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A Study of the Effects of Diet, Sex, and Mating Status on Feeding and Oviposition Preference

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Chapter 1 Introduction

Choice is a complex process, as many factors are involved in determining what food source to eat, who to mate with, and even where to raise offspring. Many internal factors, such as nutritional state, development, and physiology affect the choices that one makes. External factors also play a role in choice; these factors are more limiting. How does one choose between different alternatives? What are the mechanisms involved in those choices? In terms of nutritional decisions, which receptors and neurons are involved in the decision making process? This study takes these questions and attempts to answer them using *Drosophila melanogaster*, the fruit fly, as a model.

Seeing as taste preferences can change based on sex, mating status, and current dietary state, *D. melanogaster* is an excellent model system to use to explore the plasticity of choice. There are a variety of genetic tools and behavioral assays available for researchers to investigate choice in flies. Using these tools, the neural system of the fruit fly can be dissected relatively easily to see which receptors, neurons, pathways, and brain regions are responsible for choice in a variety of areas. Monitoring and modulation of gene expression in flies in different states can be used to easily see how choice is affected. Due to these manipulations, the process of choice, as well as its plasticity is elucidated easily.

D. melanogaster is used as a model system for a variety of processes that are conserved from yeast to flies to mice to humans. Flies can be used in this way because of the conservation of the proteins involved in these pathways. Flies have simpler systems compared to mammals; study of *Drosophila* makes the study of higher systems easier where it might be too complex to find or locate a starting point.

Gustatory Receptors

D. melanogaster can taste sugars, bitters, salts, water, acids, amino acids, amines, and pheromones. *D. melanogaster* expresses a family of 68 Gustatory receptors (Grs) that are multiple transmembrane domain proteins to detect sweet, and bitter substances, as well as pheromones (Clyne, 2000; Dunipace et al., 2001a; Scott et al., 2001; Robertson et al., 2003; Bray and Amrein, 2003; Wang and Anderson, 2010). How gustatory receptors affect choice is the focus of this thesis.

Many of the 68 Gr functions are unknown. The sweet clade of gustatory receptors consists of Gr5a, Gr61a, and Gr64a-f. Gr5a has been shown to be the receptor for trehalose (Dahanukar et al., 2001; Chyb et al., 2003). It also senses m-α-glucoside, glucose, and melezitose (Dahanukar et al., 2007a). Gr64a, on the other hand, has been shown to be necessary for responses to sucrose, maltose, turanose, maltitol, palatinose, maltotriose, stachyose, raffinose and leucrose (Dahanukar et al., 2007a; Jiao et al., 2007)., Gr64e responds to glycerol (Wisotsky et al., 2011), while Gr43a has recently been shown to be an internal fructose receptor (Sato et al., 2011; Miyamoto et al., 2012). Ligands for Gr64b, Gr64c, and Gr64d are currently unknown. Gr64f is thought to be a co-receptor for Gr5a and Gr64a (Jiao et al., 2008).

Work has been done to see how a taste-blind fly would survive. Interestingly, Slone has shown that Gr5a, Gr61a, and Gr64a-f are necessary for proper development; when these receptors are missing, the embryo arrests (Slone, 2009).

Receptors associated with detection of bitter compounds include Gr93a, Gr66a, Gr33a, and Gr32a (Moon et al., 2006, 2009; Lee et al., 2009). Gr33a is broadly required

for responses to a variety of bitter compounds (Jiao et al., 2007; Lee et al., 2009; Moon et al., 2006, 2009). All four bitter Grs are needed to provoke a response to caffeine (Moon et al., 2006; Lee et al., 2009). Carbonated water is detected through E409 neurons (Fischler et al., 2007), while ambient CO_2 is detected by Gr21a and Gr63a, which are located in olfactory organs (Kwon et al., 2007). Other aversive stimuli, such as acids, are also detected via the olfactory system through the ionotropic receptor 64a (IR64a), while amines are detected by IR92a (Ai et al., 2010; Min et al., 2013a). Carboxylic acids, such as vinegar (acetic acid) can be detected by bitter and sweet sensing Gr-neurons as well (Charlu et al., 2013).

Grs are located in the membranes of Gustatory Receptor Neurons (GRNs) (Figure 1). These GRNs are housed in external taste sensilla located on the flies' proboscis, legs, wing margins, and females' ovipositor. They are also located internally in the pharynx and labellum. A mechanosensory cell and support cells surround the GRNs in each sensillum. Each GRN in a sensillum is tuned to a different taste modality, sweet, bitter, salt, or water (Thorne et al., 2004; Hiroi et al., 2004). The majority of GRNs send their projections to the subesophageal ganglion complex (SOG), and some tarsal GRNs send their projections to the thoracic-abdominal ganglion. Sweet and bitter representation remains separate, as sweet and bittern sensing GRNs send their projections to different regions of the SOG (Wang et al., 2004; Dahanukar et al., 2007) In addition to being separated by function, GRN projections are also segregated by the location of the gustatory organ. Anterior projections to the SOG belong to GRNs from the internal mouth parts, medial projections are from the proboscis, and most posterior projections

from the tarsi (Wang et al., 2004; Dahanukar et al., 2007). As for secondary order neurons, these remain to be elucidated by the field.

In order to send a signal to the SOG, GRNs must have some way of transducing their message. There is some evidence that G-protein signaling is invovled in taste transduction (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001; Robertson et al., 2003). G-protein coupled receptors (GPCRs) interact with G-proteins, a family of heterotrimeric guanine nucleotide-binding proteins composed of α, β, and γ subunits, in order to transduce signals downstream (Neves et al., 2002). Later, it was found that Gγ1 is needed to detect sucrose, glucose, trehalose, and fructose in the labellum, as well as the possibility of a G-protein independent sugar detection pathway existing (Ishimoto et al., 2005). This study also identified the Gα and Gβ subunits present in the labellum: Gα73B, Gβ13F, and Gβ5 (Ishimoto et al., 2005). Isolation of the labellar subunits made it easier to identify the subunits involved in sugar perception (Ishimoto et al., 2005). These findings could explain how a fly chooses between stimuli. Perhaps different protein domains interact in each GRN to signal different tastes.

Most recently, researchers have found that Gαs is involved in the perception of Gr5a sugar-detection in the labellum (Ueno and Kidokoro, 2008). This suggests the cAMP pathway is transducing the Gr5a-neuronal signal from the proboscis to the SOG (Ueno et al., 2006). Two years later, Ueno et al. also discovered that the Adenylyl Cyclase gene AC78C is co-localized in GRNs with Gαs (Ueno and Kidokoro, 2008a). Both AC78C and Gαs are involved in the cAMP pathway for detection of trehalose and

sucrose at low concentrations (2008). Fatty acid taste is also attractive and utilizes the PLC pathway in some way (Masek and Keene, 2013).

There is much similarity between invertebrate and vertebrate taste systems, from organization at the periphery to the behavioral output from stimuli. Like *D. melanogaster*, mammals sense a core set of taste modalities: sweet, sour, salty, umami, and bitters. T2Rs sense bitter stimuli, T1Rs function as sweet and umami receptors, and ENaC channels respond to salty stimuli (Chandrashekar et al., 2000; Nelson et al., 2001, 2002; Zhao et al., 2003; Yarmolinsky et al., 2009). Sweet, bitter, and umami sensing TRs signal through TRPM5, which is activated by a GPCR (Zhang et al., 2003). Other receptors are responsible for sour and salty tastes. Mammalian taste receptors that confer responses to each modality are contained within a single taste receptor cell (TRC) (Zhang et al., 2003; Huang et al., 2006). These TRCs are expressed in groups that reside in papillae on the tongue, and the soft palette of the mouth, much like the GRNs that are housed in taste sensilla (Yarmolinsky et al., 2009). Stimulation of these cells through edible substances signals either an attractive or an aversive response to the central nervous system.

There are two main theories for how taste is coded in organisms, the first being label-lined, and the second being across-fiber. The label-lined theory is that a single taste cell responds to and confers a stereotyped behavior, while the across-fiber theory states that temporal coding and interaction among groups of taste cells determine the response and behavior of the organism. Currently, there is evidence for both theories, but the label-line theory will be discussed here. Much evidence has come about to support the label-

lined theory, in both mammals and *Drosophila*. In 2003, Zhao et. al. showed that expression of an opioid receptor in the T1R2 expressing cells of mice led to attractive responses to spiradoline, whereas the control mice did not respond (Zhao et al., 2003). Mueller et. al. went on to show that when the same receptor was expressed in mouse bitter cells, an aversive response was present; in addition they drove the expression of a human bitter receptor in the mouse's bitter and sweet cells, and saw an aversive and attractive response respectively (Mueller et al., 2005). The following year, another group discovered a candidate sour receptor, PKD2L1 (Huang et al., 2006). When they ablated the cells that contained this receptor, the other TRC containing sweet, bitter, and umami receptors still responded to their respective stimuli and did not respond to the sour stimuli (Huang et al., 2006). These three groups have showed that the taste neuron itself is responsible for conferring an attractive or aversive behavior in mammals, regardless of the receptors contained within the neuron.

Many researchers in the *Drosophila* field have also shown this to be the case. Neurons expressing Gr5a have been shown to induce positive responses to trehalose, while those expressing Gr66a respond negatively to bitters (Chyb et al., 2003; Wang et al., 2004; Thorne et al., 2004). These sweet and bitter neurons do not express the opposing receptor of the other neurons (Thorne et al., 2004; Wang et al., 2004). Another research group expressed a mammalian receptor for capsaicin in Gr5a positive neurons, and an attractive response to capsaicin was seen, whereas expression of this receptor in Gr66a positive neurons led to an aversive response to capsaicin (Marella et al., 2006). One group ectopically expressed the channelrhodopsin2 (ChR2) receptor in the sweet

responsive cells, and when activated by light, a positive feeding response was seen (Gordon and Scott, 2009). As in mammals, these groups have shown that the gustatory neuron itself is responsible for the behavioral output, while the receptor is responsible for signaling which compound should evoke an attractive or aversive response.

Olfactory Receptors

In addition to Grs, Olfactory receptors (Ors) help the fruit fly smell, which aids in the location and subsequent ingestion of food. *D. melanogaster* have two olfactory organs, the maxillary palp, and the antenna. Like the gustatory organs that have gustatory sensilla, olfactory organs have olfactory sensilla. These olfactory sensilla are covered in tiny pores to allow odorants to enter the sensilla, as well as accessory cells which aid the olfactory receptor neurons (Tunstall and Warr, 2012). The three kinds of olfactory sensilla, (basiconic, trichoid, and coeloconic), differ in location and function. Basiconic sensilla are found on the antenna and maxillary palp, and detect general food and environmental odors (Stocker, 2001; Zhou et al., 2010). Trichoid sensilla on the antenna detect pheromones (Stocker, 2001; Zhou et al., 2010). The last class, coleoconic sensilla, located on the antenna, detect amines, carboxylic acids, and some food odors (Stocker, 2001; Zhou et al., 2010). This sensilla class does not express Ors; instead ionotropic receptors (Irs) (see below) are expressed in coeloconic sensilla ORNs (Rytz et al., 2013).

Altogether, males have about 419 and females have around 457 olfactory sensilla located on their antennas (Tunstall and Warr, 2012). Male *Drosophila* have 30% more trichoid sensilla than females, and females have 20% more basiconic sensilla than males (Tunstall and Warr, 2012). Each sexes' maxillary palp has 60 basiconic sensilla and 120

ORNs (Tunstall and Warr, 2012). Males would be expected to have more sensilla that are used to detect pheromones and mating signals, because males use pheromones to court females and avoid males. Having more trichoid sensilla, males may be able to distinguish among mated and virgin females, as well as young male flies; this would lead to a greater mating success chance. By having more basiconic sensilla, females might have a more fine-tuned ability to find food that she and her future offspring need, as well as use for egg laying sites.

Ors are limited to one receptor per neuron plus a co-receptor, Or83b. Or83b is necessary for olfaction detection (Larsson et al., 2004). Unlike other seven-transmembrane receptors, all Ors have an inverted conformation, with their N-terminus on the cytoplasmic side of the cell, and their C-terminus positioned on the extracellular side (Benton et al., 2006). Ors can either be broadly or narrowly tuned to a number of ligands, and many Ors can respond to the same ligand (Hallem et al., 2004).

