

THE EFFECTS OF DIET AND ALTERED EXPRESSION OF
THE KEAP1/CNCC PATHWAY ON SECRETION OF
ORGANIC TOXINS BY MALPIGHIAN TUBULES OF
DROSOPHILA MELANOGASTER

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**The Effects of Diet and Altered Expression of the
Keap1/CncC Pathway on the Secretion of Organic
Toxins by Malpighian tubules of *Drosophila
melanogaster***

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Abstract

The Keap1-Nrf2 pathway is a major upstream regulator of xenobiotic detoxification. In *Drosophila*, directed activation of the protein complex of Keap1 and CncC (the homolog of human Nrf2) in principal and stellate cells of the Malpighian (renal) tubules confers resistance to lethal doses of the pesticide malathion, which is metabolized into organic anions. Dietary exposure to organic anions such as salicylate (10 mM) causes increases in fluid secretion rate and salicylate flux across Malpighian (renal) tubules. Here we used salicylate-selective microelectrodes and Ramsay assays to determine the role of Keap1/CncC in regulating these responses. Fluid secretion rate and salicylate flux across tubules isolated from adults with directed activation of Keap1/CncC in the principal cells are comparable to the values from salicylate-fed controls. Fluid secretion rate, concentration of salicylate in the secreted fluid and salicylate flux did not differ significantly between tubules isolated from adults with directed activation of Keap1/CncC in the principal cells reared on a diet containing salicylate and those reared on control media, indicating that the detoxification pathway was activated regardless of the presence of dietary salicylate. This is in contrast to the significant increase in fluid secretion rate and salicylate flux between tubules isolated from salicylate-fed adults and adults reared on a control diet with directed activation of Keap1/CncC in the stellate cells, supporting previous studies that demonstrated the inability of stellate cells to transport organic anions. Taken together, these results suggest a role for Keap1/CncC in upregulating fluid secretion in response to the presence of dietary organic anions.

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Introduction

***Drosophila* as a Model System**

Drosophila melanogaster is a model organism that has been studied for > 100 years, in part because it is a small organism requiring a simple diet and is easily reared in laboratory culture. As a complex multi-cellular organism where many aspects of its development and behavior parallel those in humans, coupled with the recent development of the UAS/GAL4 system for directed gene expression, *Drosophila* provides a powerful model for analysis of gene function and of fundamental biological processes (Beckingham et al., 2005). The Malpighian tubules, in conjunction with the hindgut, form the functional kidney and act as the primary system for ionoregulation, osmoregulation and detoxification in *Drosophila* (O'Donnell and Rheault, 2005). There are two pairs of Malpighian tubules in *Drosophila*, an anterior and a posterior pair, which are composed of a single layer of principal and stellate cells (O'Donnell et al., 1996). The relative ease of access to the tubules, their simple structure as well as their role in transporting xenobiotics makes them ideal models for studying ion and fluid movement and elimination of toxins.

Malpighian Tubules and Ion Movement

The primary active ion pump in Malpighian tubules is generally accepted to be an apical vacuolar-type H⁺-ATPase which maintains a proton gradient across the apical membrane (O'Donnell et al., 1996). This gradient drives the movement of cations, typically Na⁺ and K⁺, from the cell to lumen through proton antiporters and also produces a favourable electrical gradient for the movement of Cl⁻ from cell to lumen (O'Donnell et al., 1996). Fluid movement through aquaporin water channels is a passive osmotic consequence of the active transport of ions (O'Donnell et al., 1982).

Malpighian Tubules and Organic Anions

Given that a wide range of charged and potentially toxic xenobiotics and endogenous compounds are actively excreted by the Malpighian tubules, it was of interest to determine the transport mechanisms involved in the excretion of organic cations and anions (Bresler et al.,

1989; Linton and O'Donnell, 2000). Using the anionic dye fluorescein, Bresler et al. (1989) demonstrated the active transport of organic acids through the cells of the Malpighian tubule, and identified three steps that must occur for transport: transport across the basolateral membrane, transport through the cytosol and subsequent transport across the apical membrane (Linton and O'Donnell, 2000).

Organic anions can be categorized as carboxylates, such as fluorescein, which are characterized by negatively charged carboxyl groups and a hydrophobic moiety, or sulphonates, such as indigo carmine, which possess a negatively charged sulphonate group as opposed to a carboxylate group (Linton and O'Donnell, 2000). Although it is unclear whether or not there is a common or separate transporter for carboxylates and sulphonates in tubules of some species, it appears that in *Drosophila* there are two organic anion transporters (Linton and O'Donnell, 2000). Linton and O'Donnell further demonstrated that small negatively charged organic molecules, such as para-aminohippuric acid (PAH), have a low permeability to the Malpighian tubules of *Drosophila melanogaster*, and this, coupled with the high rates of transport of PAH by the Malpighian tubules, suggests that the tubules are extremely efficient at excreting organic acids. Given that organic acids are actively transported against their concentration gradient, their movement must be coupled to an energy-dependent pump. By using Na⁺-free saline, as well as inhibiting the Na⁺/K⁺-ATPase using either K⁺-free saline or ouabain, it has been shown that PAH transport across the basolateral membrane of *Drosophila* Malpighian tubules is consistent with a Na⁺-dependent organic anion transporter (Linton and O'Donnell, 2000).

Research on the transport of organic acids across proximal kidney cells of the killifish and the cells of the urinary bladder of the crab *Cancer borealis* indicates that transport through the cytoplasm involves both the diffusion of the organic acid as well as vesicular transport (Miller et al., 1993, 1994). Similar observations were made via fluorescent staining in the Malpighian tubules of *Drosophila*, indicate diffusion of fluorescein throughout the cytoplasm (Linton and O'Donnell, 2000). In addition a punctate staining was also observed and, coupled with inhibition of vesicular movement by microtubular disruptors, suggested that organic acids move both through diffusion and through vesicular transport (Linton and O'Donnell, 2000).

Malpighian Tubules and Salicylate

A naturally occurring organic anion, salicylic acid, or salicylate, has been shown to inhibit the growth of insect species such as the corn earworm *Helicoverpa zea* (Li et al., 2002). Salicylate is a potentially harmful compound and previous research has focused primarily on the activation of detoxification enzymes in insects, such as the cytochrome P450 enzymes (Li et al., 2007). There is evidence, however, that the renal system is also involved in the excretion not only of metabolic wastes, but of toxins acquired through food. For example following consumption of a protein-rich meal by *Rhodnius prolixus*, transport of PAH and uric acid across the Malpighian tubules has been observed to increase (Maddrell and Gardiner, 1975; O'Donnell et al., 1983). Similarly, Bijelic et al., (2005) observed higher excretion rates of tetraethylammonium (TEA) by Malpighian tubules isolated from larval *Drosophila melanogaster* following 24 hour exposure to a TEA-enriched diet.

Exposure to chronic dietary salicylate is correlated with increases in the rates of secretion of fluid and salicylate by Malpighian tubules of larval *Drosophila melanogaster*, and also with increases in the rate of elimination of salicylate from the hemolymph (Ruiz-Sanchez and O'Donnell, 2007). Salicylate flux across the Malpighian tubules was reduced when tubules were bathed in Na⁺-free saline, supporting the idea that organic anion transport is facilitated by a Na⁺ cotransporter (Ruiz-Sanchez and O'Donnell, 2007). Further, Chahine and O'Donnell (2010) demonstrated that larvae reared on a salicylate-enriched diet for as little as 24 hours increased the fluid secretion rate by the Malpighian tubules and salicylate flux across the tubules. Fluid secretion is stimulated by intracellular second messengers such as cAMP or cGMP acting on the V-H⁺-ATPase on the apical membrane, however the fluid secretion of cAMP-stimulated control tubules is comparable to the fluid secretion of unstimulated tubules from salicylate-fed larvae (Ruiz-Sanchez and O'Donnell, 2007; Broderick et al., 2003; O'Donnell et al., 1996). Moreover the addition of cAMP to tubules from salicylate-fed larvae increased fluid secretion further, which suggests that fluid secretion is modulated via insertion of more ion transporters as opposed to an increase in levels of cAMP and second messengers (Ruiz-Sanchez and O'Donnell, 2007). A suggested candidate is the V-type H⁺-ATPase on the apical membrane; an increase in the number of proton pumps would increase the secretion of Na⁺, K⁺ and Cl⁻ with a subsequent

increase in the rate of secretion of osmotically obliged water (Ruiz-Sanchez and O'Donnell, 2007). These findings indicated that dietary exposure to salicylate leads to upregulation of multiple transporters implicated not just in the transport of salicylate, but also in the transport of inorganic ions (Na^+ , K^+ , Cl^-) which drive the observed increase in the rate of fluid secretion.

