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Detection of Organic Acids by the Taste Systems in *Drosophila melanogaster* and
Drosophila sechellia

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Biomedical Sciences

by

Sandhya Shamala Charlu

December 2014

Dissertation Committee:

Dr. Anupama Dahanukar, Chairperson

Dr. Anand Ray

Dr. David Lo

Dr. Michael Adams

Dr. Morris Maduro

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The Dissertation of Sandhya Shamala Charlu is approved:

Committee Chairperson

University of California, Riverside

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Without Adriana's contribution, the behavior experiments would not have been done nearly as efficiently and her contribution made it possible for me to focus on electrophysiological characterizations. Some of the work described in Chapters 2 and 3 has been previously published in Nature Communications under the title. "Acid sensing by sweet and bitter taste neurons in *Drosophila melanogaster*". I would like to acknowledge my co-author on this work, Zev Wisotsky, whose characterization of the bitter neuron response to acids informed my studies. I would also like to thank all of my committee members, past and present: Dr. Anand Ray, Dr. Michael Adams, Dr. David Lo, Dr. Morris Maduro, Dr. Emma Wilson and Dr. Glenn Stanley. Their input has been invaluable to my progress and I am indebted to them for their contributions.

Dedication

I would like to dedicate this work to my family: my father and mother, Srinivasa Charlu and Attibele Shamala; my grandmother, Rathna Ramaswamy; my uncles Shashi Kumar and Dr. A.R. Nagendra; and my aunt Vasulakshmi Divakar.

ABSTRACT OF THE DISSERTATION

Detection of Organic Acids by the Taste Systems in *Drosophila melanogaster* and
Drosophila sechellia

by

Sandhya Shamala Charlu

Doctor of Philosophy, Graduate Program in Biomedical Sciences
University of California, Riverside, December 2014
Dr. Anupama Dahanukar, Chairperson

In this work we examine the response of two *Drosophila* species to acids found in their natural food sources. We show that acids reduce normal gustatory behavior to sweet stimuli. We find that this acid aversion behavior is mediated by both the sweet and bitter neurons in the *Drosophila* taste system. Through electrophysiological studies we determine that acids inhibit neuronal firing to sucrose independently of bitter neuron input. We show that this inhibition can be overcome by increased sucrose concentration. We also find that acid inhibition of the sweet neuron is dependent on pH, regardless of the anion of the acid. Lastly, we examined the behavior of two members of the *melanogaster* subgroup, *Drosophila sechellia* and *Drosophila melanogaster*, to acids present in the main food source of *Drosophila sechellia*, morinda fruit. Behavioral studies showed that these two species diverge in their responses to morinda fruit and its component acids. Upon further examination we find that for some of the acids the behavioral difference can be attributed to a reduced sweet neuron inhibition in *Drosophila sechellia*. Thus we propose that sweet neuron inhibition plays an important role in behavior towards acidic stimuli.

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Chapter 1: Introduction

Organization of the Taste System in Drosophila melanogaster

The taste system is vital to detecting edible food sources for animals as well as preventing them from ingesting harmful substances. The taste system detects chemicals present in food and signals to the brain the nutritive value or toxicity of these chemicals. The brain then integrates this information along with other physiological and sensory cues and directs the animal to either consume or avoid the food. Thus, by studying taste we can better understand how animals choose what and what not to consume.

The taste system in *Drosophila melanogaster* offers a simple model system in which to study taste. The *Drosophila* taste system is divided into five sensory organs: the labellum, the tarsi, the wing margins, the pharynx and the ovipositor (Stocker 1994). Taste sensilla, or hairs, are located on most of these organs and act as the main subunits of stimulus detection. Taste sensilla have a stereotypical morphology. They are trichoid sensilla with a single pore at the tip to allow the flow of chemicals into the sensilla. Within the sensilla, chemosensory neurons, support cells and mechanosensory neurons are housed (Falk 1976). In each sensilla there is one mechanosensory neuron, two-four chemosensory neurons and three support cells. Every taste sensilla houses three support cells: the trichogen cell, the thecogen cell and the tormogen cell (Liman and Montell 2014). The purpose of these cells is to produce ions and small molecules necessary for proper function of the chemosensory neurons (Thorne and Amrein 2005).

Unlike the support cells, the number of taste neurons varies based on the type of sensillum. On the labellum there are three major types of taste sensilla. There are termed

the large (L) sensilla, intermediate (I) sensilla and small (S) sensilla based on their length (Hiroi 2004). There are a total of 18 large sensilla on the labellum (Montell 2009). Each of these sensilla has four chemosensory neurons, classified as the sweet, high salt, water and low salt neurons. The large sensillum has no response to common bitter compounds, thus its main aversion neuron is the high salt neuron and not the bitter neuron (Hiroi 2002).

There are 20 intermediate sensilla on the labellum (Montell 2009). The intermediate sensilla only have two chemosensory neurons. One neuron detects attractive stimuli such as sweet and low salt compounds, and the other neuron detects aversive stimuli including bitter and high salt compounds (Hiroi 2004). The I sensilla do not contain a water neuron. The last class of sensilla is the small, or S, sensilla. There are a total of 24 small sensilla on the labellum and they are all located medially on the labellar surface (Montell 2009). The small sensilla contain four chemosensory neurons: a sweet neuron, a bitter/high salt neuron, a low salt neuron and a water neuron along with the mechanosensory neuron and the support cells.

The chemosensory, or taste, neurons are bipolar neurons (Hiroi 2004). They send their dendrites into the shaft of the sensilla such that the tip of the dendrite is close to the open pore at the tip of the sensilla (Falk 1976). At the other end of the neuron, the axons project into the subesophageal ganglion, or SOG, of the *Drosophila* brain (Nayak 1984, Kwon 2014). In the SOG, the taste projections are spatially separated based on the type of taste they signal for and the taste organ that the signal from (Wang 2004). Thus, the labellar projections are separated from the tarsal projections, and both of these are

separated from the pharyngeal projections (Stocker 1994). Second order neurons, which signal within the SOG or from the SOG to other brain regions have only recently been discovered (Flood 2013, Fei 2014, Pool 2014). Work from our lab has shown that a novel taste region in the brain, the AAMC, acts as a relay center for second taste order neurons from the SOG (Kain unpublished). This region was found to be important in integrating satiety state with sweet taste perception and is the first higher brain center to be definitively implicated in taste processing.

Taste modalities and receptors

There are six current taste modalities in *Drosophila melanogaster*. They include sweet taste, bitter taste, low salt taste, high salt taste, water taste and carbonation taste (Liman and Montell 2014). Thus far, receptors have been discovered for four of the taste modalities: sweet, bitter, low salt and water. The receptors for high salt and carbonation have yet to be found. The taste receptors identified thus far come from a diverse array of chemosensory receptor families, including the gustatory receptor, ionotropic receptor, Trp receptor and pickpocket receptor families (Liman and Montell 2014).

Sweet taste detection

The sweet taste modality was one of the first tastes to be studied in *Drosophila melanogaster*. Sweet taste involves the detection of sugars, such as sucrose and glucose, and sugar derivative compounds, such methyl alpha glucoside and glycerol (Dahanukar 2007). Sweet taste is mediated by the gustatory receptor family (Grs). A small clade of

receptors in the Gr family, known as the “sweet clade” mediates detection of sweet stimuli (Jiao 2008). Gr5a was the first member of this clade to be implicated in sugar detection (Dahanukar 2001). It was found to be the receptor for the fruit sugar trehalose. Further studies showed that it was also necessary for other sugars such as glucose, melezitose and methyl alpha glucoside (Dahanukar 2007, Freeman 2014). The necessity of Gr5a for the detection of multiple sugars as well as its broad expression in the taste sensilla of the labellum led to the theory that Gr5a is a broad co-receptor for a subset of sugar compounds (Dahanukar 2007).

Two other sugar clade Grs were also found to be widely required for multiple sweet compounds. Gr64a and Gr64f are part of the Gr64 cluster within the sweet clade (Jiao 2008). The Gr64 cluster is a group of 6 genes, Gr64a-f, that are adjacent on the third chromosome. These genes are closely linked to one another, and in fact two sets of them are encoded by bicistronic messages (Dahanukar 2007). When this entire cluster of genes was eliminated, the responses to numerous sugars were drastically reduced (Slone 2007). Selective rescue of Gr64f in this background only showed a slight increase in the sugar response, but when combined with Gr64a, the response to glucose, sucrose and maltose was rescued (Jiao 2008). Deletion of Gr64f as well as ectopic expression in the *Drosophila* CO2 neuron, showed that Gr64f is also involved in the detection of trehalose, melezitose and methyl alpha glucoside (Freeman 2014). It is also necessary for the response to glucose, though it is not sufficient to confer a response in a heterologous system.

Gr64a has been shown to be required for a complementary subset of sugars from those mediated by Gr5a and Gr64f (Dahanukar 2007, Freeman 2014). Gr64a is necessary and sufficient for the response to sucrose, maltose, maltotriose, fructose and glycerol (Dahanukar 2007, Freeman 2014). When both Gr5a and Gr64a were deleted, the response to numerous sugars was eliminated, further validating their complementary function (Dahanukar 2007).

The other Grs in the Gr64 cluster do not consistently group with Gr5a or Gr64a in sugar detection and thus appear to be promiscuous between these two co-receptors (Freeman 2014). It has been hypothesized that the sweet receptors form heteromeric complexes with these co-receptors and that different permutations and combinations of these receptors detect different sweet compounds. The only receptor not in the sweet clade that detects a sweet stimulus is Gr43a (Miyamoto 2012, Freeman 2014). Gr43a is required for the response to fructose. Interestingly, it serves this role not only in the taste system, but also in the brain.

Bitter taste detection

Majority of the Grs are proposed to be involved in bitter detection based on their expression in bitter neurons (Weiss 2011). The bitter taste modality includes detection of allelochemicals and alkaloids, such as caffeine and lobeline (Moon 2006, Moon 2009, Lee 2009). Three Grs, Gr33a, Gr66a and Gr93a, have been implicated in bitter taste (Moon 2006, Moon 2009, Lee 2009). Gr66a was the first bitter taste receptor discovered in *Drosophila melanogaster* (Moon 2006). It was found to be necessary for the normal

behavioral and neuronal response to caffeine as well theophylline. The next bitter Gr mutants studied, Gr93a mutants, showed a similar phenotype to Gr66a, and the authors proposed that these two receptors act together, along with possibly other receptors, to detect caffeine and theophylline (Lee 2009). Gr33a, on the other hand, is much more broadly needed than Gr66a and Gr93a, even though its expression largely overlaps with Gr66a (Moon 2009). Gr33a mediates the response to numerous bitter compounds, including caffeine, berberine, lobeline and denatonium amongst others.

All three of these receptors are expressed in bitter neurons located in the I and S sensilla on the *Drosophila* labellum (Weiss 2011). These sensilla have been further classified into subgroups based on their responses to a panel of bitter compounds. For instance, the S sensilla have been divided into S-a and S-b sensilla based on their differential responses to a panel of 16 bitter compounds. Though S-b and S-a sensilla respond to the same compounds, S-b sensilla respond more strongly. Thus, the two subclasses can be distinguished by how intensely they are activated by certain bitter compounds. The I-a and the I-b sensilla, however, are much more divergent than the S-a and S-b sensilla (Weiss 2011). The I-a and I-b sensilla respond to complementary sets of bitter compounds that do not show any overlap, thus these two sensillar subclasses are very easy to distinguish based on their bitter responses.

Salt taste and ionotropic receptors

The third taste category in *Drosophila melanogaster* is salt taste. Salt taste is divided into two modalities, low salt and high salt. Low salt denotes salt concentrations

below 100 mM and high salt denotes salt concentrations above 200 mM (Zhang 2013). The Montell lab has shown that Irs are involved in the detection of low salt stimuli in the taste system (Zhang 2013). They found that at lower salt concentrations, Ir76b is necessary and sufficient for the response to salt. They also found that the detection of low salt is primarily mediated by the L sensilla, while high salt detection is mediated by the S sensilla. Ir76b was only found to be necessary for the response in L sensilla, not in S sensilla. In this paper they showed that Ir76b is expressed in neurons that are neither the bitter nor the sweet neurons and that exogenous expression of Ir76b can confer a low salt response (Zhang 2013). Additionally, they predicted that Ir76b acts a constitutively open sodium channel that depolarizes the cell when the extracellular concentration of sodium is increased.

Prior to this work, ionotropic receptors were almost exclusively studied in the *Drosophila* olfactory system. Ionotropic receptors (Irs) were first described by Richard Benton and Leslie Vosshall in 2009. The Ir family is proposed to be the most ancient chemosensory family, showing presence in species across Protostomia (Croset 2010). In the olfactory system, it has been shown that Irs detect a variety of chemicals including acids such as acetic acid (the main component of vinegar), and amines such as DEET (an important repellent for another member of the dipteran family, mosquitoes) (Silbering 2011, Ai 2010, Kain 2013). The Irs are proposed to be ligand-gated ion channels, with structures similar to the glutamate receptors NMDA and kainite (Benton 2009). Their ligand-binding domains are vastly different from glutamate receptors, however,

suggesting that they do not detect glutamate but instead detect a wide variety of compounds (Croset, Benton 2010).

Only recently, a thorough characterization of Irs in the taste system was published (Koh 2014). In this study they examined the Ir20 clade. This clade of 35 members was found to be expressed in multiple taste organs on the *Drosophila melanogaster* body. Expression analysis using *GAL4* constructs showed expression of these Irs in the labellum, legs, pharynx, and wing margins of the fly (Koh 2014). Some of the receptors also showed overlap with known gustatory receptors thus suggesting a role for these Irs in taste detection. This work lays a foundation for further analysis of ionotropic receptor function in the taste system.

Water taste detection and pickpocket receptors

Water detection in the taste system of *Drosophila melanogaster* is mediated by the pickpocket receptor family (Cameron 2010). Pickpocket receptors are degenerin/epithelial sodium channels (Deg/ENaC). Pickpocket 28 (ppk28) is the main receptor responsible for water detection in the taste system of *Drosophila melanogaster* adults. PPK28 neurons have been shown to be activated by low osmolarity solutions and inhibited by high osmolarity solutions, regardless of the compound used to adjust the osmolarity (Cameron 2010). Besides water detection, pickpocket channels have also been implicated in pheromone detection and larval salt taste in *Drosophila melanogaster* (Lin 2005, Lu 2012, Vijayan 2014, Liu 2003). Thus the pickpocket family is one of the many chemosensory families with multi-modal functions in *Drosophila* chemosensation.

Trp channels

The Trp, or transient receptor potential, family has also been shown to play a role in taste detection. The Trp receptors are a very broad and well-studied family of ion channels. There are 13 Trp receptors in *Drosophila melanogaster* (Montell 2005). They have been implicated in many different sensory systems, including mechanosensory, thermosensory and chemosensory perception. Their chemosensory role is largely based on chemicals that cause a mechanical sensation in addition to a taste sensation, such as camphor, menthol, capsaicin and cinnamaldehyde (Voets 2005). Al Anzi, *et al.* showed that *painless*, a member of the Trp family, is involved in the detection of isothiocyanate, the “spicy” component of wasabi. Flies showed avoidance of isothiocyanate in behavior assays, but this avoidance was reduced in *painless* mutants. *Painless* neurons were shown to overlap with bitter, Gr66a neurons in the labella of flies thus validating their role in aversion. When a rescue construct was expressed solely in Gr66a neurons, the neuronal response to isothiocyanate was restored suggesting that *painless* function in these neurons is necessary for isothiocyanate detection (Al-Anzi, 2006).

Another Trp receptor, TrpA1, has been found to mediate the response to aristolochic acid, an aversive taste compound (Kim 2010). *TrpA1* mutants showed defects in normal aversion to aristolochic acid. Neuronal stimulation of the *TrpA1* mutants with aristolochic acid also elicited fewer action potentials than in wildtype flies. TrpA1 neurons, like *painless* neurons, showed an overlap with a known bitter receptor, Gr93a (Kim 2010, Lee 2009). Exogenous expression of TrpA1 was not sufficient for the response to aristolochic acid, however, implying that other receptors are needed for this

response (Kim 2010). Taken together, these two studies show that Trp receptors have a role in *Drosophila* taste detection of aversive compounds.

Mammalian acid taste detection

Prior to this work, acid detection in the *Drosophila* taste system was not well understood. Acid detection in the mammalian system, however, has been well studied and the receptors and cells mediating this taste have been largely elucidated (Lyll 2001, Huang 2006, Huang 2008, Wang 2010, Horio 2011, Chang 2011). Mammalian acid taste is divided into strong and weak acid detection (Lyll 2001, Wang 2010, Chang 2011). Strong acids are inorganic acids, such as HCl and HNO₃, and weak acids are organic acids, such as acetic acid and tartaric acid. These two types of acids are detected by separate mechanisms in the mammalian taste system.

Lyll *et al.* found that the unit of detection for weak acids is the undisassociated acid itself, not the hydrogen ion (2001). Organic acids at similar concentrations elicited similar cellular responses, while acids at the same pH gave very different responses. This led the authors to conclude that organic acid detection is based on the concentration of the acid itself and not the concentration of hydrogen ion. Further studies showed that the undisassociated acid crosses the cellular membrane unassisted and acidifies the cytosol of the cell (Wang 2010). This intracellular acidification was found to be independent of extracellular pH (Lyll 2001). TrpA1 was shown to be the receptor which detects this intracellular acidification and in turn causes calcium influx into the cell (Wang 2011, Huang 2008).

Strong acids, on the other hand, were found to be dependent on extracellular pH (Lyll 2001). Strong acid activation of taste cells is caused by proton conductance through a zinc-sensitive ion channel (Chang 2011). The identity of this channel is not yet known but the cells that detect this stimulus are. The Trp receptors PKD2L1 and PKD1L3 have been shown to mark cells responsive to acidic stimuli (Huang 2006). Elimination of these cells caused a marked decrease in response to acids. This led to the hypothesis that PKD2L1 and PKD1L3 are the sour receptors (Huang 2006). However, when null mutants for PKD2L1 were tested, the response to organic acids was only reduced by half (Horio 2011). PKD1L3 mutants had an even smaller effect on the sour taste response. In addition, heterologous expression of these two receptors was not able to confer a direct “on” response to sour stimuli, thus indicating that another receptor may be needed for an acid response (Horio 2011).

Fatty acid taste in Drosophila sechellia and Drosophila melanogaster

Drosophila sechellia is a closely related species to *Drosophila melanogaster* (McBride and Arguello 2007). *Drosophila sechellia* are natives of the Seychelles Islands off the coast of Africa, from which they get their namesake (Dworkin and Jones 2009). *Drosophila sechellia* appear very similar to *Drosophila melanogaster*. The most obvious difference between the two species is the length of the sensilla on their chemosensory organs; *Drosophila sechellia* have much shorter sensilla than *Drosophila melanogaster* (Dekker 2006). *Drosophila sechellia* are well known for their preference for feeding on a putrid fruit native to the Seychelles called *Morinda citrifolia* L. (R’Kha 1991).

Drosophila sechellia is the only *Drosophila* species known to feed on this fruit when ripe as well as lay its eggs on it (Legal 1994). Other *Drosophila* species, such as *Drosophila simulans* and *Drosophila melanogaster*, though closely related to *Drosophila sechellia*, avoid this fruit in nature until it has gotten rotten. Thus ripe morinda fruit is an ecological niche for the *Drosophila sechellia* flies (R’Kha 1991).

This interesting difference in natural behavior to morinda fruit has prompted many studies examining the components of morinda fruit and the behavior of different *Drosophila* species towards them. Largely researchers have focused on the acids that comprise morinda fruit, since these compounds are presumably what give morinda its distinctive smell (Legal 1994, Amlou 1998, Harada 2008). The two main compounds studied from morinda fruit are octanoic acid and hexanoic acid. Octanoic acid is the most prevalent of all acids in morinda fruit (Legal 1994, Pino 2009). It is ten times as prevalent as the next common acid, hexanoic acid. Both octanoic and hexanoic acid have an odor, thus many of the behavior studies to date have examined the olfactory behavior of *Drosophila sechellia* and *Drosophila melanogaster* to these two acids (Higa 1993, Matsuo 2007, Dekker 2006) . However, little has been done to examine the actual feeding behavior of these two species on these acids or, for that matter, the feeding on morinda fruit itself (Harada 2008).

Most of the molecular studies into morinda acids have examined the role of odorant binding proteins, or Obps (Harada 2008, Dworkin and Jones 2009, Matsuo 2007). Thus far no Obps have been able to completely account for the behavior towards octanoic and hexanoic acid, but they have shown some involvement. Obp57d has been

shown to play a role in oviposition behavior towards morinda acids (Matsuo 2007, Harada 2008). Obp57e, though originally promising, has shown no necessary part in behavior towards morinda acids (Matsuo 2007). Though both of these Obps have shown expression in the taste hairs of the *Drosophila melanogaster* tarsi, they did not have much impact on feeding on morinda acids (Dworkin and Jones 2009, Harada 2008). Only the feeding on nonanoic acid, a minor morinda acid, was affected by elimination of both Obp57d and Obp57e (Harada 2008). Another Obp, *Obp56e*, also showed a change in avoidance of morinda itself (Dworkin and Jones 2009). Knockdown of *Obp56e* caused flies to choose morinda media over regular media. This results suggest that Obps could play a role in *Drosophila sechellia* preference for morinda fruit.

Overall, much more research needs to be done to better understand the detection of acids in the taste systems of *Drosophila* species. The work presented here furthers this understanding. First, we examine the behavior of *Drosophila melanogaster* to carboxylic acids found in fruits and vinegar. We show that organic acids are aversive stimuli to *Drosophila melanogaster* and that the bitter neuron is involved in this behavior. We also analyze numerous candidate receptors for a role in acid behavior, but only find one receptor that affects this aversion. Also, we show that a second neuron, the sweet neuron, is involved in acid detection, and that acids inhibit the normal response of this neuron to sucrose. We find that the sweet neuron acts as a gauge of sucrose-acid content and can be manipulated to affect the behavior towards acids. In the third chapter, we analyze the feeding behavior of *Drosophila sechellia* and *Drosophila melanogaster* to morinda food.

We find that at lower concentrations of morinda, the two species diverge in their behavior. Further studies into the feeding and neuronal response to octanoic and hexanoic acid show that differences in the sweet neuron response to octanoic acid may contribute to this divergence in morinda preference. We also investigate the detection of other acids found in morinda and explore the molecular mechanisms underlying their detection. Together these three studies expand the understanding of the cellular and molecular mechanisms that underlie acid detection in *Drosophila*.

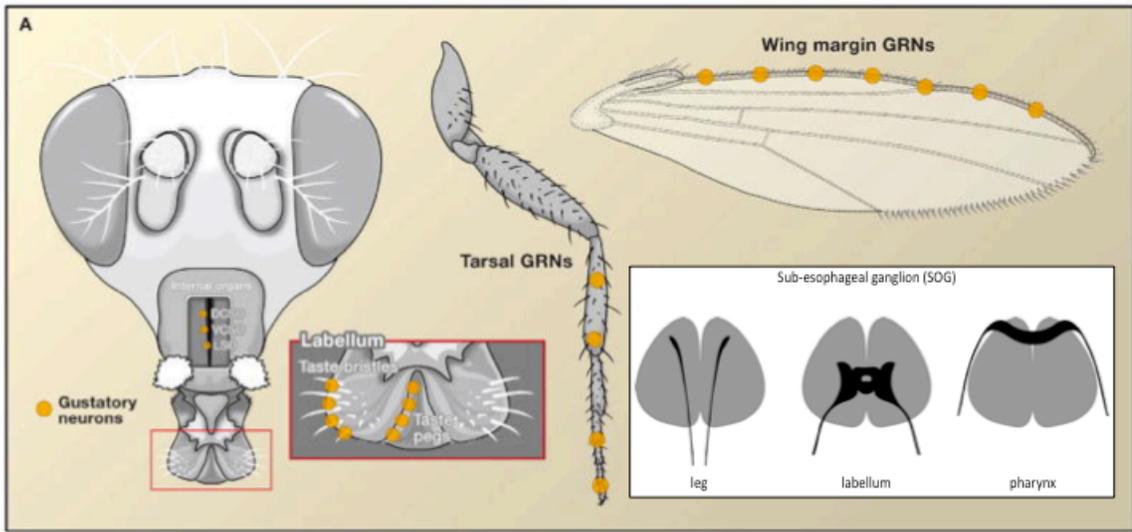


Figure 1: Taste neuron map in periphery organs and SOG (Adapted from Yamolinsky 2009)

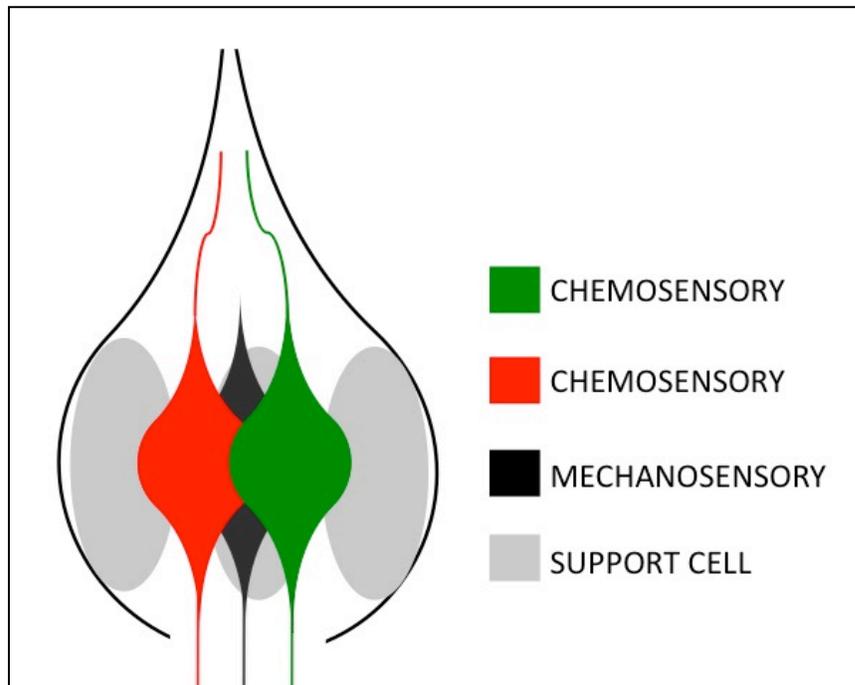


Figure 2: Taste sensilla structure

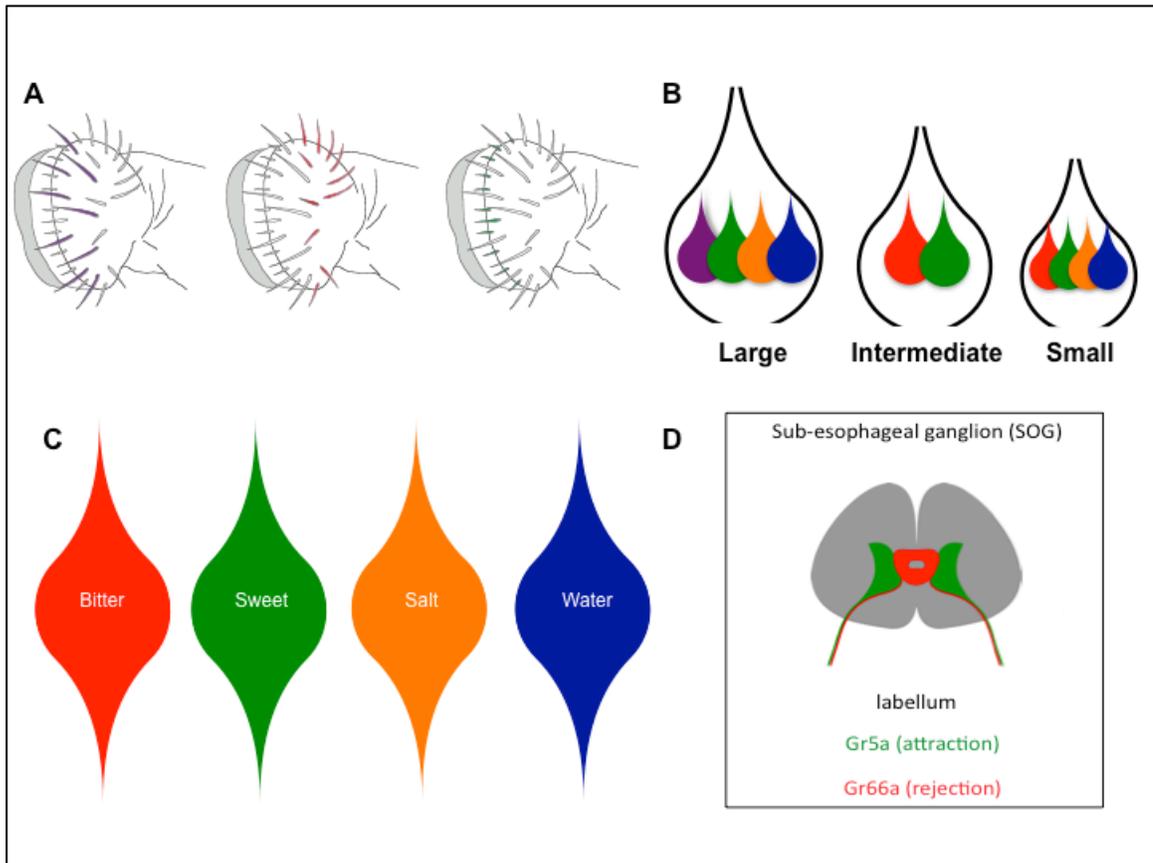


Figure 3: Labellar taste sensilla, taste neurons and central neuronal projections
a) Large, intermediate and small sensilla position on labella b) Chemosensory neurons in large, intermediate and small sensilla c) Types of taste neurons d) Projection patterns of sweet and bitter neurons in the SOG