Ors are present in olfactory receptor neurons (ORNs). ORNs are located in olfactory sensilla and send their signals to the antennal lobe. Neurons containing the same Ors send signals to a specific glomerulus in the antennal lobe. Projection neurons (PNs) mediate communication between glomeruli. PNs also send projections from the antennal lobe to the lateral horn and mushroom body, the higher olfactory centers. This system is similar in larvae, however, larval Ors in the ORNs project to the dorsal organ. From there, they project to the larval antennal lobe. Larval PNs then project to the mushroom body and lateral horn.

The glomeruli between the sexes are near identical, save for three: DA1, VAlv, and VL2a (Kondoh et al., 2003; Stockinger et al., 2005). These glomeruli are larger in the male than in the female, due to sex specific transcripts of *fruitless* (more information below) (2003; 2005). 11-*cis*-vaccenyl acetate (cVA) activates Or67d-positive neurons, which project to the DA1glomerulus (Kurtovic et al., 2007). DA1 regulates mating behavior in both males and females (2007). In males, detection of cVA inhibits mating with other males, while enhancing their receptivity to court females (2007). VAlv receives projections from Or47b neurons that respond to male and virgin female odors (Vosshall et al., 2000; van der Goes van Naters and Carlson, 2007). Recently, Or47b has been found to regulate female receptivity in mating (Sakurai et al., 2013). Lastly, IR84a positive neurons project to VL2a and respond to phenylacetic acid and phenylacetaldehyde (Grosjean et al., 2011). The male detects these compounds they increase courtship activities (Grosjean et al., 2011).

For a long time, Ors were thought to be G-protein coupled receptors. Recently, a trio of papers came out with evidence supporting Ors as being primarily ion-channels. Two of the papers conducted electrophysiological experiments in heterologous systems which showed Ors are non-selective cation channels (Sato et al., 2008; Wicher et al., 2008). All three papers used a variety of G-protein blockers to access the activity of the receptors without a G-protein signal; each saw that when the G-protein pathway was blocked, there was still a response that was due to ionotropic receptors (Smart et al., 2008; Wicher et al., 2008; Sato et al., 2008). In addition, two of the papers showed that there was some residual signal that was due to a GPCR pathway, indicating that the main

signal was ionotropic, and a slower, sustained signal may be the result of a GPCR pathway (Smart et al., 2008; Wicher et al., 2008).

Ionotropic Receptors

Ionotropic receptors (IRs) are unrelated to Grs and Ors, being part of the ionotropic glutamate receptor (iGluR) family (Benton et al., 2009). The *Drosophila* IR co-receptors, IR8a and IR25a, have the same domains as the iGluR family; an external amino-terminal domain and ligand binding domain, and an internal ion channel domain (Croset et al., 2010; Abuin et al., 2011; Benton et al., 2009). The remaining IRs have only a ligand binding domain and an ion channel domain (Croset et al., 2010). These ion channels are permeable to sodium, potassium, and calcium (Abuin et al., 2011).

IRs, like Ors, respond to olfactory signals. IRs are expressed in the coeloconic sensilla of the antenna, the sacculus and the arista (Rytz et al., 2013). IRs are expressed in one of four types of neural clusters in coeloconic sensilla, and each IRN contains between one and three IR genes, along with one or two co-receptors (Abuin et al., 2011; Benton et al., 2009). IRNs are different from ORNs in that they are tuned more narrowly, are less sensitive, take longer to respond to odors and adapt to odors, and they detect different classes of odors (Rytz et al., 2013). IRs are housed in OSNs and project from the glomeruli to the antennal lobe (Benton et al., 2009). The two IR co-receptors, Ir8a and Ir25a have similar functions as OR83b; they are broadly expressed, necessary for odor responses to take place, and are required for correct IR localization (Abuin et al., 2011).

Not much is known about *Drosophila* IRs, but over the last few years, subsets of the IR ligands have been identified, as well as the behavioral responses they induce. One

of the most exciting finds is IR76b; this IR is the low salt receptor of the taste system (Zhang et al., 2013). IR76b works in concert with the high salt receptor, still unknown, to determine attractiveness or aversion to low and high concentrations of salt, respectively (Zhang et al., 2013). Another IR, IR64a, has been found to signal to the DC4 glomeruli; its activation results in the avoidance of acids (Ai et al., 2010). IR92a detects amines and ammonia, and sends signals to the VM1 glomerulus resulting in an attractive response (Min et al., 2013). IR84a, which responds to phenylacetic acid and phenylacetaldehyde, is located in male *fruitless* (FRU^M) positive ORNs which project to one of three sexually dimorphic glomeruli, the VL2a glomerulus (Grosjean et al., 2011). Most recently, Ir40a has been shown to lead to avoidance of DEET, an insect repellant (Kain et al., 2013). This is an exciting discovery, as the other repellants Ir40a responds to, also repel mosquitoes (Kain et al., 2013).

Outside of the antenna, IRs are also found in the labellum (IR7a), the ventral cibarial sensory organ (VCSO, IR11a), and the dorsal cibarial sense organ (DCSO, IR100a) of the taste system, although their ligands have not been identified (Croset et al., 2010). As current known IRs respond to a variety of stimuli, along with the different expression patterns of IRs being reported, this implicates other IRs as playing a role in more than one taste modality detection.

Transient Receptor Potential Cation Channels

In addition to Grs, Ors, and IRs, transient receptor potential channels (TRPs), aids in taste perception. TRPs are cation channels that respond to many stimuli; they are involved in chemosensation, thermosensation, nociception, mechanosensation, and

phototransduction. TRP proteins, like the other chemoreceptors, form heteromultimers, but they can also form homomers (Venkatachalam and Montell, 2007).

The TRP proteins *Drosophila* TRPA1 (dTRPA1), Painless, and Pyrexia are needed for thermosensation at > 26°, 39-42°C, and 40°C respectively (Fowler and Montell, 2013). Interestingly, dTRPA1 has also been found in aversive GRNs in the labella, where it then signals the repulsion of aristolochic acid (a plant toxin) through phospholipase C (PLC) (Kim et al., 2010). This receptor has also been found to detect citronellal, an insect repellant, that signals through the Gq/PLC pathway (Kwon et al., 2010). Painless also signals avoidance of potent aversive chemicals (such as the compound isothiocyanate in wasabi) (Al-Anzi et al., 2006). Expression of painless in the labella partially overlaps with Gr66a neurons, suggesting that these two receptors may work together to signal repulsion of wasabi (Al-Anzi et al., 2006).

Sex Determination

Sex determination (Figure 2) in *D. melanogaster* is dependent on the number of X chromosomes available, which is relayed to the cell through X-linked signal elements (XSE) present in the embryo during nuclear cell divisions 12-14 (Erickson and Quintero, 2007). These XSE include the activators *scute*, *unpaired*, *runt*, *sisterless-A*, *daughterless*, and the repressors *deadpan*, *groucho*, and *hey* (Hairy/E(spl)-related with YRPW motif) (Duffy and Gergen, 1991; Younger-Shepherd et al., 1992; Erickson and Cline, 1993; Sefton et al., 2000; Lu et al., 2008; Paroush et al., 1994). The interplay of these activators and repressors leads to both female or male differentiation, and later male or female behavior. When the activators override the repressors, the female transcript of *sex lethal*

(SXL^F) is transcribed; SXL^F then goes on to repress *male-specific lethal-2* (MSL-2), (which is required for dosage compensation in males), and activate *transformer* (TRA^F) (Kelley et al., 1997; Robinett et al., 2010). TRA^F along with transformer-2, TRA-2, activates the female transcript of *doublesex* (DSX^F) (Garrett-Engele et al., 2002; Robinett et al., 2010). The interactions of DSX^F, *intersex*, and *her*, lead to female differentiation (Garrett-Engele et al., 2002; Robinett et al., 2010). When the XSE repressors override the activators, the male transcript of SXL is transcribed, (SXL^M). MSL-2 is then free to compensate for the single X chromosome, and the male transcript of *doublesex*, DSX^M, and *fruitless*, FRU^M, are produced (Robinett et al., 2010; Kelley et al., 1997). DSX^M leads to male differentiation and behavior, and FRU^M leads to male behavior (Robinett et al., 2010).

As mentioned above, *fruitless* (*fru*) plays a major role in sexual differentiation of *D*. *melanogaster*. *Fru* has also been shown to contribute majorly to the sexual differentiation of the central nervous system (CNS). One of the first groups to show this sexual dimorphism was Kimura et al. They found a group of medial antennal lobe neurons, mAL, which are dimorphically expressed (five neurons in females and 30 neurons in males) (Kimura et al., 2005). The large difference in cell number is due to programmed cell death that occurs in female flies during development (Kimura et al., 2005). These neurons project their axons into the superior lateral protocerebrum, and then to the SOG (Kimura et al., 2005).

Unlike males, the female axons have forked arborizations in the SOG (Kimura et al., 2005). Recently, two groups have investigated the *fru* circuitry in depth by exploring

the *fru*+ neurons and their dimorphic expression. Cachero et al. found that males have larger *fru*+ neuronal process filled brain regions compared to females (Cachero et al., 2010). They also found that a third of the *fru*+ neuronal cells are dimorphic in their dendritic arbors or axonal projections (Cachero et al., 2010). The same year, Yu et al. found four *fru*+ neurons that descend from the CNS to the ventral nerve cord (VNC). One of these neurons in particular, pMP2 has terminal processes in the SOG and the VNC; in the VNC it is in close contact with another *fru*+ motor neuron needed for flight and song during courtship, vMS2. In addition, an ascending neuron (from the VNC to SOG), vPR1, was found, suggesting there is feedback to the CNS. These two neurons were only found in males (Yu et al., 2010). This might explain why females do not appear to court males.

Drosophila Courtship

Courting behavior is complex in *D. melanogaster*. First, the male needs to be aware of the female and then orient himself towards her (Hall, 1994). The male can tap the female's abdomen to get her attention; she can either accept the courting or reject him by flicking her wings at him (1994). However, if the male senses 11-*cis*-vaccenyl acetate (cVA, a male pheromone), on the female, then courtship towards her is inhibited (Kurtovic et al., 2007; Ejima et al., 2007). This cVA transfer occurs through the male ejaculate (Ejima et al., 2007). Or65a is needed to modulate the courtship response by (2007). But if the female senses cVA on the male, she is most likely receptive (Kurtovic et al., 2007a). He can continue to follow her, and when she is receptive, he produces a courtship song by extending and vibrating a wing, actions which require FRU^{M} and

DSX^M to be present (Hall, 1994; Rideout et al., 2007; Clyne and Miesenböck, 2008). If she is not receptive to the courting, she can block courting further by extruding her ovipositor, decamping, kicking him, fluttering her wings or changing the position of her abdomen so mating is blocked (Spieth, 1974; Hall, 1994)**.** If she is receptive to the song, the male then moves on to probing the female's genitals, and copulation proceeds for up to 20 minutes (Spieth, 1974; Hall, 1994).

Detection of Pheromones by Grs, Ors, and Pickpocket Receptors

Some of the receptors involved in pheromone detection and courtship behavior are Gr32a, Gr33a, Gr39a, Or65a, Or67d, ppk23, ppk25, and ppk29 (Miyamoto and Amrein, 2008; Moon et al., 2009; Watanabe et al., 2011; Bray and Amrein, 2003; Wang and Anderson, 2010; Lu et al., 2012; Starostina et al., 2012; Thistle et al., 2012). These receptors allow the fly to choose if they will mate or not. Gr32a has been shown to prevent male-male courtship, through the function of a male inhibitory pheromone (Miyamoto and Amrein, 2008). Gr32a receptors are located in mouthparts of the fly, as well as the foreleg (Miyamoto and Amrein, 2008; Pavlou and Goodwin, 2013). Gr32a-neurons project to the SOG, pass through the antennal lobe, and terminate in the ventrolateral protocerebrum, (Miyamoto and Amrein, 2008). It is possible that some interaction between gustatory and olfactory signals occurs here. Gr33a, a bitter receptor, was also found to inhibit male-male courtship (Moon et al., 2009). The pheromone that is a possible candidate ligand for these receptors is Z-7-tricosene. Z-7-tricosene inhibits male-male courtship and activates neurons that respond to bitter stimuli (Lacaille et al., 2007). Bitter stimulation leads to aversion in most cases, which would explain inhibition

of male-male courtship. In contrast to Gr32a and Gr33a, Gr39a has been found to sustain the length and frequency of male-female courtship, and has no effect on male-male courtship (Watanabe et al., 2011). Gr32a, Gr33a, and Gr39a are present in both males and females (Miyamoto and Amrein, 2008; Moon et al., 2009; Watanabe et al., 2011) .