Upstream Regulation of Detoxification

In contrast to detailed studies on central regulators of detoxification in mammals, little is known about transcriptional regulation of xenobiotic detoxification in insects. The transcription factor cap 'n' collar isoform-C (CncC) and its negative regulator Kelch-like ECH-associated protein 1 (Keap1) have recently been identified as a major regulator of detoxification in response to oxidative or electrophilic stress (Nguyen et al., 2009). CncC proteins are conserved across vertebrate and invertebrate species including mammals, fish and insects, and function throughout embryogenesis and development or act as homeostatic regulators in response to endogenous and environmental stressors (Sykiotis and Bohmann, 2010). Given that mammalian nuclear receptors such as the pregnane X receptor (PXR) and constitutive androstane receptor (CAR) play a central role in detoxification pathways, an effort was made to characterize the role of the *Drosophila* ortholog DHR96 (Misra et al., 2011). Contrary to expectations, it was found that only approximately 10% of genes regulated by exposure to the xenobiotic phenobarbital (PB) depended on DHR96 for their proper transcriptional response whereas more than half of the genes regulated by PB and 70% of the genes upregulated by PB are also controlled by CncC and upregulated by CncC expression (Misra et al., 2011). By capitalizing on the UAS-GAL4 system, Misra et al., (2011) were able to confer resistance in *Drosophila* to lethal doses of the pesticide malathion by directing expression of Keap1RNAi in the principal and stellate cells of the Malpighian tubules. Further, since malathion is metabolized into organic anions such as malathion monocarboxylic acid, this suggests that activation of the Keap1/CncC pathway, at least through inhibition of Keap1, has a major role in organic anion detoxification (Neufeld et al., 2005; Morton and Holwerda, 1985). The components of the Keap1/CncC pathway are indicated in Figure 1.

Objectives

Given that dietary salicylate induces an increase in fluid secretion by Malpighian tubules and an increase in salicylate flux across the tubules isolated from *Drosophila melanogaster* third instar larvae, it is of interest to more closely examine the effects of dietary salicylate. Further, given that directed activation of the Keap1/CncC pathway in the principal and stellate cells of the Malpighian tubules of *Drosophila* is sufficient to confer resistance to lethal doses of malathion, coupled with the fact that malathion is metabolized to organic anions that are secreted at high rates by the tubules, it is of interest to determine what role, if any, the Keap1/CncC pathway has on the elimination of organic anions, particularly dietary salicylate.

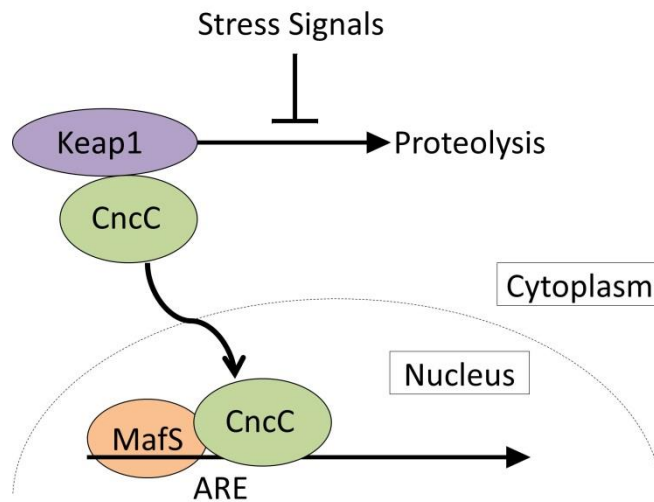


Figure 1: In the absence of stress signals, Keap1 binds CncC and designates it for proteolysis. Cellular stress due to oxidative or electrophilic damage causes Keap1 to release CncC which then translocates to the nucleus and binds to the small MafS protein and initiates transcription of AREs.

This thesis proposes to address three questions:

1. What is the effect of dietary salicylate on fluid secretion rate, concentration of salicylate in the secreted fluid and salicylate flux in Malpighian tubules isolated from adult *Drosophila melanogaster*?

2. What is the effect of directed manipulation of the Keap1/CncC pathway in the Malpighian tubules of *Drosophila* on fluid secretion rate and salicylate flux following exposure to dietary salicylate? Specifically, can activation of the pathway in flies reared on a control diet produce the same increase in fluid secretion rate and salicylate flux seen in control lines reared on salicylate-rich diet? The Keap1/CncC pathway will be manipulated in four ways to test the following four hypotheses:
- a. Inhibition of the negative regulator Keap1 in either the principal or stellate cells using the UAS-Keap1RNAi responder sequence should constitutively activate the pathway, and if Keap1/CncC has a role in upregulating fluid secretion rate and salicylate flux, we should expect to observe a similar response in activated lines reared on a salicylate-free diet relative to control lines reared on a diet containing salicylate.
 - b. Overexpression of the small MafS (muscle aponeurosis fibromatosis) protein in either principal or stellate cells, which heterodimerizes with CncC to promote transcription of ARE-dependent genes, should activate the pathway by reducing the amount of CncC needed to initiate a detoxification response. Thus we should expect a similar response in activated lines reared on a salicylate-free diet and in control lines reared on a diet containing salicylate.
 - c. Overexpression of CncC in either the principal or stellate cells using UAS-CncC should constitutively activate the pathway, assuming the increases in CncC expression are sufficient to counter the inhibiting effects of Keap1. Similar to the first two hypotheses, we should expect a response in activated lines reared on a salicylate-free diet to be comparable to control lines reared on a diet enriched in salicylate.
 - d. In contrast to the first three hypotheses, overexpression of Keap1 in either the principal or stellate cells using UAS-Keap1 should constitutively deactivate the pathway. Assuming that the presence of dietary salicylate is insufficient to produce a significant detoxification response, we expect to observe a dampened response to the presence of dietary salicylate in activated lines reared on a diet

containing salicylate compared to control lines also reared on a salicylate-rich diet.

3. Given that the Keap1/CncC protein complex regulates a broad detoxification response through upregulation of antioxidant response elements (AREs), are there xenobiotics regulated by Keap1/CncC that produce the same physiological response as dietary salicylate? Possible candidates include phenobarbital, chlorpromazine and caffeine, which have been shown to induce a coordinated transcriptional detoxification response consistent with one driven by a small number of, or single, transcription factors (Misra et al., 2011).

Materials and Methods

Insect rearing and diet preparation

Drosophila melanogaster were reared on control diet prepared as follows: 800 mL deionized water, 100 g sucrose, 18 g agar, 1 g potassium dihydrogen orthophosphate, 8 g sodium potassium tartate tetrahydrate, 0.5 g sodium chloride, 0.5 g magnesium chloride, 0.5 g calcium chloride, 0.5 g ferric sulphate, 200 mL deionized water, and 50 g dry active yeast. Following sterilization in an autoclave, the mixture was brought to 55 degrees Celsius and an acid mix (11 parts deionized water, 10 parts propionic acid and 1 part O-phosphoric acid (85%)) was added before the diet was allowed to cool. Flies were allowed to lay eggs on the diet for 2 days and were subsequently removed. Diets containing salicylate were created with the addition of the appropriate concentration of salicylate to produce diet concentrations of 10 mmol l⁻¹.

Drosophila stocks and rearing

Drosophila melanogaster Oregon R. strain (Bloomington #2376) were obtained from the Bloomington Drosophila Stock Center at Indiana University. C42 GAL4 and C724 GAL4 were provided by J. Dow of the University of Glasgow. UAS-Keap1RNAi, UAS-Keap1, UAS-MafS and UAS-CncC were provided by A. Campos of McMaster University. Males carrying the GAL4 driver for the principal (C42 GAL4) or stellate (C724 GAL4) cells of the Malpighian tubules were crossed with virgin females aged 1-2 days post eclosion carrying the UAS sequence to produce what will be termed activated lines, in contrast to control lines which were crossed back to white flies. All experiments were carried out on the adult females of the F1 aged 3-5 days post eclosion in the activated and control lines. Flies were allowed to lay eggs on the control or salicylate-rich diet for 5 days and were subsequently removed. The F1 eggs were incubated at 29 degrees Celsius to optimize UAS-GAL4 activation for 10 days after which recently eclosed adult females were used for experiments. For Oregon R, the eggs were incubated at room temperature (21-23 degrees Celsius) for approximately 12 days after which recently eclosed (3-5 days post eclosion) adult females were collected.

Chlorpromazine exposure

Adult females were collected 2-3 days post eclosion and were starved overnight for 16 hours on 1% agar. Whatman filter paper was cut into a disk approximately 2.5 mm in diameter and soaked in 150 μ L of deionized water and placed in the polystyrene vial with the flies to create a humid environment. Immediately following starvation, flies were exposed for 24 h to a 5% sucrose or 5% sucrose + 0.1% chlorpromazine solution which was administered by soaking two pieces of 2.5 mm diameter Whatman filter paper with 250 μ L of the solution. During both starvation and exposure adult females were incubated at 29 degrees Celsius to maintain optimal UAS-GAL4 activation.