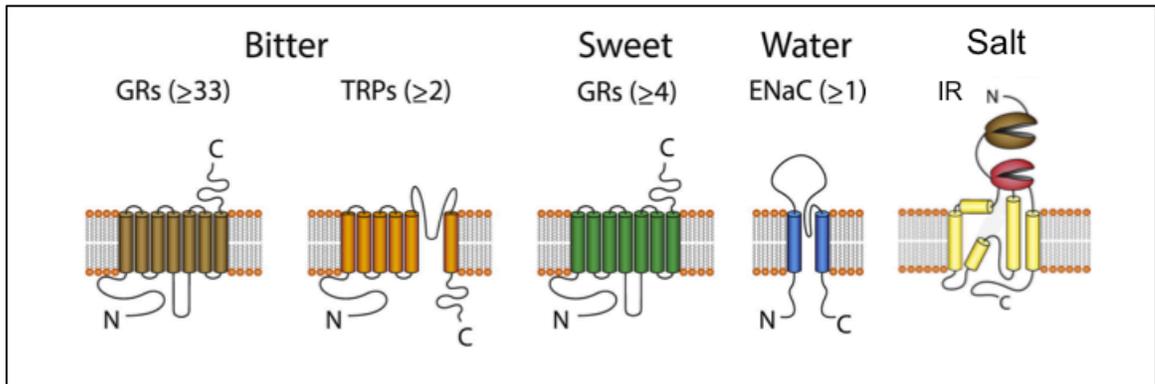


Figure 4: Grs, Irs, Trps and ENacs mediate taste in *Drosophila melanogaster* (Adapted from Liman 2014)

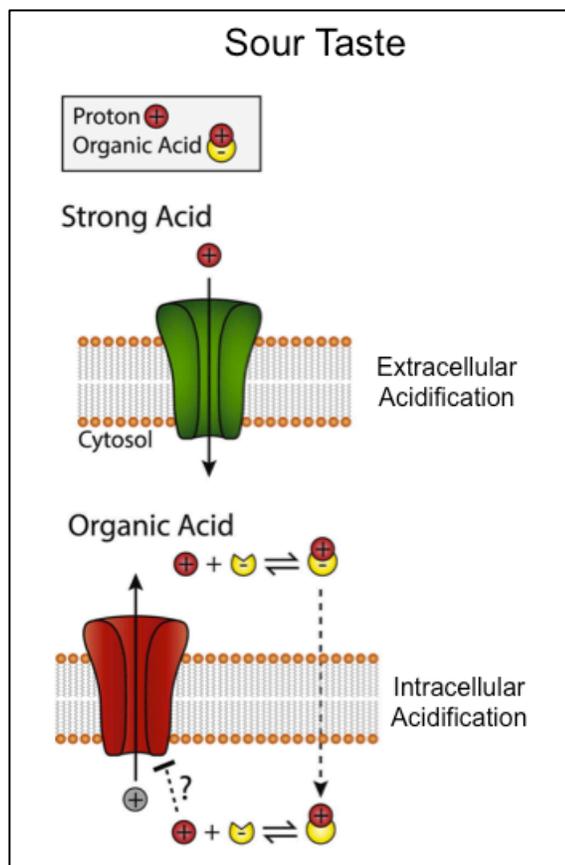


Figure 5: Acid detection in the mammalian taste system (Adapted from Liman 2014)

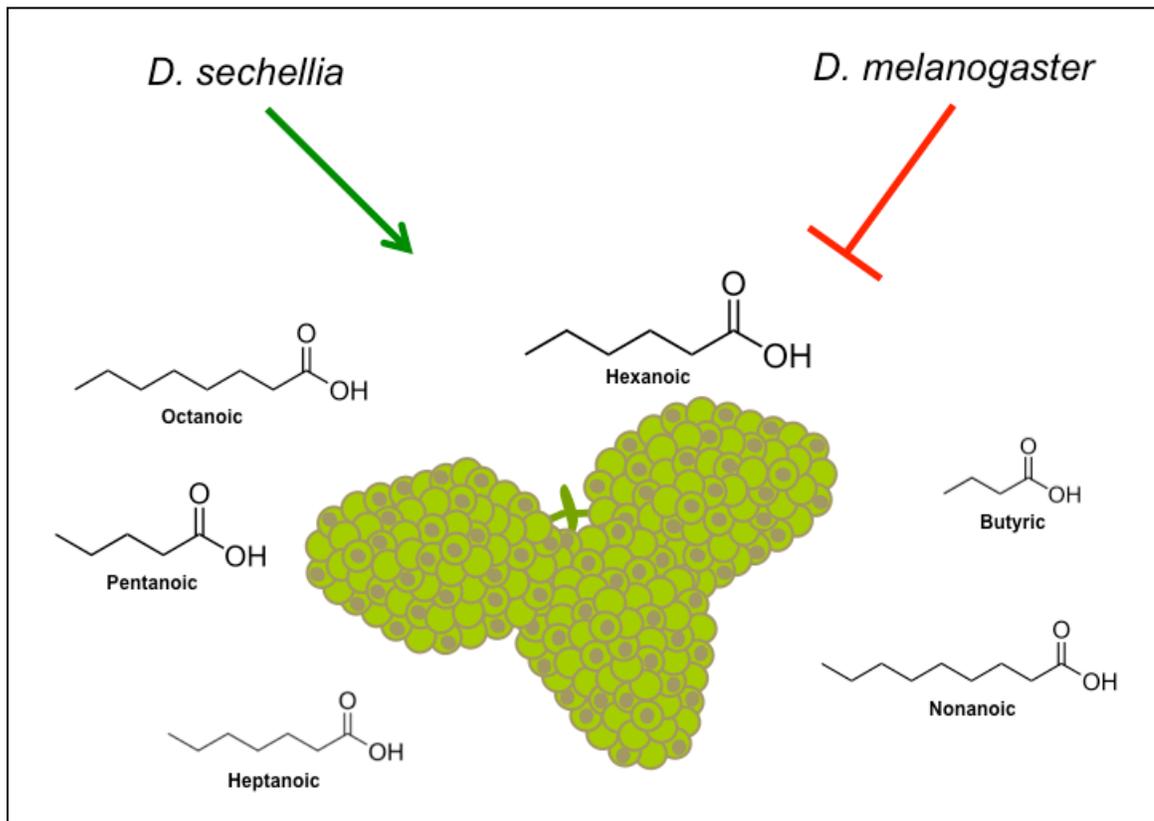


Figure 6: Attraction of *Drosophila sechellia* and aversion of *Drosophila melanogaster* to morinda fruit and its component acids

Chapter 2: The Behavioral Response of *Drosophila melanogaster* to Organic Acids

Introduction

A key question in *Drosophila melanogaster* feeding behavior is why *Drosophila* prefer overripe or rotten fruit to raw or ripe fruit. Fruits at different stages of maturity and ripening are composed of the same classes of chemicals; the difference lies in the percent composition of these chemical classes (Handbook of Fruit Ripening). The two main classes of chemicals that determine edibility of a fruit are sugars and acids. As fruit ripens, the concentrations of sugars and organic acids change. As starch is hydrolyzed and sugar is transported from the other parts of the plant to the fruit, the levels of sugars, such as sucrose, fructose and glucose, increase. Conversely, organic acid concentration decreases during fruit ripening. Many raw fruits have a pH below 3, indicating high levels of acid. However, as fruit ripens, the amount of acid is reduced (Handbook of Fruit Ripening). Ripe fruits are composed of approximately 1% acid by weight. Some citrus fruits, however, can contain up to 3% acid. The main organic acids in fruit are citric acid, malic acid, tartaric acid and glycolic acid. Both citric and malic acid are converted into other non-acidic compounds during ripening, thus contributing to the higher pH of ripe fruits.

Organic acids are an integral part of the fruits that *Drosophila melanogaster* feed on, such as bananas, grapes, melons, and oranges (University of Kentucky Agriculture). Given the pervasiveness of organic acids in the *Drosophila melanogaster* diet, it is logical to assume that the *Drosophila* taste system has a way of detecting these

compounds. Acid detection has been previously studied only in the *Drosophila* olfactory system. In the olfactory system, acids elicit aversive behavior (Ai 2010). When testing odoriferous acids such as acetic and propionic acid, Ai, *et al.* found that 80% of flies avoid these acids. In mammals, acid taste is also characterized as aversive (Yarmolinsky 2009). It is thought to represent spoiled or raw fruit, which is unappetizing to mammals.

To explore if acid taste behavior in *Drosophila melanogaster* follows this trend, we tested behavioral responses to common carboxylic acids found in fruit. The acids tested included citric acid, glycolic acid, tartaric acid and acetic acid. Citric acid is common in citrus fruits, such as oranges (Penniston J Endourol 2008). Glycolic and tartaric acid are both found in grapes (Kliwer 1966). Acetic acid is not very common in raw and ripe fruit, but is actually a byproduct of fermentation, and thus is often found in rotten fruit (Mira 2010, Chakir 1993). Considering the ubiquity of organic acids in *Drosophila* food sources, we were interested to determine whether *Drosophila* find acids attractive or aversive.

In addition to determining the valence of acids in *Drosophila* behavior, we wanted to determine which receptors mediate the behavioral response. No specific acid taste receptors have been found in *Drosophila* to date. Thus we composed a list of candidate receptors to test for necessity in behavioral assays. These candidates included members from the Ir, Trp and Gr families. The candidates from these families were chosen due to their previously described roles in chemosensory detection and/or their expression in taste tissue (Benton 2009, Ai 2010, Al-Anzi 2006, Moon 2009).

Both the Ir and Trp families have been implicated in acid detection in other systems (Ai 2010, Huang 2006, Wang 2011). The Ir family has been found to be involved in acid detection in the *Drosophila* olfactory system. Ir64a, in particular, was found to be necessary and sufficient for detection of organic acids by the *Drosophila* olfactory system (Ai 2010). The Trp family has also been found to have a role in organic acid detection. The Trp receptor, PKD2L1, is partially necessary for detection of strong acids, while TrpA1 is involved in the detection of weak acids by the mammalian taste system (Huang 2006, Horoi 2011, Wang 2011). The Gr family is important for the primary aversive taste category, bitter taste (Moon 2006, Lee 2009, Moon 2009). Specifically, Gr33a has been shown to be necessary for many bitter compounds and is considered a broad bitter co-receptor (Moon 2009).

Here we show that *Drosophila melanogaster* have an aversive behavioral response to organic acids in taste chemosensory assays. We observed this aversion in two separate behavioral assays, one that measures instantaneous response and another that measures long-term feeding. In both of these assays, carboxylic acids inhibited the normal response to sucrose. Using bitter-silenced flies, we also found that this behavior is partially mediated by the bitter neuron. Additionally, when testing receptor mutants from the Ir, Trp and Gr families, we found that though some receptor mutants showed a reduction in the aversion to acids, though none showed a complete elimination of the aversive response. Thus, we conclude that *Drosophila melanogaster* avoid organic acids and that this behavior is mediated, at least in part, by the bitter taste neuron.

Results

Drosophila show an aversive behavioral response to organic acids

To identify *Drosophila* taste responses to acids, we examined behavior to four organic acids commonly found in fruit and vinegar. These acids included citric, glycolic, tartaric and acetic acid. We tested all four acids at three concentrations: 0.1%, 1%, and 10%. These concentrations were tested because they covered an ecologically relevant range of acid found in ripe fruit, which typically ranges from 1% in non-citrus fruits to 3% in citrus fruits (Handbook of Fruit Ripening). We chose concentrations one order of magnitude above and below these concentrations to test a pH range that includes the pH of raw and overripe fruits.

First we tested whether acids could evoke proboscis extension, a sign of food acceptance, upon stimulation of taste sensilla on the surface of the fly labellum. Preliminary tests showed that organic acids failed to evoke proboscis extension when tested alone, similar to what has been observed for other classes of noxious stimuli including allelochemicals and alkaloids (Dethier 1976). We therefore tested if the presence of acids could suppress acceptance of sucrose, a characteristic of noxious stimuli. All four of the tested organic acids were capable of blocking proboscis extension to sucrose (Figure 2.1a). The inhibition of the normal sucrose response was dose dependent for all of the acids, with the 0.1% mixture showing the least suppression and the 10% mixture showing the most suppression. For acetic acid, the 1% mixture had a similar inhibition to the 10%, whereas for the other three acids the 1% mixture was closer in value to the 0.1%.

Taste aversion to organic acids was confirmed using an independent feeding preference assay. In a series of binary choice experiments in which wild-type flies were tested for preference between 1 mM sucrose and mixtures of 5 mM sucrose with acids, we found that sucrose-acid mixtures were rejected as the concentration of acid was increased (Figure 2.1b). Flies were starved for 24 hours prior to the experiment to motivate them to feed. For the assay, flies were presented with a choice between 5 mM sucrose alone (control) or with acids (test) versus 1 mM sucrose. The 5 mM sucrose mixtures were laced with pink dye while the 1 mM sucrose solution was laced with blue dye. This color choice was consistent throughout all experiments, due to a slight bias observed for pink dye (not shown). Abdomens were scored for blue, pink, purple or no color to determine which solutions were consumed. Both male and female flies were tested, but little difference was observed between their responses. Addition of 10% acid to 5 mM sucrose completely reversed the normal preference for 5 mM sucrose alone ($PI = 0.91 \pm 0.04$, s.e.m., $n = 10$) for three of the four acids (acetic: -1 ± 0 , s.e.m, $n = 6$, glycolic: -0.90 ± 0.09 , s.e.m., $n = 6$, tartaric: -1 ± 0 , s.e.m, $n = 6$), suggesting a strong aversion to feeding on acids. It should be noted that different acids caused different degrees of aversion. This difference could be attributed to differing molar concentrations at the same percent acid (Table 2.1). Taken with the proboscis extension results, these two assays show that *Drosophila melanogaster* have an aversive behavioral response to acids and that this aversion is dose-dependent.

Antennal olfactory neurons are not involved in acid feeding behavior

Many acids have pungent odors and a recent study identified acid-sensing olfactory neurons in the *Drosophila* antenna (Ai 2010). Numerous acids are detected by the *Drosophila* olfactory system (Ai 2013 and Siberling 2011). We therefore tested surgically antennectomized flies in the feeding preference assay to determine the extent to which feeding aversion is dependent on acidic volatiles. Although antennae-less flies retain olfactory function in the maxillary palps, responses to acidic volatiles appear to be largely mediated by olfactory neurons in the antenna (Ai 2010). Age-matched flies were collected, of which half were antennectomized and the others were used as controls. After surgery, flies were allowed to recuperate for two days before being starved for feeding assays. Antennae-less flies and wild-type flies were tested for their response to 5 mM sucrose alone and as well as to mixtures of 5 mM sucrose with various acids at 10% versus 1 mM sucrose. Only the highest concentrations of acid were tested because they had the strongest odors. Also, if the antennae were not needed for aversion at the highest concentrations then it could be extrapolated that they were not involved with detection at lower concentrations. It is well known in *Drosophila* olfactory research that even attractive stimuli are often averse at higher concentrations, so testing 10% acids seemed the most appropriate.

Antennae-less flies avoided ingesting acid-laced sucrose to the same degree observed for their control siblings (Figure 2.2a). Flies with and without antennae had an average preference index within 0.2 units of each other for all four acids tested and no statistical difference was seen for any of the acids. Mean participation rates were also

similar for control (37–89%) and antennae-less (36–78%) flies. Antennae-less flies showed a strong preference for 5 mM sucrose in the absence of acid (antennae-less: $PI = 0.8 \pm 0.05$, s.e.m., $n=9$), though there was a observable reduction in response compared to the control flies ($PI = 0.95 \pm 0.03$, s.e.m., $n=8$) (Figure 2.2). Overall, these results indicate that feeding aversion to carboxylic acids appears to be largely independent of olfactory input.

Acid taste behavior is mediated by the bitter neuron

We next asked whether the main aversion neuron in the taste system, the bitter neuron, is involved in the behavioral response to acid tastants. There are only 2-4 chemosensory neurons in each sensillum, and since they have each already been assigned to a taste modality, it stood to reason that one of these neurons detects acids in addition to its previously ascribed class of chemicals. The bitter neuron was the primary candidate for acid detection, since acids were aversive and the bitter neuron signals deterrence. In order to determine if the bitter neuron was mediating the behavioral response to acids we tested flies whose bitter neurons were silenced by hyperpolarization (Figure 2.2b). These flies had all of their bitter taste neurons genetically silenced using UAS-Kir2.1 driven by Gr89a-GAL4. The Gr89a-GAL4 driver is broadly expressed in all bitter neurons of the labellum (Weiss 2011). UAS-Kir2.1 is an inwardly rectifying potassium channel that hyperpolarizes the neuron it is expressed in (Fischler 2007). Combining these constructs together causes hyperpolarization of all of the bitter neurons of the labellum, thus preventing the bitter neurons from responding.

Proboscis extension trials with sucrose-caffeine mixtures verified that aversion to a well-characterized bitter stimulus, caffeine, was impaired in bitter-silenced flies, indicating that the neurons were silenced (Figure 2.2b). However, bitter-silenced flies continued to reject sucrose-acid mixtures to the same extent observed for controls, with the exception of tartaric acid at the highest concentration tested (Figure 2.2b). Conversely, in the binary feeding assay, we observed that bitter-silenced flies had a reduced sensitivity towards sucrose-acid mixtures as compared to control siblings (Figure 2.2c). Consistent with our previous results, feeding avoidance was linked to acid tastant concentration and was largely independent of antennal olfactory input (Figure 2.2c). These findings indicate that the bitter neuron is at least partially involved in behavioral aversion to acids. Work from other members of our lab has shown that the bitter neuron is able to detect organic acids, thus further validating the role of the bitter neuron in acid taste detection (Charlu 2013).

Trp channel mutants are not involved in acid taste behavior

In order to determine the identity of the receptor(s) mediating acid behavior, we examined a diverse list of candidate receptors. These candidates were chosen from various sources. They included receptors that are involved in aversive taste in *Drosophila melanogaster*, receptors that have been implicated in acid detection in other animals, as well as receptors that have shown expression in *Drosophila* taste tissue. These candidates belong to three major chemosensory families: the gustatory receptors (Grs), the ionotropic receptors (Irs) and the transient receptor potential (Trp) channels. Though not

an extensive list, these candidate receptors include broad co-receptors that have previously been shown to be involved in responses to numerous compounds, as well as receptors specifically implicated in acid taste detection.

Trp channels have been implicated in both aversive taste in *Drosophila* and acid detection in mammals (Al-Anzi 2006, Huang 2006, Horoi 2011, Wang 2011). The Trp channels we tested were *painless* and *Pkd2*. *painless* has been shown to mediate detection of isothiocyanate by the *Drosophila* taste system (Al-Anzi 2006). Isothiocyanate is found in wasabi and is a strong aversive stimulus to adult flies. *painless* is expressed in multiple taste organs, including the labellum, pharynx, legs and wing margins (Al-Anzi 2006). Neurons that express *painless* partially overlap with those that express the bitter gustatory receptors, Gr66a, Gr47a and Gr32a (Al-Anzi 2006). Thus, *painless* seemed to be a good candidate for mediating aversive behavior to acids.

In order to determine the role of *painless* in acid behavior, we tested a *painless* mutant with a *GAL4* insertion in the first exon of the *painless* gene (*w[*]*; P{w[+mW.hs]=GawB}pain[GAL4]) (Al-Anzi 2006). We examined the *painless* mutants for proboscis extension to 100 mM sucrose with caffeine, citric, glycolic and tartaric acid. *painless* mutants showed no significant difference in the response to 100 mM sucrose alone, but the 10 mM sucrose response appeared slightly lower than the wild type (Figure 2.3a). The mutants showed no significant difference in proboscis extension to 100 mM sucrose with caffeine. When presented with sucrose-acid mixtures, however, the mutants showed a reduced sensitivity compared to wild-type (glycolic= 0.72 ± 0.09 , s.e.m., n=16, citric= 0.69 ± 0.12 , s.e.m., n=16, tartaric= 0.79 ± 0.10 , s.e.m., n=16). Due to the

variability of the assay, though, no statistical significance was observed between the wild-type and *painless* mutant flies, and thus no conclusions could be drawn from this assay alone.

Thus, we tested *painless* mutants in feeding preference assays with medium concentrations of acid and sucrose (1% for glycolic and citric, 0.1% for tartaric). This assay showed no deviation in behavioral responses of *painless* mutants from wild-type flies (Figure 2.5a). In fact, the average PIs of the *painless* mutants were more negative than those of wild-type flies for citric and tartaric acids, though these differences were not significant. Taken together, these results suggest that *painless* does not play a significant role in acid detection at the periphery, nor does it have an impact on feeding rejection of sucrose-acid mixtures, and thus is not the key receptor for acid taste behavior.

The other Trp channel that we tested for involvement in acid behavior was Pkd2. *Pkd2* is a homologue of the mammalian gene *PKD2L1*. *PKD2L1* was found to mark acid detecting cells in the mammalian taste system (Huang 2006). *PKD2L1* is co-expressed with *PKD1L3* in the circumvallate and foliate taste cells. It was shown that when these cells were silenced using diphtheria toxin the peripheral response to acids was eliminated (Huang 2006). Another group showed that knocking out the *PKD2L1* gene partially reduced the acid response in the fungiform papillae of the anterior tongue (Horoi 2011). However, knocking out *PKD1L3* had no effect on acid detection. Thus, *PKD2L1* is only partly responsible for the response to acids in mammals. It does, however, label acid detecting cells and so we thought that testing its *Drosophila* homologue may give us some insight into the mechanism of *Drosophila* acid behavior.

Pkd2 mutants were only tested in the PER assay, though two mutant lines were examined. One line was a null mutant of *Pkd2* (BL 24495) and the other an insertion mutant (BL 25244). Neither of these mutants showed significant changes in behavior from wildtype (Figure 2.3b and c). We tested 100 mM sucrose alone as well as 100 mM sucrose mixed with 10% glycolic acid, 10% citric acid or 1% tartaric acid. These concentrations were selected because they caused strong aversion in wild-type flies (Figure 2.1a). We observed a trend upwards in the null mutant for tartaric and citric acids, indicating that the flies were less averse to these acids (Figure 2.3b and c). However, these initial findings were not supported by analysis of the insertion mutant, which showed a slightly lower but not significant sensitivity to glycolic acid, and aversion to citric and tartaric acids to the same extent as wild-type flies. Given that no significant changes were observed in either mutant, we conclude that any difference between the two lines and wildtype is due to background effects and that *Pkd2* is not involved in acid behavior.

Ir76b is involved in peripheral taste detection of organic acids

In addition to testing Trp channel mutants, we also tested members of the ionotropic receptor (Ir) family (Figure 2.4 and Figure 2.5). The Ir family is composed of 61 receptors and 2 pseudogenes (Benton 2009). The Irs are structurally related to ionotropic glutamate receptors. They have an ion pore as well as a ligand-binding domain (Benton 2009). However, only three Irs retain the residues needed for binding glutamate, suggesting that Irs bind ligands other than glutamate. In fact, the ligand-binding domain

is not very similar amongst the Irs (Benton 2009). This lack of similarity is predicted to allow the Irs to detect a diverse array of chemicals. Since their discovery, these receptors have been well studied in the *Drosophila* olfactory system (Benton 2009, Croset 2010, Silbering 2011, Ai 2010, Ai 2013, Kain 2013). However, only recently has their role in the *Drosophila* taste system been elucidated (Zhang 2013, Carlson 2014). Thus, receptors from this family seemed to be good candidates for mediating taste aversion to acids.

To examine if the Irs are involved in acid taste behavior, we tested mutants for three Irs: *Ir21a*, *Ir76b* and *Ir64a*. The first Ir candidate examined was *Ir76b*, the receptor for low salt taste in *Drosophila melanogaster* (Zhang 2013). *Ir76b* was one of the first Irs to be found in the taste system (Benton 2009). The original characterization of this receptor, however, was in the *Drosophila* olfactory system. *Ir76b* is expressed in the coeloconic sensilla of the *Drosophila* antennae. It is the only Ir expressed in all four of the coeloconic sensilla types (Benton 2009). It has not, however, been implicated in detection of any specific ligand in the olfactory system. In 2013, the Montell lab found that *Ir76b* is necessary and sufficient for detection of low salt stimuli (<400 mM) (Zhang, Science, 2013). *Ir76b* was found to be expressed in non-sweet, non-bitter neurons in the labellum as well as in the tarsi and the wing margins. It was predicted to act as a sodium ion leak channel that activates depolarization when sodium levels increase in the sensillar lymph.

When tested for their feeding preference for organic acids, *Ir76b* mutants showed no significant difference compared to wildtype (Figure 2.5a). In the proboscis extension assay, however, they did have a reduction in aversion to acids (Figure 2.4a). At the

highest concentrations tested of glycolic and tartaric acid, Ir76b mutants did not detect the presence of the acids. Instead, they responded to the sucrose-acid mixture as if it were sucrose alone (Figure 2.4a). Wildtype flies, in comparison, showed a strong inhibition of the normal extension to sucrose. This insensitivity was also observed for the 1% citric acid mixture, though it was not significantly different from wildtype (Figure 2.4a). These results suggest that Ir76b may be partially necessary for the peripheral rejection of acids, but not sufficient to prevent ingestion of acids.

Ir21a and Ir64a are not necessary for acid taste behavior

Ir21a has been shown to be expressed in olfactory tissue, but its role in ligand detection is still undiscovered (Benton 2009, Silbering 2011). Ir21a mutants had no behavioral differences from wildtype in feeding or peripheral detection of acids (Figure 2.4b, 2.5a). The mutant flies were similarly affected by the sucrose-acid mixtures and had no deficit in their rejection of acids. Accordingly, it can be concluded that Ir21a does not play a role in acid tastant behavior.

The last Ir mutant tested was Ir64a. Ir64a mediates acid detection in the olfactory system of *Drosophila* (Ai 2010). Ir64a surrounds the third chamber of the sacculus in the *Drosophila* antennae. It has been shown to be necessary for olfactory detection of both organic and inorganic acids. It is believed to act as a co-receptor for detection of inorganic acids, since alone it is not sufficient to elicit a response to these acids. Conversely, Ir64a is able to confer specificity for organic acids in a heterologous system and thus is believed to be the main receptor for these stimuli (Ai 2010). Thus, a role for

Ir64a in organic acid detection by the taste system in *Drosophila* is a reasonable hypothesis.

When *Ir64a* mutants were tested, there was no reduction in aversion to feeding on acids (Figure 2.5b). In feeding preference assays, we observed that *Ir64a* mutants showed no shift compared to wildtype flies in their aversive behavior towards all three acids tested. This was seen for almost all of the concentrations of acids tested. For glycolic acid and tartaric acid, the response was almost identical to that of wildtype flies. Mutant flies showed a dose dependent response, indicating that they were still able to detect the presence of the acids. For the citric acid response, however, the wildtype flies themselves had a very different response from previous assays (Figure 2.5b and Figure 2.1b). The wildtype flies had an unusually weak aversion to citric acid at the highest concentration. This difference could occur if the flies were hungrier than usual due to being held on dry food. This increased hunger would have led them to choose the more nutritious food (5mM sucrose) regardless on acid content. When compared to results of previous behavioral assays with wildtype flies (Figure 2.1b) responses of *Ir64a* mutants were significantly different at 10% citric acid. The proboscis extension assay also showed little change in *Ir64a* mutant flies. Only at 1% glycolic acid and 10% tartaric acid was there a difference between the mutants and wildtype flies, however this difference was not significant. Taken together, these results indicate that there is not a role for *Ir64a* in the aversion to feeding on acidic tastants.

Discussion

Acid detection is very important for *Drosophila melanogaster*. Many of the foods that *Drosophila* feed on contain acidic compounds. Organic acids are found in both ripe and overripe fruit. It has long been known that *Drosophila* are attracted to overripe fruit as well as vinegar. Both of these substances contain acidic compounds such as acetic acid and glycolic acid. Thus, determining the behavior of *Drosophila melanogaster* to organic acids is vital to understanding their feeding habits.