One of the most common ligands detected during mating, 11-*cis*-vaccenyl acetate (cVA), is detected by Or67d and Or65a (Wang and Anderson, 2010; Ejima et al., 2007). These two Ors are located in trichoid sensilla and respond only to fly pheromones (van der Goes van Naters and Carlson, 2007). cVA is transferred from the male's ejaculatory bulb to the female during procreation, leaving a distinct chemical scent on the female (Butterworth, 1969; Brieger and Butterworth, 1970; Ejima et al., 2007). Activation of the Gr65a neuron by cVA suppresses courtship, activation of the OR67d neuron invokes male-male aggression (Ejima et al., 2007; Wang and Anderson, 2010). When activated, Or65a-expressing neurons block courting of mated females, but not virgin females since they have not received cVA (Ejima et al., 2007a; van der Goes van Naters and Carlson, 2007).

The last group of receptors that are involved in courting are the *pickpocket* (*ppk*) receptors. The ppk receptors belong to the degenerin/epithelial sodium channel family of *Drosophila*. The three ppk receptors that have been identified in courtship processes are ppk23, ppk25, and ppk29. ppk23 has recently been found to be important for initiation and length of courtship, as well as the prevention of courtship of other males (Lu et al., 2012; Thistle et al., 2012). ppk23 is dimorphically co-expressed with ppk29 in chemosensory neurons of the foreleg in males and females, and as a result, the neuronal

projections to the thoracic ganglion are dimorphic as well (Lu et al., 2012; Thistle et al., 2012). ppk23 neurons respond to the female "aphrodisiac pheromone," 7(Z), 11(Z)-heptacosadiene; activation of these neurons lead to male initiation of courtship towards females (Lu et al., 2012). ppk29 inhibits male courtship as well as stimulating courtship towards female flies (Thistle et al., 2012). ppk25 is also sexually dimorphically expressed in gustatory neurons that are positive for the male version of fruitless in leg neurons (Starostina et al., 2012). ppk25 positive neurons projects to two of the sexually dimorphic glomeruli, VAlv and VL2a (2012). ppk25 has been found to be necessary and sufficient for the progression of male courtship of females (2012).

Post-Mating Response in the Female *Drosophila*

After female flies have mated, they go through many behavioral changes referred to collectively as the post-mating response (PMR); which is maintained by sperm storage (Liu and Kubli, 2003; Schnakenberg et al., 2011). The PMR includes changes in food intake and preference (Carvalho et al., 2006; Ribeiro and Dickson, 2010; Vargas et al., 2010), increase in oviposition rate, decrease in receptivity to further mating (Chen et al., 1988), the stimulation of juvenile hormone synthesis (which leads to egg production) (Soller et al., 1999), and inhibition of siesta sleep (Isaac et al.,2010). Post-mating, female flies also see a reduction in lifespan (Wigby and Chapman, 2005; McGraw et al., 2008). These changes occur due to the accessory gland proteins (Acps) that are transferred from the male to the female after he inseminates her. The main Acp that causes these changes is Acp70A, or sex peptide (SP), (Chen et al., 1988). Additional Acps that affect the mated female include Acp26Aa (ovulin), which stimulates egg laying in mated females for 24

hours (Kalb et al., 1993; Herndon and Wolfner, 1995), and Acp36DE, which allows for sperm storage (Neubaum and Wolfner, 1999; Qazi and Wolfner, 2003). Acps CG1652/CG1656, CG9997, and CG17575, maintain egg laying, reduced sexual receptivity, and release of sperm from storage (Ram and Wolfner, 2007). Acp29AB and Acp62F may induce sperm competition and affect the female's lifespan (McGraw et al., 2008). Seminase, a seminal fluid proteolytic cascade protein, is needed for long term regulation of the PMR; it might also be needed for SP to bind to sperm, since knock down of seminase leads to phenotypes that are similar to SP-null flies (LaFlamme et al., 2012).

In 2006, it was shown that SP is necessary to induce increase in food intake post-mating (Carvalho et al., 2006). The receptor for SP was unknown, so the pathways involved for the changes in feeding behavior could not be elucidated. Two years later, the sex peptide receptor (SPR) was found in reproductive organs, the CNS, as well as being active in *fruitless*+ neurons (Yapici et al., 2008b) (Figure 3). SPR is a GPCR that couples to $G_{\alpha i}$ or $G_{\alpha o}$, meaning it regulates via the cAMP pathway (2008). The subsequent year, two groups found SPR is specifically expressed in a set of 3–4 *fru*+/*ppk*+ internal sensory neurons located on either side of the uterus (Figure 3) (Häsemeyer et al., 2009; Yang et al., 2009). These neurons are necessary and sufficient for the PMR behavioral changes (increase in egg laying and reduced receptivity) (Häsemeyer et al., 2009; Yang et al., 2009). Recently, the gene *doublesex* was found to also be expressed in the *fru*+/*ppk*+ neurons that induce these PMR behavioral changes (Figure 3) (Rezával et al., 2012).

In 2010, two groups showed that females change their feeding preferences after mating; the TOR/S6K pathway is most likely involved in this change and maintenance of feeding preference (Ribeiro and Dickson, 2010). Ribeiro and Dickson (2010) showed that SPR paracrine signaling in *ppk*+ cells is necessary for females to modulate their food choice; when SP is absent, (i.e., in virgins), females choose sugar, and when SP is present (after mating) females choose to eat yeast extract (2010). When they tested females mated to males lacking SP, the females still had some preference for yeast extract, leading them to believe that there was another pathway involved, which they showed to be the TOR/S6K (2010). Vargas et al. (2010) looked at the role of serotonin in this post-mating feeding preference change and found ingestion of serotonin in the presence of dominant negative S6K enhances virgin females' preference for yeast. This group also showed that nutrient preference is fluid; if a fly's diet is lacking in sugar, the fly will choose carbohydrates when presented with a choice between carbohydrates and protein, regardless of its mating status (2010).

Summary

Drosophila uses many receptors to let them sense the world around them. The mechanism, location, and pathways of subsets of these receptors are well studied, while others are just beginning to be understood. In particular, there is still a lot to learn about gustatory receptors. This manuscript discusses work that has been done to clarify certain aspects of the taste system in different aspects of the fly's life. First, Gr61a's role in diet and preference will be discussed. Mated females preference to yeast is then examined, and the preferences of male, mated, and virgin female flies are examined to try to

determine why they differ. Finally, genetic means are used in order to glean more information about how oviposition sites are chosen.

These studies show that Gr61a may direct diet modulation in male flies and nutrient detection in females. The sexes may have different thresholds for nutrients they can tolerate, which may play a role in their feeding preferences. In addition, males and virgin females are shown as being able to detect yeast extract, even though they do not prefer to ingest it. Mated females preferences to sucrose is concentration dependent; this behavioral shift in the mated females preference to yeast is most likely not due to changes in neuronal responses in taste neurons, although they may play a small role. It has been assumed that mated females are attracted to yeast and yeast extract because of its protein content; here, it is shown that she is attracted to amino acids, and perhaps other peptides that help compose yeast. Lastly, these studies show that Gr5a plays a role in oviposition site selection when Gr5a dependent sugars are present, but appear not to when they are absent.

Figures

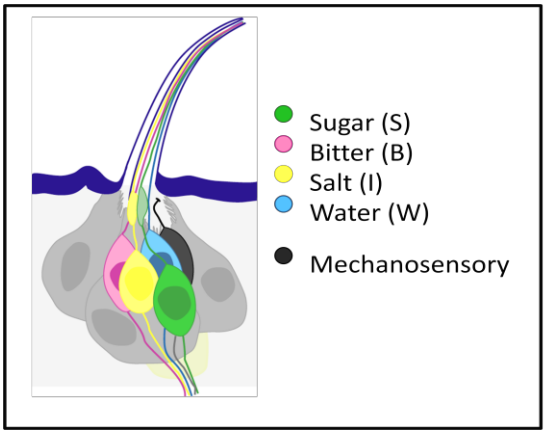


Figure 1 Gustatory Receptor Neurons (GRN). GRNs are located inside gustatory sensilla, taste hairs, which are present on the proboscis, labellum, wing margins, tarsi, and the ovipositor of the female. There are four types of GRNs, a sugar, a bitter, a salt, and water. A mechanosensory neuron is also present to detect movement of the taste bristle. Support cells insulate the GRNs and the mechanosensory cell. Image is courtesy of A. Dahanukar.

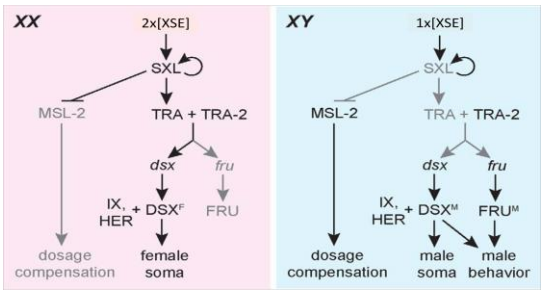


Figure 2 Sex Determination Pathways. The female sex determination pathway is on the right; the male sex determination pathway is on the left. Activation pathways are bolded, repression pathways are grayed out. See text for details. Image modified slightly from Robinett et al 2010.

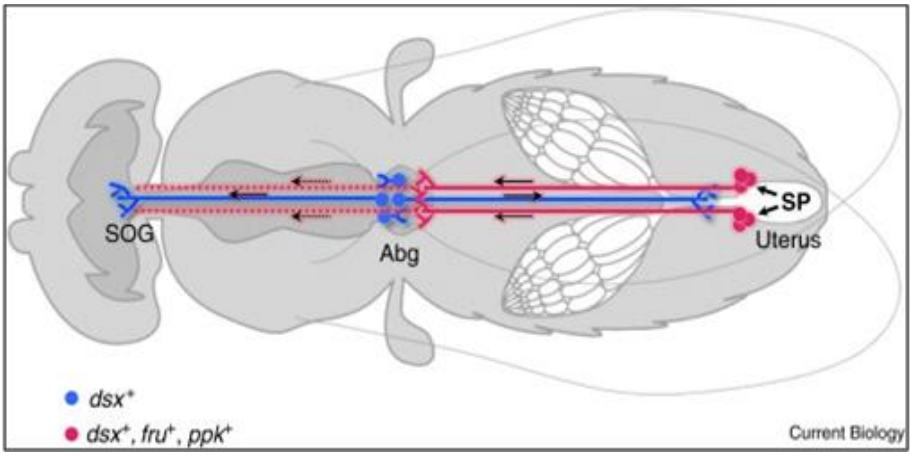


Figure 3 The Sex Peptide/Sex Peptide Receptor Pathway (SP/SPR Pathway). Sex Peptide binds to the *dsx+/fru+/ppk+* neurons located near the uterus and relays information to the abdominal ganglion (Abg). *Dsx+* neurons then relay information to the primary taste-processing center in the brain, the subesophageal ganglion (SOG) and back down to the reproductive organs. Image is courtesy of (Kubli and Bopp, 2012).

Chapter 2 The role of Gr61a in nutrient regulation

Abstract

Like humans, *Drosophila melanogaster* use their senses, touch, taste, smell, hearing, and sight to survive. The taste system in particular is quite complex, being made of a family of 68 gustatory receptor proteins (Grs) that form multimers to sense different sugars, acids, bitters, and pheromones. The particular sub-clade of receptors that confers attraction to sugar is one of the most vital to a flies' survival; the fly gets its carbohydrates from sugars. Sugar detection is mediated through the sweet clade of Grs. This clade is composed of Gr5a, Gr61a, and Gr64a-f. Gr5a is necessary for responses to trehalose, Gr61a for responses to glucose, Gr64a for sucrose, Gr64e for glucose, and Gr64f is thought to be a co-receptor for Gr5a and Gr64a. The role of Gr61a in diet modulation is investigated in this chapter. Gr61a appears to play a role in diet modulation in males and nutrient detection in females. These differences between the sexes may be due to the role of the Gr61a receptor, since there is no significant difference in their mean number of Gr61a positive neurons. Differences in diet regulation and modulation thresholds may also play a role.