Ramsay assay

Fluid secretion rate and salicylate flux across isolated Malpighian tubules were measured using the Ramsay assay (see Figure A1 in the Appendix). A pair of Malpighian tubules connected by a common ureter was dissected in standard bathing media (SBM) (117.5 mM NaCl, 20 mM KCl, 2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 8.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10.2 mM NaHCO_3 , 4.3 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 8.6 mM HEPES, 20 mM glucose, 10 mM glutamine titrated to pH 7.0 with 1 M NaOH). Dissected pairs of tubules were transferred to an 18 μ L bathing saline droplet pipetted into a well under paraffin oil in a Sylgard-lined petri dish. The distal portion of one tubule was drawn into the bathing solution. The distal portion of the other tubule was wrapped around a metal pin in the dish such that the common ureter of the tubule pair was sufficiently far from the saline droplet so as to allow a secreted droplet to form at the ureter. Secreted droplets were collected after 30 or 40 minutes and the diameter (d) was recorded using an eye-piece micrometer. The diameter was used to calculate the volume of the spherical secreted droplets ($\text{volume} = \pi d^3/6$). Fluid secretion rate (nl min^{-1}) was calculated by dividing the droplet volume over the time it formed. Tubules were bathed either in 18 μ L of SBM as the control or 18 μ L of SBM with the addition of 2 μ L of 500 μ M salicylate to bring the concentration of salicylate in the bathing droplet to 50 μ M.

Salicylate selective and reference microelectrodes

The concentration of salicylate in the secreted droplets was measured using a salicylate selective microelectrode previously described (O'Donnell and Rheault, 2005). Micropipettes were prepared by pulling borosilicate glass capillaries (TW150-4, World Precision Instruments Inc., Sarasota, FL) on a Narishige puller. Reference microelectrodes were back-filled with 500 mM KCl. Salicylate selective microelectrodes were prepared from micropipettes that were first silanized. N, N-dimethyltrimethylsilylamine (0.35 μL) was pipetted onto the inner surface of a 15 mm glass petri dish that was immediately inverted over 16 – 20 micropipettes on a hot plate at 200 degrees Celsius for 30 minutes. Salicylate-selective microelectrodes were prepared by back-filling each micropipette with 5 mM salicylate, 128 mM NaCl and 14 mM KCl, then front-filling with a salicylate selective cocktail (9% TDMA and 91% NPOE). Both the salicylate selective and reference microelectrodes were connected through chlorided silver wires to an A-M Systems pH/ion amplifier (Model 2000), which in turn was connected to a computerized data acquisition and analysis system (PowerLab 4/26 with Chart 5 software, ADInstruments, Inc., Colorado Springs, CO, USA). A microelectrode calibration curve relating voltage output to salicylate concentration was used to convert the microelectrode voltage into salicylate concentration. Three calibration solutions of 10, 1 and 0.1 mmol l^{-1} of salicylate were made up in 150 mM KCl. The slopes for a change from 10 to 1 mmol l^{-1} were on average 58 mV, and for a change from 1 to 0.1 mmol l^{-1} the slopes averaged 35 mV. Salicylate flux (pmol min^{-1}) across the Malpighian tubules was calculated as the product of the fluid secretion rate (nl min^{-1}) and the concentration of salicylate (mmol l^{-1}) in the secreted fluid droplet.

Results

Effects of dietary salicylate on secretion of fluid and salicylate by isolated Malpighian tubules

Previous studies have shown that *Drosophila melanogaster* larvae reared on a diet containing 10 mM salicylate for 24 hours increase fluid secretion by the Malpighian tubules and salicylate flux across the tubules. By contrast, the response of tubules of adult flies to dietary salicylate was unknown. Recently eclosed (< 1 day) adults were reared on media containing 10 mM salicylate for 1 day (24 hours), 2 days, 5 days and 7 days. There were no observed changes in fluid secretion rate, concentration of salicylate in the secreted fluid or salicylate flux between tubules isolated from salicylate-fed adults and tubules isolated from adults reared on control media (data not shown, $N = 12-13$ adults per group).

Adults reared from eggs on a diet containing 10 mM salicylate increase fluid secretion by 1.7-fold from 0.36 nl min^{-1} to 0.61 nl min^{-1} compared to tubules isolated from adults reared on a control diet. Salicylate flux across the tubules similarly increased 2.1-fold from $0.61 \text{ pmol min}^{-1}$ to $1.25 \text{ pmol min}^{-1}$ compared to tubules isolated from adults reared on control media. There was no change in the concentration of salicylate in fluid secreted by tubules isolated from adults reared on a salicylate-rich diet relative to tubules isolated from adults reared on control media (Figure 2).

Effects of manipulations of the Keap1/CncC pathway on fluid regulation and salicylate secretion

UAS-Keap1RNAi

Inhibition of Keap1 was predicted to increase fluid secretion rate and salicylate flux in the absence of dietary exposure to salicylate. There was a significant increase in fluid secretion rate and salicylate flux seen in control lines when reared on a diet containing salicylate compared to a salicylate free diet (Figure 3). When tubules isolated from salicylate-fed adults were compared to tubules isolated from adults reared on control media, fluid secretion rate increased 1.4-fold from 0.33 nl min^{-1} to 0.45 nl min^{-1} in the white control line, increased 1.4-fold from 0.35 nl min^{-1} to 0.49 nl min^{-1} in the C42 GAL4 control line, increased 1.1-fold from 0.33 nl min^{-1} to 0.37 nl min^{-1} in the C724 GAL4 control line and increased 1.2-fold from 0.36 nl min^{-1} to

0.44 nl min⁻¹ in the UAS-Keap1RNAi control line (Figure 4). Salicylate flux similarly increased 1.5-fold from 0.54 pmol min⁻¹ to 0.84 pmol min⁻¹ in the white control line and increased 3.1-fold from 0.35 pmol min⁻¹ to 1.11 pmol min⁻¹ in the C42 GAL4 control line when tubules isolated from adults reared on a diet containing salicylate and were compared with adults reared on salicylate-free media (Figure 3). The concentration of salicylate in the secreted fluid increased significantly by 2.4-fold in the C42 GAL4 control line from 1.03 mmol l⁻¹ to 2.47 mmol l⁻¹. In contrast to the findings with multiple control lines, there was no further increase in fluid secretion rate and salicylate flux in activated Keap1RNAi lines reared on a diet containing salicylate compared to a salicylate-free diet when expression was directed to principal or stellate cells (Figure 4). Fluid secretion rate and salicylate fluxes for tubules isolated from activated Keap1RNAi lines reared on control media were comparable to fluid secretion rate and salicylate flux values from control lines reared on a diet enriched with salicylate.

UAS-MafS

Overexpression of MafS was predicted to increase fluid secretion rate and salicylate flux in the absence of dietary exposure to salicylate. As described above, fluid secretion rate in tubules isolated from flies from the white, C42 GAL4 and C724 GAL4 control lines increased in response to dietary salicylate relative to flies fed salicylate-free diet. There was also an increase of 1.4-fold from 0.32 nl min⁻¹ to 0.44 nl min⁻¹ in the UAS-MafS control line between tubules isolated from flies reared on the salicylate-rich diet relative to those of flies reared on the control diet (Figure 5). There was no further increase in fluid secretion rate and salicylate flux in activated MafS lines except when expression was driven in the stellate cells. Salicylate flux increased 1.5-fold from 0.78 pmol min⁻¹ to 1.14 pmol min⁻¹ in tubules isolated from adults overexpressing MafS in stellate cells fed on a diet enriched with salicylate compared to tubules isolated from adults overexpressing MafS in stellate cells fed on salicylate-free media (Figure 5).

UAS-CncC

Overexpression of CncC was predicted to increase fluid secretion rate and salicylate flux in the absence of dietary exposure to salicylate. Fluid secretion rate also increased significantly in the control line UAS-CncC (see Figure 3 for the other control line changes) by 1.5-fold from

0.31 nl min⁻¹ to 0.46 nl min⁻¹ when comparing tubules isolated from adults reared on a salicylate-rich diet and tubules isolated from adults reared on a salicylate-free diet. As seen in the responses of tubules from the control lines (see *UAS-Keap1RNAi* section), there was an increase of 1.3-fold and 1.2-fold in fluid secretion rate from 0.35 nl min⁻¹ to 0.45 nl min⁻¹ and from 0.31 nl min⁻¹ to 0.39 nl min⁻¹ in activated lines overexpressing CncC directed to principal and stellate cells respectively when compared to activated lines reared on salicylate-free media (Figure 6). Further, salicylate fluxes for tubules from activated lines reared on either a salicylate-free or salicylate-rich diet are not comparable to salicylate fluxes for tubules from control lines reared on a salicylate-rich diet. This is also in contrast to activated MafS and Keap1RNAi lines where salicylate flux values from salicylate-free or salicylate-rich diets are comparable to control lines reared on a diet containing salicylate.