In this study, we have shown that *Drosophila melanogaster* reject acid-laced solutions of sucrose. Typically, when presented with a choice between feeding on a high sugar stimulus and a low sugar stimulus, the fly chooses to feed on the high sugar stimulus. However, when presented with the choice between a low sugar solution and a high sugar solution laced with acid, the fly consistently chooses to feed on the low sugar solution, in turn avoiding the acid. The same is seen when the fly is presented with a sucrose-acid mixture to its proboscis. Sucrose alone elicits a strong extension of the proboscis, but when acid is added to this solution the extension probability is greatly decreased. Both of these behaviors occur in a dose dependent manner, indicating that it is the amount of acid that controls the response.

We also discovered in this study that feeding on acids is largely independent of olfactory input. This is interesting considering that in the feeding assay the flies are able to smell the compounds and that organic acids are detectable by the olfactory system (Ai 2010, Silbering 2011). In fact, not only does the olfactory system sense organic acids, it assigns the same valence to organic acids as the taste system. Both systems sense acids as

aversive stimuli. *Drosophila* avoid organic acids to a level of 60-80% just using olfactory input (Ai 2010). Thus, one would assume that olfactory input would be more important in an organic acid feeding assay. This is not the case however. For the less aromatic acids, such as citric and tartaric acid, this lack of olfactory necessity stands to reason, since these compounds are not volatile enough for olfactory detection. However, for acids such as acetic acid, which has been shown to cause olfactory avoidance, this lack of difference between antennectomized flies and normal flies is perplexing. This lack of difference could be due to the fact that there is no gradient of organic acid aroma in the plate. In a standard olfactory assay, such as a t-maze, the tested compound is only applied to one side of the testing chamber so that an olfactory gradient is established (Ai 2010). This gradient allows the compound to be more saturated at one end of the chamber versus the other, and thus allows the fly to make a choice whether to move towards or away from the compound. In the feeding assay, however, no such gradient is established. The dots with the organic acid are evenly distributed throughout the plate without any side bias. This distribution likely causes the plate to have a uniform smell, and may prevent the fly from using its olfactory system for choosing one stimulus over the other. In this situation, the fly can only use its taste system for discrimination between the two stimuli and thus removing the olfactory system does not change its choice. It is also possible that the flies do not use their olfactory sense to decide between which stimuli to feed on, and thus removing the antennae have no effect.

Though taste aversion to acids is olfactory neuron-independent, it is bitter neuron-dependent. The bitter neuron is the main aversion neuron in the *Drosophila* taste system.

When the bitter neurons were silenced, the flies showed reduced aversion to acids. This suggests that the bitter neuron is involved in acid detection and is necessary for part of the aversion observed in wildtype flies. However, it should be noted that aversion to feeding on organic acids is not completely eliminated when the bitter neurons are silenced. In behavior assays with other aversive stimuli, such as caffeine, silencing of all bitter neurons eliminates the aversion to the stimulus. In contrast, this is not the case for behavioral responses to organic acids. These findings suggest that there is another mechanism involved in the aversion to acids. As we will show in the next chapter, acids are also detected by the sweet neuron. Unlike for the bitter neuron, however, acids do not activate the sweet neuron but rather inhibit its activity. By inhibiting the sweet neuron, organic acids are able to prevent the flies from feeding on sweet stimuli that they would normally be attracted to. We postulate that the additive effects of bitter neuron activation and sweet neuron inhibition result in behavioral aversion to organic acids.

None of the receptor mutants tested showed a complete loss of aversion to acids in both assays. However, in the proboscis extension assay, *Ir76b* mutants showed a lack of acid detection for all acids and at all concentrations tested. This suggests that *Ir76b* is necessary for peripheral detection of organic acids. No phenotype was seen in the feeding assay, thus indicating that another receptor expressed internally may be able to sense acids and prevent the flies from feeding on them. As mentioned previously, *Ir76b* is a broad co-receptor in the olfactory system and thus it is possible that *Ir76b* acts as a co-receptor in other chemosensory systems, and that other *Irs* are responsible for conferring specificity. Further studies with other *Irs* could reveal an acid receptor.

Stimulus	0.1%	1%	10%
Tartaric	6.66 mM	66.6 mM	666 mM
Glycolic	13.1 mM	131 mM	1.31 M
Citric	5.20 mM	52.05 mM	520 mM
Acetic	16.6 mM	166 mM	1.66 M

Table 2.1: Molar concentrations of fruit acids

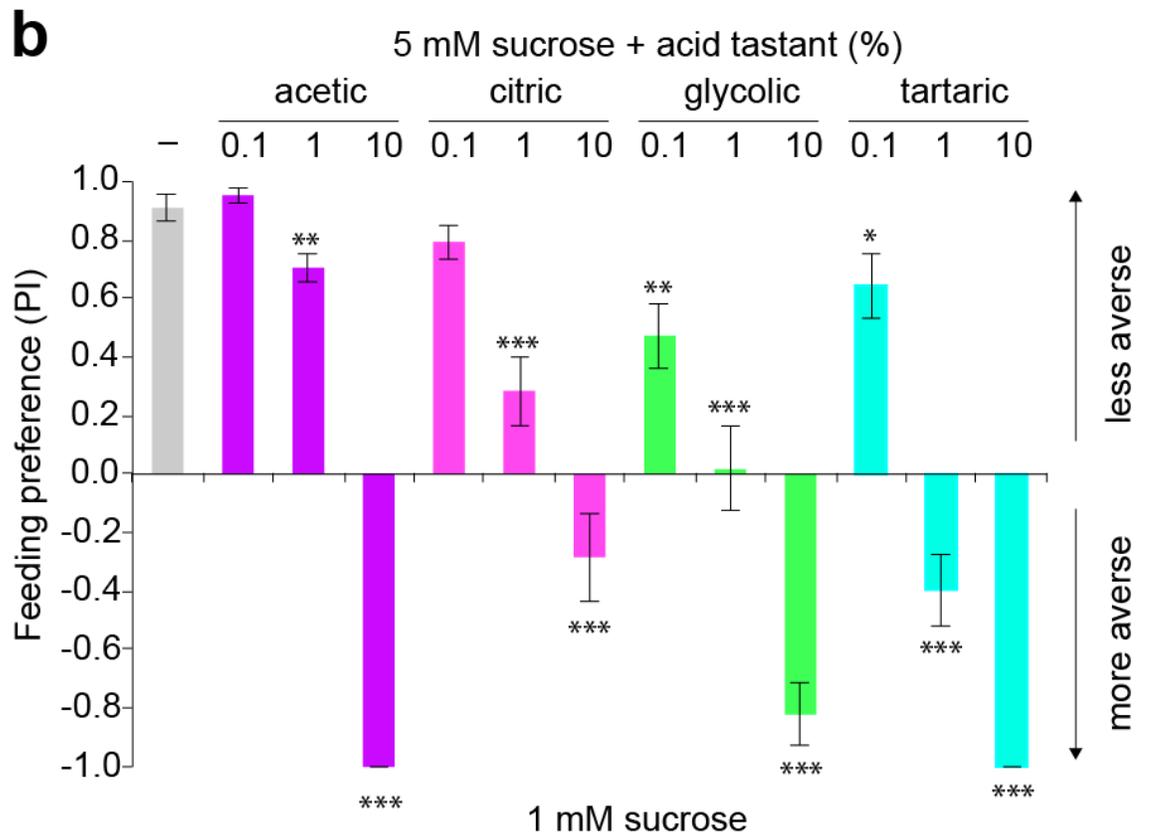
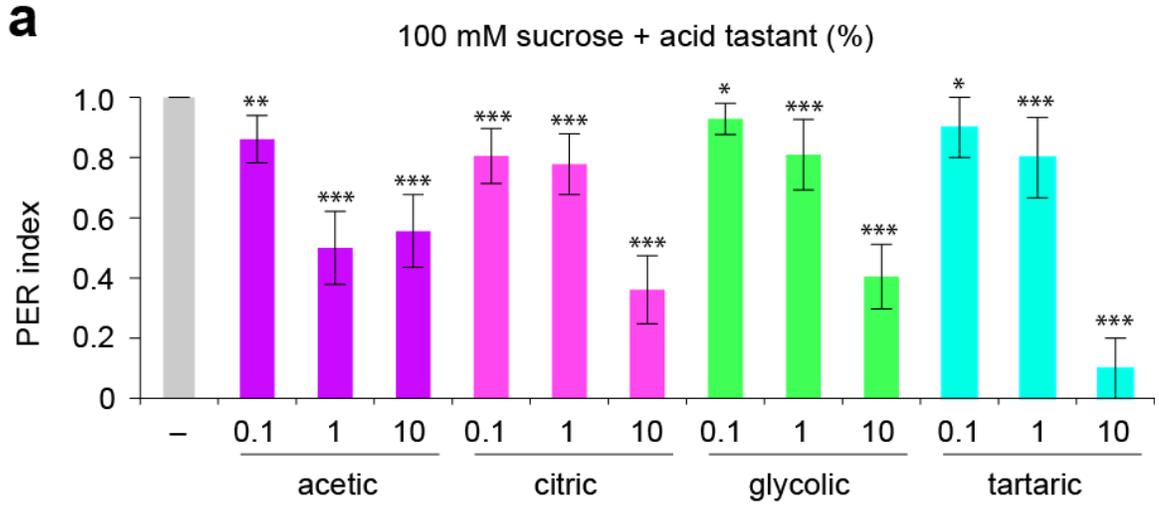


Figure 2.1 Common fruit acids inhibit *Drosophila* taste behaviors.

a. Proboscis extension responses of wild-type flies to 100 mM sucrose alone (–) or in mixtures with indicated acids. $n=32$ (–), $n=18$ (acetic), $n=18$ (citric), $n=21$ (glycolic), $n=10$ (tartaric). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, versus 100 mM sucrose, Student's t -test. For each stimulus, independent trials were conducted over 2–6 days. **b.** Results of binary choice assays using indicated mixed stimuli tested against 1 mM sucrose. $n=10$ (sucrose control), $n=6–10$ (acetic), $n=16–17$ (citric), $n=7$ (glycolic), $n=7$ (tartaric). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, versus 5 mM sucrose, Student's t -test. For each acid tastant series independent trials were performed on 2–5 days.

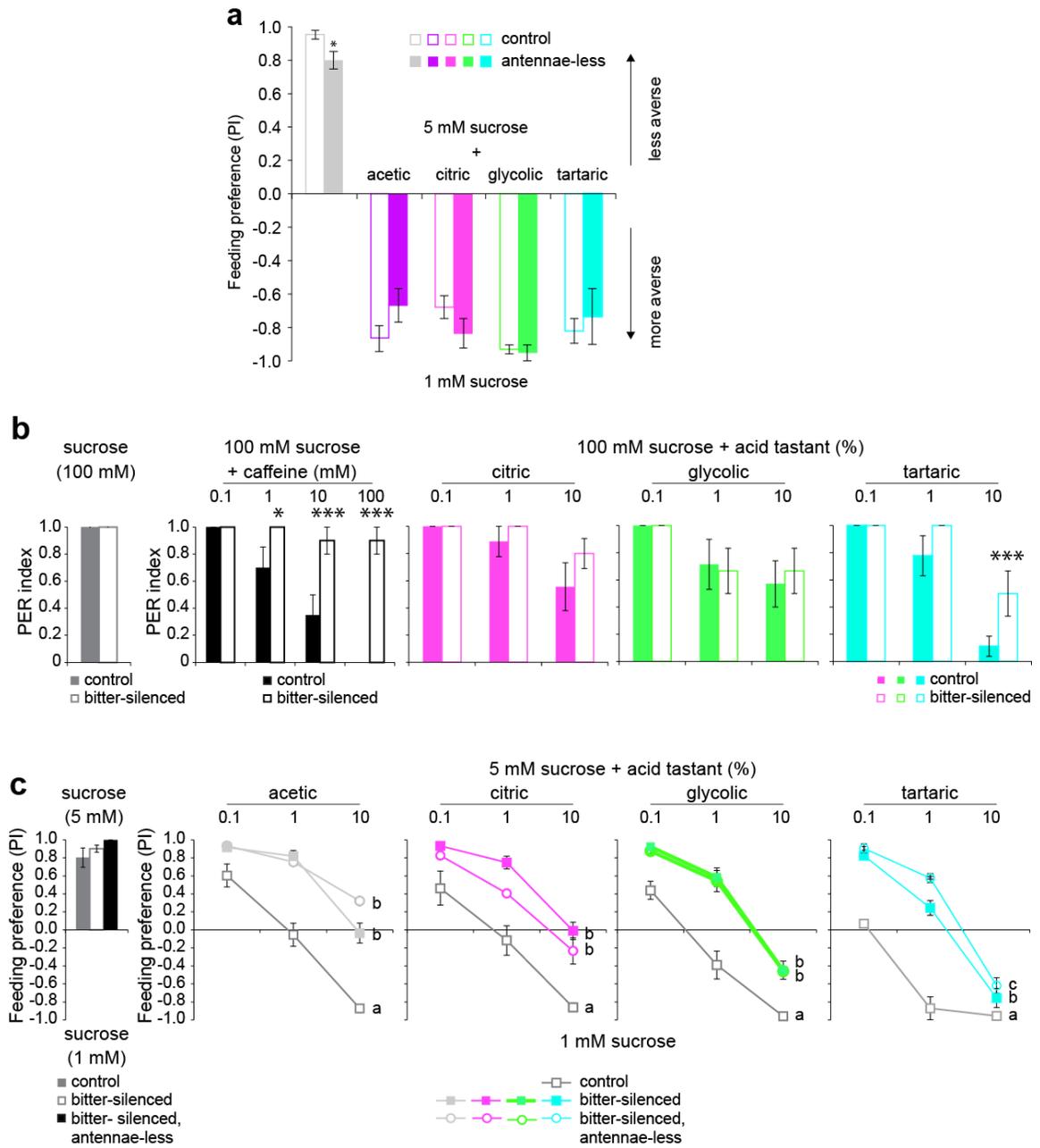


Figure 2.2 Antennae-less and bitter-silenced flies are able to reject acidic stimuli

a. Feeding preference of wild-type flies with antennae (control) and those with antennae removed surgically (antennae-less). Named acids were tested at 10%. For control, antennae-less: $n=8, 9$ (sucrose); $n=6, 6$ (acetic); $n=6, 7$ (citric); $n=6, 6$ (glycolic); $n=8, 6$ (tartaric). $*P < 0.05$, Student's *t*-test. For each stimulus, independent trials were performed over 1–3 days. Experiment was conducted by A.M. Lomelli. **b.** Proboscis extension responses of control (*Gr89a-GAL4/+;+/GFP*) and bitter-silenced (*Gr89a-GAL4/+;UAS-Kir2.1/GFP*) flies to 100 mM sucrose alone or in mixtures with caffeine or acid tastants. Sucrose: $n=44$ (control), $n=46$ (bitter-silenced); caffeine: $n=10$ (control), $n=10$ (bitter-silenced); citric: $n=9$ (control), $n=10$ (bitter-silenced); glycolic: $n=7$ (control), $n=9$ (bitter-silenced); tartaric: $n=9$ (control), $n=9$ (bitter-silenced). $*P < 0.05$, $***P < 0.005$, bitter-silenced versus control, 2-way ANOVA with pairwise comparisons. For each genotype-stimulus combination, independent trials were performed on two days. **c.** Feeding preferences of control (*Gr89a-GAL4/+;+/TM3*), bitter-silenced (*Gr89a-GAL4/+;UAS-Kir2.1/TM3*), and bitter-silenced, antennae-less (*Gr89a-GAL4/+;UAS-Kir2.1/TM3* with antennae removed surgically) flies. Stimulus and assay conditions were as in Fig. 1b. $n=11, 13, 3$ (sucrose control); $n=7-8, 7-8, 3$ (acetic); $n=7, 7-8, 3$ (citric); $n=7, 7, 3$ (glycolic), $n=8-10, 8-10, 3$ (tartaric). Lines with different letters are significantly different, $P < 0.001$ for all comparisons, except $P = 0.031$ for b versus c lines for tartaric acid, 2-way ANOVA with Tukey's *post hoc* analysis. For each genotype-stimulus combination, independent trials were performed on two days. Experiment was conducted by A.M. Lomelli. Error bars = s.e.m.

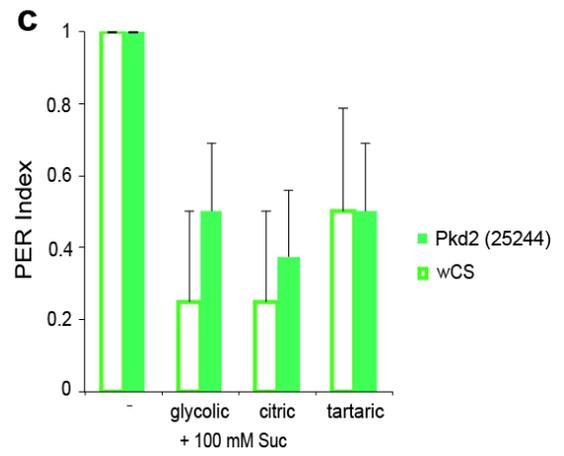
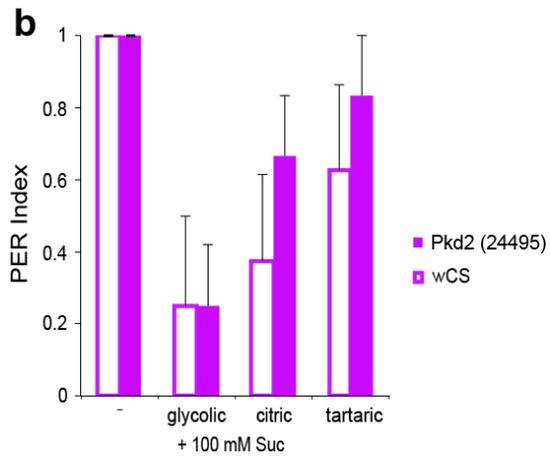
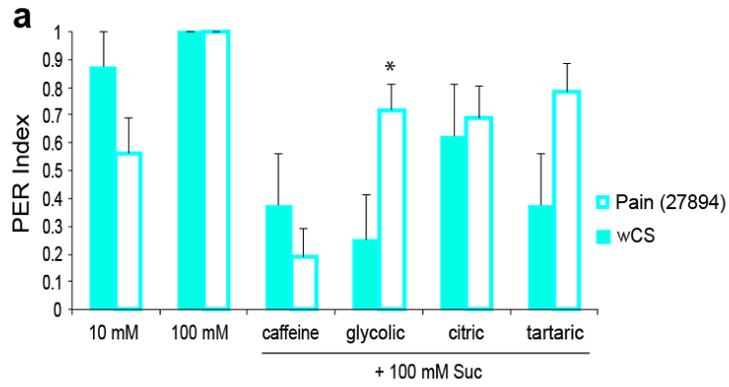


Figure 2.3 Trp receptors do not mediate peripheral detection of sucrose-acid mixtures

a. Proboscis extension response of *painless* mutants and wildtype flies. Control stimuli were 10 mM and 100 mM sucrose. Test stimuli were 100 mM sucrose mixed with 100 mM caffeine, 10% glycolic acid, 10% citric acid and 1% tartaric acid. n=8 (control), n=16 (mutant). * $P < 0.05$, Student's t-test. **b.** Proboscis extension response of *Pkd2* null mutant and wildtype flies. Control stimulus was 100 mM sucrose alone. Test stimuli were 100 mM sucrose with 10% glycolic acid, 10% citric acid and 1% tartaric acid. n=4 (control), n=6 (mutant). **c.** Proboscis extension response of *Pkd2* insertion mutant and wildtype flies. Control stimulus was 100 mM sucrose alone. Test stimuli were 100 mM sucrose with 10% glycolic acid, 10% citric acid and 1% tartaric acid. n=4 (control), n=8 (mutant). Error bars = s.e.m.

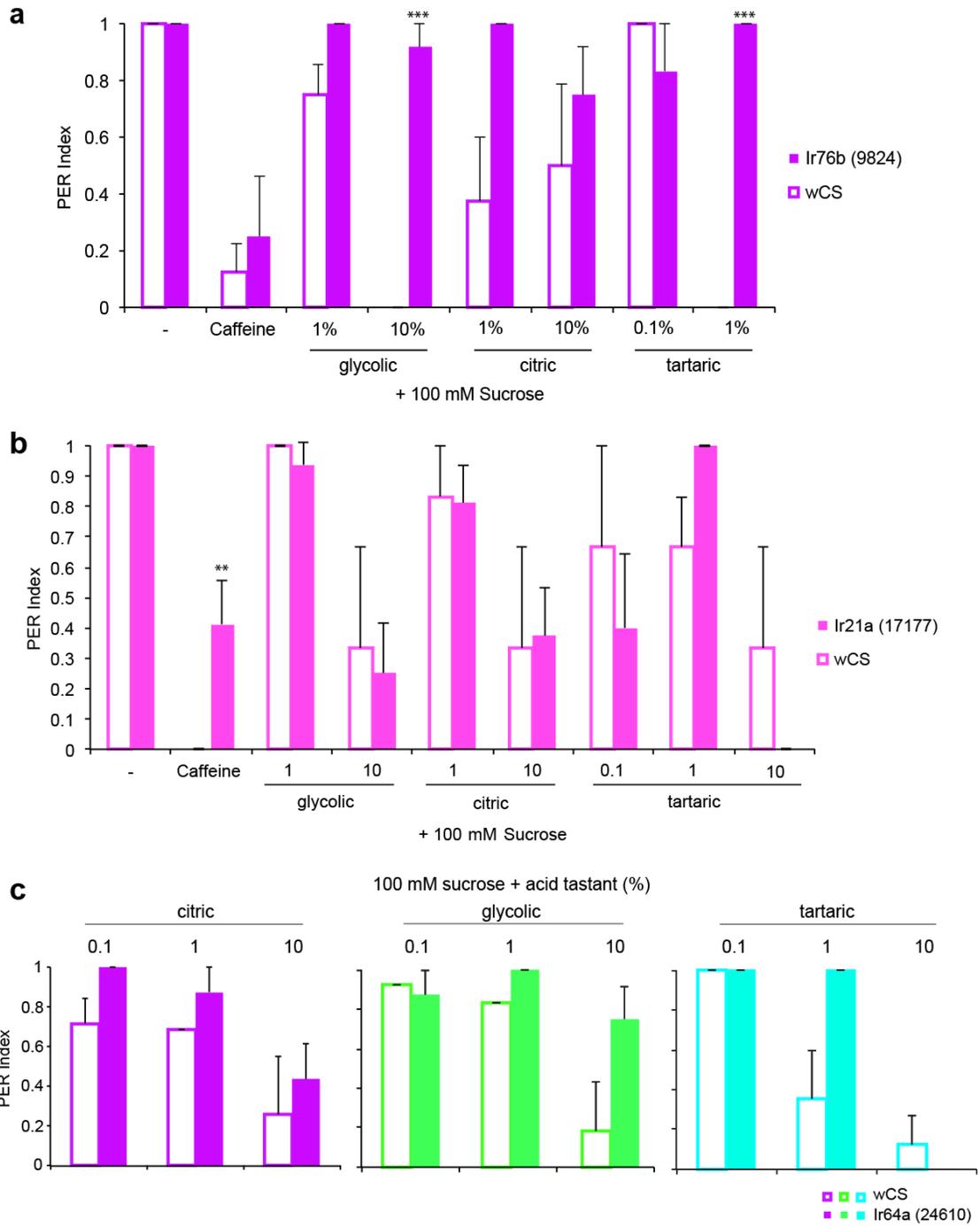


Figure 2.4 Ir receptors are partially necessary for peripheral response to sucrose-acid stimuli

a. Proboscis extension response of *Ir76b* mutant and wildtype flies. Control stimuli were 100 mM sucrose alone and 100 mM sucrose with 100 mM caffeine. Sucrose: n=6 (wildtype), n=8 (mutant); caffeine: n=5 (wildtype), n=6 (mutant); glycolic: n=6,4 (wildtype-1%, 10%), n=8,6 (mutant-1%, 10%); citric: n=6,4 (wildtype-1%, 10%), n=8,6 (mutant-1%, 10%); tartaric: n=4,6 (wildtype-0.1%, 1%), n=6,8 (mutant-0.1%, 1%). ***P<0.001, Student's t-test. **b.** Proboscis extension response of *Ir21a* mutant and wildtype flies. Control stimuli were 100 mM sucrose alone and 100 mM sucrose with 100 mM caffeine. Sucrose: n=6 (wildtype), n=13 (mutant); caffeine: n=6 (wildtype), n=13 (mutant); glycolic: n=6,3 (wildtype-1%, 10%), n=13,8 (mutant-1%, 10%); citric: n=6,3 (wildtype-1%, 10%), n=13,8 (mutant-1%, 10%); tartaric: n=3 (wildtype), n=5,8,8 (mutant-0.1%, 1%, 10%). **P<0.01, Student's t-test. **c.** Proboscis extension response of *Ir64a* mutant and wildtype flies. Control stimuli were 100 mM sucrose alone and 100 mM sucrose with 100 mM caffeine. Sucrose: n=4 (wildtype), n=8 (mutant); caffeine: n=4 (wildtype), n=8 (mutant); glycolic: n=4 (wildtype), n=8 (mutant); citric: n=4 (wildtype), n=8 (mutant); tartaric: n=4 (wildtype), n=10 (mutant). Error bars= s.e.m.

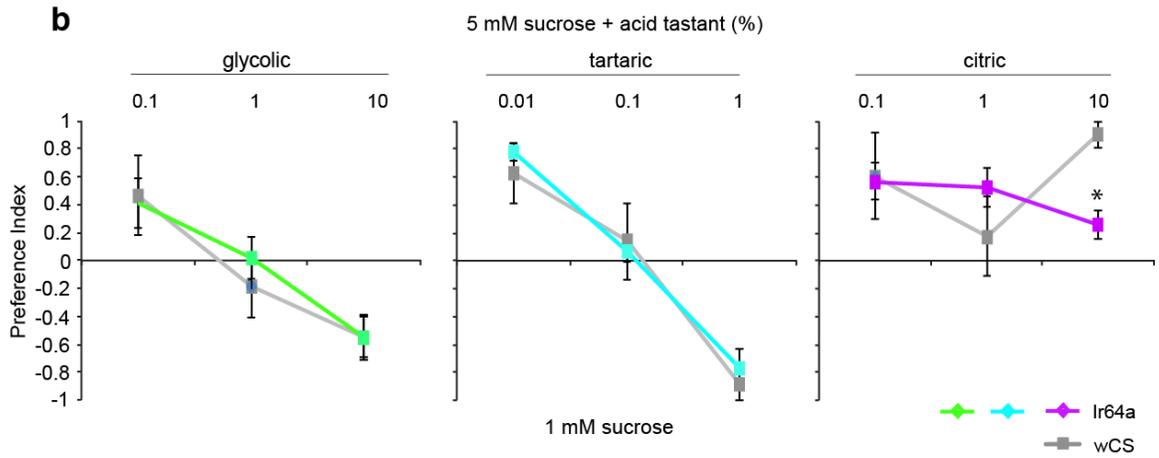
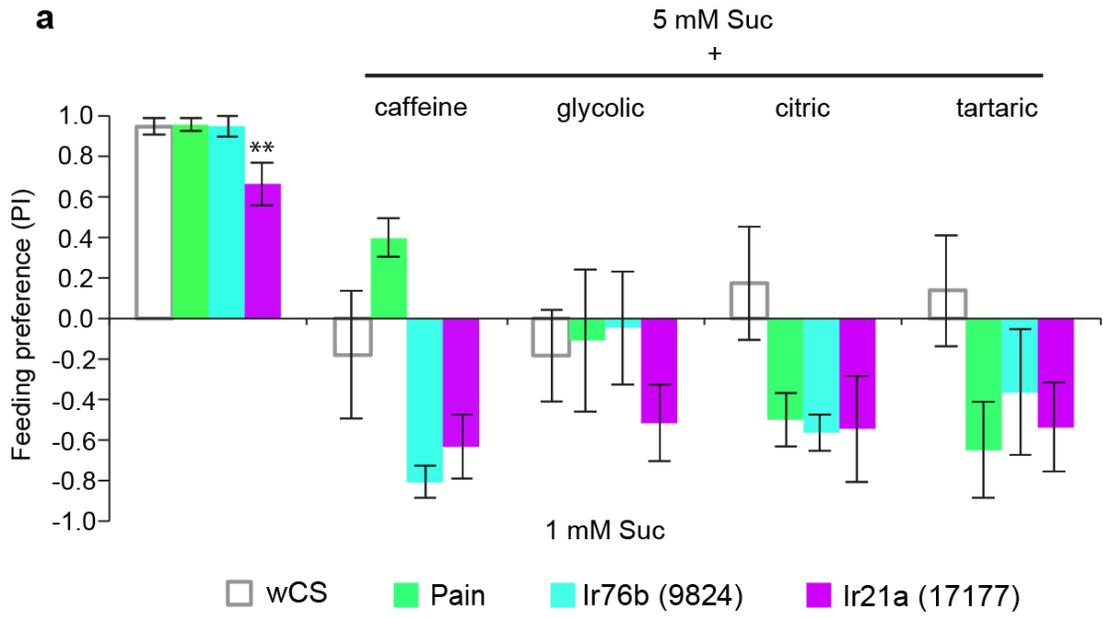


Figure 2.5 Ir receptors are necessary for feeding avoidance of acidic sucrose stimuli

a. Feeding preference assay results for wildtype, *painless* mutant, *Ir76b* mutant and *Ir21a* mutant flies. The control experiment was 5 mM sucrose vs. 1 mM sucrose. The test stimuli were 5 mM sucrose mixed with 10 mM caffeine, 1% glycolic acid, 1% citric acid and 0.1% tartaric acid vs. 1 mM sucrose. Sucrose: n=13 (wCS), n=5 (Pain), n=6 (Ir76b), n=6 (Ir21a); caffeine: n=5 (wCS), n=3 (pain), n=5 (Ir76b), n=6 (Ir21a); glycolic acid: n=7 (wCS), n=5 (pain), n=6 (Ir76b), n=6 (Ir21a); citric acid: n=7 (wCS), n=4 (pain), n=4 (Ir7b), n=6 (Ir21a); tartaric acid: n=8 (wCS), n=4 (pain), n=5 (Ir76b), n=6 (Ir21a). **P<0.01, Student's t-test. **b.** Dose response for binary choice assay with glycolic, tartaric and citric acid mixed with 5 mM sucrose vs. 1 mM sucrose. Glycolic acid: n=3, 7, 2 (wCS-0.1%, 1%, 10%), n=6 (Ir64a); tartaric acid: n=4, 8, 2 (wCS-0.01% 0.1%, 1%), n=6 (Ir64a); citric acid: n=3, 7, 3 (wCS-0.1%, 1%, 10%), n=9 (Ir64a). *P<0.01, Student's t-test. Error bars= s.e.m.