Introduction

In 2007, Dahanukar et al discovered that Gr5a is necessary for the responses to trehalose and glucose, while Gr64a is necessary for responses to sucrose and maltose. This study also investigated the role of Gr61a in sugar perception. They found that this particular sugar mutant presented no phenotype to the compounds tested, which included a number of sugars and a sugar alcohol (2007). There was, however, some reduction in m-alpha-glucoside and sucrose response (2007). This led them to believe that Gr61a

played a small role in the sugar sensing neurons of the labella. Later, Slone showed that Gr61a is responsible for the partial response to glucose and arabinose through proboscis extension response (PER) assays (Slone, 2009). Gr61a has recently been shown to be needed in the tarsi to detect glucose (Miyamoto et al., 2013).

An alumnus of the Dahanukar lab performed experiments on wild type and mutant Gr61a male flies that were raised on different foods, (standard lab cornmeal, and molasses) (Figure 4A). The ΔGr61a flies had a defective PER to 100mM sucrose. He found that there was a significant reduction in PER to 100mM sucrose on mutant Gr61a flies raised on molasses food. Ribeiro and Dickson, as well as Vargas et al., found that a flies' diet preference is dependent on its nutrient state (Ribeiro and Dickson, 2010; Vargas et al., 2010). One explanation for this defective PER may be that mutant Gr61a flies may not be able to compensate behavior for the nutrients they need to consume. In addition, they may be repelled by sucrose (molasses is heavy in sucrose, compared to glucose and fructose), and drawn to yeast extract (since it is rich in proteins). In other words, a nutritional balance may come into play. Here, the role of Gr61a, a sugar receptor, in nutrient modulation is explored. Findings indicate that the number of Gr61a-neurons between males and females appears normal, and Gr61a itself appears to play a small role in nutrient detection in females and diet modulation in males.

Materials and Methods

Fly stocks

Flies used for two-choice preference index tests were wCS (white Canton-S, used as wild type) and ΔGr61a. Flies were kept on standard a standard cornmeal-dextrose food or molasses food at 25°C and ≥ 50% humidity on a 12:12 hour light: dark cycle

Two-choice Feeding Preference Assays

Fifteen to twenty 0- to 1-day old flies were collected and maintained on fresh fly food for 3-4 days. Flies were sorted into male and female groups. On the subsequent day, they were transferred to starvation vials' containing wet Kim wipes for 24 ± 2 hours. Flies were assayed the following day. Assays were run using tight-fit Petri dishes (Falcon 35-1006). Solutions containing the stimuli and either 0.25 mg/mL indigo carmine (Sigma I8130) or 0.5 mg/mL sulforhodamine B (Sigma 230162) were prepared before the start of the experiment in 0.75% agarose and spotted in the Petri dishes. Starved flies were mildly anesthetized with carbon dioxide, transferred to the dishes (Figure 5A), and placed in a humidified box at 25°C for 2 hours in the dark. After, they were frozen at -80°C for twenty minutes, moved to -20°C, and their abdomens scored within 72 hours (Figure 5B). Data analysis includes trials where at least 50% of the flies participated, unless otherwise stated. Experiments were performed between two and 6 PM. Preference index values were calculated using the formula:

$$(Nr + (Np)/2) / (Nr + Nb + Np)$$

where Nr is the number of red flies, Np is the number of purple flies and Nb is the number of blue flies.

Starvation assay

Five sets of twenty 0-1 day old flies were obtained and kept on fresh fly food for two to three days. Flies were then sorted into male and female groups so there were around twenty flies of a single sex in each vial. On the subsequent day, the flies were starved at 8 p.m., and checked again twelve hours later at 8am. The number of alive and dead flies was recorded. The process of checking and recording the live and dead flies continued every one to two hours until all of the flies succumbed to starvation. The time to 50% survival/ 50% starvation was then calculated from the graphed data.

Proboscis Extension Response (PER)

Flies were collected from eclosion to one day old. At three-four days old, flies were sorted based on sex, and starved the following day. On the day of the experiment, flies were placed in a pipette jacket in a room with 30-51% humidity. Flies were allowed to satiate with water before the experiment began. Alternating stimulants and blanks (water) were offered to the flies, and their response was recorded as one, full proboscis extension, 0.5, partial proboscis extension, and zero, no proboscis extension. Experiments were performed from 2-7pm in the same room. Multiple flies were tested and their responses averaged. If flies responded to water, they were allowed to drink until full and the assay continued.

Confocal

Confocal images were obtained on a Zeiss 510 confocal microscope using 40x magnification.

Chemicals

All chemicals were obtained from Sigma-Aldrich.

Statistics

Statistics were performed using IBM SPSS Statistics 20 software. Outliers were included in the analysis.

Results

ΔGr61a flies can sense a wide range of sucrose concentrations.

It was first determined if ΔGr61a flies could detect a wide range of sucrose concentrations. The proboscis extension response (PER) assay was used because it indicates whether a fly can sense peripheral stimuli. This assay measures the direct behavioral measure of gustatory receptor neuron stimulation. A wild-type control was tested alongside ΔGr61a to compare its responses. Wild-type males and females could detect sucrose at all levels tested (10, 50, 100, and 250mM). The ΔGr61a males had a small reduction in sucrose response, while the ΔGr61a females were lower than the control at all concentrations tested (Figure 4B).

Apparent diet modulation by Gr61a

In order to see if Gr61a was involved in modulation of nutritional needs of flies, the wild type and mutants were raised on standard lab food, and a second set were maintained on molasses food, which has reduced protein content. Molasses food contains 1.7% yeast extract and 9.4% molasses (sugar), while standard laboratory fly food has a balanced amount of sugars (9.6% dextrose) and protein (4.8%). When the molasses line

was established, the wild type and mutant flies' ability to modulate nutritional sources were assayed with the two-choice feeding preference test (Figure 5).

As seen in Figure 6 A, wild type males raised on either dextrose or molasses food both chose sucrose to 1% yeast extract at all concentrations tested. This choice persisted as the flies aged to ten days (Figure 6B). However, the Gr61a mutant males show an increased preference to 1% yeast extract when offered 5mM sucrose; this occurs for both flies raised on dextrose and molasses food, indicating that Gr61a may somehow be invovled in diet regulation. When offered higher concentrations of sucrose, the mutant Gr61a males increased their preference to sucrose, preferring both yeast extract and 10mM sucrose, and going on to prefer the higher concentrations of sucrose to yeast extract. Seeing as how there is this reduction in preference to sucrose at lower concentrations, Gr61a may be needed to determine which compounds to ingest, while at higher levels of sucrose offered, another receptor compensates for the lack of Gr61a. As the Gr61a mutants age, this stark difference in preference is still observed compared to the wild type, but compensation appears to be occurring faster now (the mutants prefer 10mM sucrose to yeast extract now). Over time, other chemosensory receptors or proteins may overcome the loss of the Gr61a receptor.

As for females, Gr61a does not appear to be invovled in diet preference when lower levels of sucrose are present, as their feeding preference matches that of the wild type on both foods (Figure 7A). However, when offered 10 or 25mM sucrose, the wild type females raised on molasses have an increased preference to yeast extract, as do the Gr61a mutant females. These differences appear to disappear at higher levels of sucrose.

Older wild type females raised on molasses again show an increase in preference to yeast extract compared to their dextrose raised counter parts (Figure 7B). This time, the change in preference appears earlier and persists over the range of concentrations tested. Molasses raised ΔGr61a females also show an increased yeast extract preference, which appears to remain at all concentrations. These results infer that Gr61a is not a main player in diet preference in molasses raised young or old female flies.

There is indication of a female specific Gr61a phenotype

Since the molasses raised males indicated that there may be a ΔGr61a phenotype and there was evidence of that in females at 5mM sucrose, it was decided that a lower concentration of yeast extract be offered with the low sucrose concentration pairings. This was so that any phenotype that was hidden by the high yeast content could be observed. Wild type males preferred sucrose at all three concentrations tested, one, five, and 10 mM, as well as the ΔGr61a males at 5 and 10 mM concentrations of sucrose (Figure 8B). Male ΔGr61a preferred the 0.1% yeast extract to the 1mM sucrose, potentially showing a decrease in regulation. Meanwhile, female wild type preferred all three concentrations of sucrose over yeast extract, while the ΔGr61a females preference was towards either substance (Figure 8A), indicating that Gr61a is necessary for this behavior.

In order to parse out Gr61a's phenotype more, trehalose, a sugar present in yeast extract, was presented opposite yeast extract (Figure 9). Wild type males preferred trehalose at all concentrations (Figure 9B). The ΔGr61a males have no preference over trehalose or yeast extract at any concentration at 1 or 5mM, the same as ΔGr61a females

at 1 and 10mM trehalose (Figure 9A). Wild type females preferred trehalose to yeast extract. This is interesting, since trehalose, is a Gr5a ligand. It would be intriguing if the Gr61a receptor aided in the detection of trehalose, and thereby regulation of dietary needs. Next, m-a-glucoside, which needs Gr61a to confer a response, was tested to see how preference and modulation would be affected.

Male wild type preferred both m-α-glucoside at 1mM and 5 mM and yeast extract equally. They preferred m-α-glucoside at 10 mM; male ΔGr61a preferred yeast extract to this sugar at 1mM and 5mM, but showed a small reduction in preference to the m-α-glucoside at 10mM (Figure 10B). The Gr61a receptor seems to be playing some role in the modulation of this sugar and yeast extract, which makes sense, as Gr61a is necessary for responses to m-a-glucoside. Wild type females' preference went from indifferent between the two to slightly preferring the m-α-glucoside at 10mM. The ΔGr61a female preferred the yeast in place of 1mM m-a-glucoside and were indifferent about the 5mM and 10mM sugar and the yeast (Figure 10A). Gr61a seems to play a small part in regulation of m-a-glucoside. In summary, the loss of the Gr61a receptor appears to lead to a female specific phenotype to sucrose (loss of attraction), and a partial phenotype to trehalose and sucrose (slight loss of attraction), while loss of Gr61a seems to affects males perceptions at low levels of sucrose and m-a-glucoside.

The number of Gr61a-neurons is similar in males and females

Before moving on to perform additional behavioral experiments, it was essential to see if there were any differences in the number of Gr61a-postive labellar neurons in between male and female flies. After performing antibody staining, there appears to be no

difference in the mean number of Gr61a positive labellar neurons between the sexes (Figure 11A and B). This indicates that males and females may receive a similar number of signals to the brain, but their difference in sugar preference is due to regulation of this, and perhaps other receptors. Any differences in how males and females perceive their surroundings could be due to the differing composition of their neurons, or different connections, inputs, signals, etc. in each sexes central and peripheral nervous system, as well as different regulation of amounts of gustatory or other chemosensory receptors.

The Gr61a phenotype appears to be due to the loss of the receptor

The question remained: is this female Gr61a phenotype to sucrose neuronal or receptor specific? In order to answer this question, Kir2.1, an inward rectifying potassium channel, was expressed in Gr61a positive neurons to prevent neuronal firing. With these neurons silenced, it became clear that the phenotypes being observed were due to the receptor. Both males and females who had their Gr61a neurons silenced had an increased preference to yeast extract, whereas the wild type males and females highly preferred sucrose. Silenced female Gr61a neurons showed a reduction in preference to 50 and 100 mM sucrose compared to wild type females; silenced males preference was not affected at these levels. Since females showed a reduction in sucrose preference at all sucrose concentrations tested and males only showed a reduction in sucrose preference up to 50mM, the diet regulation thresholds are likely different between males and females.

Food sources high in protein and low in carbohydrates reduce flies lifespan

In order to see how a fly's preference changed when sufficient qualities of macronutrients were lacking, the right combination of food that would allow the flies to

survive during starvation needed to be found. Survival-ship assays were performed to see how different ratios of protein to sucrose affected the flies' lifespan. Three types of food with different ratios of casein (a protein source) and sucrose (carbohydrate) were tested in order to determine how they affected the flies. They were, regular lab cornmeal-dextrose food with 4.8% yeast extract and 9.6% dextrose, with differing ratios of protein to carbohydrates: 2% casein and 10%, 6% casein and 6% sucrose, and, 10% casein and 2% sucrose.

As can be seen in Table 1, the ΔGr61a male flies had a similar 50% death time as the wild type flies. In the case of the ΔGr61a female flies, their survival ship was much higher than the wild type flies on all types of food, sans the 10% casein and 2% sucrose food. This interesting phenotype may warrant further investigation. In fact, the 50% survival ship for both male and female wild type and ΔGr61a when fed the 10% casein 2% sucrose food is under 24 hours. Since the 50% survival ship for the other three remaining diets was at least a day or more, it was decided that these should be used, while the 10% casein and 2% sucrose diet would be dropped. Foods heavily enriched with protein could potentially be detrimental to the fly because carbohydrates are needed for energy. Diets or foods rich in carbohydrates but low in protein appear to be sufficient in sustaining fly life.