UAS-Keap1

Overexpression of Keap1 was predicted to repress any increases in fluid secretion rate and salicylate flux in the presence of dietary exposure to salicylate. Fluid secretion rate significantly increased in the control line *UAS-Keap1* (see Figure 3 for the other control line changes) by 1.4-fold from 0.36 nl min⁻¹ to 0.52 nl min⁻¹. Fluid secretion rate and salicylate flux increased 1.2-fold and 1.8-fold respectively from 0.32 nl min⁻¹ to 0.38 nl min⁻¹ and from 0.58 pmol min⁻¹ to 1.05 pmol min⁻¹ when Keap1 overexpression was driven in stellate cells (*C724>Keap1*) on a salicylate-rich compared to salicylate-free diet (Figure 7). In contrast there was no significant increase in fluid secretion rate and salicylate flux when Keap1 overexpression was driven in principal cells (*C42>Keap1*). Moreover, salicylate fluxes for tubules across control lines reared on a salicylate-rich diet are comparable to salicylate fluxes for tubules of flies where Keap1 overexpression is driven in stellate cells and were reared on a diet containing salicylate.

Effects of manipulation of the Keap1/CncC pathway in conjunction with chlorpromazine exposure:

UAS-Keap1RNAi

Knockdown of Keap1 was predicted to increase fluid secretion rate and salicylate flux in the absence of exposure to chlorpromazine. Exposure to dietary chlorpromazine did not alter

fluid secretion rate, the concentration of salicylate in the secreted fluid, or salicylate flux in tubules isolated from any of the control lines, relative to tubules isolated from flies reared on chlorpromazine-free diet. In contrast, exposure to dietary chlorpromazine increased fluid secretion rate and salicylate flux significantly by 1.2-fold and 1.9-fold respectively, when Keap1 expression in the principal cells was inhibited using Keap1RNAi (Figure 8).

UAS-Keap1

Overexpression of Keap1 was predicted to repress fluid secretion rate and salicylate flux in the presence of exposure to chlorpromazine. There was no change in fluid secretion rate or salicylate flux in tubules isolated from control lines upon exposure to dietary chlorpromazine. When Keap1 was overexpressed in the principal cells, fluid secretion rate of tubules isolated from flies reared on diet containing chlorpromazine increased by 1.2-fold from 0.29 nl min⁻¹ to 0.34 nl min⁻¹ and salicylate flux increased by 1.8-fold from 0.49 pmol min⁻¹ to 0.89 pmol min⁻¹ (Figure 8). The concentration of salicylate in the fluid secreted by tubules isolated from flies from the UAS-Keap1 control line increased significantly by 1.5-fold from 1.56 mmol l⁻¹ to 2.33 mmol l⁻¹ in response to dietary exposure to chlorpromazine.

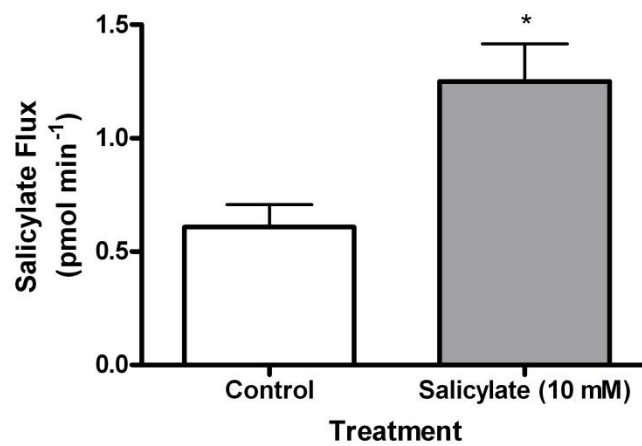
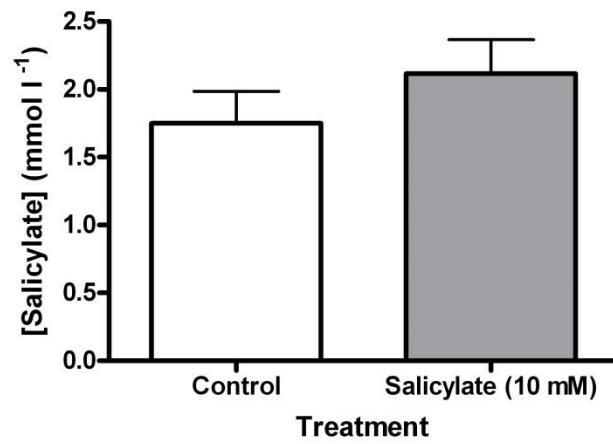
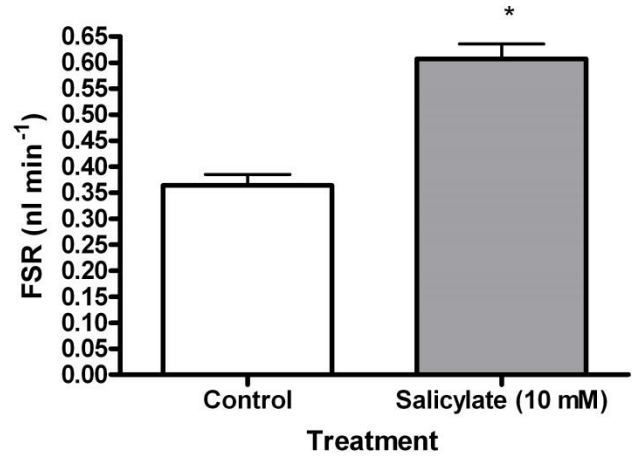


Figure 2: Adults reared from eggs on a diet containing dietary salicylate show a similar response to that seen in 3rd instar larvae. Fluid secretion rate (A) and salicylate flux (C) increased significantly in tubules isolated from adults reared from eggs on a diet containing 10 mM salicylate compared to tubules isolated from adults reared on control media. (*Unpaired t-test, $P < 0.05$, $N = 8-22$ adults per group*).

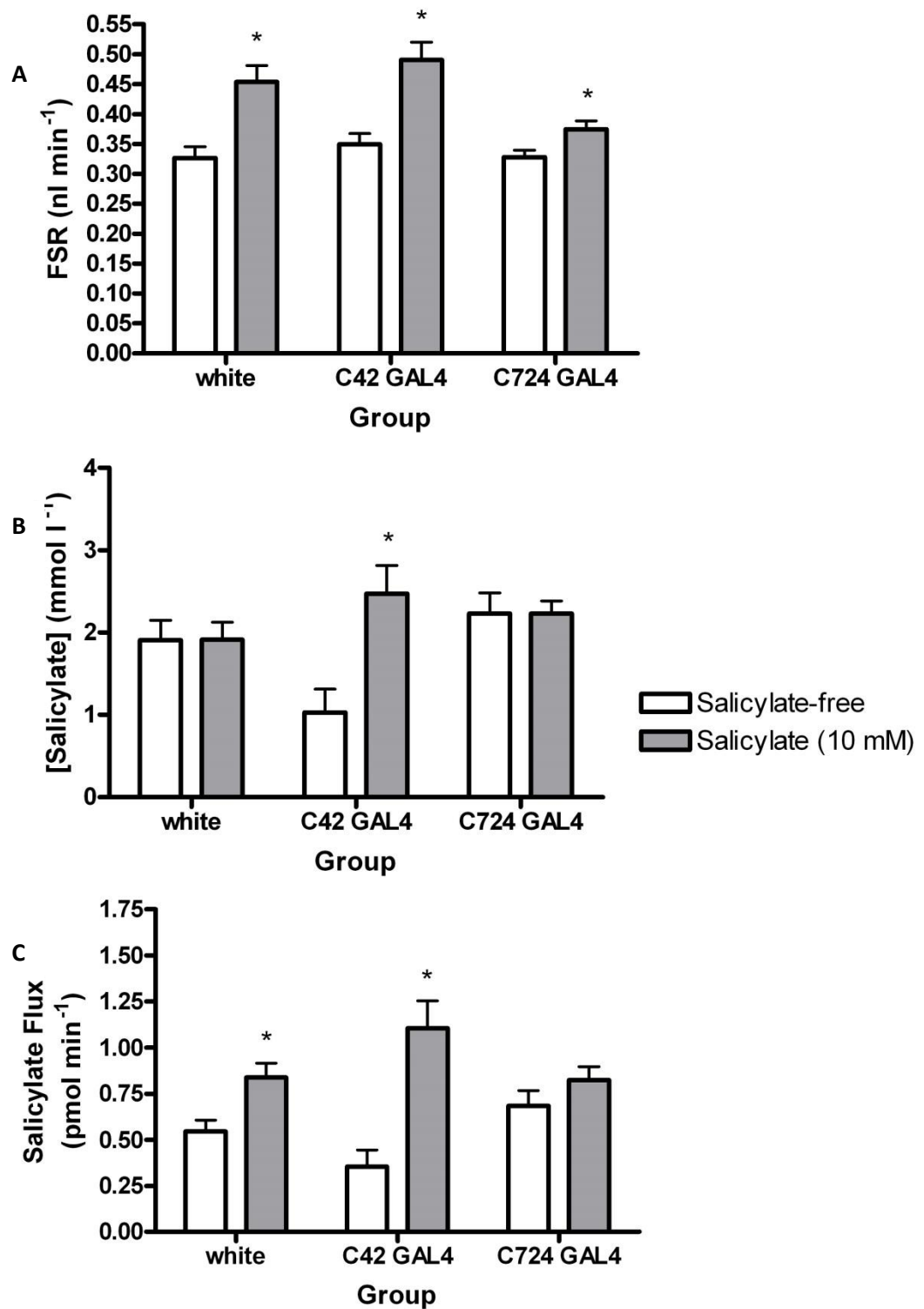


Figure 3: Consistent with larval responses, exposure to dietary salicylate increases fluid secretion rate and salicylate flux across isolated Malpighian tubules. Fluid secretion rate (A) increased significantly across all groups and salicylate flux (C) increased significantly in all groups except C724 GAL4. (Unpaired *t*-test, $P < 0.05$, $N = 10-25$ adults per group).