Chapter 3: Organic Acid Detection by the Sweet Neuron in *Drosophila melanogaster*

Introduction

Once we discovered that fruit acids are able to inhibit normal behavior towards sucrose we wanted to find which neurons mediated this behavioral response. Silencing all bitter neurons had shown us that acid inhibition of normal sucrose behavior is partially mediated by the bitter neuron. However, there was not a complete elimination of the aversive behavior when the bitter neurons were silenced (Figure 2.2b and c). Thus, we wanted to further evaluate which other neurons were mediating this aversive behavior.

In larger flies it has been shown that low pH can inhibit the neuronal response to sucrose as well as salt (Gillary 1966, Shiraishi 1969, Bernays 1998). Bitter inhibition of the sweet and water cells has been seen in the tarsal taste neurons of *Drosophila* (Meunier 2003). This cross-modality function of neurons allows for peripheral integration of taste information independent of central processing. This allows the fly to make an immediate decision about the palatability of a substance without having to wait for integration of input from multiple neurons. In other insects, where bitter receptors are few, this dual function of the sweet neuron can compensate for lack of bitter detection diversity. The animal may not tell exactly which compound it is, but it can detect how unappetitive it is based on its affect on the sweet neuron alone.

In the olfactory system, the idea of ephaptic coupling has been proposed. This idea suggests that when one neuron in a sensilla fires, it prevents the other neuron in the cell from firing at its normal rate. This could explain why in the I and S sensilla recordings,

we did not see any bitter neuron spikes to acids, though acids are known to activate neurons in these sensilla.

It is important to test mixed stimuli because animals rarely come in contact with a pure stimulus. In nature, food sources are usually complex and contain numerous types of stimuli, including acids, sugars, salt and bitter compounds. Thus, understanding how the taste system codes mixed stimuli and how these different tastes interact is vital to comprehending natural taste detection. As mentioned previously, the “sugar-acid ratio” in fruits is a critical component of their palatability to animals. Thus determining how this ratio affects *Drosophila* taste can lend insight into *Drosophila* feeding on fruits.

Since the behavior we had seen was in the context of sucrose-acid mixtures, we decided to test neuronal responses to sucrose-acid mixtures using electrophysiology in order to investigate the cellular mechanisms of acid aversion. We recorded from the three morphological classes of sensilla, L, I and S, in the labellum and found that acids inhibit the response of the sweet neuron to sucrose. This response was not affected by silencing the bitter neuron, and thus is a cell-autonomous mechanism for acid detection. This inhibition is pH dependent and is not reliant on the anion of the acid. Moreover, inhibition can be overcome to some degree by increasing the concentration of sucrose. None of the major taste co-receptors are involved in this inhibition, nor are any previously implicated odorant binding proteins.

Results

Sweet neurons in the large sensilla are inhibited by addition of organic acids

Having discovered that the aversive behavioral response to sucrose-acid mixtures is retained when the bitter neurons are silenced, we sought to examine the response of the sweet neuron to sucrose-acid mixtures. We tested mixtures of 100 mM sucrose with increasing amounts of the four fruit carboxylic acids on all three morphological types of labellar sensilla (Figure 3.1). First we examined the large, or L, sensilla on the labellum. The large sensilla are not activated by any bitter stimuli, and thus are predicted to not possess a canonical bitter neuron (Weiss 2011). The large sensilla, then, are a convenient means by which to test the affects of fruit acids on the sweet neuron response to sucrose without any interference from the accompanying bitter neuron.

Compounds were tested from lowest to highest concentration of acid mixed with 100 mM sucrose (Figure 3.1). In between each concentration of sucrose-acid mixture, 100 mM sucrose was tested to ensure continued neuronal function. The recovery bar represents the response to 100 mM sucrose after the acid sequence and was performed to show that no damage was done to the neuron by the acids (Figure 3.1). This response was typically not significantly different from the initial 100 mM sucrose recording, however, for glycolic and tartaric acid there was a slight decrease in the recovery to sucrose (Figure 3.1a).

We found that when increasing concentrations of acids were added to the 100 mM sucrose solution, the number of action potentials (spikes) per second was drastically reduced (Figure 3.1a). In fact, the response decreased to less than 20 spikes per second in

L sensilla upon addition of 10% acid, for three out of the four compounds tested (Figure 3.1a). All four acids caused a dose dependent decrease in the firing of the sweet neuron to sucrose. The control sucrose recordings for all of the acid sequences showed a similar firing frequency amongst themselves to 100 mM sucrose alone (100 spikes/sec). It should be noted, however, that this rate is much higher than what has been previously reported (60-80 spikes/sec) (Dahanukar 2007). This discrepancy is due to the difference in the counting window for our recordings. In the previous study, spikes were counted for 500 ms starting 200 ms after application of the stimulus. However, in this study we counted spikes in the first 500 ms upon contact with the stimulus. We chose this window because the fast adaptation of the neuron to sucrose-acid mixtures required that spikes be counted from contact to capture the true spike rate. Since, the firing frequency is higher in the first 200 ms of a recording than it is after the first 200 ms, including this window increases the spike count.

Notably, though the firing frequencies to the 100 mM sucrose controls were higher than what has been previously shown, the addition of acids was able to reduce the firing by as much as 84% in the L sensilla (Figure 3.1a). This inhibition was seen for 10% glycolic acid with 100 mM sucrose. There was some divergence in the extent to which each acid inhibited the sucrose response. The acetic acid inhibition was the weakest, with a 48% inhibition at the highest concentration. The glycolic acid inhibition was the strongest, as mentioned, at 84%, and the citric and tartaric acid inhibition values fell closer to the glycolic value, with 75% and 80%, respectively (Figure 3.1a).

I and S sensilla show a similar inhibition profile to the L sensilla

Once we had determined that the organic acids inhibited the sweet neuron response to sucrose in L sensilla, we wanted to evaluate whether this inhibition occurred in the other two sensilla types, the I and S sensilla. Unlike the L sensilla, the intermediate, or I, sensilla possess a bitter neuron, in addition to a sweet neuron (Hiroi 2004). However, these are the only two taste neurons the I sensilla possess. The sweet neuron, besides detecting sweet compounds, also acts as the low salt neuron and detects salt solutions below 400 mM (Hiroi 2004). Likewise, in addition to canonical bitter compounds, the bitter neuron in I sensilla detects high salt compounds, which are also aversive stimuli for *Drosophila*. Thus the sweet and bitter neurons in the I sensilla are more appropriately deemed the attractive and aversive neurons.

As in the L sensilla, the attractive neurons in the I sensilla showed a dose dependent inhibition by acids (Figure 3.2a). There was only one exception to this pattern. When 0.1% citric acid was added to 100 mM sucrose there was a slight increase in spike frequency, though the increase was not significant. One possibility is that the sweet neuron in the I sensilla being primed by the previous sucrose recording. If the 0.1% citric acid has no inhibitory affect on the I sensilla sweet neuron, then the neuron would respond as if it is responding to sucrose alone. Sometimes when a neuron is presented with a stimulus two consecutive times, it will respond more strongly to the second presentation because it is primed from the first. This would explain the slight increase in average neuronal response when 0.1% citric acid is added to 100 mM sucrose.

When comparing the spike ratios of the I and L sensilla there was no significant difference (Figure 3.2b). A spike ratio normalizes the response of the sucrose-acid mixture to that of the preceding sucrose recording, thus indicating the level of inhibition. For instance, a spike ratio of 1 would indicate no inhibition, while a spike ratio of 0.2 would indicate high, or to be exact, 80%, inhibition. This measure shows that there is no difference in acid-dependent inhibition of I sensilla and L sensilla. There was a difference, however, between the small sensilla and the intermediate sensilla. Sweet neurons housed in S sensilla were more strongly inhibited than those in I sensilla by tartaric acid (Figure 3.2b). Sweet neurons in S sensilla showed a steep dose dependent decrease in spike firing as acid concentration was increased, with less than 10 spikes per second at the highest concentrations (Figure 3.2b). A significant difference was not observed between the L and S sensilla.

Sweet neuron inhibition is independent of bitter neuron activation

The occurrence of acid-mediated sweet neuron inhibition in sensilla that lack bitter neurons (L-type) suggests that it is independent of bitter neuron activation. To determine whether this is indeed the case, we examined sucrose response inhibition by citric and tartaric acids in I-type sensilla of bitter-silenced flies, in which the sweet neuron remains the sole functional neuron in the sensillum (Hiroi 2004, Weiss 2011). We measured responses to sucrose-acid mixtures and normalized each response in order to calculate the spike ratio. A comparison of spike ratios across the two genotypes showed that acid-dependent inhibition was not significantly different in bitter-silenced flies as

compared to sibling controls (Figure 3.3a). Thus we propose that the sweet neuron can detect acids independently of the bitter neuron. This also further explains why little difference was seen in sucrose inhibition between the sensillar types that contain bitter neurons and those that do not. If the inhibition is only dependent on the sweet neuron, then the presence, in the case of the I and S sensilla, or absence, in the case of the L sensilla, of a bitter neuron should not affect the response. These results then substantiate an independent role for sweet taste neurons in assessing the value of acidic food sources.

pH does not change with fruit fermentation

Initially we hypothesized that the reason for *Drosophila* preference for overripe fruit is that the acid content of overripe fruit is less than ripe fruit. However, our experiments, as well as others' work, has shown that over-ripe and rotten fruits can have an acid content comparable to that of ripe fruit (Palma, J Proteomics 2011). In our experiments, we measured the pH of multiple fruits in ripe or rotten states (Table 3.1). Rotten fruits were prepared by fermenting the pulp of the ripe fruits for 7 days with yeast. We measured the pH of the fruits before and after fermentation. From these recordings we discovered that the acid content of fruits does not change with the fermentation process. On average, most of the fruits tested had a pH of 4 at both the ripe and rotten stages. It can be concluded then that pH value does not change with rotting as it does with ripening. Thus other mechanisms must allow the flies to distinguish between ripe and rotten fruit other than the detection of acid content alone.

Acid aversion can be overcome by increased sucrose content

The other major class of chemicals in fruit that changes with ripening and rotting is sugar. The “sugar-acid ratio” is an important determinant of palatability (Handbook of Fruit Ripening). Given that we have been examining how acids affect the response to a standard concentration of sucrose, we decided to test how sucrose affected the response to a standard concentration of acid. We wanted to determine whether an increase in sugar concentration could overcome acid-evoked inhibition in sweet neurons. To do this, we recorded from sweet neurons in L-type sensilla using mixtures of 10% citric acid with varying concentrations of sucrose (Figure 3.3b). From these recordings we observed an increase in the firing rate of the sweet neuron with increasing concentrations of sucrose in the sucrose-acid mixtures (Figure 3.3c). For each stimulus mixture, we normalized the response to that of sucrose at the same concentration, which was obtained immediately prior to the sucrose-acid recording. A comparison of the spike ratios for each concentration of sucrose showed that the degree of acid-evoked inhibition grew weaker with higher sugar content (Figure 3.3c). These results suggest that the activity of the sweet neuron reflects both the sugar and acid content of the mixed stimulus. If the acid outweighs the sugar, then the neuron will fire weakly. However, if the sugar outweighs the acid, then the neuron will respond with more strongly.

We next wanted to test whether flies would choose to consume low pH foods if the sugar content were higher. We performed a series of feeding choice experiments in which flies were tested for preference between 1 mM sucrose alone and 10% citric acid with concentrations of sucrose from 5 mM to 1 M. As before, we found that flies rejected

a mixture of 10% citric acid and 5 mM sucrose (Figure 3.3d). The same was seen for 10mM sucrose with 10% citric acid. However, increasing the amount of sucrose past this point caused a shift in behavior, and flies displayed a positive behavioral valence for mixtures of 10% citric acid with sucrose at concentrations $\geq 50\text{mM}$ (Figure 3.3d). In fact, when the sucrose concentration was increased to 500 mM, all of the flies chose to feed on the acid-laced stimulus. This indicates that if the sucrose concentration is increased sufficiently, the flies will overlook the low pH and feed on the stimulus. This is possibly what happens in overripe fruit and thus explains why flies prefer this type of fruit. Taken together, our results suggest that sweet taste neurons can evaluate the palatability of food sources by integrating information about sugar and acid content, and thus drive behavior towards feeding on high sugar substances regardless of acid content.

Acid inhibition of the sweet neuron is pH-dependent

Once we determined that the sweet neuron acts as a master gauge of sucrose and acid content, we wanted to elucidate whether acid detection by the sweet neuron is based on the pH of the solution alone or if the anion component is important. When the acids are dissolved in water, a percentage of the acid disassociates and the hydrogen from the carboxyl group(s) separates from the structure, thus producing an anion and a hydrogen ion (which goes on to associate with water to make H_3O^+). Though organic acids are weaker than inorganic acids, at high concentrations they can create very acidic solutions. Thus, it is possible that either of the components of the carboxylic acid, the dissolved hydrogen ion or the anion, could be responsible for inhibition of sweet neuron activity.

In order to determine if there was a correlation between the percent inhibition of the sweet neuron and the pH of the solution, we graphed a best-fit plot of the average percent inhibition recorded for each concentration of each acid and the average pH for that sucrose-acid mixture (Figure 3.4a). The pH of the sucrose-acid mixture was determined empirically using a pH meter. The recordings used were those obtained from the L sensilla of wild-type flies (Figure 3.1a). Upon graphing this data, we found that there is a strong correlation between pH and inhibition ($R^2 = 0.84405$). Lower pH solutions showed stronger inhibition of the sweet neuron than higher pH solutions. Thus, percent inhibition does correlate with the acidity of the solution.

Correlation, however, does not necessarily mean causation. In order to prove that the inhibition of the sweet neuron was indeed pH-dependent, we tested solutions buffered to a higher pH to see if we could reduce inhibition. We buffered a solution of 10% citric acid and 100 mM sucrose from pH 2 to pH 4 using 400 mM sodium citrate. When sodium citrate is dissolved in water it separates into Na^+ and citrate anion. The citrate anion is the same as that released when citric acid is dissolved in water. By adding more citrate to the citric acid-sucrose solution, we shifted the equilibrium in the direction of the undisassociated acid, and thus reduced the hydrogen ion concentration. When we tested the buffered solution, we saw that there was a significant reduction in the inhibition of the sucrose response (Figure 3.4b). The inhibition of the buffered solution was still significant when compared to the response to sucrose alone, but it was dramatically reduced from inhibition observed with unbuffered citric acid-sucrose solution. This

evidence suggests that the pH of the solution is what determines the inhibition of the sweet neuron.

We further verified this by testing the anion salts of the organic acids mixed with 100 mM sucrose on L sensilla (Figure 3.4c). As expected, there was no inhibition when the anion salt-sucrose mixture was tested. We recorded with three anions salts at concentrations equivalent to 10% acid and none of them caused inhibition of the normal sucrose response. The spike ratio for these anion salts was 1.0, 0.9 and 1.1 for sodium tartrate, sodium acetate and sodium citrate, respectively (Figure 3.4c). It should be noted that since there is a low salt neuron in the L sensilla and sodium is added to the solution with the anion, we tested *Ir76b* mutant flies to eliminate the firing of the low salt neuron. This allowed us to clearly distinguish the sweet neuron spikes without concern that we could be counting salt neuron spikes. In fact, when the anion salts were tested alone on the *Ir76b* mutants, they elicited an average neuronal response of 3.25 (tartrate), 2 (acetate) and 1.2 (citrate) spikes per second, indicating that the response to salt was indeed shut down. Taken together, these results indicate that inhibition of the sweet neuron is pH-dependent and that the anion alone does not cause inhibition.

Acid inhibition of the sweet neuron is not mediated by Ir25a or Ir64a

After determining that pH causes inhibition of the sweet neuron, we wanted to find which receptor family could be mediating this inhibition. To do this we tested two broad co-receptors of the Ir and Gr chemosensory families as well as a receptor previously implicated in acid detection (Figure 3.5 and Figure 3.6). Ir25a is co-receptor

for the ionotropic receptor (Ir) family (Siberling 2011). It is the most ancient and conserved of all Ir receptors (Croset 2010). It has previously been implicated in olfactory detection of amines and has been shown to have expression in taste tissue (Siberling 2011, Benton 2009). Thus, we tested *Ir25a* null mutants for their acid-dependent sweet neuron inhibition. However, there was no significant difference between *Ir25a* null mutants and wildtype flies for any of the acids tested (Figure 3.5a). Citric, glycolic and tartaric acids all evoked the same degree of sweet neuron inhibition in *Ir25a* mutant flies as they did in wildtype flies. These results, along with the findings of feeding preference assays described in Chapter 2, rule out the involvement of *Ir25a* in acid inhibition of sucrose detection.

The other Ir tested, *Ir64a*, also did not show a significant difference in sweet neuron inhibition (Figure 3.5b). Though *Ir64a* mutants appeared to have a higher spike ratio than wildtype flies for citric and glycolic acids, no significant reduction in the inhibition of the sweet neuron was observed. All three acids were tested at a 10% concentration with 100 mM sucrose. Of the three acids, tartaric acid showed the strongest inhibition in the *Ir64a* mutants (Figure 3.5b). Tartaric acid at a 10% concentration has a lower pH than the other two acids, 2.14 versus 2.42 (citric) and 2.52 (glycolic), which could account for the stronger inhibition (Figure 3.5b). Consistent with what we observed in behavioral assays, *Ir64a* was not necessary for acid-dependent inhibition of the sweet neuron. Thus, neither of the *Ir* mutants tested with single sensillum recordings showed a change in sweet neuron inhibition.

Gr33a and Obp49a mutants are not involved in acid inhibition of the sweet neuron

The gustatory receptor, or Gr, family is involved in more than one taste modality in the *Drosophila melanogaster* taste system. Grs have been shown to mediate the responses to both sweet and bitter compounds (Dahanukar 2007, Moon 2006, Moon 2009, Lee 2009). To date three Grs have been implicated in the detection of bitter compounds. Among them is Gr33a, which had the broadest effect on bitter compound detection (Moon 2009). *Gr33a* mutants showed a loss in response to numerous bitter stimuli in electrophysiology assays. Since acids are aversive stimuli, like bitter compounds, it is possible that Gr33a could be mediating acid inhibition of the sweet neuron.

Upon evaluation, however, we found that Gr33a is not involved in acid inhibition of the sweet neuron (Figure 3.6a). We recorded responses of I sensilla, in which Gr33a is expressed, to sucrose-acid mixtures. Only concentrations of 1% and 10% were tested, since these showed the highest inhibition in wildtype flies. We used Gr33a heterozygotes as the control and Gr33a homozygotes as the test group. When tested with sucrose-acid solutions, both groups had similar responses (Figure 3.6a). For all three acids tested, the two genotypes showed dose dependent responses. Little variation was seen and none of it was significant. From this we can conclude that Gr33a is not necessary for acid inhibition of the sweet neuron.

Sweet neuron inhibition is not only caused by acids, but can also be caused by bitter compounds. Obps, also known as odorant binding proteins, have been implicated in inhibition of the sweet neuron by bitter compounds (Jeong 2013). It was found that at low

concentrations of bitter stimuli, *Obp49a* mutants showed a reduction in sweet neuron inhibition. *Obp49a* was an obvious candidate then, for mediating acid inhibition of the sweet neuron. However, when we tested *Obp49a* mutants we found no obvious difference from wildtype flies (Figure 3.6b). *Obp49a* mutant flies had a percent inhibition of 76%, 89% and 90% for 10% concentrations of citric, glycolic and tartaric acid, respectively. This was not statistically different from wildtype flies' response at this concentration, so we conclude that *Obp49a* is not necessary for acid inhibition of the sweet neuron.

Discussion

Until recently, it was believed that animals coded the palatability of a food source based on a labeled-line model (Yarmolinsky 2009). This model proposed that different tastes are detected by distinct cell populations and that integration of different tastes occurs in the brain. The work described here repudiates this model. We have shown that acids, the main components of sour taste, can be detected by the sweet neuron independent of input from the bitter neuron. This cross-modality function of the sweet neuron has been found with other aversive stimuli besides acids (Jeong 2013). Bitter compounds have also been shown to inhibit the sweet neuron independent of the bitter neuron. These results together establish the sweet neuron not only as a sensor of appetitive compounds, but also as a sensor of non-appetitive stimuli. The ability of the sweet neuron to be inhibited directly by aversive compounds allows the fly to detect the presence of aversive stimuli even in the absence of a functional bitter neuron.

This mechanism can serve two purposes. First, it allows for redundancy in the *Drosophila* taste system in detection of aversive stimuli. This redundancy may be important for adding a level of protection against ingestion of harmful chemicals. It also, however, can act to allow flies to ingest complex stimuli which contain non-appetitive substances but have high nutrition value. As observed in both the behavioral and electrophysiological assays, increasing the nutritive value of a mixed stimulus can overcome the inhibition caused by the non-appetitive compound. This ability could be vital if the fly can only choose between foods that contain aversive compounds and it needs to determine which source is more nutritious.

We have also found that acid inhibition of the sweet neuron is pH-dependent. Buffering of the sucrose-acid mixture to a higher pH causes a reduction in inhibition. Also, the anion salts of the organic acids are unable to inhibit the normal response to 100 mM sucrose. These results validate the correlation we observed between pH of the tastant solution and inhibition of the sweet neuron. pH was also found, by other members of our laboratory, to be important for activation of the bitter neuron (Charlu 2013). What remains to be seen, however, is if pH-dependent inhibition of the sweet neuron is due to extracellular acidification or intracellular acidification. In mammals, it is known that detection of organic acids by taste cells is caused by intracellular acidification (Wang 2011). Acetic acid crosses the membrane of the cell as an undisassociated acid and then disassociates in the cytoplasm. The resulting drop in intracellular pH inhibits the Trp channel, TrpA1, and this in turn causes activation of the cell (Liman and Montell 2014). In mammals, however, organic acid detection is not pH dependent. Instead it is based on

the concentration of the undisassociated acid (Lyall 2001). Strong acid detection, on the other hand, is pH-dependent and acts through extracellular acidification (Chang 2010, Lyall 2001). Since we observed that it is indeed pH that mediates *Drosophila* acid detection, I would predict that in the *Drosophila* taste system organic acids act via extracellular acidification.

The other part of the molecular mechanism that needs to be elucidated is whether hydrogen ions directly inhibit the sucrose taste receptor or whether they inhibit the sweet neuron from firing via a more general mechanism. Functional expression of the sucrose receptor in an exogenous neuron, such as the ab1C neuron (Freeman 2014), will allow us to examine whether acid inhibition is receptor dependent. In the event that acid-dependent inhibition appears to occur in a receptor specific manner, mutational analysis of the sucrose receptor might reveal regions of the protein that are involved in acid inhibition.

Alternatively, to test whether the hydrogen ion inhibits neuronal activity in a non-specific manner, one could express a different acid-insensitive receptor in the sweet neuron and determine if the response of the receptor to its endogenous ligand is affected by addition of acid. I hypothesize that the acid inhibition of the sweet neuron is based on the neuron and not the receptor. In preliminary experiments, acids were able to inhibit responses to low salt solutions in L sensilla. Unfortunately, this experiment was not fully completed because it was difficult to recover low salt responses after acids were tested, even after giving the neuron an extended amount of time to recover. Thus I propose that acid inhibition of taste neurons is caused by direct effects on the ability of the neuron to

generate action potentials. Further studies shall help to elucidate the mechanism by which acids inhibit the response of the sweet taste neuron.

Fruit	pH (ripe)	pH (rotten)
Cantaloupe	6.48	4.26
Banana	5.35	5.27
Cherry	4.39	4.36
Red Apple	4.25	4.23
Orange	4.05	4.21
Grape	3.80	3.93
Pineapple	3.67	4.11
Green Apple	3.58	3.79
Lemon	2.73	2.38

Table 3.1 pH recordings of ripe and rotten fruits

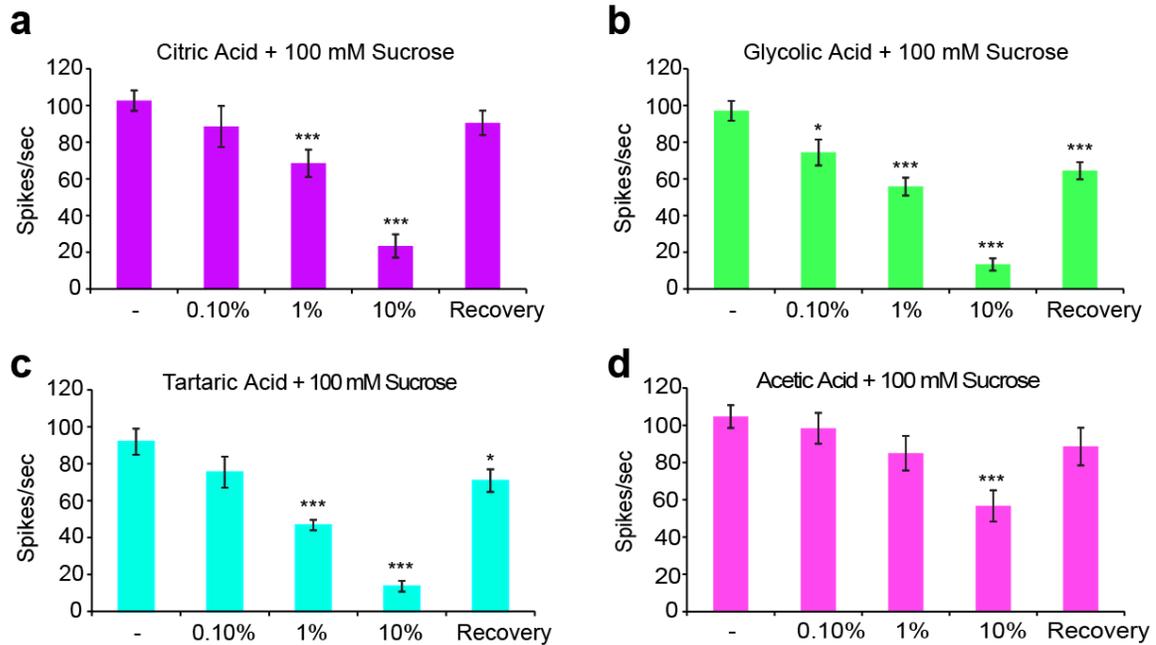


Figure 3.1 Fruit carboxylic acids inhibit the sweet neuron in all L sensilla

a,b,c,d. Sweet neuron response to sucrose-acid mixtures in large (L) sensilla. Recovery recordings were done with 100 mM sucrose alone. Tricholine citrate was the electrolyte used. Citric: n=20 (100 mM sucrose), n=12 (0.1%), n=12 (1%), n=14 (10%), n=19 (recovery); glycolic: n=13 (100 mM sucrose), n=14 (0.1%), n=13 (1%), n=10 (10%), n=13 (recovery); tartaric: n=21 (100 mM sucrose), n=15 (0.1%), n=11 (1%), n=15 (10%), n=21 (recovery); acetic: n=18 (100 mM sucrose), n=10 (0.1%), n=12 (1%), n=13 (10%), n=13 (recovery). Student's t-test against initial sucrose. *P<0.05, ***P<0.001. Error bars= s.e.m.