Gr61a may be needed to maintain nutritional balance in males

With the survival curve in hand, the relative starvation time of the flies were calculated; 24 hours for regular food for both wild type and mutant, 22hr 24 minutes for wild type and 20hr 16min for mutant on 2% casein; 10% sucrose, and 19hr 44min for

wild type and 14hr 56min for mutant on 6% sucrose: 6% casein. After spending four to five days on the different food, the flies were presented with a choice between different concentrations of sucrose and 1% yeast extract (Figure 12). At 1mM sucrose, flies preferred the 1% yeast extract, however, the ΔGr61a flies, on each of the three food sources, were heavily drawn to the yeast extract more than their wild type counterparts were. This difference is exacerbated when given a choice between 5mM sucrose and 1% yeast extract. The wild type flies on regular food strongly prefer the sucrose; while those flies that had low protein and high sugar diets had a small decrease in preference to 5mM sucrose, and those raised on an equal amount of protein and sugar had equal preference to both tastants. As for the ΔGr61a flies, they all strongly preferred yeast extract on each of the three food types. This indicates that Gr61a is required for sensitivity to low levels of sucrose. However, at higher concentrations of sugars tested, both wild type and ΔGr61a male flies had a high preference for sucrose. At 10mM sucrose, ΔGr61a flies on regular and 2% casein: 10% sucrose diets had a small reduction in sucrose preference. This indicates that Gr61a may be needed to regulate nutritional balance in males at low concentrations of sugar either by itself, or with a co-receptor.

Discussion

Ribeiro and Dickson have shown that when given a choice between a substance in their diet and a needed macronutrient absent from that diet, a fly will prefer the macronutrient that was absent (2010). Experiments performed here indicate this as well. Here, the Gr61a receptor is shown to perhaps play a small role in this macronutrient regulation in female and male flies (nutrient detection in females, and diet regulation in

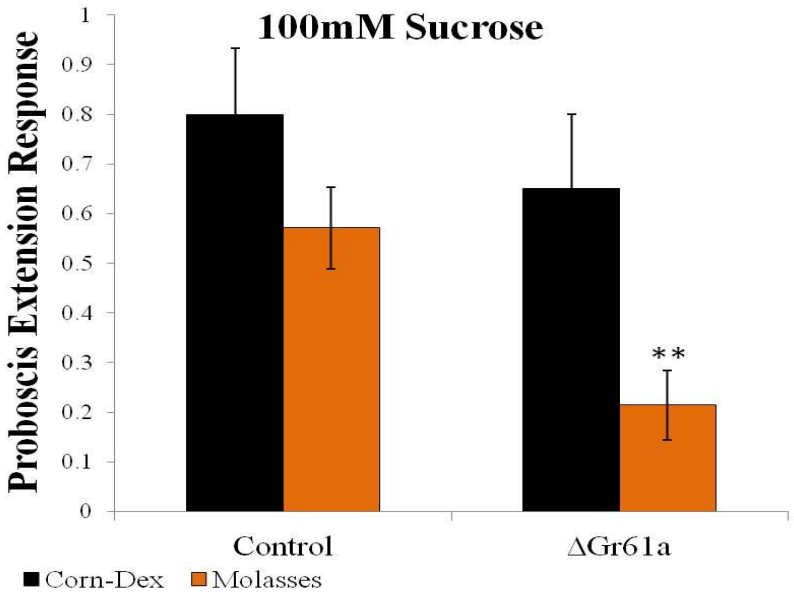
males). This may be due to differences in Gr61a neural signals between the two sexes. When Gr61a neurons are silenced, both sexes show a reduction in preference to sucrose, but the silenced males are able to overcome this when higher levels of sucrose are present. Each sex's sensitivity thresholds to sugars may be different due to the complexes that Gr61a is a part of, or due to other factors that take over the higher concentrations of the threshold. As female flies needs are more complex, their nutritional regulations and modulations have to account for changes in their food source for their own needs and their offspring's needs. This may be why a difference is seen when the neurons are silenced. The number of Gr61a neurons in both sexes is also similar, which supports role for other factors that may help females and males regulate their needs in addition to Gr61a.

Since the mutant Gr61a phenotype for females appears to be a defect in diet detection and in males a defect in diet regulation, it could be said that Gr61a helps play a role in choice. Alternatively, the thresholds for diet regulation could be different in males and females. This receptors role in female choice allows her to pick out and ingest the nutrients that she needs. Gr61a's role in males indicates that it is needed for diet regulation between low levels of sucrose and yeast extract. Flies, in general, are able to adjust their needs when their diet is lacking. Wild type flies raised on 2% casein: 10% sucrose and 6% casein and 6% sucrose shifted their preference towards yeast extract, to make up for the lack of protein in their diet. However, this was only seen when presented with 5mM sucrose, and slightly with 10mM sucrose, while at higher concentrations of sugar, the wild type flies preferred sugar, no matter what their diet. This is interesting to

see; apparently, yeast extract is needed (or perhaps preferred) at low levels, and higher levels of sucrose seem to override this need to compensate for the lack of protein in the diet. Further investigation of these phenomena would be interesting.

Figures and Tables

A)



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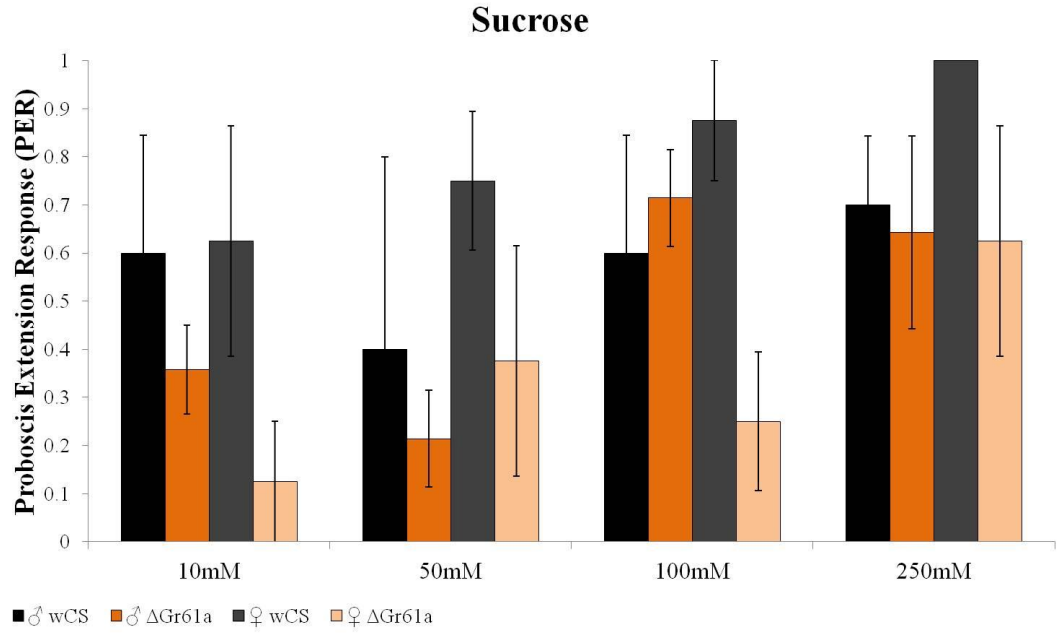


Figure 4 A) ΔGr61a males exhibit a diet-induced behavioral defect. B) Gr61a mutants can detect sucrose at the periphery.

Figure 4A) ΔGr61a males exhibit a diet-induced behavioral defect. Error bars are S.E.M. n = 35, 28 for molasses, 24, 14 for Formula 4-24, and 10, 10 for (regular) corn-dextrose (n = wCS, ΔGr61a). Data is courtesy of Kyle Risser. There is a significant difference in proboscis extension response at 100mM sucrose between control (wCS) and ΔGr61a, when raised on molasses food, $p = 0.002$. Assessed by an independent sample t test. Results obtained with the Mann-Whitney U test indicated similar results. **4B) Gr61a mutants can detect sucrose at the periphery**. Error bars are S.E.M. n = 5, 7, 4, and 7 for wCS ♂, ΔGr61a ♂, wCS ♀, and ΔGr61a ♀ for all concentrations. wCS = control. There was no significant difference as determined by a Mann-Whitney U test.

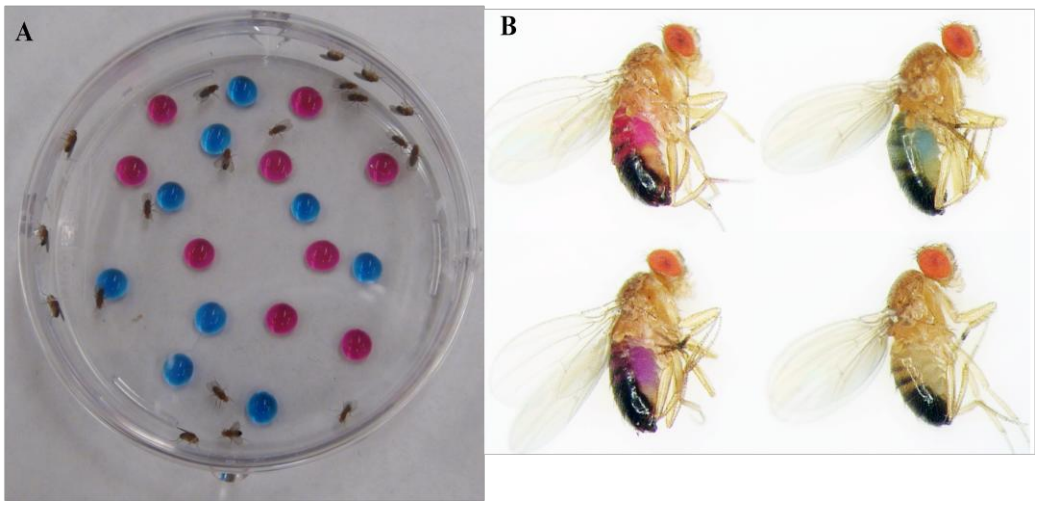
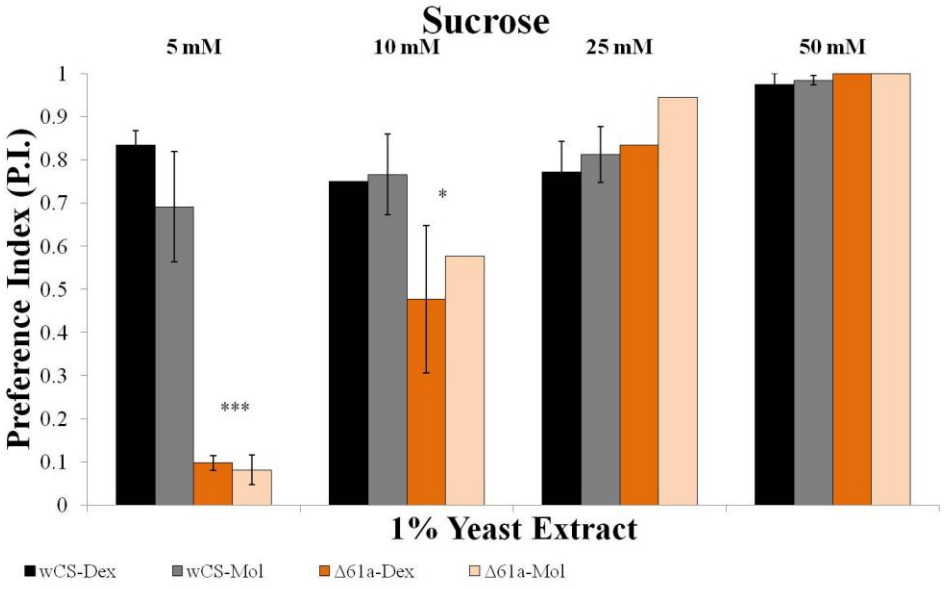
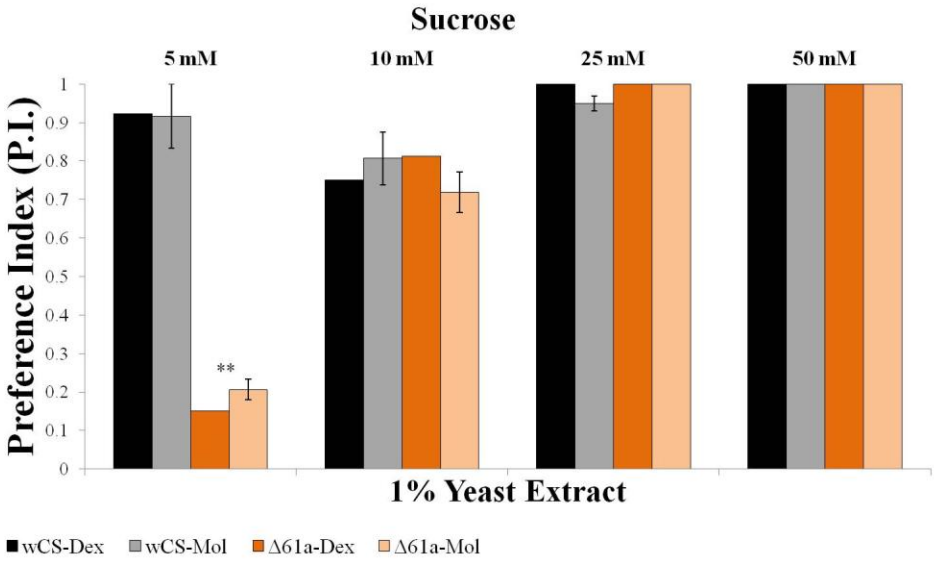


Figure 5 A) Two-choice feeding preference assay chamber. **B)** Male flies with different colored abdomens indicating which stimulus they consumed. From top left, counter clockwise, males preferred the stimulus in the red dye, blue dye, none (white), and both dyes (purple). Image is courtesy of A. Dahanukar.