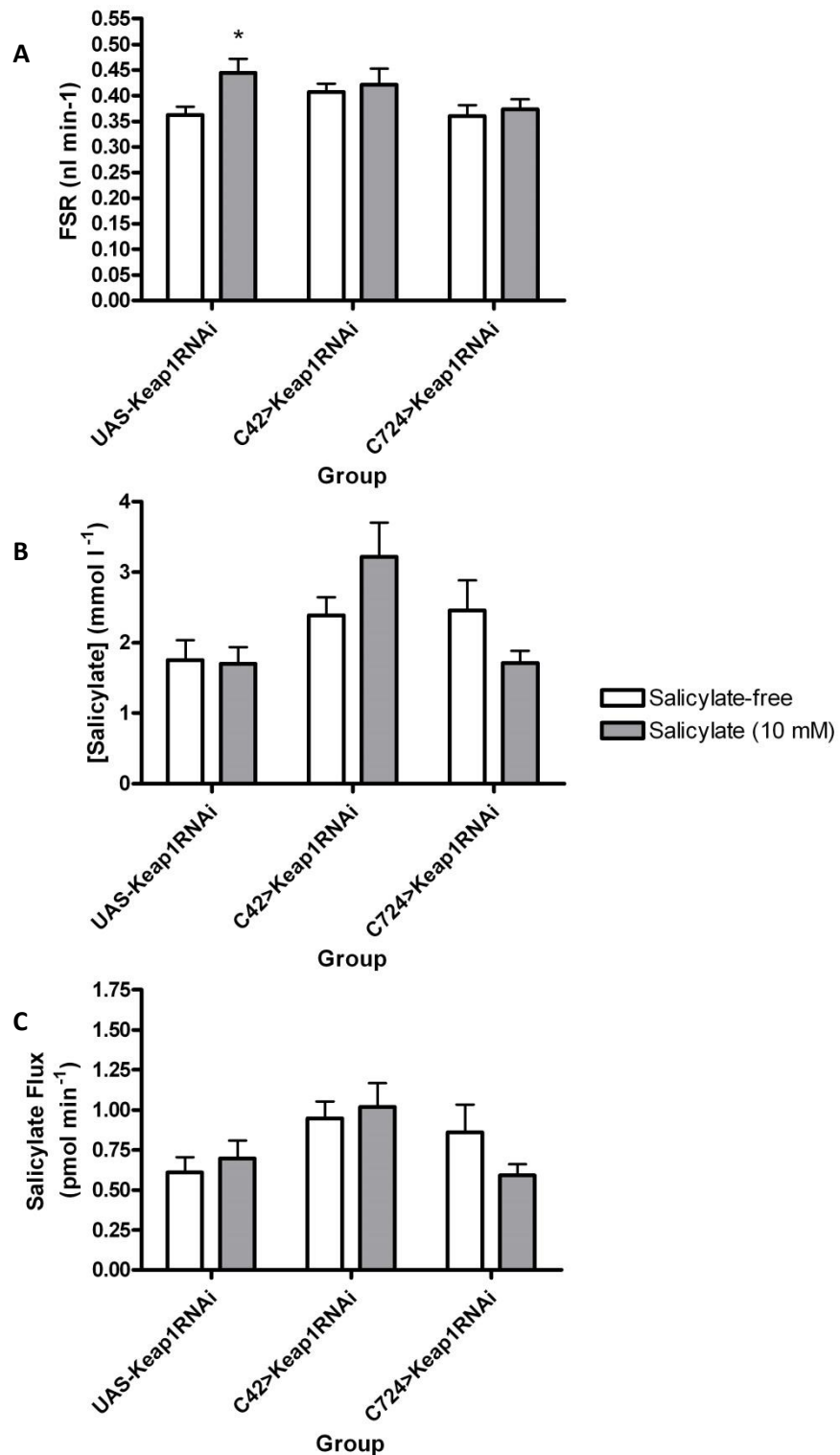


Figure 4: Inhibition of Keap1 in principal or stellate cells is sufficient to produce a comparable physiological response to control lines reared on a diet containing salicylate. Fluid secretion rate (A) and salicylate flux (C) across control lines (Figure 3) increases significantly when reared on a diet containing salicylate compared to a salicylate-free diet. Fluid secretion rate (A) and salicylate flux (C) do not further increase across activated lines (last two groups) when reared on a salicylate-rich diet. (Unpaired *t*-test, $P < 0.05$, $N = 10-25$ adults per group).

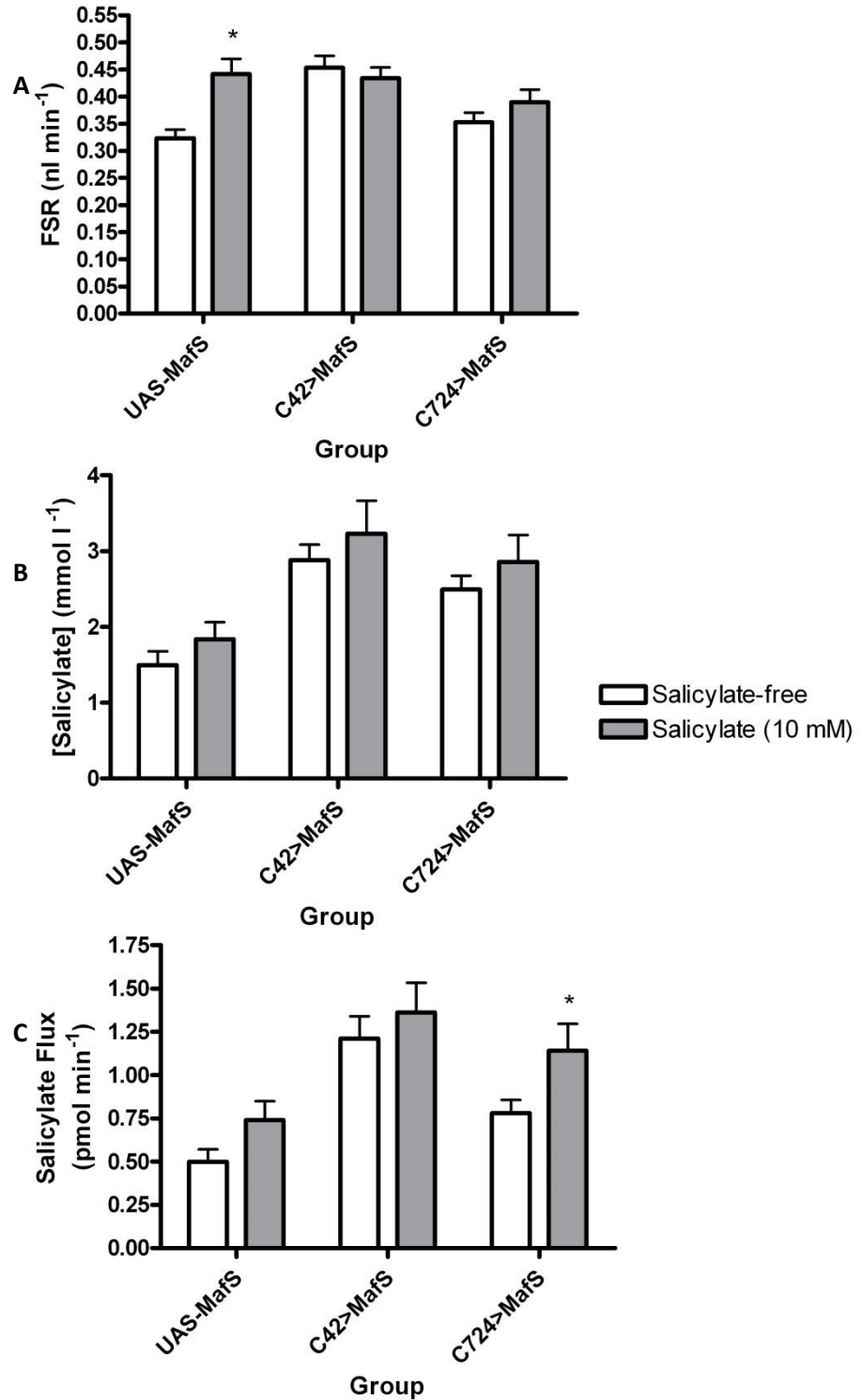


Figure 5: Overexpression of MafS has differing effects on salicylate flux when expressed in stellate cells compared to principal cells. Fluid secretion rate (A) and salicylate flux (C) increase significantly across control lines (Figure 3). The presence of dietary salicylate further increases salicylate flux (C) when MafS is overexpressed in the stellate cells (C724>MafS), however dietary salicylate did not induce further increases in fluid secretion rate (A) across both activated lines. (Unpaired *t*-test, $P < 0.05$, $N = 10-25$ adults per group).