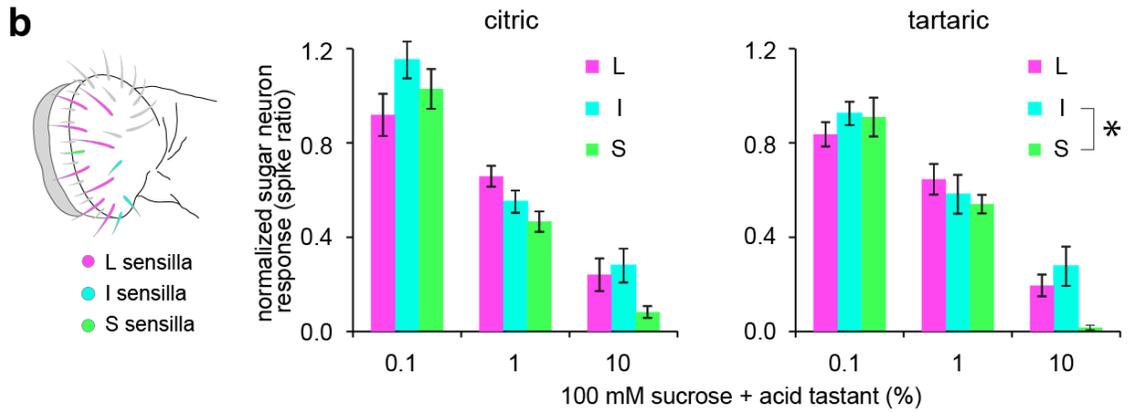
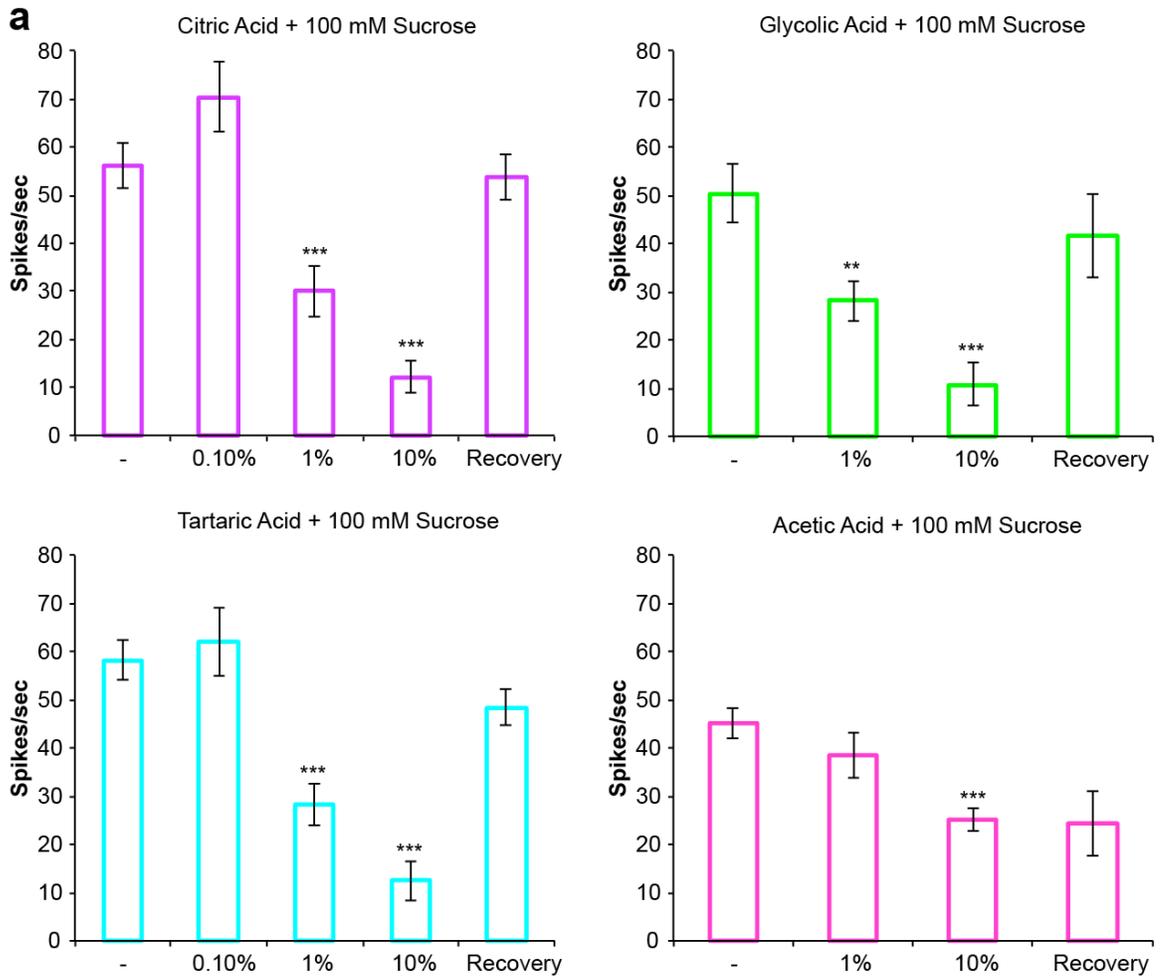


Figure 3.2 All sensillar types show similar sweet neuron inhibition by fruit acids

a. Sweet neuron response to sucrose-acid mixtures in intermediate (I) sensilla. Recovery recordings were done with 100 mM sucrose alone. Tricholine citrate was the electrolyte used. Citric: n=21 (100 mM sucrose), n=10 (0.1%), n=10 (1%), n=11 (10%), n=21 (recovery); glycolic: n=10 (all stimuli); tartaric: n=18 (100 mM sucrose), n=10 (0.1%), n=8 (1%), n=8 (10%), n=18 (recovery); acetic: n=10 (all stimuli). Student's t-test against initial sucrose. **P<0.01, ***P<0.001. **b.** Spike ratio of L, I and S sensilla to 100 mM sucrose with citric and tartaric acid. Citric (0.1%,1%,10%): n=12,12,14 (L), n=10,10,11 (I), n=9,9,9 (S); tartaric (0.1%,1%,10%): n=15,11,15 (L), n=10,8,8 (I), n=10,10,10 (S). *P<0.05, two-way ANOVA with Tukey's *post hoc* analysis. Error bars=s.e.m.

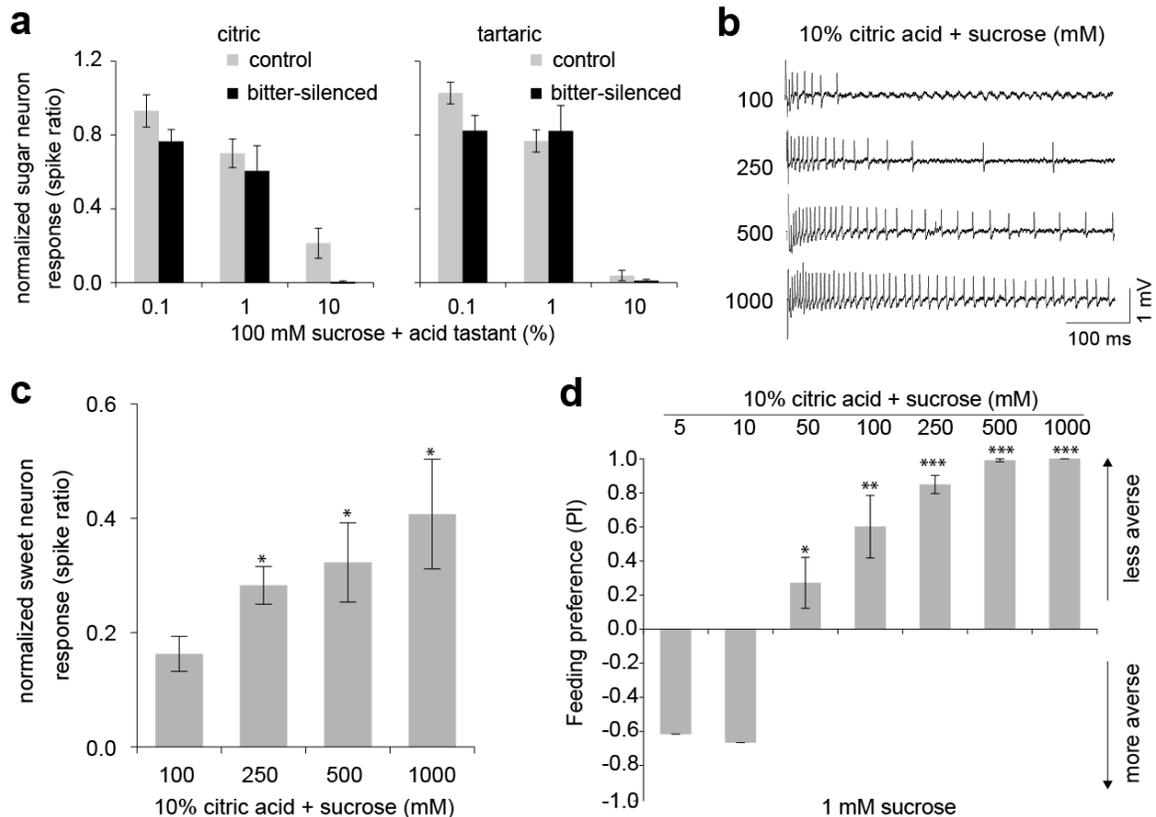


Figure 3.3 The sweet neuron is a master gauge of sucrose and acid content

a. Normalized responses of sweet taste neurons in control (*Gr89a-GAL4/+;+/GFP*) and bitter-silenced (*Gr89a-GAL4/+;UAS-Kir2.1/GFP*) flies. Recordings were obtained from I-b sensilla. $n = 4-14$. $P = 0.853$ (citric), $P = 0.294$ (tartaric), ANOVA with pairwise comparisons. **b.** Representative traces of recordings from a single L-type sensillum of a wild-type fly. **c.** Responses normalized to the corresponding concentration of sucrose obtained for sweet taste neurons in L-type sensilla. $n = 10-15$. $*P < 0.05$, $***P < 0.005$, versus mixture with 100 mM sucrose, Student's *t*-test. **d.** Results of binary choice assays using indicated mixed stimuli tested against 1 mM sucrose. $n = 3-10$. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$, Student's *t*-test. Experiment conducted by A.M. Lomelli. Error bars = s.e.m.

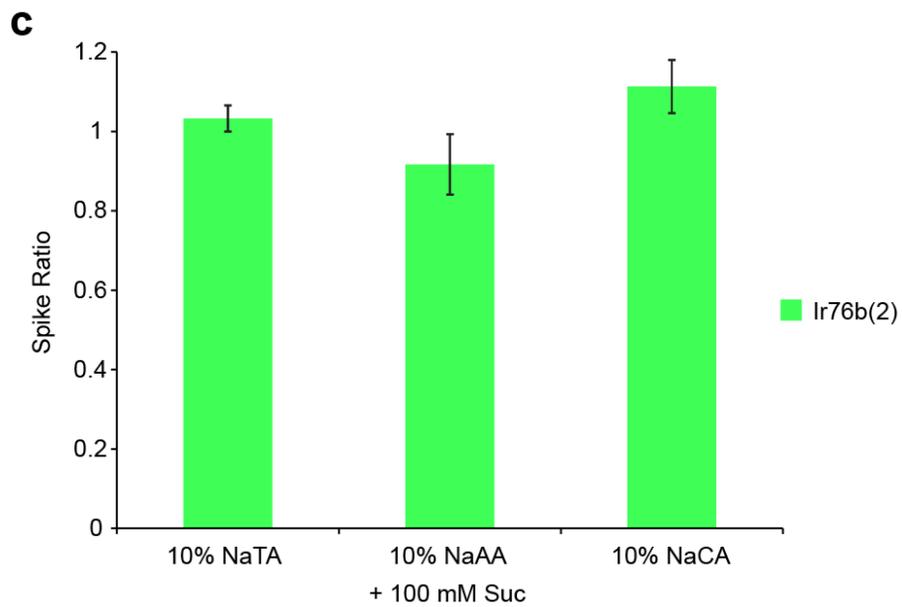
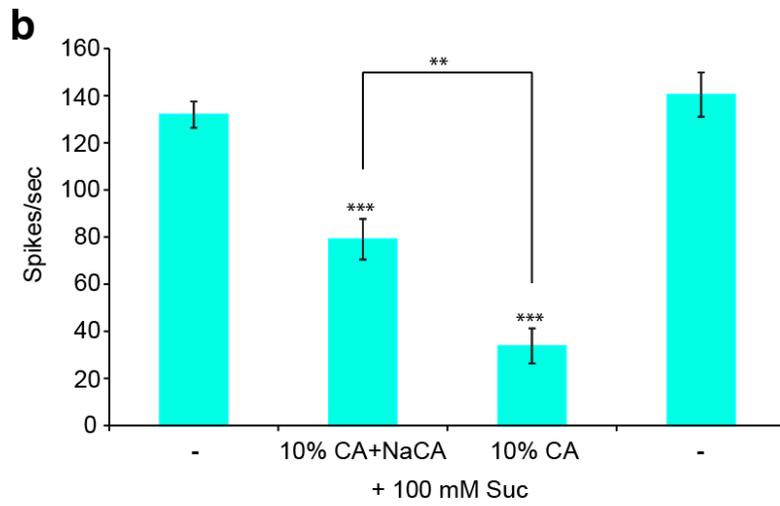
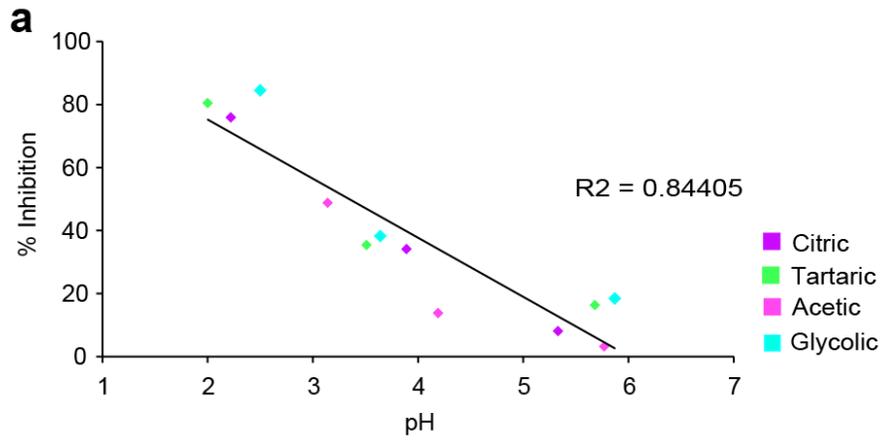


Figure 3.4 Acid inhibition of the sweet neuron is pH dependent

a. Best fit plot of percent inhibition of the sweet neuron versus pH of the test solution. R squared value of 0.84405. Test solutions comprised of acid with 100 mM sucrose and 30 mM tricholine citrate. **b.** Average response from the sweet neuron to buffered citric acid-sucrose solutions. Recordings obtained from L sensilla. Buffered solution contained 400mM sodium citrate. n=9. ** $P < 0.01$, *** $P < 0.001$, versus initial 100mM sucrose. Student's *t*-test. **c.** Normalized response of sweet neuron to sucrose-anion salt mixture with corresponding sucrose response as control. Recordings obtained from L sensilla. n=10 (sodium tartrate), n=6 (sodium acetate), n=5 (sodium citrate). Student's *t*-test. Error bars = s.e.m.

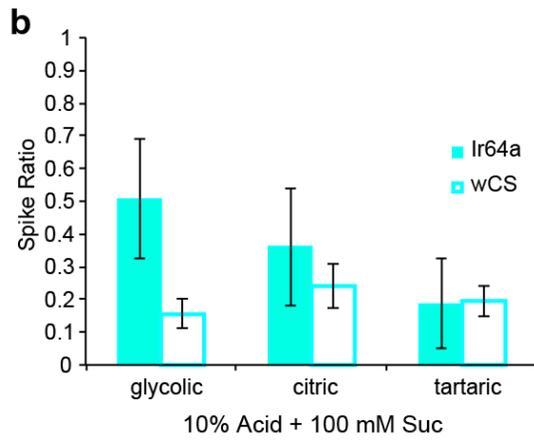
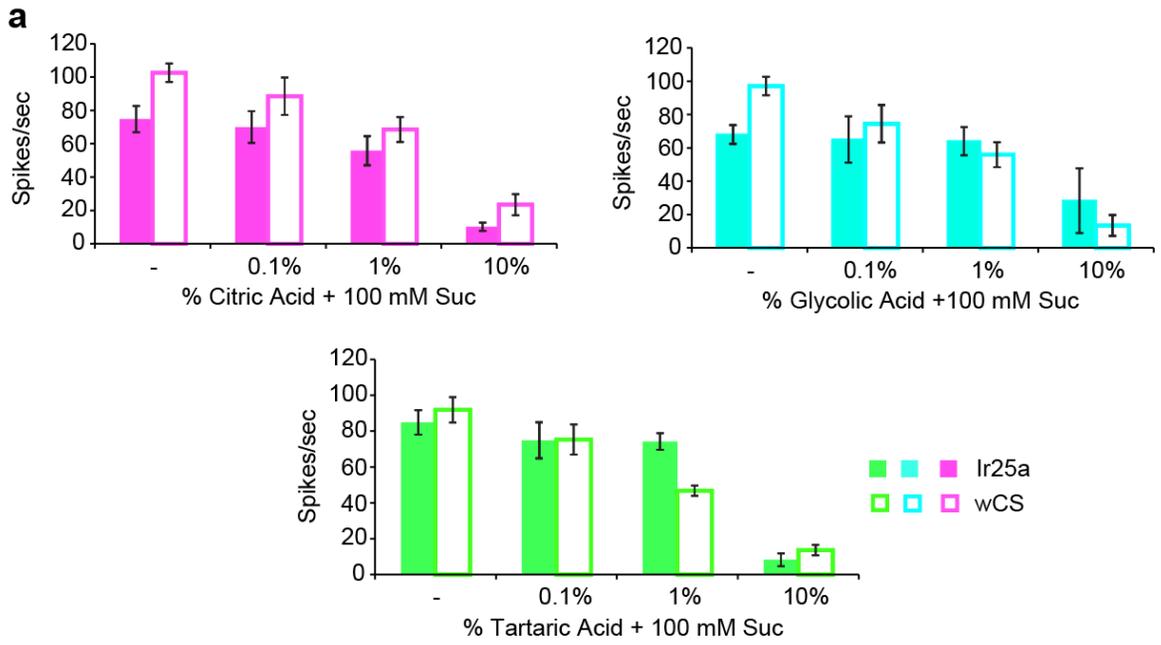


Figure 3.5 Ionotropic receptors are not involved in acid inhibition of the sweet neuron

a. Average neuronal response of Ir25a mutants to sucrose-acid mixtures. Only L sensilla used for recordings. Ir25a, citric: n=11, 8, 11, 10 (sucrose, 0.1%, 1%, 10%); glycolic: n=7, 6, 7, 6 (sucrose, 0.1%, 1%, 10%); tartaric: n=9. wCS, citric: n=20 (100 mM sucrose), n=12 (0.1%), n=12 (1%), n=14 (10%); glycolic: n=13 (100 mM sucrose), n=14 (0.1%), n=13 (1%), n=10 (10%); tartaric: n=21 (100 mM sucrose), n=15 (0.1%), n=11 (1%), n=15 (10%); acetic: n=18 (100 mM sucrose), n=10 (0.1%), n=12 (1%), n=13 (10%). **b.** Spike ratio of Ir64a mutant and wild-type flies to 100 mM sucrose with 10% citric, glycolic and tartaric acid. Ir64a: n=8 (glycolic), n=8 (citric), n=4 (tartaric); wCS: n=10 (glycolic), n=14 (citric), n=15 (tartaric).

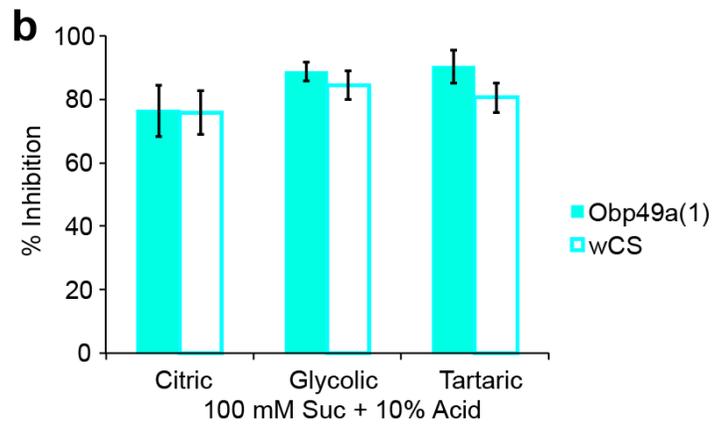
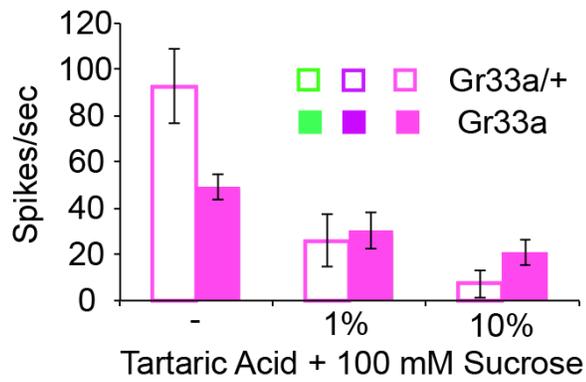
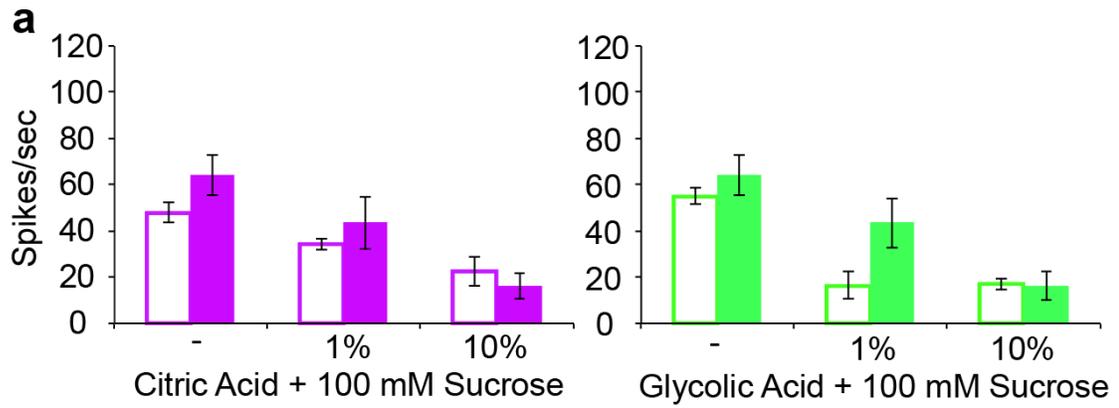


Figure 3.6 Gr33a and Obp49a are not necessary sweet neuron inhibition

a. Average neuronal response on Gr33a heterozygotes (Gr33a/+) and homozygotes (Gr33a) to sucrose-acid mixtures. Recordings obtained from I sensilla. Gr33a/+, citric: n=5, glycolic: n=4, tartaric: n=5; Gr33a, citric: n=8, glycolic: n=7, tartaric: n=6. **b.**

Percent inhibition of sweet neuron of Obp49a mutants. Recordings obtained from L sensilla. Citric: n=8, glycolic: n=9, tartaric: n=8. Error bars = s.e.m.

Chapter 4: Behavioral and Electrophysiological Responses of *Drosophila melanogaster* and *Drosophila sechellia* to Carboxylic Acids Found in *Morinda citrifolia*

Introduction

In previous chapters we examined the response of *Drosophila melanogaster* to carboxylic acids found in fruit, a primary food source of *Drosophila melanogaster*. As mentioned before, though *Drosophila melanogaster* have a preference for fruit, they are technically generalist insects. This is not true of all *Drosophila* species, however. Other *Drosophila* species, such as *Drosophila sechellia*, are specialist insects. It is well known that *Drosophila sechellia* are particularly attracted to one type of fruit that other *Drosophila* species find unappealing. This fruit is the *Morinda citrifolia* L., or morinda, fruit (R’Kha 1991). Morinda fruit is found on many islands throughout the Indian and Pacific Oceans, including Australia, Tahiti, Hawaii and the Seychelles (Legal 1994). It is in the Seychelle Islands off the coast of Africa that *Drosophila sechellia* are predicted to have first come in contact with the morinda fruit (Jones 2005).

Behavior studies have shown that *Drosophila sechellia* are attracted to the smell of ripe morinda fruit and that it is their preferred egg-laying substrate (R’Kha 1991). *Drosophila sechellia* can smell the morinda fruit from a distance of ~150 meters (Matsuo 2007). Other *Drosophila* species, such as *Drosophila melanogaster* and *Drosophila simulans*, however, are driven away by the smell of morinda fruit and do not prefer to lay their eggs on the ripe fruit (Amlou 1998). In fact, when tested for survival on morinda fruit, most non-*sechellia* species died within the first few hours of exposure to the ripe

fruit (R'Kha 1991). The one exception to this was *Drosophila melanogaster* who survived up to two days on the ripe morinda fruit. Rotten morinda fruit is a different story however. It is well known that when the morinda fruit begins to rot, other *Drosophila* species are able to colonize it (R'Kha 1991, Legal 1994). This timing difference between the attraction of *Drosophila sechellia* and other *Drosophila* species is a selective advantage for the *Drosophila sechellia*. The lack of aversion of *Drosophila sechellia* to ripe morinda allows it to colonize the fruit before the other species are able to do so (R'Kha 1991). It has been proposed that the ripe morinda fruit contains chemicals that are aversive to the other *Drosophila* species but not to *Drosophila sechellia*.

The main class of chemicals implicated in this aversion is short chain fatty acids (C4-C10). As with other fruits, the chemical composition of the morinda fruit changes with ripening and rotting (Legal 1994). Raw morinda contains small amounts of hexanoic acid, octanoic acid, palmitic acid and 1-octadecenoic acid (Legal 1994). With ripening, the concentrations of palmitic and 1-octadecenoic acid recede whereas those of hexanoic and octanoic acid increase. When the fruit begins to rot, hexanoic and octanoic acids are converted to hexanoic ethyl ester and octanoic ethyl ester, respectively. This conversion reduces the amount of the two acids to levels intermediate between raw and ripe fruit (Legal 1994). Other labs have also found butyric acid, pentanoic acid, heptanoic acid, nonanoic acid, citric acid and methyl hexanoate to be present in ripe morinda fruit (Pino 2009, Higa 1993, Dekker 2006). The most abundant of all these compounds, however, are hexanoic and octanoic acids (Legal 1994, Pino 2009).

Consequently, many behavior experiments have been carried out using hexanoic and octanoic acid (Amlou 1998, Matsuo 2007, Higa 1993, Legal 1994, Dekker 2006, Harada 2008). A majority of these experiments have examined oviposition or olfactory behaviors. The results for oviposition assays show that *D. sechellia* flies prefer to lay their eggs on media containing hexanoic or octanoic acid and that *D. melanogaster* avoid laying their eggs on this media (Amlou 1998, Matsuo 2007, Harada 2008, Higa 1993). Similarly, olfactory assays indicate that *D. sechellia* are attracted to butyric, pentanoic, hexanoic and octanoic acids, while *D. melanogaster* are only attracted to butyric acid and averse to the other compounds (Dekker 2006, Harada 2008, Higa 1993). Only one study examined feeding on octanoic and hexanoic acid substrates (Harada 2008). In this study, the authors report that *D. melanogaster* choose to feed on 2 M sucrose solutions laced with less than 1 microliter of acid versus food with 2 M sucrose alone. On the contrary, when the food was laced with 1 microliter or more of acid, this behavior reversed completely. This experiment did not test the feeding behavior of *D. sechellia*, though, so that remains to be examined.

Some molecular investigation into the mechanisms behind the olfactory and oviposition preferences of *D. sechellia* has been reported. Dekker, *et al* hypothesized that behavioral differences between *D. sechellia* and *D. melanogaster* were due to proliferation of ab3 sensilla at the expense of ab1 and ab2 sensilla in the antennae of *Drosophila sechellia*. However, EAD recordings revealed little response to butyric, hexanoic and octanoic acid in the two species (Dekker 2006). In addition, when single sensillum recordings were performed from large basiconics, no response to acids was

seen even in *D. melanogaster* flies. We now know that it is in fact the coeloconic sensilla of the antennae that respond to acids (Siberling 2011, Benton 2009). Neurons in the coeloconic sensilla of the *Drosophila melanogaster* respond to butyric, pentanoic, hexanoic, heptanoic and octanoic acids to varying degrees (Siberling 2011). *Ir64a+* neurons, which were extensively studied for their response to acetic acid, are also partially necessary for avoidance of butyric and hexanoic acid (Ai 2010). To date, nothing is known about which sensilla and neurons are involved in the olfactory response of *D. sechellia* to these acids.