A)



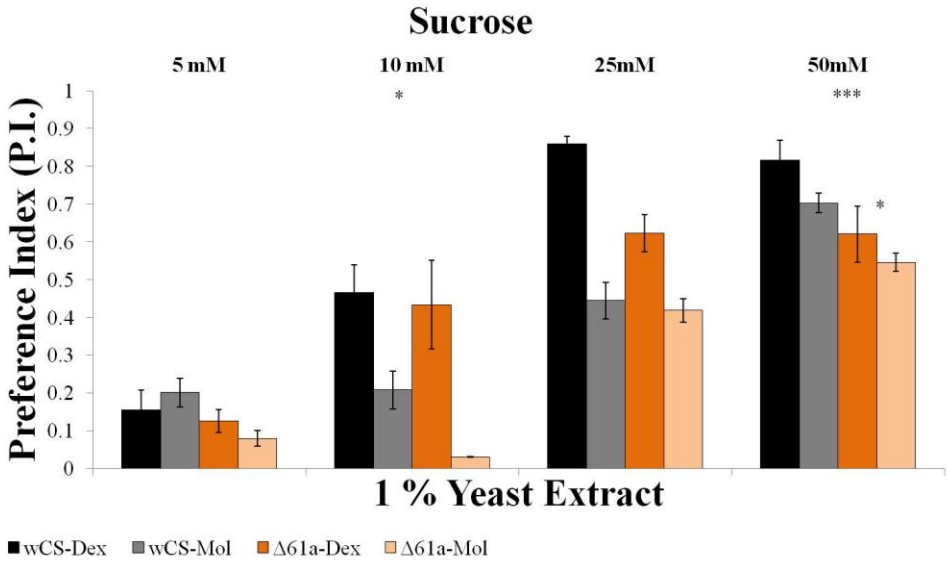


B)

wCS-Dex wCS-Mol Δ61a-Dex Δ61a-Mol

Figure 6 A) Male flies, aged 5-6 days. n = 5-6 for wCS (control) on dextrose food; n = 4-10 for wCS (control) on molasses food; n =6,3, 1, and 1 for ΔGr61a on dextrose food; and n = 7, 1, 1, 5 for ΔGr61a on molasses food. Error bars are S.E.M. There are statistical differences between control and mutant flies at 5 and 10 mM ($p \leq 0.0005$, and $p \leq 0.05$). There were no statistical differences between the flies when raised on different foods. Mann-Whitney U test. **B) Male flies aged 9-10 days.** n = 1-3 for wCS (control) on dextrose food; n = 3-4 for wCS (control) on molasses food; n =1-3 for ΔGr61a on dextrose food; and n = 3-5 for ΔGr61a on molasses food. Error bars are S.E.M. There is a statistical difference between control and mutant flies at 5mM mM ($p \leq 0.05$). There is no statistical difference between the flies when raised on different foods. Mann-Whitney U test. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.005$.

A)



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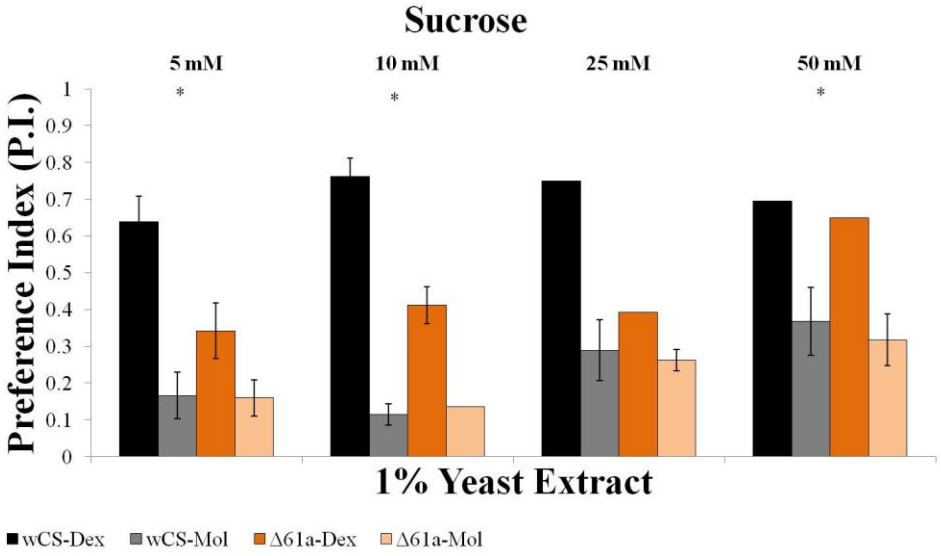
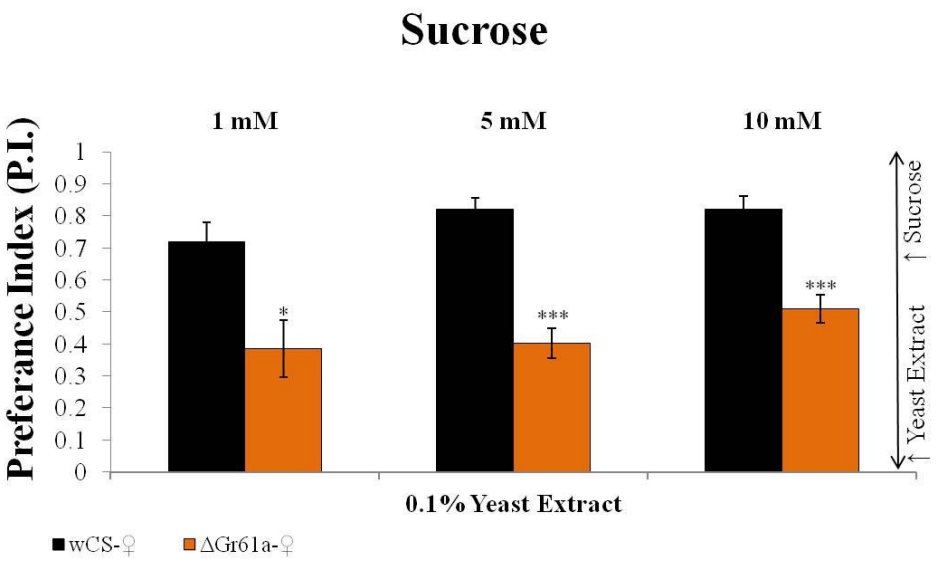
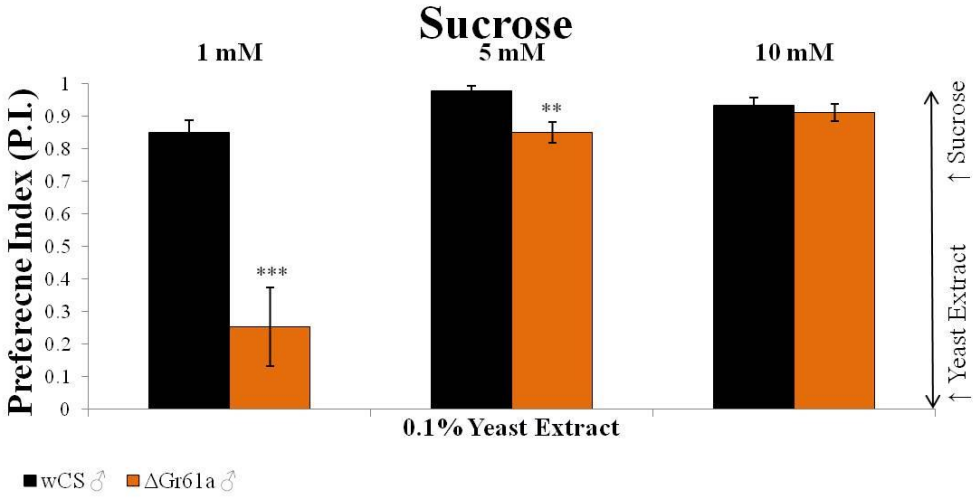


Figure 7 A) Females aged 5-6 days. n = 6-12 for control (wCS) on dextrose; n = 4-12 for control (wCS) on molasses; n = 7 for ΔGr61a on dextrose; n = 2-7 for ΔGr61a on molasses. Error Bars are S.E.M. There is a statistical difference between control and mutant flies at 50mM (p = 0.001). There is a statistical difference between flies raised on different foods at 10mM ($p \leq 0.05$) and 50mM ($p \leq 0.0005$). Mann-Whitney U test. **B) Females aged 9-10 days**. n = 3, 3, 1, 1 for control (wCS) on dextrose; n = 3-4 for control (wCS) on molasses; n = 3, 3, 1, 1 for ΔGr61a on dextrose; n = 3-5 for ΔGr61a on molasses. Error Bars are S.E.M. There is a statistical difference between flies raised on different foods at 5mM ($p \leq 0.05$), 10 mM ($p \leq 0.05$), and 50 mM ($p \leq 0.05$). Mann-Whitney U test. * = $p \leq 0.05$, *** = $p \leq 0.001$

A)

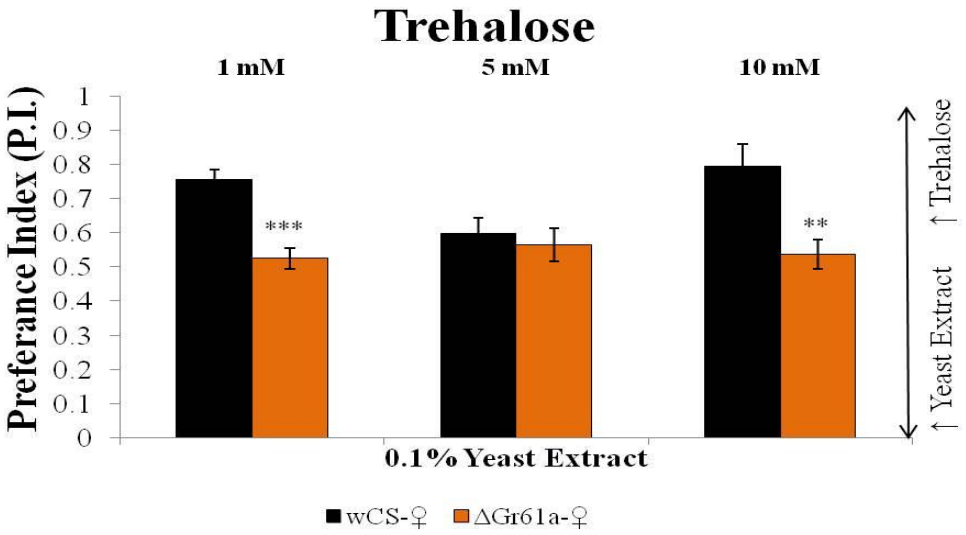




B)

Figure 8 A) Sucrose preference in females. There was a statistically significant difference in sucrose preference between control (wCS) and ΔGr61a females at 1mM , $p = 0.009$, 5mM, as assayed by an independent sample t test. (Similar results were achieved with the Mann-Whitney U test, $p = 0.025$), 5mM, $p < 0.0005$,, and 10mM $p < 0.0005$, as assessed by an independent sample t test. n = 7-12 for control (wCS) and n = 11-17 for ΔGr61a. **B) Sucrose preference in males.** There was a statistically significant difference in sucrose preference between control (wCS) and ΔGr61a males at 1mM, $p = 0.001$, and 5mM, $p = 0.002$, as assessed by an independent sample t test. (Similar results were achieved with the Mann-Whitney U test). n = 8-15 for wCS and n = 9-16 for ΔGr61a. Error bars are S.E.M. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.005$.