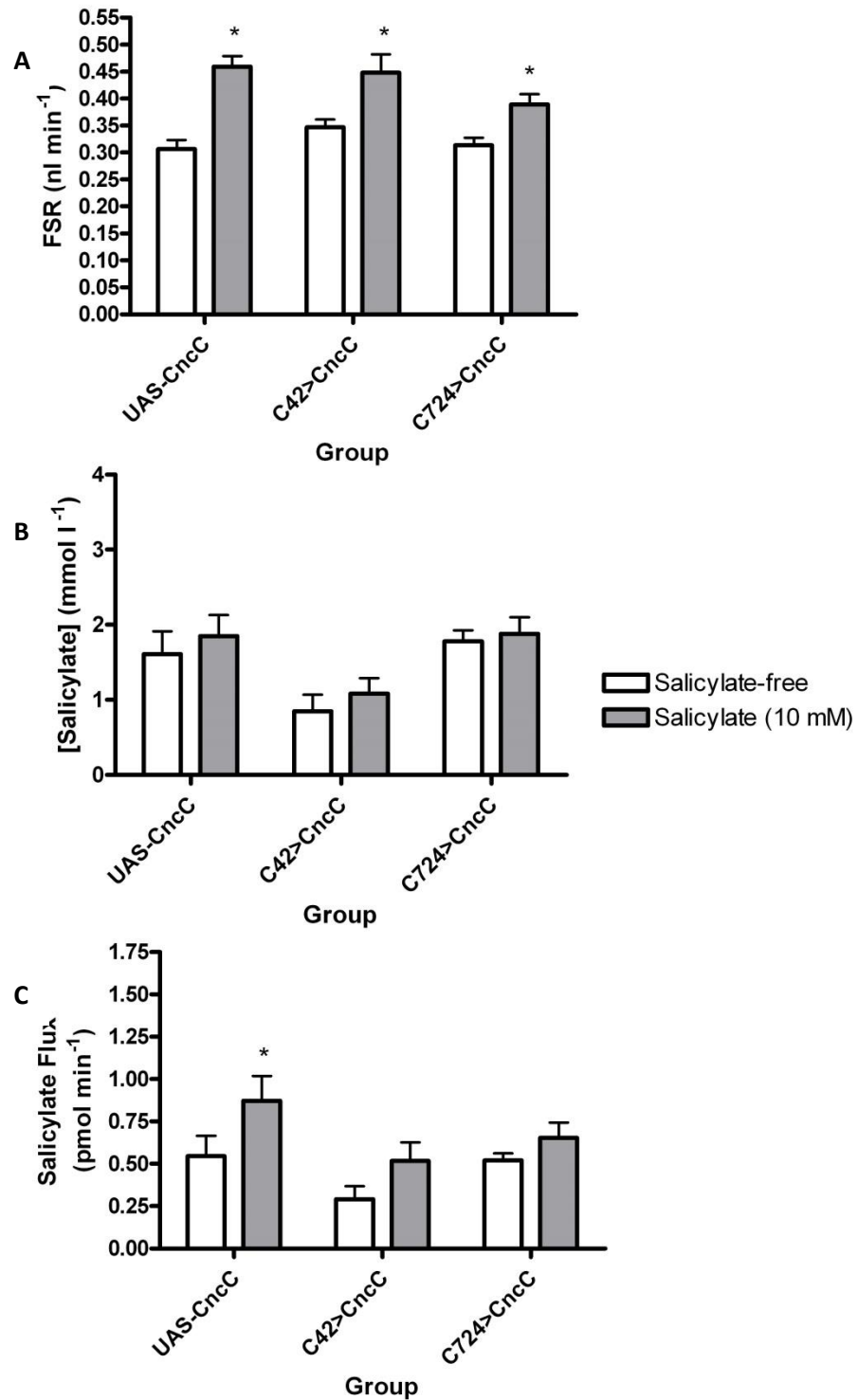


Figure 6: Overexpression of CncC in principal or stellate cells appears to have a detrimental effect on salicylate flux. Fluid secretion rate (A) increased significantly across all groups when reared on a diet containing salicylate compared to salicylate-free media. Salicylate flux (C) conversely did not increase in activated lines. (*Unpaired t-test, P < 0.05, N = 10-25 adults per group*).

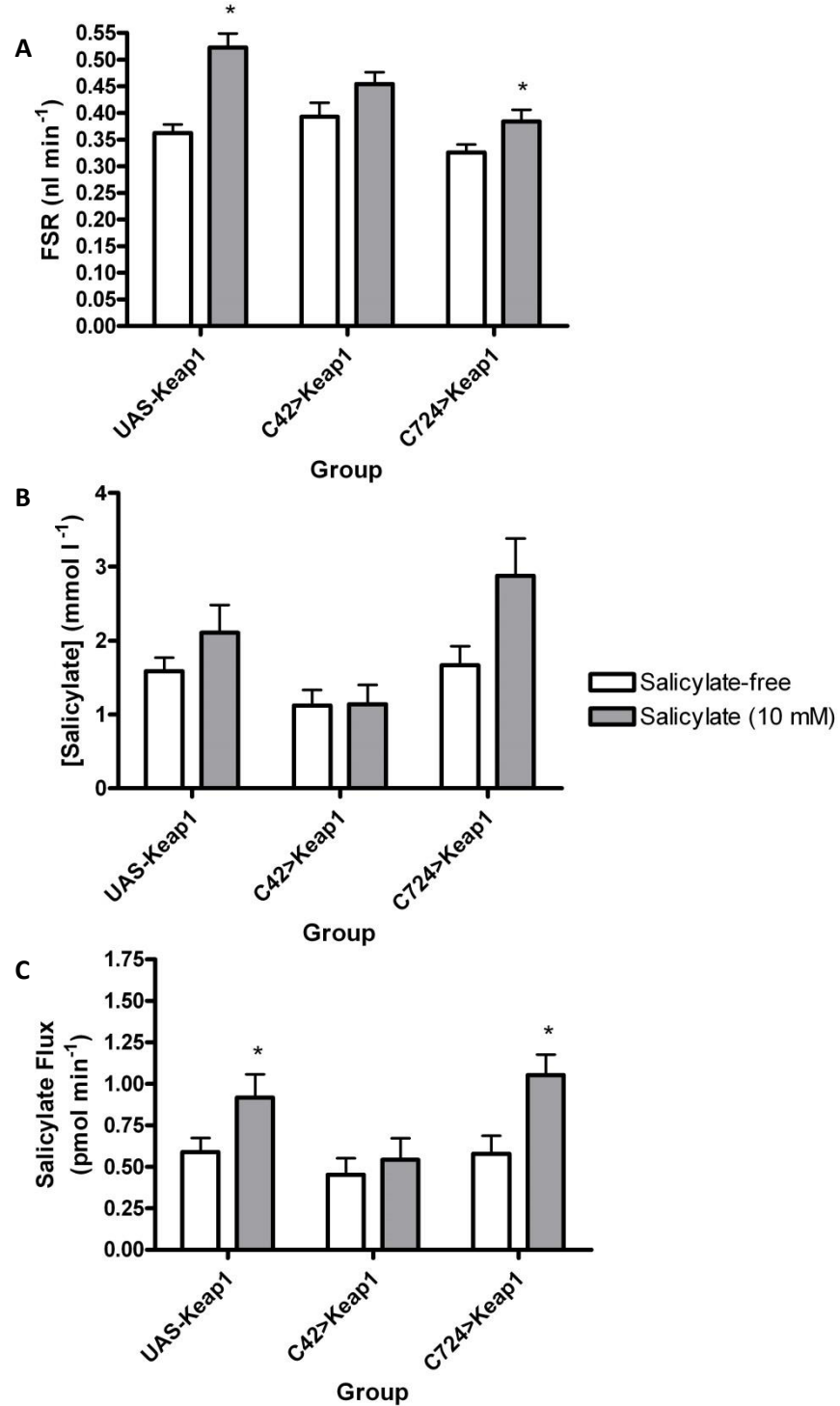


Figure 7: Inhibition of the pathway via overexpression of Keap1 appears to support previous research indicating stellate cells do not transport organic anions. Fluid secretion rate (A) significantly increased across all control lines (Figure 3) and when overexpression of Keap1 was directed to stellate cells. Salicylate flux (C) similarly increased significantly when activation was directed to stellate cells. (*Unpaired t-test, P < 0.05, N = 9-25 adults per group*).