In oviposition behavior, the main candidate proteins for the divergence between *D. melanogaster* and *D. sechellia* are the odorant binding proteins (Obps) (Matsuo 2007, Harada 2008, Dworkin and Jones 2009). Odorant binding proteins are hydrophilic compounds in the sensillar lymph that are thought to transport hydrophobic compounds to and from the membranes of chemosensory neurons (Harada 2008). Obps examined for roles in behavior to morinda fruit acids are *Obp57d*, *Obp57e* and *Obp56e* (Matsuo 2007, Harada 2008, Jones and Dworkin 2009). *Obp57d* and *Obp57e* are expressed in the bitter neurons of the legs of *Drosophila melanogaster* (Harada 2008). *Obp56e* is expressed in the antennae and labellum (Dworkin and Jones 2009). *Obp57d* null mutants showed less aversion to laying their eggs on hexanoic acid, heptanoic acid, octanoic acid and nonanoic acid (Matsuo 2007, Harada 2008), while *Obp56e* mutants showed less aversion to morinda fruit itself.

Since behavioral studies with morinda fruit have primarily focused on oviposition and olfactory behaviors, we decided to examine feeding behavior of both *Drosophila*

sechellia and *Drosophila melanogaster* to morinda fruit and its component acids. As a specialist, *Drosophila sechellia* not only seeks out morinda fruit and lays its eggs on it, but it also feeds on the morinda fruit. Thus a study to evaluate the feeding response of *Drosophila sechellia* and *Drosophila melanogaster* to morinda fruit and its chemical components is needed. We found that *Drosophila sechellia* prefer to feed on morinda juice at lower concentrations than those at which *Drosophila melanogaster* do. They are also less susceptible to the aversive feeding behavior caused by short chain fatty acids present in morinda fruit. In this study we were able to evaluate the neurons and receptors involved in the response of *D. sechellia* and *D. melanogaster* to the chemicals found in morinda fruit. We found that S-a class of labellar sensilla showed the strongest responses to all of the 6 short chain fatty acids found in morinda. Responses in these sensilla were found to be dependent on the broadly expressed bitter taste receptor, Gr33a. We also discovered that, as with the fruit carboxylic acids, some of the morinda fatty acids were able to inhibit neuronal responses to sucrose. For some of these acids, this inhibition was seen in *D. melanogaster*, but not in *D. sechellia*. This suggests that the difference in the feeding responses of *D. sechellia* and *D. melanogaster* to morinda fruit and its chemical components is due to a change in sweet neuron sensitivity.

Results

D. sechellia showed a greater preference for morinda juice than *D. melanogaster* at low concentrations

In order to examine feeding behavior of *D. melanogaster* and *D. sechellia* to morinda fruit, we performed two-choice feeding preference assays with increasing

concentrations of morinda juice versus 1 mM sucrose (Figure 4.1a). In our assay we tested 1%, 10%, and 20% morinda juice-agar media versus 1 mM sucrose-agar media. Our results showed that *D. sechellia* had a stronger preference for morinda juice-agar solutions than *D. melanogaster* at low concentrations. At 1% morinda juice, *D. sechellia* flies had a preference for the morinda juice almost equal to that of 5 mM sucrose (PI sucrose= 0.71 ± 0.13 n=6, PI 1% Morinda= 0.62 ± 0.15 , n=6). At this same concentration, *D. melanogaster* flies showed a preference towards the 1 mM sucrose solution (PI= -0.23 ± 0.16 , n=6). This difference in feeding preference between the two species was only seen at this concentration of morinda juice. At a higher concentration of 10%, *D. sechellia* flies still showed a strong preference but the difference between the two species was less pronounced (Figure 4.1a). At 20% morinda juice, both species had similar preferences. From this data we conclude that *D. sechellia* display a stronger preference for feeding on low concentrations of morinda juice than *D. melanogaster*.

It is worth noting that unlike what has been reported for oviposition and olfactory assays, *D. melanogaster* are not completely averse to feeding on morinda. It appears that as the amount of morinda juice is increased, the *D. melanogaster* flies prefer to feed on it more, to the point of consistently choosing it over a pure sucrose solution. This attraction is contrary to what is known about *D. melanogaster* behavior to morinda fruit. One possibility is that in our assay we are testing the morinda juice and not the morinda fruit, as others have (R'Kha 1991, Amlou 1998). Thus it is possible that there are unfavorable stimuli lost in the processing of the fruit into juice. Another possible explanation for this is that, unlike in the olfactory assays, in the feeding preference assay the *D. melanogaster*

are able to come into contact with the morinda and are sensing something in this fruit that they find appetizing. Thus, though *D. melanogaster* may be averse to the smell of morinda fruit, it is attracted to the taste at higher concentrations. This concept has also been proposed by other labs (Amlou 1998). In addition, as mentioned in the previous chapters, the two choice feeding preference assay places the two test solutions in close proximity and thus the fly may not be able to use its olfactory system to distinguish between the two compounds. Thus, its contact chemosensory system is more important in this behavior.

In addition to what has been reported previously (Higa 1993, Amlou 1998, Matsuo 2007, Harada 2008), we also saw that *D. melanogaster* flies, though they chose to feed on the morinda juice, did not choose to lay their eggs on it (Figure 4.1b). In fact there was an overwhelming difference between the *D. sechellia* and *D. melanogaster* in this regard. *D. sechellia* flies laid almost all of their eggs on the morinda food, with an average of 25 eggs per plate on 20% morinda food. The *D. melanogaster* flies laid almost no eggs on the morinda food or on the sucrose alternative (Figure 4.1b). Thus, we can confirm that indeed *D. sechellia* lay more eggs on morinda than on other substrates and that this behavior is not seen in *D. melanogaster*.

D. sechellia are less averse to feeding on octanoic and hexanoic acid

Once we established that *D. sechellia* had a stronger preference for feeding on low concentrations of morinda food than *D. melanogaster*, we wanted to determine the response of these two species to the most prominent short chain fatty acids in morinda fruit, hexanoic and octanoic acids. These two acids are the most prevalent acids in

morinda fruit and are most concentrated at the ripe stage (Legal 1994). Given that *D. sechellia* and *D. melanogaster* have the strongest difference in behavior to ripe morinda, we hypothesized that they would exhibit differences in their taste sensitivity to these two acids.

Indeed this is what we found. First we examined the feeding behavior of the two species to hexanoic acid (Figure 4.2a). In this assay, we tested hexanoic acid mixed with 5 mM sucrose versus 1 mM sucrose. We found that with increasing concentrations of hexanoic acid, both species avoided the sucrose-hexanoic acid mixture. The aversion of *D. melanogaster* flies, however, was stronger than that of the *D. sechellia* flies at all concentrations tested. Both species showed a shift from positive PI to negative PI between 0.1% and 0.5%. In fact, *D. melanogaster* showed a complete reversal, with the PI for 0.5% being very close to -1. Interestingly, both species had no change in response between 0.5% and 1%. Based on preliminary results not shown here (due to lack of participation by *D. melanogaster*) it appears that if 2% hexanoic acid is added to the 5 mM sucrose, the PI for *D. sechellia* goes down to -0.93 ± 0.04 (n=3). This implies that *D. sechellia* cannot distinguish between 0.5% and 1% hexanoic acid but can distinguish between 1% and 2%. From these results we can conclude that the *D. melanogaster* flies are more averse to hexanoic acid than the *D. sechellia* flies. *D. sechellia* flies are able to detect hexanoic acid and avoid it, but they do so at a higher concentration than *D. melanogaster*, thus suggesting that they are less sensitive to hexanoic acid.

A similar, but much more drastic phenotypic difference was seen with octanoic acid (Figure 4.2b). For every concentration of octanoic acid tested, the *D. sechellia* flies

preferred to feed on the octanoic acid-containing solution over the 1 mM sucrose solution. This attraction did decrease as the concentration of octanoic acid increased, but even at 1% majority of *D. sechellia* flies fed on the octanoic acid-sucrose solution rather than 1 mM sucrose. Conversely, for every concentration of octanoic acid tested we saw an aversion in the *D. melanogaster* flies (Figure 4.2b). It should be noted that the control response of the *D. melanogaster* flies to 5 mM sucrose alone versus 1 mM sucrose was lower than usual. In order to correct for this defect, we calculated the delta PI for each concentration and for both species. The delta PI quantifies the change in PI between the 5mM sucrose control and the sucrose-octanoic acid test solution. Using this parameter we found that the change between the control sucrose response and the responses to the sucrose-octanoic acid mixtures was still greater for *D. melanogaster* compared to *D. sechellia* at all concentrations. The delta PI for *D. melanogaster* was 0.998, 0.770 and 1.09, for 0.1%, 0.5% and 1%, respectively. The delta PI for *D. sechellia* was 0.138, 0.520 and 0.483 for the same concentrations. These results prove that *D. sechellia* is less averse to feeding on octanoic acid than *D. melanogaster*. Together with the hexanoic acid results, we can conclude that the difference between *D. sechellia* and *D. melanogaster* in morinda feeding behavior corresponds with a reduced aversion to feeding on hexanoic and octanoic acids in *D. sechellia*.

D. sechellia and *D. melanogaster* differ in their feeding on morinda acids

There are numerous other acids present in morinda fruit, though at much lower concentrations than octanoic and hexanoic acids (Pino 2009). These acids include

butyric, pentanoic, heptanoic and nonanoic acids. In order to evaluate the response of *D. sechellia* and *D. melanogaster* to these acids we decided to first test them in our behavioral feeding assay. All of the acids were tested at a 1% concentration. Each acid was mixed with the 5 mM sucrose and presented versus 1 mM sucrose. Two of these acids, butyric and nonanoic acid, evoked significant differences in feeding behaviors of *D. sechellia* and *D. melanogaster* (Figure 4.2c). *D. melanogaster* flies had a stronger aversion to these acids (butyric= 0.1 ± 0.3 , n=5, pentanoic= 0.06 ± 0.37 , n=6, nonanoic= -0.97 ± 0.02 , n=6) than the *D. sechellia* flies (butyric= 0.82 ± 0.08 , n=6, pentanoic= 0.38 ± 0.2 , n=6, nonanoic= 0.78 ± 0.04 , n=6). *D. sechellia* showed a strong preference for the sucrose-acid mixture with butyric acid and nonanoic acid. In fact, for the nonanoic acid, the PI values were almost completely reversed for the *D. melanogaster* and *D. sechellia* flies (melanogaster= -0.96 , sechellia= 0.78). Control responses to sucrose were similar for both species. Given the large difference between the two species one possibility is that *D. sechellia* do not detect butyric and nonanoic acids at 1% concentration and thus behave as if the acids are not present.

Pentanoic acid, on the other hand, did not elicit a significant difference in behavior between the two species (figure 4.2c). Both *D. melanogaster* and *D. sechellia* showed a similar intermediate response to feeding on pentanoic acid. Heptanoic acid was not included in the final data because there was very low participation from *Drosophila melanogaster*. In fact some fly death was observed on the heptanoic acid plates, which may be explained by possible noxious activity of heptanoic acid on neuronal activity (see

below). Thus, of the four minor morinda acids tested, only two showed a notable difference between *D. sechellia* and *D. melanogaster* flies.

Sensillar subclass responses to morinda acids

Having established the behavioral paradigm for *D. sechellia* and *D. melanogaster* to morinda fruit and its component acids, we wanted to evaluate the neuronal mechanism underlying the differences in behavior. As described in Chapter 3, there are two mechanisms for acid detection by the taste system: 1) activation of bitter neurons and 2) inhibition of sweet neurons. Based on our previous findings, we hypothesized that the underlying mechanism for morinda acid behavior is either a change in the level of activation by these acids or a change in the level of inhibition, or both. To test which if any of these mechanisms were involved we performed single sensillum electrophysiological recordings with all six of the morinda acids. We only analyzed the component acids of the morinda fruit, but not the fruit juice itself, because complex stimuli can often evoke complex responses, thus making it difficult to identify which neurons are firing.

For this experiment, we decided to examine a broad range of sensilla types for their neuronal responses to morinda acids. In *D. melanogaster*, extensive work has been done to classify the L, I and S sensilla into subclasses based on their electrophysiological responses to bitter compounds (Weiss 2011). Thus we decided to examine the response of these different subclasses to all six morinda acids in *D. melanogaster* (Figure 4.3). Of all of the sensillar subclasses, the S-a sensilla responded the most strongly to most number

of acids (Figure 4.3). The S-a sensilla had the highest response to all of six of the acids tested. In addition, the S-b sensilla responded equally strongly but only to four out of six of the acids tested (pentanoic, hexanoic, heptanoic and octanoic). The I-a sensilla were the weakest and the I-b were intermediate. These results are very different from what we observed with fruit acids and inorganic acids (Charlu 2013). In those recordings, the S-b sensilla was more strongly activated than the S-a sensilla, thus suggesting that these two classes of acids are mediated by different groups of sensilla.

To further examine the response of the S-a sensilla to morinda acids, we tested increasing doses of the acids on these sensilla. Pentanoic, hexanoic and octanoic acid all elicited a dose response, with each concentration causing a higher response than the concentration preceding it (Figure 4.4b,c,e). Heptanoic acid showed a weaker response at the lowest concentration and an equivalent response at the two higher concentrations, but not a complete dose-dependence (Figure 4.4d). Butyric and nonanoic acids generally did not show much variation from concentration to concentration (Figure 4.4a,f). Overall the less abundant morinda acids did not evoke strong dose-dependent responses as compared to the two prominent acids, hexanoic and octanoic acids, with pentanoic acid as the one exception to this (Figure 4.4). It is possible that we did not test the dynamic range for these minor morinda acids and thus did not observe dose dependence.

Gr33a is partially necessary for the response to morinda acids in S-a sensilla

Once we had determined which sensilla were important for the bitter neuron response to morinda acids, we wanted to test which receptor was mediating this response.

Given that we saw aversive behavioral responses in *D. melanogaster* for all of the acids tested, we hypothesized that a receptor that mediates aversive taste would be involved. The most obvious candidate for this detection was the broad bitter co-receptor Gr33a. Gr33a is a member of the gustatory receptor family that is expressed in all labellar bitter neurons and has been implicated in the detection of a wide array of bitter compounds (Moon 2009). It was not found to mediate the response to HCl (Charlu 2013), but given the difference in sensillar responses between morinda acids and HCl it is possible that Gr33a may play a role in morinda acid detection.

Indeed for all of the acids, except nonanoic acid, the Gr33a mutants showed a significant difference from wild-type flies in least one sensillar type (Figure 4.5). Notably, octanoic and hexanoic acid both showed a significant difference in S-a sensilla between wildtype and Gr33a mutant flies (Figure 4.5c,e). This finding suggests that Gr33a is involved in the detection of octanoic acid and hexanoic acid in the most responsive sensillar subclass. Octanoic acid also had a reduction in response in the S-b and I-b sensilla of the Gr33a mutants, implying a pan-sensillar necessity for Gr33a in the response to octanoic acid (Figure 4.5e). Response in I-a sensilla did not change for octanoic acid in the Gr33a mutants, but this response was weak to begin with and thus allowed little room for a difference to be seen.

Butyric acid showed a significant change in I-b sensilla, while pentanoic acid showed a phenotype in S-b and I-a (Figure 4.5a,b). Interestingly, the response to pentanoic acid was slightly higher in I-a sensilla of *Gr33a* mutants (Figure 4.5b). This slight increase could be attributed to a difference in genotypic background between the

two lines. Heptanoic acid had a significant loss in response in S-b sensilla, but not in any other sensilla (Figure 4.5d). In fact, four out of five of the acids that had reduced responses in the *Gr33a* mutant flies, showed this reduction in the S-a and S-b sensilla. This and the fact that for all of the acids, except nonanoic acid, the S-b and S-a sensilla were the highest responders suggests that the S sensilla are the broad detectors of the morinda acids. In turn, based on these results, we can conclude that Gr33a is at least partially necessary for the response of S sensilla to most morinda acids.

D.sechellia and *D. melanogaster* show similar bitter neuron responses to octanoic and hexanoic acids

Having established the bitter neuron response of *D. melanogaster* to morinda acids, we wanted to determine the response of *D. sechellia* to these acids and compare the results of the two species. To do this, we first tested S-a sensilla of *D. sechellia* flies with dose curves of octanoic acid and hexanoic acid. Single sensillum recordings with octanoic acid showed no difference between the two species (Figure 4.6a). *D. sechellia* showed a dose dependent response to octanoic acid, with 0.1% evoking the lowest firing rate (~8 spikes per second) and 1% causing the highest rate (~20 spikes per second). There was also no difference in the response to hexanoic acid between the species (Figure 4.6b). Though *D. sechellia* appear to have a slightly higher mean response to hexanoic acid at 0.5% and 1%, no significant difference was observed. Both *D. melanogaster* and *D. sechellia* showed dose dependent activation by hexanoic acid. These results suggest

that the divergence in behavior between the two species is not due to differences in bitter neuron activation by octanoic and hexanoic acids.

D. sechellia and D. melanogaster differ in morinda acid inhibition of the sweet neuron

We next tested the possibility that the distinction in the behavioral response to octanoic and hexanoic acids between *Drosophila sechellia* and *Drosophila melanogaster* is based on the inhibition of the sweet neuron. First, we tested both species for their responses to mixtures of 100 mM sucrose and octanoic acid. At all concentrations tested, *D. sechellia* had a reduced inhibition of the sweet neuron (Figure 4.7a). The spike ratio for *D. sechellia* was significantly higher than that for *D. melanogaster* for 0.1%, 0.5% and 1% octanoic acid. The spike ratio is the same as that calculated in the previous chapter (Figure 3.2b), with the number of spikes elicited by the sucrose-acid mixture normalized to the response to 100 mM sucrose alone. The spike ratio at 0.1% for *D. sechellia* was close to 1, while that for *D. melanogaster* was close to 0.6. This difference was also observed for the other concentrations. These results show that sweet taste neurons in *D. sechellia* are less susceptible to inhibition by octanoic acid. Taken with the behavior results to octanoic acid, we propose that this reduced sweet neuron inhibition is the underlying cause for the difference in behavior to octanoic acid between *D. sechellia* and *D. melanogaster*.

The same cannot be said for the behavior to hexanoic acid however. As shown in Figure 4.7b, there was little to no sweet neuron inhibition observed in either species when hexanoic acid was added to 100 mM sucrose. Though there was a significant difference

at 0.5% hexanoic acid, the *D. sechellia* spike ratio was still quite high (0.867 ± 0.03 , $n=7$). Furthermore, there was no dose dependence observed with either species. These results indicate that the sweet neuron is not inhibited by hexanoic acid. Thus, unlike for octanoic acid, a difference in sweet neuron inhibition cannot explain the difference in behavioral sensitivity to hexanoic acid.

The question then remains, what is causing the behavioral difference between *D. sechellia* and *D. melanogaster* to hexanoic acid? One possibility is that the difference is caused by detection in another taste organ. All of our single sensillum recordings were performed on taste hairs on the labellum. It is possible that another taste organ, such as the tarsus or the pharynx, may be contributing to hexanoic acid behavior. Calcium imaging, or when feasible single sensillum recordings, from these organs with mixtures of hexanoic acid and sucrose as well as hexanoic acid alone may help elucidate mechanisms for the observed behavioral divergence between the two species.

Based on our results with hexanoic and octanoic acid, we were curious to test whether behavioral differences to butyric and nonanoic acids in the *D. sechellia* and *D. melanogaster* flies are based on the inhibition of the sweet neuron. We tested 1% butyric acid and 1% nonanoic acid mixed with 100 mM sucrose in our single sensillum recordings (Figure 4.7c). Indeed, for nonanoic acid we saw a significant difference in the inhibition of the sweet neuron response to sucrose. The sweet neuron of *D. sechellia* was not inhibited by 1% nonanoic acid (spike ratio= 0.99 ± 0.1 , $n=10$). The *D. melanogaster* sweet neuron, on the other hand, was inhibited by 70%. Conversely, 1% butyric acid did not cause much inhibition in either species (Figure 4.7c). This is similar to what we saw

for hexanoic acid. As with hexanoic acid, we can conjecture that observed differences to butyric acid in behavioral assays may arise from variations in responses within other taste organ(s).

Interestingly, heptanoic acid inhibited the sweet neuron in the *D. melanogaster* flies (Figure 4.7c). This inhibition was not as drastic in the *D. sechellia* flies but the difference was not statistically significant. It is worth noting, that in line with the behavioral assay results, in which heptanoic acid caused fly death, a recovery sucrose after heptanoic acid application was often messy or weak. A recovery sucrose recording is performed after the application of the sucrose-acid mixture in order to verify that the neuron is not damaged by the acid. In the case of heptanoic acid, it appears that some damage occurred. This corresponds with the observation that the *D. melanogaster* flies showed low participation, and even died, when allowed to feed on heptanoic acid.

Pentanoic acid had a very different affect on the sweet neuron (Figure 4.3b). The spike ratio for pentanoic acid mixed with 100 mM sucrose was above 1 (mel: 1.12 ± 0.14 , $n=11$, sech: 1.36 ± 0.14 , $n=10$), implying that pentanoic acid actually caused activation of the sweet neuron, not inhibition. A spike ratio above 1 indicates that there were more spikes when the acid was added than when the sucrose alone was presented. All of the spikes counted were of similar amplitude and no doublets were observed (Meunier 2003). This would suggest that the same neuron is responding to both the sucrose and the pentanoic acid, thus causing the increased spike count. Based on the amplitude of the action potentials in these recordings, and the fact that the recordings were obtained from

the L sensilla, we can deduce that the sweet neuron was the neuron firing to the sucrose-pentanoic acid mixture.

These results for pentanoic acid do not tally with the behavior results, however, which show that addition of pentanoic acid causes a shift from feeding on 5 mM sucrose to feeding on 1 mM sucrose (Figure 4.2c). This shift indicates that the flies are averse to the addition of pentanoic acid and thus when it is added, they avoid eating the 5 mM sucrose mixture. Instead they choose to feed on the 1 mM sucrose, which does not contain any acid. If indeed the sweet neuron was the only neuron mediating the response to pentanoic acid, we would not expect to see this shift in behavior. This shift tells us that in addition to the involvement of the sweet neuron, there must also be involvement of an aversion neuron in this behavior. Thus further evaluation of other bitter neuron-containing sensilla may reveal the mechanism underlying this behavior.

Overall, our results suggest that morinda acids are divided into three groups: 1) acids to which *D. melanogaster* and *D. sechellia* exhibit similar behavioral sensitivity, 2) acids to which *D. melanogaster* and *D. sechellia* exhibit different behavioral sensitivities and that inhibit sweet taste neuron activity to a far greater extent in *D. melanogaster* as compared to *D. sechellia*, and 3) acids to which *D. melanogaster* and *D. sechellia* exhibit different behavioral sensitivities and that inhibit sweet neuron activity to a similar extent in both species. For the acids whose behavioral results cannot be explained by sweet neuron inhibition, further electrophysiological experiments need to be done with other taste organs to find the neuronal difference underlying the behavior.

Discussion

In this study we examined the feeding and taste responses of *Drosophila sechellia* and *Drosophila melanogaster* to morinda fruit and its short chain fatty acids. We found that *D. sechellia* flies have a greater attraction to morinda juice than *D. melanogaster*, particularly at lower concentrations. We discovered that this difference is more pronounced when the two primary morinda acids were tested. For both octanoic and hexanoic acids, *D. melanogaster* flies showed an aversive feeding response. *D. sechellia*, however, did not show this same level of aversion to these acids. When evaluating the underlying neuronal mechanism of this difference in behavior we found that in fact it was the inhibition of the sweet neuron that accounts for the reduced aversion to octanoic acid in *D. sechellia*. *D. sechellia* had a significantly higher spike ratio than *D. melanogaster* to sucrose-octanoic acid mixtures. This result corroborates our previous findings (Charlu 2013) that suggest that inhibition of the sweet neuron may be the underlying cause for adaptive changes in feeding behavior. No difference was seen between *D. sechellia* and *D. melanogaster* in the bitter neuron responses to octanoic acid. For hexanoic acid, though, neither bitter neuron activation, nor sweet neuron inhibition, in the labellum, could account for the difference in behavior. More studies with other taste organs may reveal the cause for the hexanoic acid feeding difference.

We also evaluated the response of *D. sechellia* and *D. melanogaster* to other short chain fatty acids found in morinda fruit, such as butyric, pentanoic, heptanoic and nonanoic acids. The two species showed little difference in their behavior towards pentanoic acid, but they did diverge in their feeding on butyric and nonanoic acids. As in

the case of octanoic acid, the nonanoic acid phenotype appears to be a consequence of a difference in sweet neuron inhibition between the two species. For butyric acid, however, like hexanoic acid, no change in labellar response to butyric acid was observed between the two species. Heptanoic acid was not evaluated to the full extent because it caused fly death in behavior and neuronal damage in recordings.

When all sensillar subclasses were tested for their response to 1% concentrations of the six morinda acids, S-a sensilla stood out as the most consistent and robust responders. S-b sensilla were also an important for at least four out of the six acids. Response in S-a sensilla to octanoic and hexanoic acids were greatly impaired by the elimination of Gr33a, a bitter receptor. Loss of Gr33a also caused deficiencies in the S sensilla for two of the remaining four acids. A broad elimination in response in the Gr33a mutants was not seen however. It is possible that since the responses in some sensillar subclasses were already quite low, removing Gr33a did not have much affect. Taken together these results suggest that Gr33a plays a partial role in detection of the morinda acids.

One of the most interesting findings from our study was that *Drosophila melanogaster* flies chose to consume morinda-containing food over sucrose food. Not only that, but as the concentration of morinda was increased, the *Drosophila melanogaster* chose to consume more of it. The theory that *Drosophila melanogaster* are averse to morinda has largely been based on oviposition and olfactory assays. Little work has been done prior to this study examining the feeding behavior of *D. melanogaster* to morinda fruit. In most olfactory assays, the flies are not allowed to contact the morinda

fruit and thus their aversion of the morinda fruit is purely based on the smell of the fruit. It is possible then, that in contact chemosensation assays, such as our feeding preference assay, the flies are detecting a compound that they find attractive and that this is overcoming the olfactory aversion. Interestingly, another lab has also proposed that *D. melanogaster* have an attraction to morinda fruit in their taste system (Amlou 1998). When testing two methods for oviposition on morinda fruit, the authors report that contact chemosensation may be the deciding factor between whether flies lay their eggs on morinda or not. In their assay, when the oviposition plates were separated, *D. melanogaster* flies showed a strong aversion to laying eggs on the morinda fruit (% eggs=0). However, when the two media were presented in conjunction on the same plate, the *D. melanogaster* flies chose to lay 40% of their eggs on the morinda fruit. In the two-plate assay, the flies use their olfactory system alone to decide which plate to move towards and thus lay their eggs on. In the one plate assay, the flies can also use their gustatory system to instruct them which media to lay their eggs on. The authors attribute this transition towards laying eggs on the morinda fruit to an attraction in the gustatory system for some compound found in the fruit (Amlou 1998).

This brings up another interesting point. Amlou *et. al.* saw egg laying by *Drosophila melanogaster* on the morinda food when contact was allowed, but we saw no egg laying in this scenario. This discrepancy between our results and what was found in the one plate assay of Amlou, *et. al.* could be attributed to the lack of laboratory fly food in our assay. In the Amlou, *et. al.* one plate assay the flies were presented with a plate filled with mostly standard fly food and a small section covered in morinda food. Thus

the flies could detect the chemicals present in both stimuli. It is well known that the yeast in standard fly food stimulates egg production and in turn egg laying. If this signal was combined with an attractive compound in the morinda food then this could stimulate the *D. melanogaster* flies to lay eggs. In our assay there was no fly food present, and thus the *D. melanogaster* flies did not lay eggs.

Examining the neuronal and molecular mechanisms that underlie the detection of the morinda acids raises the distinction between the taste of morinda acids and the taste of the fruit acids studied in the previous chapters. As mentioned before, one main distinction between the detection of these two classes of acids is the type of sensilla that respond to them. In the case of the fruit carboxylic acids, the main sensilla mediating the response are the S-b sensilla, and S-a sensilla are considered non-responding cells (Charlu 2013). In the case of the morinda fatty acids the strongest responders are the S-a sensilla, though the S-b sensilla may also play a significant role. Response in S-a sensilla appear to be the biggest distinguishing factor between the two classes of acids, since morinda acids activate these sensilla and other fruit acids do not.

There is also a difference in the inhibition profiles of the fruit carboxylic acids and morinda short-chain fatty acids (Chapter 3, Figure 4.7). For fruit acids, though the structures diverged in carbon length and number of hydroxyl groups, all of them caused inhibition of the sweet neuron (Chapter 3). However, this was not the case for the morinda acids. Only three of the acids, heptanoic, octanoic and nonanoic acid, caused strong sweet neuron inhibition (less than 0.5 spike ratio). The other three acids did not inhibit sweet neuron firing to sucrose. Morinda acids that caused inhibition do not differ

much in structure from those that did not cause inhibition. The acids in the two groups are only 1 or 2 carbons apart in structure. Thus, morinda acids can also be distinguished from other fruit acids by their ability to inhibit the sweet neuron.