A)



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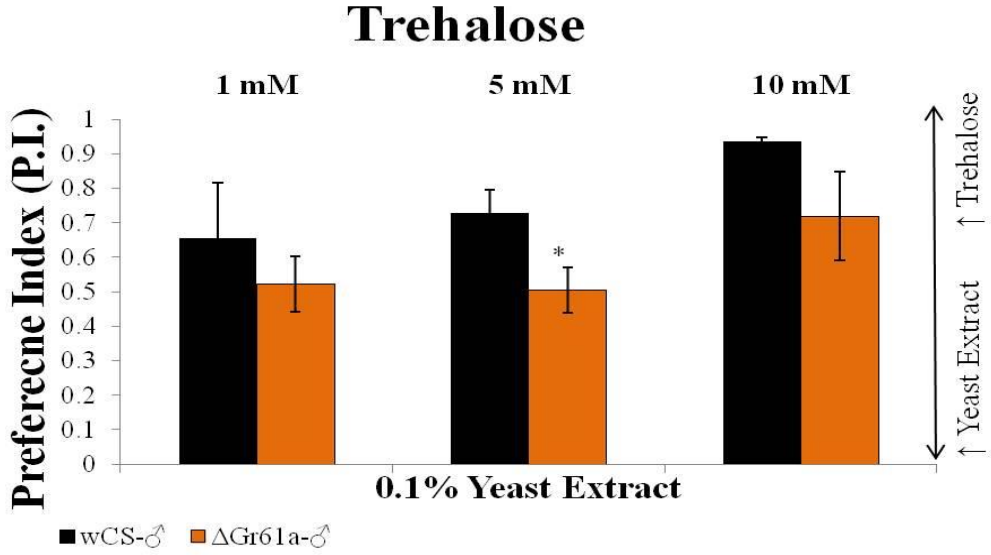
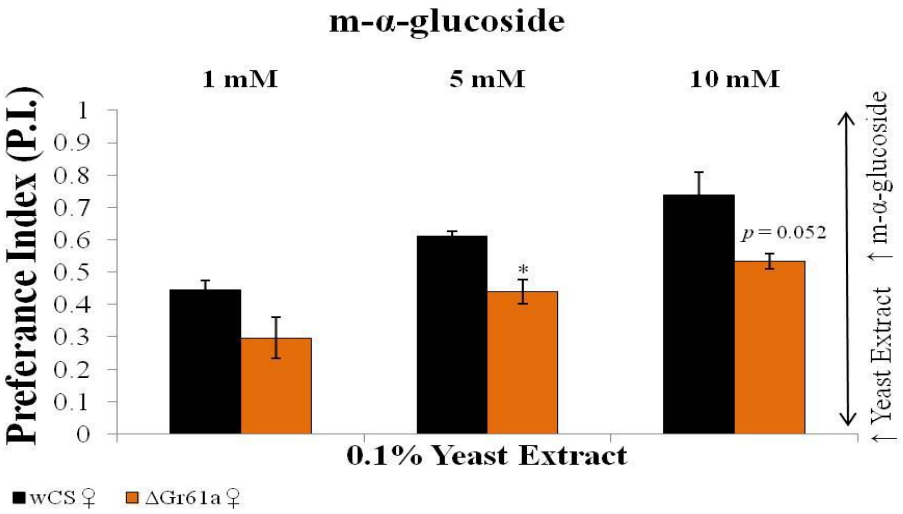


Figure 9. **A) Trehalose preference in Females.** There was a significant difference in sucrose preference between wCS and dGr61a females at 1mM $p < 0.0005$, as assessed by an independent sample t test, at 10 mM ($p = 0.005$, as assessed by an independent sample t test. (Similar results were obtained using a Mann-Whitney U test). n = 5-6 for control (wCS), and n = 7- 10 for ΔGr61a. **B) Trehalose preference in males.** There was a significant difference in sucrose preference between wCS and dGr61a males at 5mM, $p = 0.041$, as assessed by an independent sample t test. n = 3-6 for (Control) wCS, and n = 3-8 for ΔGr61a. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.005$.

A)



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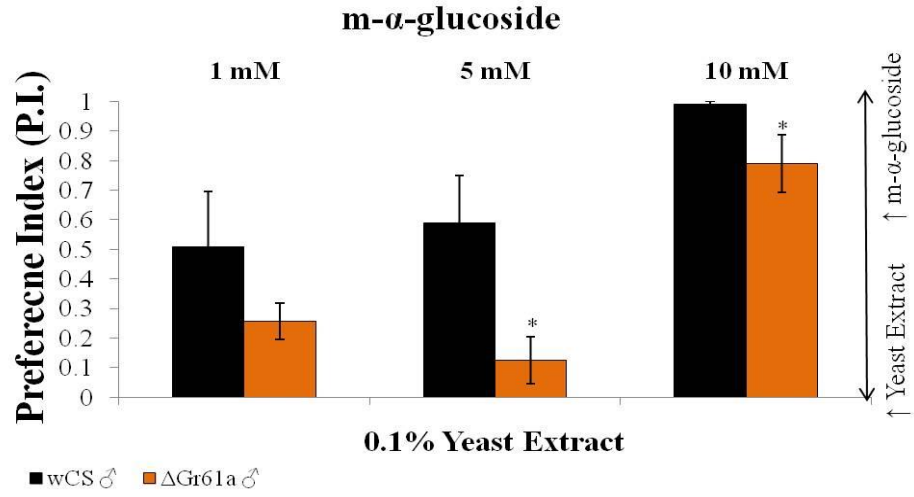
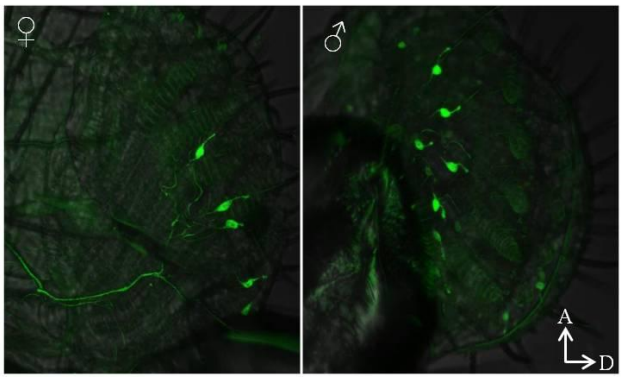


Figure 10. **A) Gr61a m-a-glucoside preference in females.** A significant difference in sucrose preference between control (wCS) and ΔGr61a was found at 5mM for females, p = 0.019, as assessed by an independent sample t test. Similar results were obtained using a Mann-Whitney U test. An almost significant difference in sucrose preference between wCS and dGr61a was found at 10mM for females; Results obtained using a Mann-Whitney U (p = 0.052). n = 3-5 for wCS; n = 6 for ΔGr61a. **B) Gr61a m-a-glucoside preference in males.** A significant difference in sucrose preference between control (wCS) and ΔGr61a was found in males at 5mM; p = 0.040, as assessed by an independent sample t test. A Mann-Whitney U test A statistically significantly difference in preference scores was present between wCS and ΔGr61a males at 10mM, p = 0.015, using an exact sampling distribution for U (Dineen & Blakesley, 1973). n = 2-6 for wCS; n = 4-6 for ΔGr61a. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.005$.

A)



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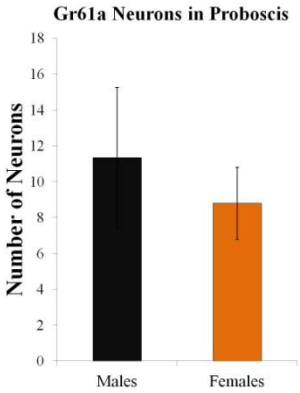


C)

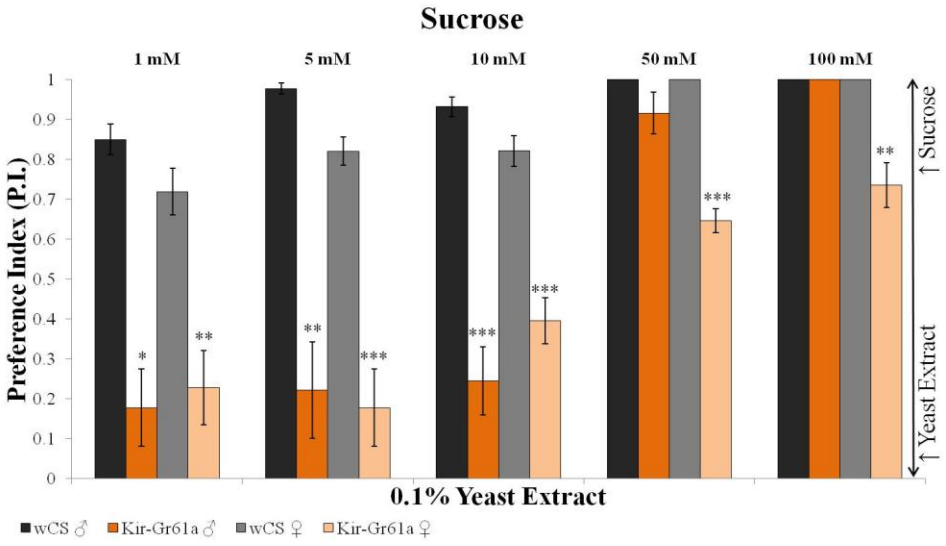


Figure 11 A) Confocal image of labellum. Section of a female (left), and a male (right) labellum **B) Number of Gr61a positive neurons.** Female, n =5, and male, n = 3. Error bars are S.E.M. There is no significant difference, as computed by an independent-samples-t-test, $p = 0.543$. **C) Silencing of Gr61a neurons**. Control (wCS males), n = 8-15. Silenced (Kir) males, n = 3-5 and 1 for 100mM sucrose. Control (wCS) females, n = 7-14. Silenced (Kir) females, n = 3- 6. Error bars represent S.E.M. There is a statistical difference between the preferences of control and Gr61a positive neuron silenced flies at all concentrations of sucrose tested. See text for details. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.005$.

50% Death	wCS ♂	ΔGr61a ♂	wCS ♀	ΔGr61a ♀
R	45	45	51.5	88
2C:10S	42	38	36	49
6C:6S	37	28	23	33
10C:2S	19	18	19	13.5

Table 1. Survival times of male and female wCS and ΔGr61a flies on different diets. R = dextrose food, n = 72-90. 2C:10S = 2% casein and 10% sucrose, n = 75- 94. 6C: 6S = 6% casein and 6% sucrose, n = 65-123. 10C:2S = 10% casein and 2% sucrose, n = 80-110. (n = Control (wCS) ♂, ΔGr61a ♂, Control (wCS) ♀, ΔGr61a ♀). N represents how many flies were used to find the fifty percent survival rate of the flies on each food source.