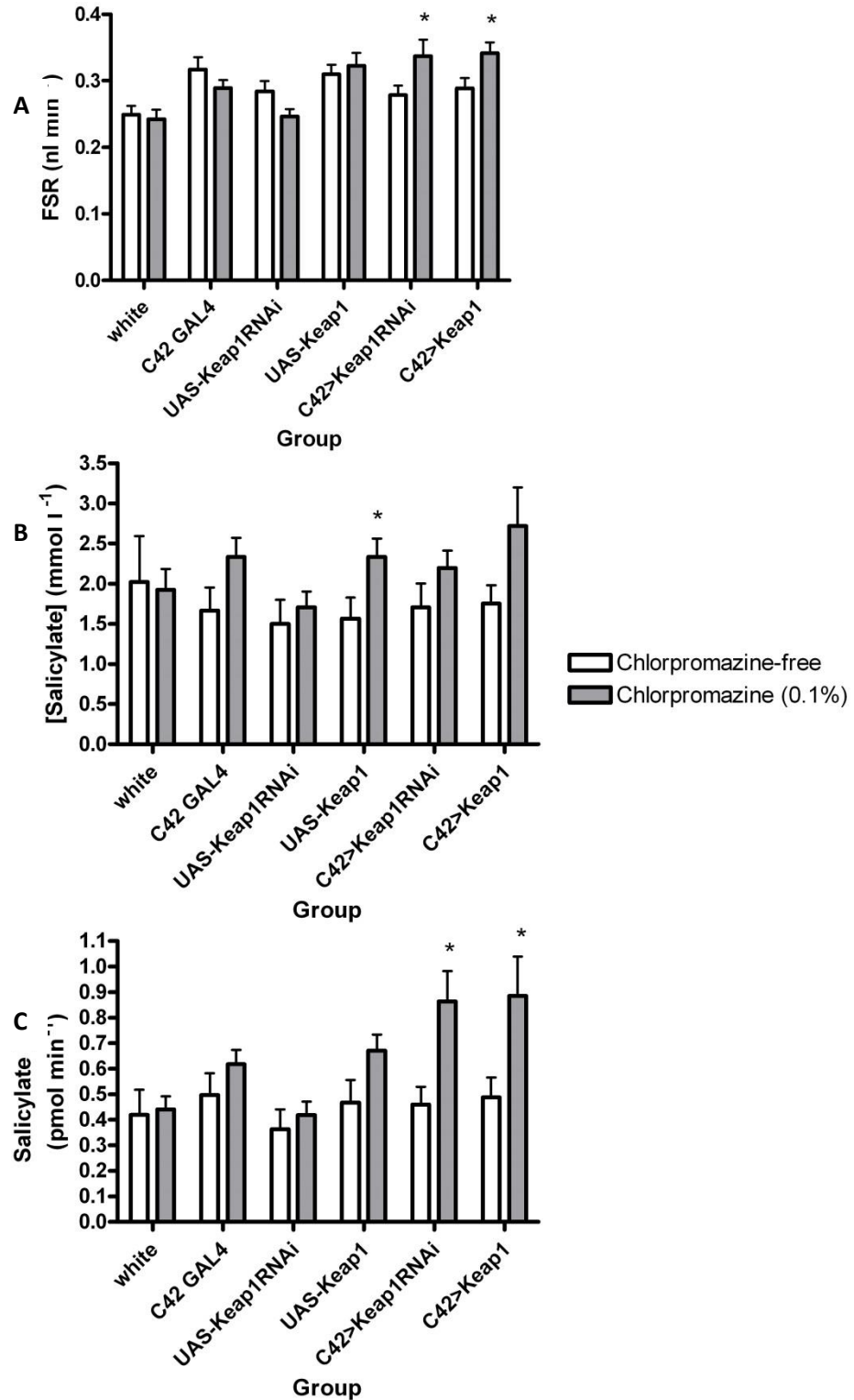


Figure 8: Exposure to chlorpromazine does not elicit the same effects as exposure to dietary salicylate across control lines. Fluid secretion rate (A) and salicylate flux (C) increase significantly when the pathway is constitutively activated or constitutively deactivated in principal cells via inhibition and overexpression of Keap1 respectively, however exposure to chlorpromazine had no effect on control lines. (*Unpaired t-test, $P < 0.05$, $N = 10-25$ adults per group*).

Discussion

Previous studies have shown that Malpighian tubules isolated from third instar *Drosophila melanogaster* larvae upregulate rates of fluid secretion in as little as 24 hours following exposure to dietary salicylate (Chahine and O'Donnell, 2010; Ruiz-Sanchez and O'Donnell, 2007). Here we show for the first time that tubules isolated from adult females aged 3-5 days post eclosion show significantly increased rates of fluid secretion across isolated tubules when reared from eggs on a diet containing salicylate (Figure 2A). Similar to the response seen in third instar larvae, salicylate flux also increased significantly across tubules isolated from adult females when reared on a diet containing salicylate (Figure 2C). It is important to note that salicylate flux is calculated as the product of fluid secretion rate and the concentration of salicylate in the secreted fluid, and that any increases in fluid secretion rate should thus be met by corresponding decreases in concentration of salicylate in the secreted fluid. Maintenance of the concentration of salicylate in conjunction with increased rates of fluid secretion is thus indicative of increased transport of salicylate (Ruiz-Sanchez and O'Donnell, 2007).

Our results also indicate that larval exposure to salicylate affects how adults respond to the same diet. While larvae respond to acute and chronic exposure to dietary salicylate by increasing fluid secretion rate and salicylate flux, adults respond to chronic dietary salicylate only if they have been reared from eggs on a diet containing salicylate. Exposure to dietary salicylate for up to 7 days post eclosion had no effect on fluid secretion or salicylate flux across tubules isolated from adult females. The inability of adults to respond to dietary salicylate following eclosion may indicate subtle differences in strategies for responding to dietary toxins between larvae and adults. Highly mobile adults are able to move from one source of food to another which could reduce pressure for organ or cell mediated detoxification responses, in contrast to relatively immobile larvae.

The results of this study indicate that the protein complex Keap1/CncC may play a role in regulating fluid secretion and organic anion detoxification. When reared on a diet containing salicylate, fluid secretion rate increased significantly across all control lines. This is consistent with known larval and adult responses to dietary salicylate (Chahine and O'Donnell, 2010; Ruiz-

Sanchez and O'Donnell, 2007). In contrast to our predictions and previous work on larvae and adults, there was no increase in salicylate flux observed in C724 GAL4, UAS-Keap1RNAi and UAS-MafS control lines when reared on a diet containing salicylate (Figures 3C, 4C and 5C). While it is possible that the genetic background as a whole could contribute to the unexpected physiological response, it's more likely that P-element insertion of the GAL4 driver and UAS responder disrupted normal gene transcription and by extension the expected response, especially given the fact that expected increases in salicylate flux were observed in the C42 GAL4, UAS-CncC and UAS-Keap1 control lines (Figures 3C, 6C and 7C) (Spradling et al., 1999).

Directed manipulation of Keap1RNAi to principal and stellate cells of the Malpighian tubules provides the clearest evidence for Keap1/CncC involvement in upregulating fluid secretion and salicylate flux, insofar as our results matched our predictions. There was no further increase in fluid secretion rate or salicylate flux across tubules isolated from adults when Keap1 inhibition was driven in principal or stellate cells when reared on a salicylate-rich diet (Figure 4). This suggests that CncC may have a role in regulating inorganic ion transport and osmotically-obliged fluid secretion through the tubules as well as organic anion detoxification. In contrast to the specialized functions of different isoforms of the homologous vertebrate transcription factor NFE2 (nuclear factor erythroid-derived 2) related factor 2 (Nrf2), CncC's structural homology to Nrf1 and Nrf3 indicate that the diversified functions of Cnc proteins may be encoded in a single locus in *Drosophila* (Sykiotis and Bohmann, 2010). In vertebrates, transcript products of Nrf2 include thioredoxins, glutathione-synthesizing enzymes, glutathione S-transferases and proteasome subunits, all of which are involved in what is known as the "electrophile counterattack" (Sykiotis and Bohmann, 2008). The role of CncC as an important central regulator of fluid secretion, specifically via increased transcription of inorganic ion transporters or increased activity of second messengers involved in diuresis, will need to be further investigated.

