibrium constant ($E_k$; based on the Nernst equation), which ultimately reduces the efficacy of intracellular $K^+$ channel inhibitors, such as Kir channel blockers [29,54]. The loss of VU041 potency (Fig. 3) supports the notion that Kir channels function as a means to provide a pathway for rapid influx of $K^+$ ions after depolarization events, a phenomenon oftentimes referred to as $K^+$-spatial buffering. Therefore, we hypothesize that Kir channels in *Drosophila* salivary glands are responsible, at least in part, for maintaining the high intracellular $K^+$ concentration through a buffering-like action, which provides the $K^+$ ion gradient to enable the outward flow of potassium ions, presumably through $Ca^{2+}$-activated $K^+$-channels as is seen in mammals. However, further studies rooted in cellular electrophysiology and membrane physiology are needed to provide additional support for this hypothesis.

Although the data of this study suggest Kir channels are likely the primary mechanism for $K^+$ spatial buffering within insect salivary gland cells, it is also evident that it is not the only transport pathway facilitating inward flow of $K^+$ ions. Genetic depletion of Kir1 channels yielded a reduction of feeding at days 2, 3, and 4, but not on day 1 (Fig. 6A) and similarly, the time spent feeding was not statistically different to controls at day 1 when compared to subsequent days (Fig. 6B). These data suggest the presence of a compensatory mechanism that accounts for the reduced expression of Kir1 in the genetically depleted animals, but one that is lost after day 1. Compensatory mechanisms are commonly observed in animals with genetic depletions of Kir channels and most oftentimes arise through upregulation of a different Kir gene. For instance, Wu and colleagues showed individual knockdown of any of the three Kir channel genes in *Drosophila* Malpighian tubules had no effect on the organ function, yet simultaneous knockdown of *irk*1 and *irk*2 had significant effects on transpeithelial $K^+$ transport [55], suggesting Kir1 and Kir2 play redundant roles in Malpighian tubule function. Due to the expression of Kir1 and Kir2 mRNA in the *Drosophila* salivary gland [24], albeit at dramatic differences in mRNA expression level, it is plausible that the Ir2 gene is upregulated after genetic depletion of Kir1, which may account for the absence of an effect to feeding at day 1. Furthermore, the Malpighian tubules partially rely on the $Na^+$-$K^+$-$2Cl^-$ cotransporter and $Na^+$-$K^+$-ATPase pump to establish a high intracellular $K^+$ ion gradient [56,57]. The compensatory systems of the Malpighian tubules and the expression of the same conductance pathways in the salivary glands highlights the possibility that the *Drosophila* salivary gland is capable of utilizing these same pathways for establishing the intracellular $K^+$ ion concentration as well as providing redundancy into the system for salivary gland $K^+$ excretion. However, additional studies are required to validate this notion.

This study provided the first insight into the role of $K^+$ ion channels in arthropod salivary gland physiology. Such knowledge helped understand the machinery arthropods evolved in the salivary gland to facilitate food acquisition. A significant amount of research remains to be performed to elucidate all the mechanisms of $K^+$ ion transport that is required for proper gland function, but we provide clear evidence that inward rectifying potassium channels expressed in the insect salivary gland are critical for its proper function as evidenced by alterations in feeding ability after chemical- or genetic- depletion. This study serves as a proof-of-concept that VU041 could serve as a lead compound for the development of new/novel vector control agents aimed at disrupting blood feeding and pathogen transport. Therefore, future studies will aim to expound on these data to characterize the functional relationship between Kir channels, $Na^+$-$K^+$-$2Cl^-$ cotransporter and $Na^+$-$K^+$-ATPase pumps as well as identify the role of Kir channels in the salivary glands of arthropod disease vectors as a means for the development of new/novel vector control agents aimed at disrupting blood feeding and pathogen transport.

## Author Contributions

Conceived, designed, and performed experiments: DRS, ZL. Analyzed the data: DRS, ZL. Participated in writing of the manuscript: DRS, ZL, APDL, FG, LDF.

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