This diversity in activation and inhibition amongst morinda acids and in comparison to the fruit acids will allow for more precise investigations into the molecular components mediating these two responses. From our results we can predict that multiple receptors are mediating the response to morinda acids and that not all of the acids act of the same receptor. We can also hypothesize that these are not the same as the receptor(s) responsible for fruit acids. From our study we know that Gr33a is partially necessary for the response to some of the acids. Further investigations will elucidate other receptors that are more specific to each acid.

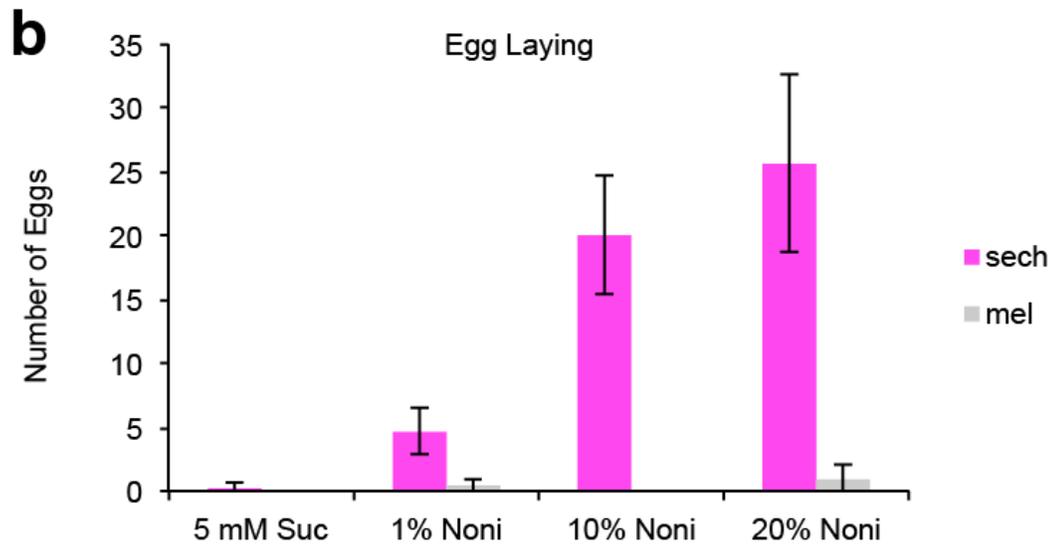
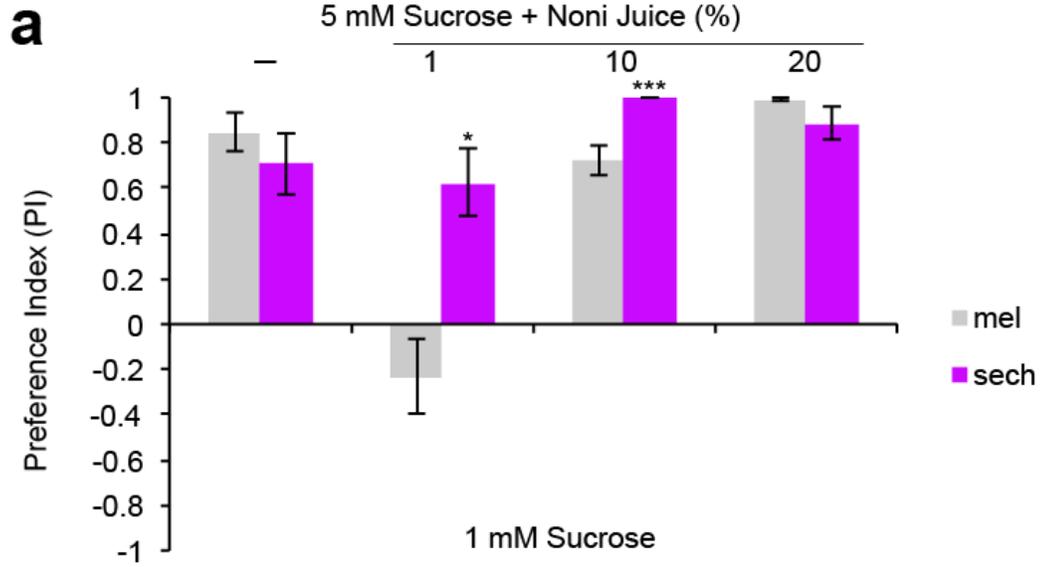


Figure 4.1 *Drosophila sechellia* and *Drosophila melanogaster* diverge in behavioral response to morinda fruit

a. Feeding preference responses for *D. melanogaster* and *D. sechellia* flies to morinda juice versus 1 mM sucrose. 5 mM sucrose: n= 6; 1% morinda: n= 6,7 (*D. mel*, *D. sech*); 10% morinda: n= 6,7 (*D. mel*, *D. sech*); 20% morinda: n= 6. *** $P < 0.001$, Student's *t*-test. **b.** Eggs counts for *D. melanogaster* and *D. sechellia* on 5 mM sucrose and morinda juice. 5 mM sucrose: n= 3; 1% morinda: n= 2,4 (*D. mel*, *D. sech*); 10% morinda: n= 2,4 (*D. mel*, *D. sech*); 20% morinda: n= 2,4 (*D. mel*, *D. sech*). Error bars= s.e.m.

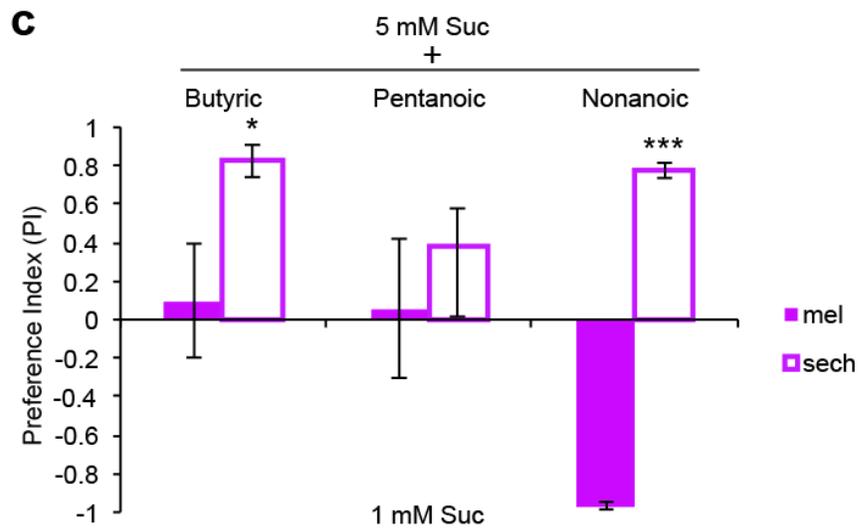
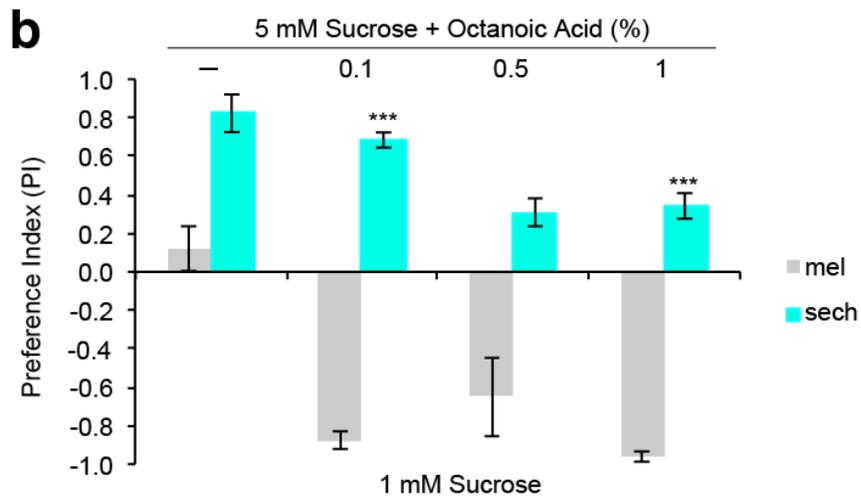
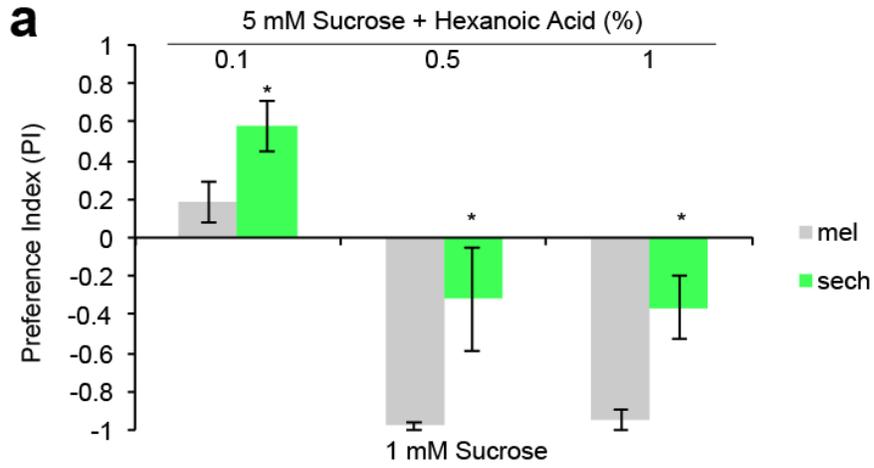


Figure 4.2 *Drosophila sechellia* are less averse to feeding on morinda acids than *Drosophila melanogaster*

a. Feeding preference results for *D. melanogaster* and *D. sechellia* flies to 5 mM sucrose mixed with hexanoic acid versus 1 mM sucrose. 0.1% hexanoic: n= 6,7 (*D. mel*, *D. sech*); 0.5% hexanoic: n= 6,8 (*D. mel*, *D. sech*); 1% hexanoic: n=3,7 (*D. mel*, *D. sech*). * $P < 0.05$, Student's *t*-test. **b.** Feeding preference results for *D. melanogaster* and *D. sechellia* flies to 5 mM sucrose mixed with octanoic acid versus 1 mM sucrose. 5 mM sucrose: n= 5,6 (*D. mel*, *D. sech*); 0.1% octanoic: n= 6; 0.5% octanoic: n= 3,6 (*D. mel*, *D. sech*); 1% octanoic: n=5,6 (*D. mel*, *D. sech*). *** $P < 0.001$, Student's *t*-test. **c.** Feeding preference results of *D. melanogaster* and *D. sechellia* to morinda acids. Butyric: n=5,6 (*D. mel*, *D. sech*); pentanoic: n=6,6 (*D. mel*, *D. sech*); nonanoic: n=6,6 (*D. mel*, *D. sech*). A.M. Lomelli contributed to this data. * $P < 0.05$, *** $P < 0.001$, Student's *t*-test. Error bars=s.e.m.

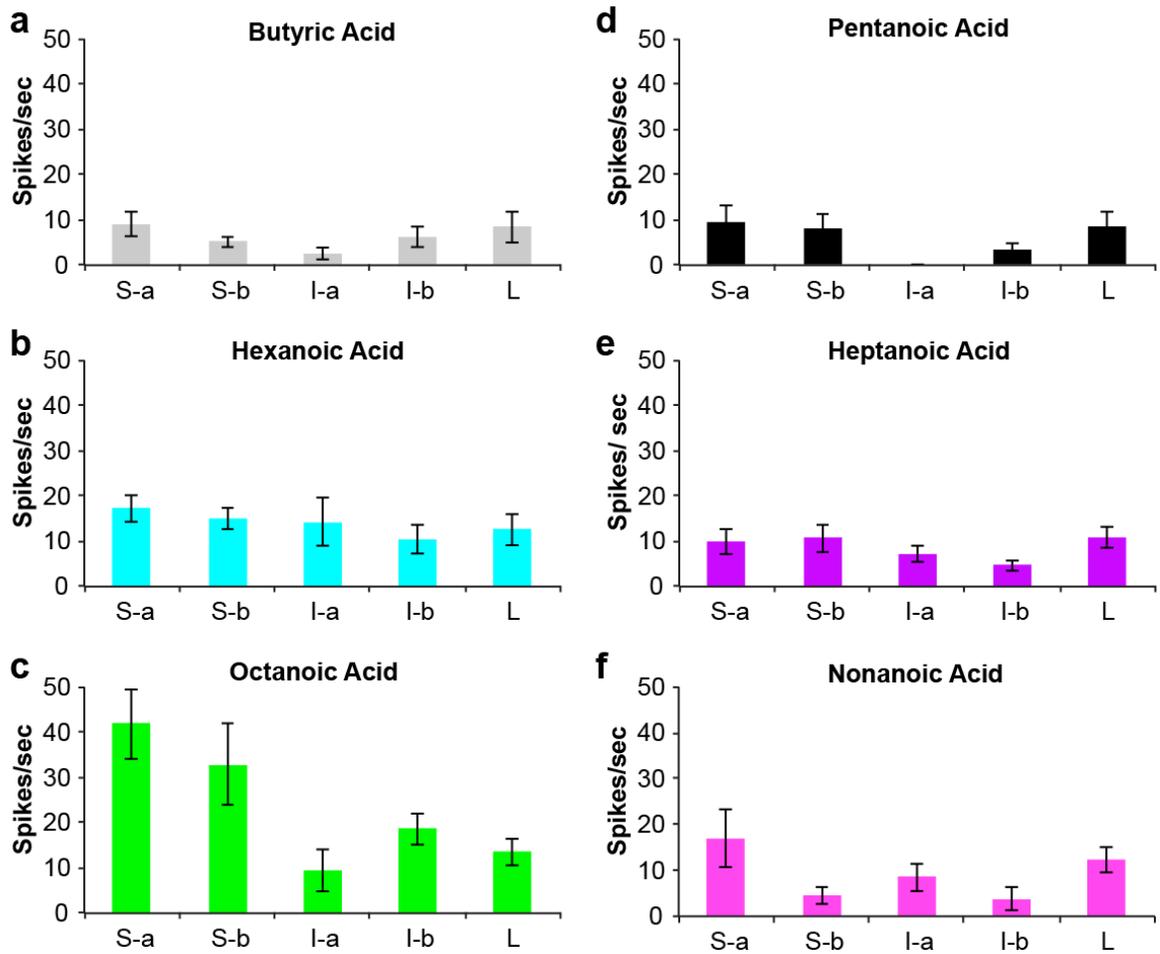


Figure 4.3 Sensillar subclasses show a varied response to morinda acids

a,b,c,d,e,f. Neuronal response of *D. melanogaster* to morinda acids using single sensillum recordings. All five subclasses tested. Recordings obtained from three or more flies. Butyric: n=7 (S-a), 8 (S-b), 4 (I-a), 12 (I-b), 9 (L). Pentanoic: n=7 (S-a), 9 (S-b), 7 (I-a), 6 (I-b), 14 (L). Hexanoic: n=22 (S-a), 15 (S-b), 6 (I-a), 9 (I-b), 10 (L). Heptanoic: n=13 (S-a), 11 (S-b), 12 (I-a), 16 (I-b), 8 (L). Octanoic: n=9 (S-a), 9 (S-b), 4 (I-a), 6 (I-b), 9 (L). Nonanoic: n=5 (S-a), 6 (S-b), 5 (I-a), 5 (I-b), 9 (L). Error bars=s.e.m.

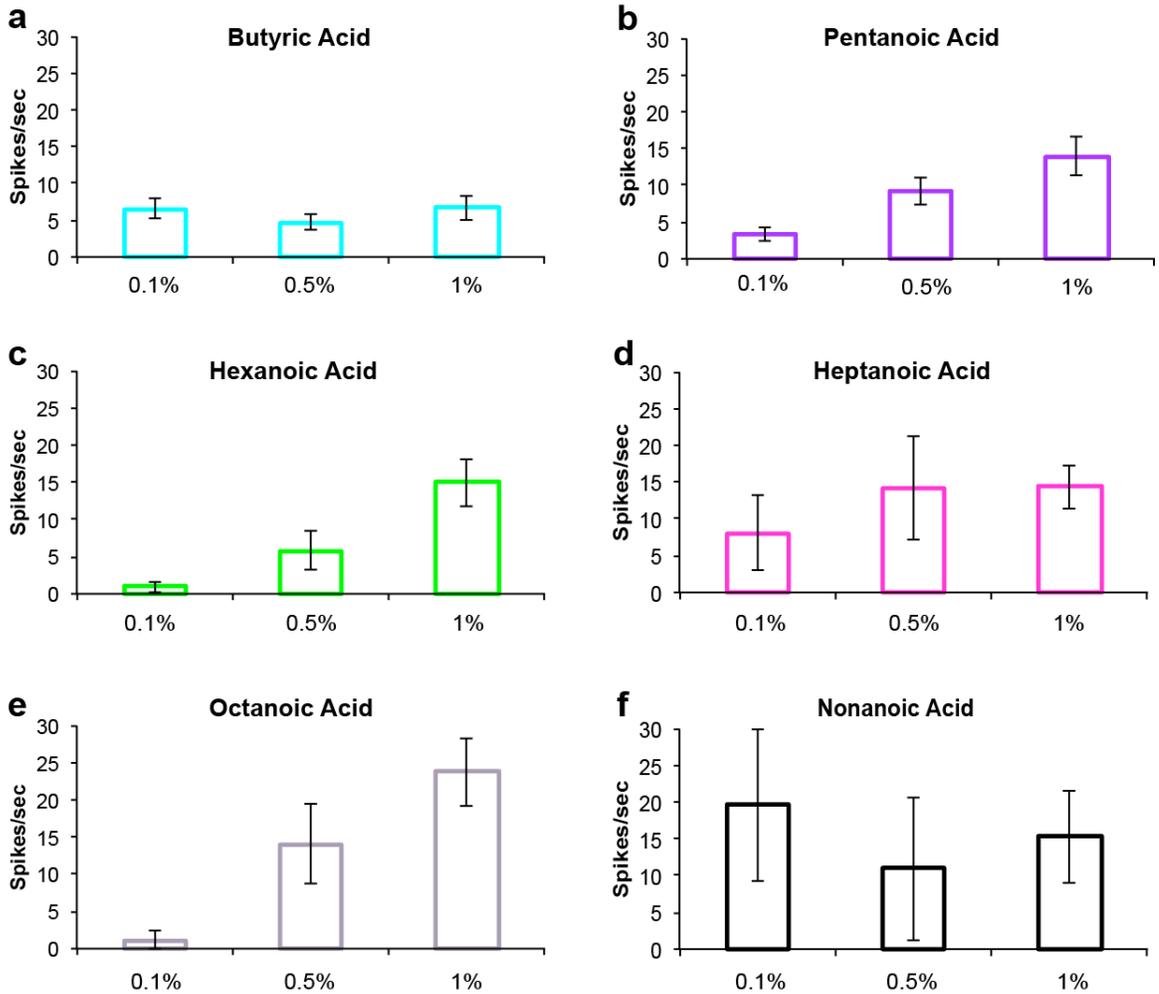


Figure 4.4 *D. melanogaster* flies show dose dependent neuronal responses to some morinda acids

a,b,c,d,e,f. Neuronal response of S-a sensilla to increasing doses of morinda acids.

Butyric: n=9, pentanoic: n=10, hexanoic: n=8,10,18, heptanoic: n=5, octanoic: n=6,7,9, nonanoic: n=5,5,6 (0.1%, 0.5%, 1%). Error bars=s.e.m.

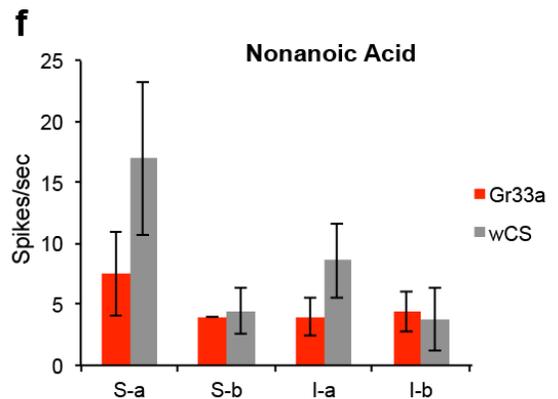
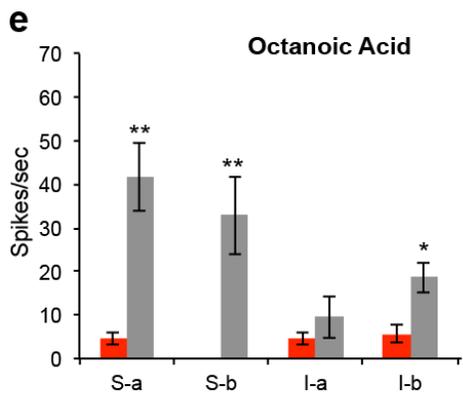
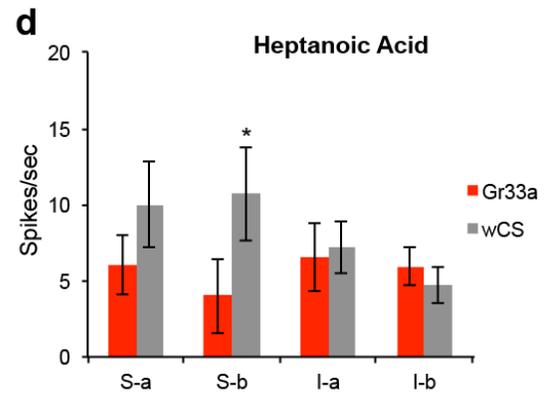
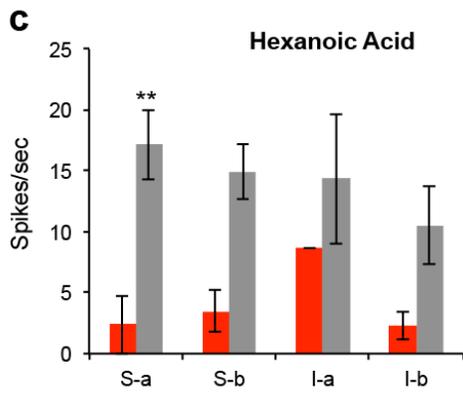
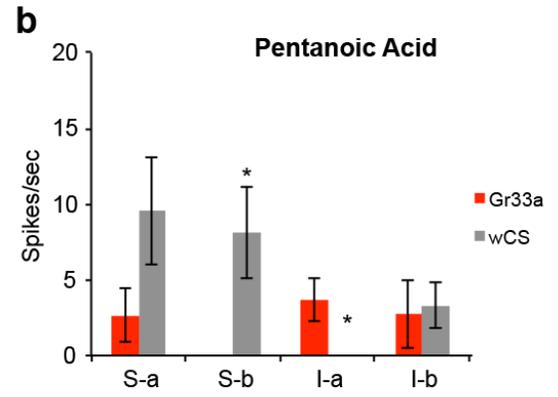
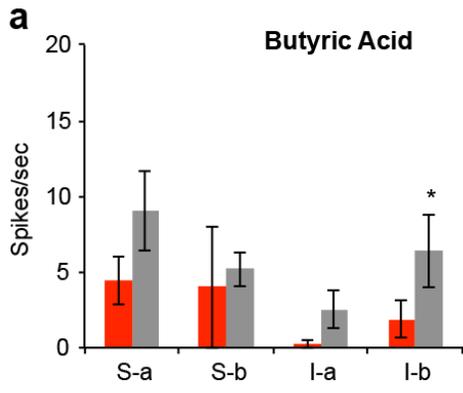


Figure 4.5 Gr33a is partially necessary for the neuronal response to morinda acids

a. Neuronal responses of wild-type and Gr33a mutant sensillar subclasses to butyric acid. S-a: n=7,9 (wCS, Gr33a); S-b: n=8,5 (wCS, Gr33a); I-a: n=4,4 (wCS, Gr33a); I-b: n=12,10 (wCS, Gr33a). * $P < 0.05$, Student's t -test. **b.** Neuronal responses of wild-type and Gr33a mutant sensillar subclasses to pentanoic acid. S-a: n=7,9 (wCS, Gr33a); S-b: n=9,5 (wCS, Gr33a); I-a: n=7,6 (wCS, Gr33a); I-b: n=6,8 (wCS, Gr33a). * $P < 0.05$, Student's t -test. **c.** Neuronal responses of wild-type and Gr33a mutant sensillar subclasses to hexanoic acid. S-a: n=22,5 (wCS, Gr33a); S-b: n=15,7 (wCS, Gr33a); I-a: n=6,5 (wCS, Gr33a); I-b: n=9,9 (wCS, Gr33a). * $P < 0.05$, ** $P < 0.01$, Student's t -test. **d.** Neuronal responses of wild-type and Gr33a mutant sensillar subclasses to heptanoic acid. S-a: n=13,7 (wCS, Gr33a); S-b: n=11,6 (wCS, Gr33a); I-a: n=12,4 (wCS, Gr33a); I-b: n=16,8 (wCS, Gr33a). * $P < 0.05$, Student's t -test. **e.** Neuronal responses of wild-type and Gr33a mutant sensillar subclasses to octanoic acid. S-a: n=9,7 (wCS, Gr33a); S-b: n=9,4 (wCS, Gr33a); I-a: n=4,8 (wCS, Gr33a); I-b: n=6,9 (wCS, Gr33a). * $P < 0.05$, ** $P < 0.01$, Student's t -test. **f.** Neuronal responses of wild-type and Gr33a mutant sensillar subclasses to nonanoic acid. S-a: n=5,6 (wCS, Gr33a); S-b: n=6,3 (wCS, Gr33a); I-a: n=5,6 (wCS, Gr33a); I-b: n=5,6 (wCS, Gr33a). Error bars=s.e.m.

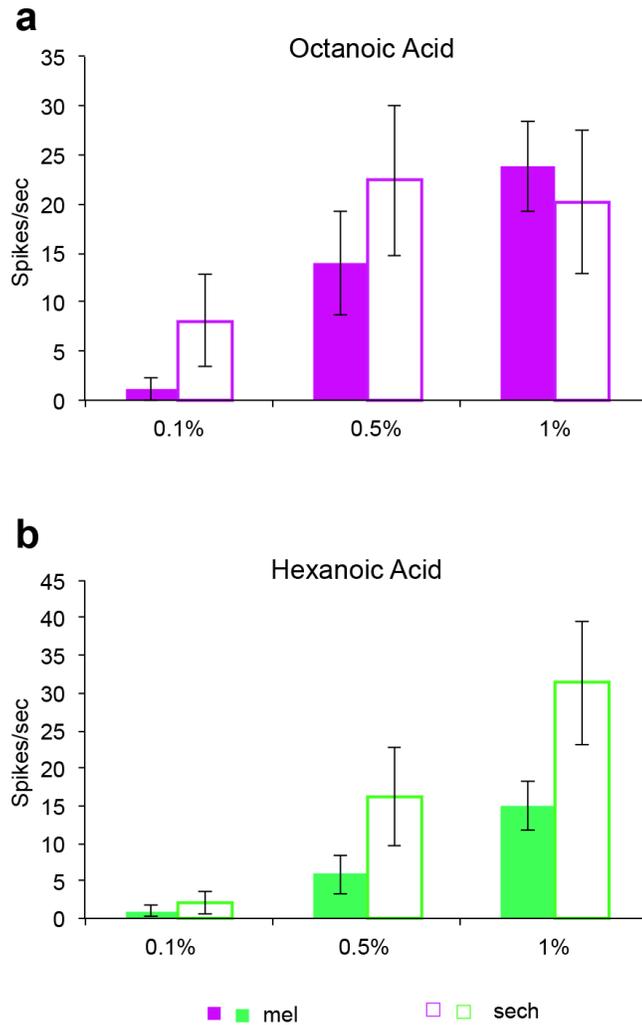


Figure 4.6 *D. sechellia* and *D. melanogaster* show similar bitter neuron responses to hexanoic and octanoic acid

a. Octanoic acid single sensillum recordings in S-a sensilla. 0.1% octanoic: n=6,8 (*D. mel*, *D. sech*); 0.5% octanoic: n=7,5 (*D. mel*, *D. sech*); 1% octanoic: n=9,8 (*D. mel*, *D. sech*). **b.** Hexanoic acid single sensillum recordings in S-a sensilla. 0.1% hexanoic: n=8,5 (*D. mel*, *D. sech*); 0.5% hexanoic: n=10,5 (*D. mel*, *D. sech*); 1% hexanoic: n=8,6 (*D. mel*, *D. sech*). Error bars= s.e.m.