Directed overexpression of MafS in the principal cells, insofar as the response matched our prediction, similarly provides evidence for Keap1/CncC involvement in regulating fluid and organic ion secretion. The presence of dietary salicylate did not further increase fluid secretion rate or salicylate flux across isolated Malpighian tubules (Figure 5). In contrast, there was a

significant increase in salicylate flux when MafS overexpression was driven in the stellate cells (Figure 5C). This response more closely resembles the expected control line responses to the presence of dietary salicylate. Given that an extensive number of homo and heterodimers can be formed with small Maf proteins to regulate transcription at the Maf recognition element (MARE; aka CncC binding site), overexpression of MafS may promote dimerization that has negative regulatory effects on transcription (Motohashi et al., 2000). Moreover, it has been argued that maintaining an optimal quantitative balance between Maf and Cnc is vital for Cnc-dependent transcriptional activation, even under non-stressed circumstances where basal levels of Nrf2 in mice were shown to maintain housekeeping expression of the same antioxidant and detoxification genes (Sykiotis and Bohmann, 2008; Motohashi and Yamamoto, 2004). Consequently our attempts at experimentally manipulating levels of MafS and CncC individually may not have had the predicted transcriptional effects.

Directed overexpression of CncC to principal and stellate cells does not provide evidence for the role of Keap1/CncC in regulating fluid secretion and organic ion detoxification, insofar as our results did not match our predictions. When overexpression of CncC was driven in principal and stellate cells, no further increase in salicylate flux was observed when tubules were isolated from salicylate-fed adults compared to tubules isolated from adults reared on control media (Figure 6C). This is in contrast to an unexpected increase in fluid secretion rate when overexpression of CncC was driven in principal and stellate cells (Figure 6A). Studies on *Drosophila* and mammals have demonstrated that CncC/Nrf2 has an autoregulatory function on the production of Keap1. Levels of Keap1 were reduced by inhibition of CncC using UAS-CncCRNAi and were increased by overexpressing CncC using UAS-CncC (Sykiotis and Bohmann, 2008). Further, overexpression and inhibition of CncC when driven in all cell types using an ubiquitous tubulin-GeneSwitch-GAL4 have been shown to increase and decrease lifespan respectively when exposed to semi-lethal doses of paraquat compared to controls (Sykiotis and Bohmann, 2008). It is possible that threshold effects on CncC are responsible for the increase in fluid secretion rate and lack of change in salicylate flux in activated CncC lines, especially given the activity of an autoregulatory feedback loop between CncC and Keap1. Specifically, overexpression of CncC in the absence of dietary salicylate may drive increased expression of Keap1 which would ultimately lower experimentally induced levels of CncC. This is in contrast to

overexpression of CncC in the presence of dietary salicylate, where Keap1 binding will be in competition between CncC and salicylate or oxidative damage following salicylate ingestion. Alternatively, there may be separate, or at the very least nuanced, differences in fluid regulation via inorganic ion movement and salicylate transport.

While the first three manipulations of the Keap1/CncC pathway upregulated ARE transcription, the fourth manipulation of the pathway using UAS-Keap1 acts antagonistically to downregulate ARE transcription. Insofar as the observed results matched our predictions, directed overexpression of Keap1 to the principal cells of the Malpighian tubule provides evidence for Keap1/CncC involvement in regulating fluid secretion and organic ion detoxification (Figure 7). Contrary to our predictions, there was an increase in fluid secretion rate and salicylate flux across isolated Malpighian tubules when Keap1 overexpression was driven in the stellate cells, a response that is supportive of previous research highlighting functional differences between principal and stellate cells (Figure 7). The *Drosophila* Integral Protein (DRIP) is localized to the stellate cells of the Malpighian tubules and has a sequence most similar to the water-specific human aquaporin hAQP4, suggesting stellate cells may transport water at very high rates (Kaufmann et al., 2005; Spring et al., 2007). Further, only principal cells were observed to fluoresce following exposure of Malpighian tubules from *Drosophila melanogaster* to fluorescein, indicating that stellate cells do not transport small type one organic anions such as fluorescein and salicylate (Linton and O'Donnell, 2000). Taken together, these data suggest a specialized role for stellate cells as primarily involved in fluid transport and kinin-regulated chloride transport whereas the larger and more mitochondrial-dense principal cells act as the primary iono and xenobiotic regulators (O'Donnell et al., 1998; Cabrero et al., 2014). Our data are similarly indicative of the inability of stellate cells to transport organic anions. Specifically, downregulation of the Keap1/CncC pathway in stellate cells would have little effect on active ion transport, the majority of which would be taking place in the principal cells, consequently inducing the physiological response expected in control lines when reared on a diet containing salicylate.

Interestingly both an increase in Keap1 and inhibition of Keap1 elicit similar responses in fluid secretion rate and salicylate flux following exposure to chlorpromazine, while there is no

effect on control lines (Figure 8). Chlorpromazine has been shown to inhibit adenylate cyclase activity as an antagonist to dopamine stimulation, which consequently can modify cAMP levels and ultimately affect fluid regulation (Uzzan and Dudai, 1982; Ruiz-Sanchez and O'Donnell, 2007). Alternatively chlorpromazine may be detoxified in the hemolymph via cytochrome P450 enzymes, as studies on humans have revealed CYP2D6 is involved in phase I detoxification of chlorpromazine, however no homologs have been identified in *Drosophila* (Yoshii, et al., 2000).

Future Studies

It is of interest to quantify gene expression by measuring levels of relevant mRNA sequences using qPCR. Quantification of basal levels of genes involved in the Keap1/CncC pathway as well as quantification of increases due to UAS-GAL4 manipulation could provide additional evidence for the role of Keap1/CncC in regulating xenobiotic detoxification and its role in upregulating fluid secretion rate.

Similarly, given that Keap1/CncC responds to oxidative or electrophilic damage, it is of interest to more closely examine the effect of directed manipulation of the Keap1/CncC pathway in the Malpighian tubules of *Drosophila* on fluid secretion rate and salicylate flux following exposure to potent oxidizing agents such as paraquat. In addition to different xenobiotics, it would be useful to determine if Keap1/CncC is present in other insect species. SKN-1 (Skinhead family member 1) has been identified as the CncC homolog in *C. elegans*, however thus far there has been no effort to identify and characterize possible homologs in other invertebrate species (Sykiotis and Bohmann, 2010). It would be of particular interest to compare the effect of manipulation of Keap1/CncC on fluid secretion rate and organic anion elimination in hemipteran species, if such a homolog exists, specifically *Rhodnius prolixus* whose Malpighian tubules appear to be comprised of one cell type, in contrast to the specialized principal and stellate cells in *Drosophila*.

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Appendix

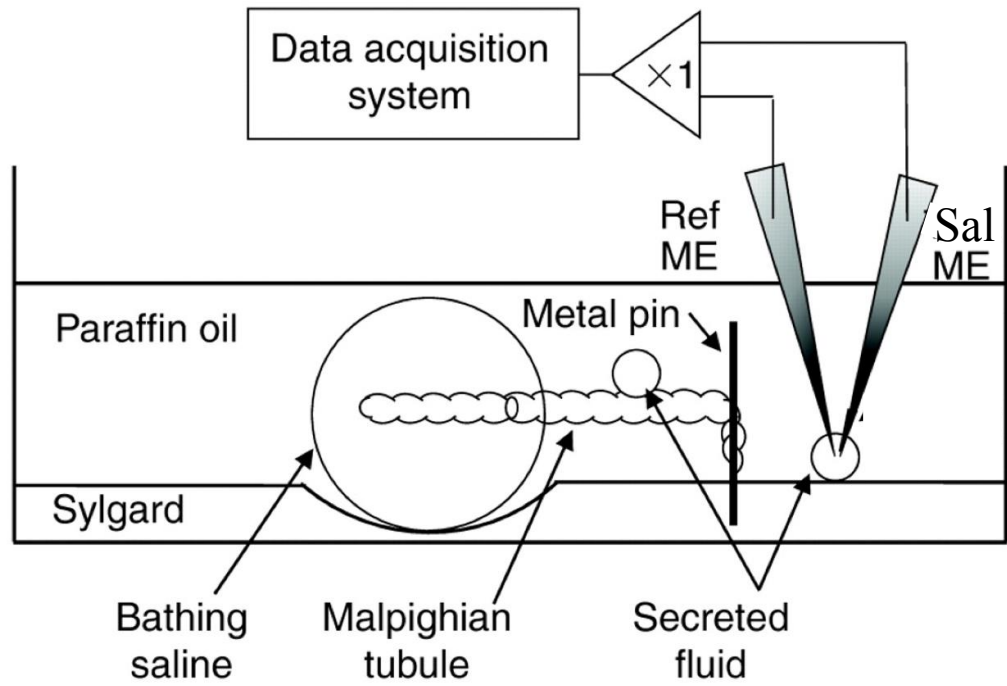


Figure A1: Schematic of the Ramsay assay. Importantly, fluid secretion rate and concentration of salicylate in the secreted fluid are directly measured whereas salicylate flux is calculated as the product of those two measurements.