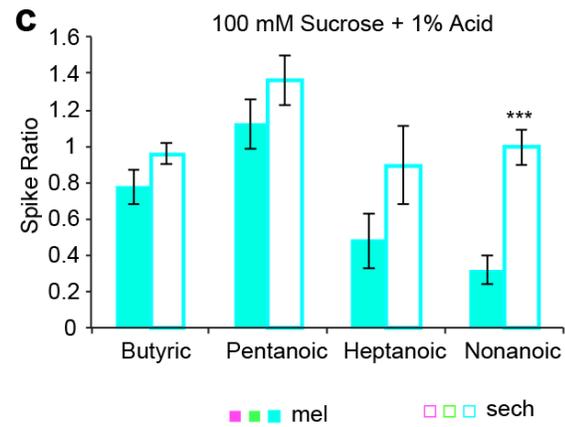
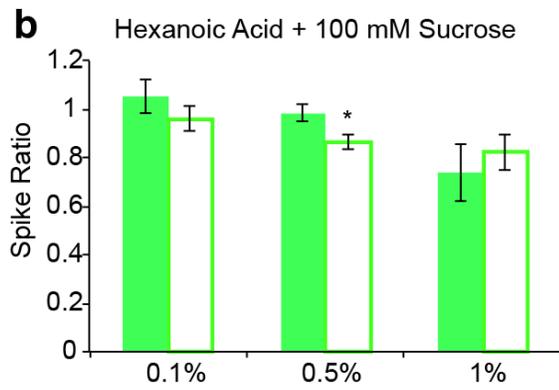
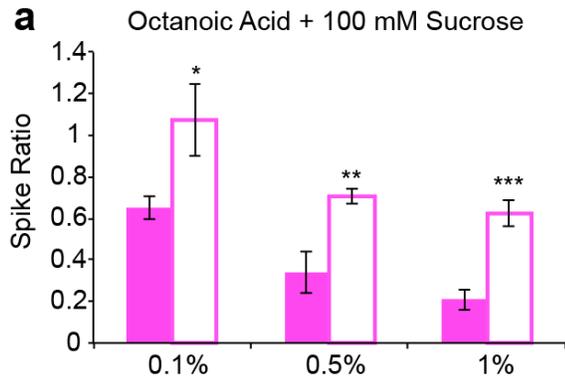


Figure 4.7 Morinda acids evoke different levels of sweet neuron inhibition in *D. sechellia* and *D. melanogaster* flies

a. Single sensillum recordings with 100 mM sucrose and octanoic acid. All recordings obtained from L sensilla. 0.1% octanoic: n=10,8 (*D. mel*, *D. sech*); 0.5% octanoic: n=10,8 (*D. mel*, *D. sech*); 1% octanoic: n=8,9 (*D. mel*, *D. sech*). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test. **b.** Single sensillum recordings with 100 mM sucrose and hexanoic acid. All recordings obtained from L sensilla. 0.1% hexanoic: n=12,8 (*D. mel*, *D. sech*); 0.5% hexanoic: n=12,7 (*D. mel*, *D. sech*); 1% hexanoic: n=10,8 (*D. mel*, *D. sech*). * $P < 0.05$, Student's *t*-test. **c.** Normalized neuronal response of *D. melanogaster* and *D. sechellia* to 100 mM sucrose with morinda acids. L sensilla used for recordings. Butyric: n=9,9 (*D. mel*, *D. sech*); pentanoic: n=11,10 (*D. mel*, *D. sech*); heptanoic: n=11,8 (*D. mel*, *D. sech*); nonanoic: n=10,10 (*D. mel*, *D. sech*). *** $P < 0.001$, Student's *t*-test. Error bars=s.e.m.

Chapter 5: Discussion

In this study examined the responses of two *Drosophila* species to two groups of carboxylic acids, alpha-hydroxy acids and short chain fatty acids. First, we found that *Drosophila melanogaster* are averse to fruit alpha-hydroxy acids and that this aversion is mediated by two independent mechanisms: the activation of bitter neurons and the inhibition of sweet neurons. In our experiments we focused on the inhibition of the sweet neuron by acids. Using buffered solutions and anion salts we determined that acid inhibition of the sweet neuron is pH-dependent and not based on the anion structure (Figure 3.4). We also found that we could overcome this pH-dependent inhibition by increasing the sugar content in the mixture (Figure 3.3).

When evaluating the behavioral and cellular response to short chain fatty acids, we discovered that they are also detected by dual pathways. In *Drosophila melanogaster* flies we observed aversion to feeding on short chain fatty acids and determined that, as with alpha-hydroxy acids, this behavior was mediated by activation of the bitter neuron and inhibition of the sweet neuron (Figure 4.3 and Figure 4.7). Interestingly, when testing *Drosophila sechellia* flies, which reside on a morinda fruit that contains many of these short chain fatty acids, we found that they were drastically less averse to feeding on these acids (Figure 4.2). This reduced aversion was at least partially explained by a reduction in sweet neuron inhibition for two of the acids tested (Figure 4.7).

Further studies examining the molecular changes underlying this phenomenon still need to be done. We have yet to determine the exact mechanism for acid inhibition of the sweet neuron for either alpha-hydroxy acids or short chain fatty acids. Thus far, we

know that the inhibition by alpha-hydroxy acids is pH-dependent. It remains to be seen, however, how the hydrogen ion inhibits the sweet neuron from firing at its normal frequency. Do the acids directly inhibit the receptor by preventing it from binding to its ligand? Or does it inhibit the neuron from firing at a normal frequency by affecting the channels necessary for producing action potentials?

There are three possible ways that the hydrogen ion is preventing the neuron from firing to sucrose. The first possibility is that the hydrogen ion is competing with sucrose for binding to its receptor, Gr64a. This competition would reduce the ability of sucrose to bind and activate Gr64a and thus the firing rate would be reduced. The drastic differences in structure between the sugars and the hydrogen ion, though, makes this possibility somewhat unlikely.

The second possibility is that the hydrogen ion is allosterically inhibiting the Gr64a receptor from binding to sucrose by changing the conformation of Gr64a. When the pH is been changed by the alpha-hydroxy acids, it is possible that the conformation of the protein could be affected. This type of change, however, might not reverse in time to give a recovery sucrose response that is similar to the initial sucrose response. As shown in Chapter 3, for most of the acids the recovery sucrose was close to the response for the initial sucrose. This indicates that the mechanism by which the acids are inhibiting the sweet neuron is rapid and can reverse in a matter of minutes.

Given this time constraint, the third possibility then seems the most likely. The third possibility is that the hydrogen ion is effecting one of the numerous ion channels that are needed for eliciting action potentials, such as the sodium channels or the

potassium channels, and that this in turn is preventing the neuron from firing with the same strength and duration. We hypothesize that a sodium channel is the most likely receptor. Experiments with salt-acid solutions caused the neuron to stop firing to salt after the acid solution was removed (Appendix Figure 4). Even after waiting for 10-20 minutes the salt response was not recovered. This effect was more drastic at higher concentrations of acid. These results suggest that one of the sodium channels that is responsible for the detection of low salt solutions is blocked irreversibly by acids. We do not predict that the low salt receptor, Ir76b, is inhibited by these acids because we saw inhibition in neurons where Ir76b is not present, those neurons being the sweet neurons (Zhang 2013). Instead we hypothesize that another sodium channel that is common to both neuron types is inhibited by alpha-hydroxy acids.

More research also needs to be done to examine the molecular changes underlying the differences in short chain fatty acid inhibition of the sweet neuron between *Drosophila sechellia* and *Drosophila melanogaster*. Previous research examining taste differences between the two species has largely focused on bitter chemosensory receptors and odorants binding proteins (Dworkin and Jones 2009, Matsuo 2007, Harada 2008, McBride and Arguello 2007). Odorant binding proteins, which have recently been shown to be necessary for inhibition of the sweet neuron by bitter compounds, are obvious candidates for *D. sechellia* adaptation to morinda acids (Jeong 2013). Dworkin and Jones recently compiled a list of chemosensory proteins, including odorant binding proteins, which differ in expression between *D. sechellia* and *D. simulans* (another generalist in the melanogaster subgroup). From this list there are numerous candidates including Obp50e,

Obp56d, Obp56e, Obp57d/e and Obp83cd. All of these candidates are expressed in taste tissue such as the tarsi and labellum and have different levels of expression in *Drosophila sechellia* compared to *Drosophila simulans*. We did examine one odorant binding protein, Obp49a, for involvement in sweet neuron inhibition. When we tested Obp49a mutants for their acid inhibition response to both alpha-hydroxy acids (Figure 3.6) and short chain fatty acids (data not shown) we found that the mutants did not differ from wildtype in their sweet neuron inhibition. We cannot rule out the involvement of other Obps, though, and thus tests with other Obp mutants may elucidate the mechanism for the differences in sweet neuron inhibition.

Another family of candidate proteins are the gustatory receptors (Grs). We found in Chapter 4 that Gr33a, a bitter co-receptor, is necessary for the response to some of the morinda acids (Figure 4.5). Work from others has shown that many bitter Grs are lost or rendered non-functional in the *D. sechellia* taste system, thus we had originally hypothesized that *D. sechellia* adaptation to morinda acids could be based on a change in the bitter receptor repertoire (Mcbride and Arguello 2007). However, upon closer examination we found that the bitter neuron activation by the morinda acids is not different between the *D. sechellia* flies and *D. melanogaster* flies (Figure 4.6).

Sweet Grs are also possible candidates for this taste adaptation. Given that the differences we saw between the two species was partially based on the inhibition of the sweet neuron, it would stand to reason that the sweet Grs mediating this response could be involved. The main Gr involved in sucrose detection is Gr64a (Dahanukar 2007). Comparison of the Gr64a amino acid sequences of *D. sechellia* and *D. melanogaster*

revealed ten amino acid changes in the *D. sechellia* sequence. Four of these amino acid changes are specific to only *D. sechellia* when compared to other members of the melanogaster subgroup. Thus we hypothesize that the differences in sweet neuron inhibition may be due to changes in the Gr64a protein in *D. sechellia*. These changes could prevent the morinda acids from inhibiting the binding of the receptor to sucrose. In order to test this hypothesis, we plan to express the *D. sechellia* Gr64a receptor in the sweet neurons of *D. melanogaster* flies and test if the level of inhibition is similar to that seen in the endogenous *D. sechellia* system.

Alternatively, the inhibition differences between the two species could be due to another protein expressed in the sweet taste neurons. To find these protein differences we plan to analyze the labellar transcriptomes of *D. melanogaster* and *D. sechellia*. This will allow us to see what protein changes may have occurred in this taste organ when *D. sechellia* adapted. This method allows us to perform an unbiased screen for potential molecular candidates and could reveal proteins that have not been previously implicated in taste.

Taste detection of mixed or complex stimuli is an under-studied topic in taste research. Most molecular studies are done with pure stimuli in order to identify the cells that they activate and their behavioral valence. Food sources, however, are usually complex stimuli. Understanding how these different types of stimuli interact is vital to understanding how the taste system reacts in a natural environment. By testing acids mixed with sucrose we were able to discover a new pathway for acid detection that would have been overlooked with testing acids alone. Given that acids are often present in

conjunction with sugars in numerous food sources it is necessary to understand how these two tastes are incorporated (Handbook of Fruits and Fruit Processing).

In this work we showed that acids are detected through two separate pathways. This is one of the few instances in the taste system where a single stimulus is detected by two types of cells. High salt detection in mammals also occurs through two cells, the bitter cell and the sour cell (Oka 2013). Likewise, bitter compounds in the *Drosophila melanogaster* taste system have been shown to act on bitter and sweet cells (Jeong 2013). Most of these taste modalities that are detected by multiple cells are aversive tastes. Detection by more than one type of cell allows for redundancy in the taste system when detecting aversive stimuli. This redundancy is more important for aversive tastes than attractive tastes because it prevents ingestion of harmful chemicals that could cause damage.

Until recently the theory in the taste field was that one cell detected one taste, or, at least, that one cell detected attractive tastants and one cell detected aversive tastants. This theory was termed the “labeled-line” model (Yarmolinsky 2009). In this model different tastes are spatially segregated at the periphery and only come together in the brain. Our findings show that tastes can overlap in the periphery and that a single cell can detect both attractive and aversive stimuli. This dual role allows a single cell to gauge the presence of multiple stimuli. Having a cell with this capacity is useful in a system with a limited receptor repertoire.

This work then lays the foundation for further molecular investigations into acid inhibition of the sweet neuron. To our knowledge this is the first time that differences in

inhibitory properties of a taste stimulus have been proposed to underlie adaptive changes in host preference. By better understanding this inhibitory mechanism we hope to elucidate the molecular changes that contribute to the adaptive behavioral changes in specialist insects.

Materials and Methods

Fly stocks: Flies were raised on standard cornmeal–dextrose–agar diet at 25 °C. Wild-type flies were w¹¹¹⁸. Mutant fly stocks were obtained from the Bloomington Stock Center: Ir76b[05] (BL9824), Ir21a[EP526] (BL17177), Ir64a[MB05283] (BL24610), Gr33a¹ (BL31427), Ir25a[2] (BL41737), Obp49a¹(BL55033) and pain[GAL4] (BL27894), Pkd2[MB06703] (BL25244), and Pkd2¹ (BL24495). UAS-Kir2.1 flies were kindly provided by K Scott (University of California, Berkeley).

Solutions: Chemicals were obtained from Sigma Aldrich at the highest purity available. Morinda juice was purchased from Juice it Up® as 100% pure juice. All solutions were made daily from stock solutions for same day use.

Behavior: For proboscis extension response assays, 5–10 day old male flies were starved for 24 h in vials with water-soaked Kimwipes. Individual flies were then trapped in cut 20-ml pipette tips such that their heads were exposed. Flies were first allowed to drink water to satiation; any flies that did not cease drinking were discarded and only those that subsequently responded to 100 mM sucrose were selected for further experimentation. Acid–sucrose mixtures were tested from lowest to highest acid concentration with water presented between stimuli. Scores were assigned as follows: 1- proboscis extension with drinking, 0.5- partial extension or extension with immediate retraction and 0- no extension. One hundred millimolar sucrose was also tested at the end of the acid series and only flies that responded were used for data analysis. Mutant experiments were done

with wildtype and mutant flies tested simultaneously. Responses were not pooled for wildtype flies across different mutant experiments. Each wildtype set was specific to the mutant experiment.

For feeding preference assays, adult flies aged 3–7 days were sorted and housed in fresh food vials for 1–2 days; one vial was prepared for every trial and contained ten males and ten females. Flies were starved as above. Feeding assay plates were prepared a few hours before the experiment and dotted with 9 spots of 10 ml of each stimulus solution in 0.75% agarose. Tastants were mixed in with melted agarose and dispensed immediately. Flies were anesthetized momentarily with CO₂, transferred to feeding plates and allowed to feed for 2 h in a dark, humidified chamber, after which they were frozen and scored within 48 h for the color of their abdomens. For experiments with antennectomized flies, antennae were removed surgically and flies were allowed to recover for 48 h before they were starved and tested as above. Flies found dead in the agarose were not scored. Trials in which fewer than 25% flies had participated in feeding were discarded.

Preference index was calculated as:

$$\frac{\# \text{pink} - \# \text{blue}}{\# \text{pink} + \# \text{blue} + \# \text{purple}}$$

Participation was calculated as:

$$\frac{\# \text{pink} + \# \text{blue} + \# \text{purple}}{\# \text{pink} + \# \text{blue} + \# \text{purple} + \# \text{uncolored}}$$

indicates the number of flies with indicated abdomen color.

Electrophysiology: Single-sensillum recordings with acid tastants were performed using the tip-recording method with 30 mM tricholine citrate as the electrolyte. Recordings were obtained from male flies aged 3–10 days. Neuronal responses were quantified by doubling the number of spikes in the 0–500 ms window upon contact with the stimulus. For sucrose-acid mixtures, sensilla were first tested with 100 mM sucrose and only those that had initial responses ≥ 50 spikes per second (L-type) or ≥ 30 spikes per second (I- and S-type) were used. Response to 100 mM sucrose was tested in between each stimulus in the acid–mixture series, which was presented from lowest to highest acid concentration. A sucrose recording was also taken at the end, and only those sensilla which retained a response were used for data analysis.

Spike ratio was calculated as:

$$\text{spike ratio} = \frac{\text{spikes for sucrose-acid mixture}}{\text{spikes/sec of preceding sucrose}}$$

Buffered solution recordings were performed with 100 mM sucrose applied between the buffered and unbuffered solutions. Anion salt recordings were also performed with 100mM sucrose interspersed. Same wildtype recordings were used for comparison with Ir25a and Ir64a mutants.

% Inhibition was calculated as:

$$\% \text{ Inhibition} = \frac{\text{spikes for sucrose-acid mixture}}{\text{spikes/sec of preceding sucrose}} \times 100$$

D. melanogaster and *D. sechellia* recordings were performed on the same days.

Recordings with acid alone were performed only on sensilla that responded to 5 mM lobeline or 1 mM caffeine control solutions. Sensillar subclasses were determined based

labellar position of the sensilla. Acid dose response recordings were followed by lobeline or caffeine application at the end. Only sensilla that responded to the final lobeline or caffeine stimulus were used. 100 mM sucrose was used as the initial test for Gr33a mutant sensilla.

pH recordings: pH recordings of sucrose-acid solutions were obtained using a pH meter. Each sucrose-acid mixture was tested at least three times. The pH meter was standardized before each recording to ensure accuracy. Ripe and fermented fruits were measured as juices with pulp. Prior to recordings, fruits were peeled and blended by a handheld blender until homogenized. Fermented fruits solutions were prepared from the blended fruit solutions. Yeast was added to the homogenized fruit solutions and allowed to ferment for 7 days. After this process, the fermented solution was stirred to homogenize the solution and the pH was measured. Ripe fruit pH recordings were performed three times. Fermented fruit pH recordings were performed 1-5 times.

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Appendix

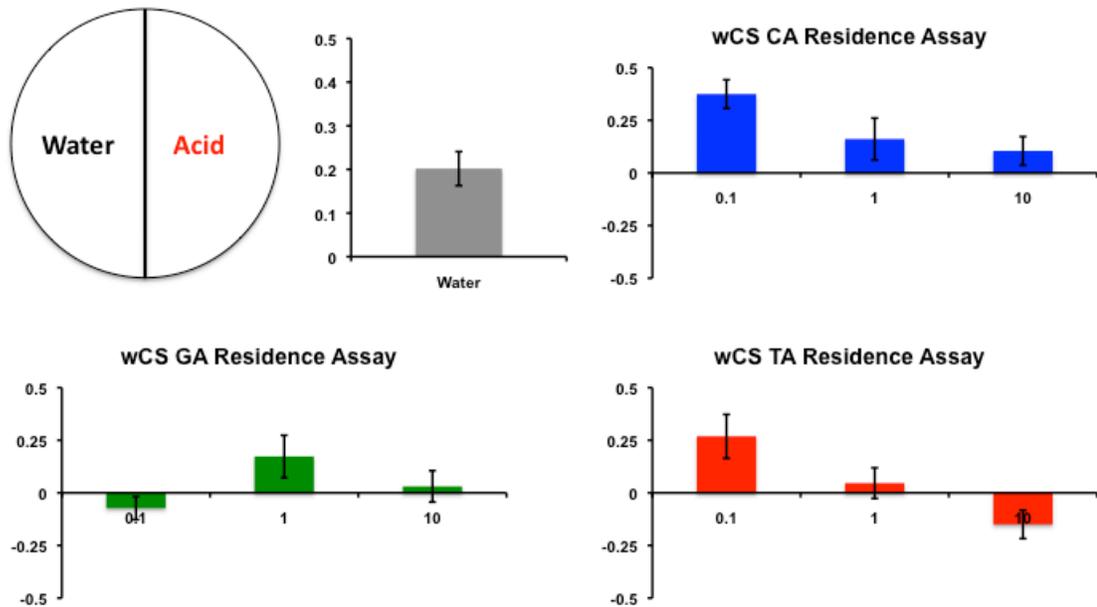
Acid residence assays

Both behavior assays used in this study to examine the response to acids were done in the context of sucrose. Thus, we wanted to determine how *Drosophila melanogaster* would respond to acids alone with no sugar present. We adapted an assay, termed the residence assay, which examines fly preference for residing on different stimuli (Marella 2006). This assay involves contact assay by allowing the flies to walk around and possibly taste the stimuli before deciding where to stay. Presumably the flies will choose to reside on a stimulus they find appealing and avoid a stimulus that they deem aversive.

The assay is set up by placing a divider through the center of a petri dish (with a tight-fitting lid) and filling the two halves of the petri dish with different stimuli mixed with agar. Once the agar solidifies, the divider is removed and adult flies aged 3-10 days are placed on the plate. The petri dish is then covered with its tight-fitting lid and placed on a surface for imaging. The flies are allowed to walk about on the plate while they are photographed every minute for 15 minutes. A diagram was made indicating which side of the plate contained which stimulus, so that conclusions could be drawn.

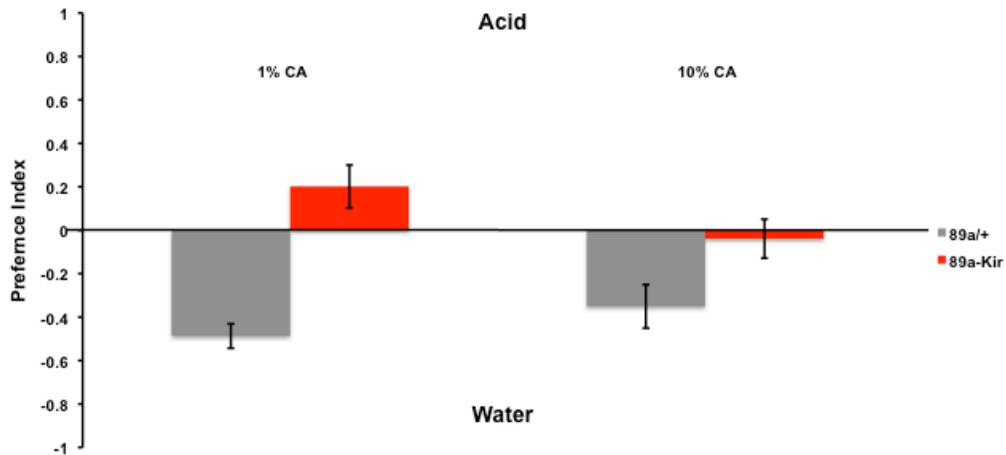
For simplicity, only photos from every 5 minutes were counted to determine the number of flies on each side of the plate and the time course of this behavior. The final photo at 15 minutes was used for comparison across multiple concentrations and multiple genotypes. In our assay we placed an acid agar mixture on one side and plain agar on the other side (Appendix Figure 1.1a). As a control we placed plain agar on both sides

(“water”) to determine if there was any side bias. As with the feeding preference assay, a value of 1 indicated preference for the acid side, a value of 0 indicated no preference and a value of -1 indicated an avoidance of the acid. We tested wildtype flies as well as bitter silenced flies and their controls in this assay. The wildtype flies showed some movement away from the acid as the concentration was increased but too few n’s were performed to make a definitive conclusion. Likewise for the bitter-silenced flies (89a-Kir) the controls showed stronger avoidance of the acid side than the bitter-silenced flies, but too few n’s were performed to draw a conclusion.



Appendix Figure 1: Residence assay with fruit carboxylic acids

Positive values indicate preference for acid side and negative values indicate preference for water side. Water: n=7; citric: n= 3-6; glycolic: n=2-6; tartaric n=3-6. Error bars = s.e.m.

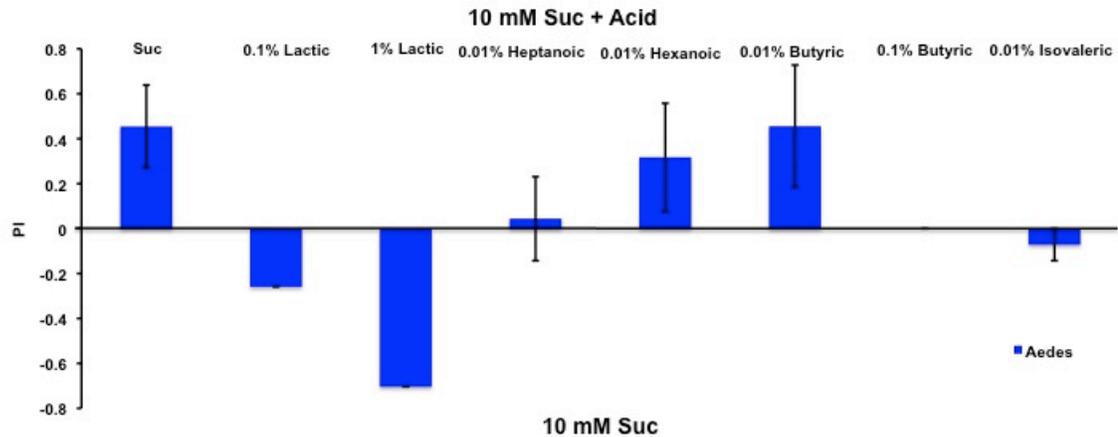


Appendix Figure 2: Response of bitter-silenced flies and controls to citric acid in residence assay
 n=4, 2 (89a/+, 89a-Kir)

Mosquito feeding preference assay

In order to test the taste response of *Aedes aegypti* mosquitoes to acids found in human sweat and on human skin, we decided to perform a two-choice feeding preference assay. This assay was adapted from () and was combined with the set-up for our usual *Drosophila* feeding preference assay. To test the mosquitoes, we first sorted them into cages at similar densities and then water starved for () hours prior to testing. The solutions were made with 10mM sucrose plus either blue (indigo carmine) or pink (sulforhodamine) dye. In the pink solution, acid was added at a calculated concentration. These solutions were then placed into the feeding receptacles for the mosquitoes and put inside the cages. The mosquitoes were allowed to feed for two hours, before being frozen in a -20 deg Celsius freeze. Once the mosquitoes were killed, the carcasses were removed from the cages and the colors of the abdomens were scored. The number of mosquitoes in

each gender were also determined at this stage. In this assay, the lactic acid phenotype seems promising but only one trial was performed with this compound so no conclusions can be made.



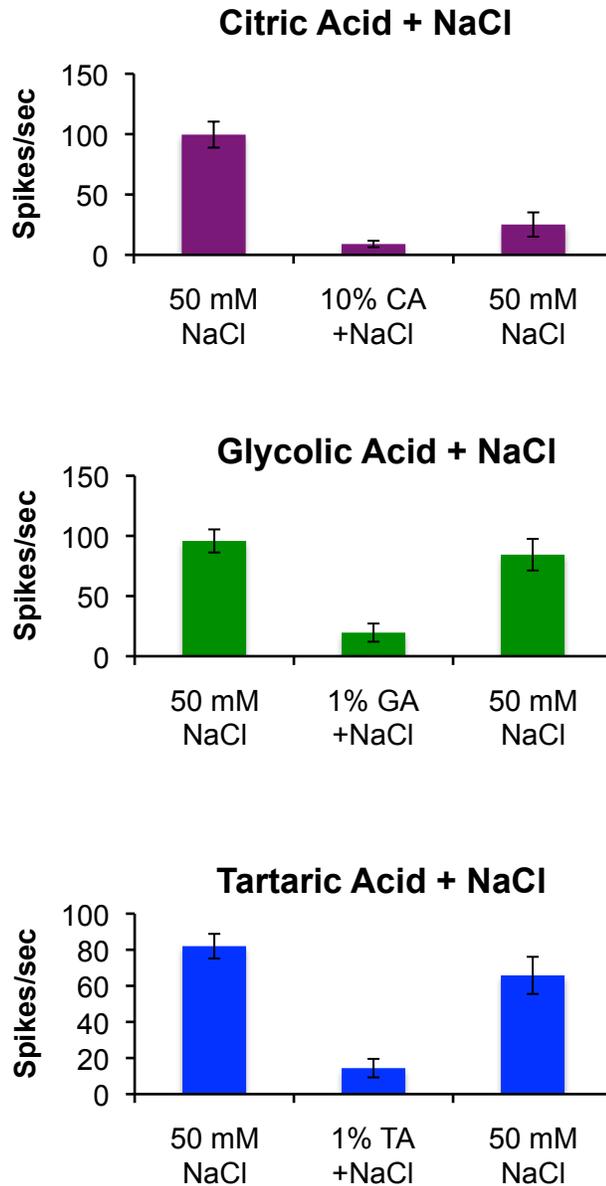
Appendix Figure 3: Feeding preference assays with *Aedes aegypti*

Sucrose: n=4; lactic: n=1; heptanoic: n=2; hexanoic: n= 3; butyric: n=1-3; isovaleric: n=3. Error bars = s.e.m.

Acid-salt recordings

Having determined that organic acids are able to inhibit the sweet neuron response to sucrose, we wanted to find if acids are able to inhibit other attractive taste modalities. Thus we tested solutions of 50 mM NaCl with three of the fruit carboxylic acids. Tartaric acid and glycolic acid were mixed with NaCl at concentrations of 1%, and citric acid was added at a concentration of 10%. For these recordings, the 50 mM NaCl was presented first and then followed by the salt-acid solution. Finally, 50 mM NaCl was presented again in order to check for neuronal damage. The 10% citric acid solution appeared to have an effect on the salt neuron because it prevented the neuron from firing normally to NaCl after acid application. The 1% solutions did not cause this same issue. This

permanent neuronal inhibition was very different from what we observed in sucrose-acid recordings. We hypothesize that high concentrations of acid damage the salt neuron and thus these recordings were not included in the main text.



Appendix Figure 4: Acids inhibit the response to low salt
Citric: n= 10, 10, 7 (initial salt, 10% citric acid, final salt); glycolic: n=10; tartaric: n=10. Error bars = s.e.m.