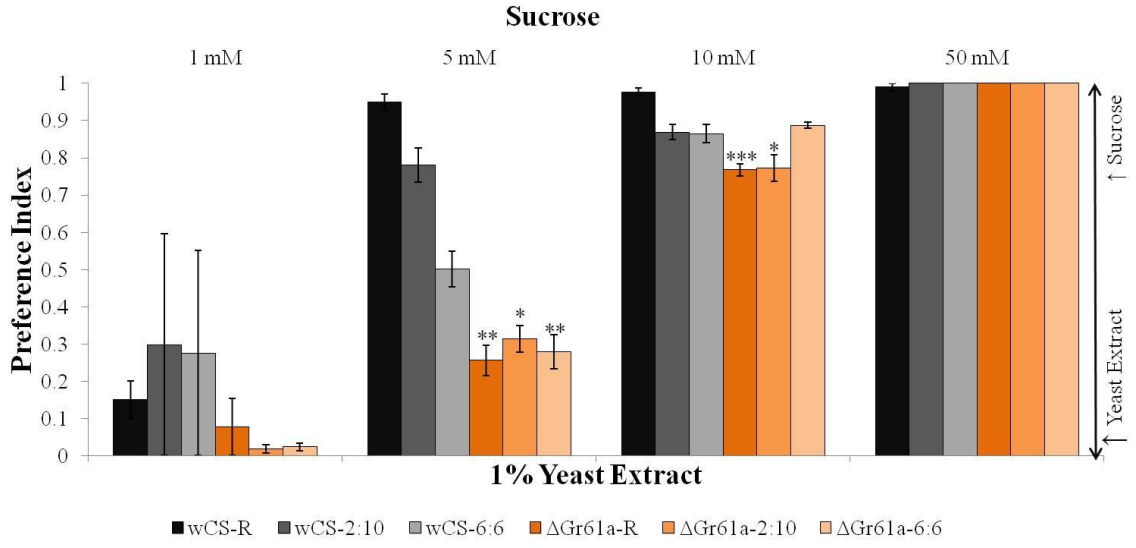


Figure 12 Males show a Gr61a phenotype on lower concentrations of sucrose when raised on molasses. For, 1mM n = 2-4 and 5, 5mM, n = 3-5 and 5, 10mM, n =5 and 5, 50mM, n = 5-13 for both, and 100mM, n = 4-10, and 4-11. (n = Control (wCS) and ΔGr61a). Error bars are S.E.M. Significance was determined through the Kruskal-Wallis test and Pairwise comparisons. Significance is determined in relation to wCS on dextrose food; at 5mM, ΔGr61a $p = 0.003$, dGr61a 2:10 $p = 0.026$, and ΔGr61a 6:6 $p = 0.009$. For 10mM, ΔGr61a is $p = 0.001$, and ΔGr61a 2:10 $p = 0.003$. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.005$.

Chapter 3 Mated Females are attracted to amino acids

Abstract

Drosophila melanogaster obtain their nutrients from a variety of sources. The two main components of the flies' diet are carbohydrates (sugars) and proteins. Flies show preference to one compound or the other based on internal state, sex, and mating status. After the female fly has mated, she begins exhibiting behaviors known as the post-mating response (PMR); these include a reduced receptivity to future mating, an increase preference to yeast extract, and an increase rate of oviposition (egg laying). Previous studies have shown that mated females do in fact prefer yeast or yeast extract, and the injection of sex peptide in virgins can trigger mated female like preferences. The following data indicate that male and virgin female flies can detect yeast extract, mated female preference to sucrose is concentration dependent, and neuronal firing dynamics may play a role in sucrose preference. Lastly, the components of yeast are broken down and amino acids and/or peptides in the yeast extract are shown to be what the mated female fly is attracted to in yeast extract.

Introduction

Fruit flies obtain their nutrients from a variety sources to obtain needed nutrients, such as sugars and proteins. Flies need sugar to provide them with energy, as well as proteins, so protein synthesis can continue. Mated females are more drawn to yeast, which is rich in proteins, after they have mated because they are about to restart oogenesis, and make many other proteins that they need in order to survive during this process (Soller et al., 1999). Nutrient balance is partially regulated through the TOR/S6K pathway and partially through the insulin like receptor pathway (Figure 13).

Ribeiro and Dickson, along with Vargas et al, have shown that virgin females and male flies prefer sucrose over yeast extract, while mated females prefer yeast extract over sucrose (2010; 2010). This indicates that virgin females and male flies need and/or prefer more carbohydrates in their diet, while mated females need/prefer more protein in theirs. Further analysis by Ribeiro and Dickson showed that the sex-peptide receptor (SPR), which is located in *ppk*+ neurons in the reproductive tract, are necessary for modulation of female food choice once mating has occurred (2010). The pair also showed that sex peptide (SP), the primary ligand for SPR, is needed to impart a partial preference towards yeast extract, but does not fully induce this response; for a full response, SP and other SPR ligands may be needed (2010). Signaling through the TOR/S6K (Target of Rapamycin/ RPS6-p70-protein kinase) pathway is needed to regulate nutrient preference, balance, and homeostasis (Ribeiro and Dickson, 2010; Vargas et al., 2010). Ribeiro and Dickson have showed that activation and repression of the TOR/S6K pathway activate yeast feeding (2010). Vargas et al also found that S6K played a role in nutrient preference, by showing overexpression of S6K leads to virgins having a mated female preference for yeast; however overexpression had no effect on mated females feeding preference (2010). S6K interacts with serotonin somehow to induce the shift in preference to yeast extract in virgin flies (2010). What attracts mated females and induced virgins to yeast? Here, for the first time, it is shown that mated female preference to sucrose is shown to be concentration dependent, neuronal firing dynamics may play a role in sucrose preference, and virgin females and males can detect yeast extract at the

periphery. Lastly, the amino acid components of yeast are shown to be what the mated flies prefer.

Materials and Methods

Fly stocks

Flies were raised on standard dextrose-cornmeal-agar diet at 24-25°C at ≥ 50% humidity on a 12:12 hr light: dark cycle. Flies used were wCS, (white-Canton S).

Two-choice Feeding Preference Assays

Fifteen to twenty 0- to 1-day old flies were collected and maintained on fresh fly food for 3-4 days. Flies were then sorted into male and female groups, and virgins were transferred to fresh vials. On the subsequent day, they were transferred to starvation vials', containing wet Kim wipes, for 24 ± 2 hours. Flies were assayed the following day. Assays were run using tight-fit Petri dishes (Falcon 35-1006). Solutions containing the stimuli and either 0.25 mg/mL indigo carmine (Sigma I8130) or 0.5 mg/mL sulforhodamine B (Sigma 230162) were prepared before the start of the experiment in 0.75% agarose and spotted in the Petri dishes (Figure 5A). Starved flies were mildly anesthetized with carbon dioxide, transferred to the dishes, and placed in a humidified box at 25°C for 2 hours in the dark. Next, they were frozen at -80°C for twenty minutes, moved to -20°C, and their abdomens scored within 72 hours (Figure 5B). Data analysis includes trials where at least 50% of the flies participated, unless otherwise indicated. Experiments were performed between 2 and 6 PM. Preference index values were calculated using the formula:

$$(Nr + (Np)/2) / (Nr + Nb + Np)$$

where Nr is the number of red flies, Np is the number of purple flies and Nb is the number of blue flies.

Tastants

All chemicals were obtained from Sigma Aldrich.

Dose-curves

Dose curves were carried out through two-choice preference assays as described above with the stimulants sucrose and yeast extract. Participation rates were 50% and above, unless otherwise stated.

PER

Flies were collected from eclosion to one day of age. At three-four days old, flies were sorted based on sex, and starved the following day. On the day of the experiment, flies were placed in a pipette jacket in a humidified room. Flies were satiated with water before the experiment began. Alternating stimulants and blanks (water) were offered to the flies, and their responses recorded as 1, full proboscis extension, 0.5, partial proboscis extension, and 0, no proboscis extension. Experiments were performed from 2-7 pm in the same room. Multiple flies were tested and their responses averaged. If a fly responded to water, it was allowed to drink until full, and the assay continued.

Electrophysiology

Extracellular single-unit recordings were performed as in Dahanukar et al. (2001). Newly eclosed flies were transferred to fresh vials with standard cornmeal agar medium, and flies were aged for 5–10 days at 25°C. Action potentials were recorded from L-type sensilla of male, mated, and virgin female flies using TasteProbe (Syntech, Hilversum,

Netherlands). Neural response was quantified by counting the number of impulses generated in the 500-ms period beginning 200 ms after onset of stimulation. All tastants were dissolved in an aqueous solution containing 0.03 M tricholine citrate (Sigma) as the electrolyte. Stock solutions were stored in glass vials at −20°C. For recordings, aliquots of 500 – 1,000 µl were stored at 4°C and kept no longer than one week.

Statistics

Statistics were performed using SPSS software version 20, or Excel for student t-tests. Outliers were included in the analysis.

Results

Mated females show a dose dependent preference to sucrose

A dose curve of varying sucrose concentrations against 1% yeast extract was performed in order to see the responses of the three groups of flies, males, mated and virgin females, to sucrose. Males, mated and virgin females were compared at 1, 5, 10, 25, 50, and 100 mM sucrose. Male flies showed a strong preference to sucrose at all concentrations tested, with a plateau reached at 25mM sucrose. Virgin females were indifferent to sucrose or yeast extract when tested with 1mM sucrose. From 5mM to 100mM sucrose though, virgin females had a high preference for sucrose. The mated females were the only group that showed a dose-dependent preference to sucrose in this range of tested concentrations. The dynamic range of the males and virgin females could be different than what was tested here. At low concentrations of sucrose, 1 and 5 mM sucrose, they preferred yeast extract. At 10mM sucrose, they preferred each equally, while the higher concentrations of sucrose, 25, 50, and 100 mM, mated females showed a

high preference to sucrose. (Figure 14 A) This indicates that male and virgin female flies do prefer sucrose, and mated females prefer yeast extract at lower concentrations, as has been shown previously in the literature (Ribeiro and Dickson, 2010; Vargas et al., 2010). However, Ribeiro and Dickson used 20mM sucrose and 5% yeast extract in their tests, a very strong concentration of yeast with a strong smell (2010), and Vargas et al used 4% yeast extract and 146 mM sucrose (2010). Here, we used a lower concentration of 1%, which nevertheless induced a robust PER. Mated females are still highly attracted to the lower concentrations of yeast extract, indicating that the mating switch is very robust.

Males can detect yeast extract

With multiple sources showing that males strongly prefer sucrose, and none showing their preference to yeast extract (unless they had been deprived) (Ribeiro and Dickson, 2010), it was hard to tell if they could detect the yeast extract when presented with sugar. A proboscis extension response (PER) assay was performed to answer this question. A PER assay measures the direct behavioral stimulation of gustatory receptor neuron stimulation by touching a Kim wipe soaked in the solution of choice either to the labellum or the tarsi of the fly. Here, labellar PERs were performed on male, mated, and virgin flies. As can be seen in Figure 14B, males and virgin females can detect yeast extract at a range of concentrations. However, they appear to have a drive to prefer/ingest sucrose in place of yeast extract. Mated females can also detect yeast extract at a range of concentrations, as was expected, since mated females they have a strong preference for this compound after mating (Ribeiro and Dickson, 2010; Vargas et al., 2010). However, detection is variable at 0.1% yeast extract for mated and virgin females.

Differences in preference to sucrose may be due to neuronal firing rate

Since there are some differences in preference to sucrose, it was possible that the peripheral taste system may have an influence on neuronal input and detection across sexes and mating statuses. Male, mated, and virgin females have similar initial firing rates to 100mM sucrose, with males having (14 ± 1, n =11), mated (15 ± 1, n =12), and virgins (10 ± 2, n =11) spikes per second (Figure 15A). Mated females and males have similar firing rates when stimulated with 100mM sucrose, (males have (119 ± 18, n =11) spikes/sec, mated females (137 ± 12, n =12) spikes/sec, and virgins have an initial firing rate of (95 ± 18, n =11) spikes/sec (Figure 15B).

Electrophysiological dose-curve dynamics

After looking at electrophysiological dynamics for one concentration of sucrose, the neural dynamics when stimulated with a variety of other concentrations of sucrose was tested. The same concentrations of sucrose tested earlier in the behavioral dose curve were tested with electrophysiology (1, 5, 10, 25, 50, and 100 mM sucrose), along with 250 mM sucrose to see how a much higher concentration would affect dynamics (Figure 16A). Tricholine citrate (TCC) was a negative control, since it was used as a buffer in the solution. There was some spiking in TCC by virgin neurons, probably either due to mechanical stimulation or contamination. At 1 and 5mM sucrose, virgins had a higher spike rate than mated females and males. At 10 and 25 mM sucrose, all three groups had similar spike rates. Mated females started surpassing the virgin and male spike rates at 50 mM sucrose, and this continued on to 100 and 250 mM sucrose; interestingly, virgin and male spike rates were about the same at these higher concentrations. These results

indicate that mated females could need some form of temporal coding in order to be as attracted to sucrose as virgin female and male flies.

The initial firing rate was also calculated for all three groups (Figure 16B). Virgin females had a higher initial firing rate from 1-25 mM sucrose. All three had similar initial firing rates at 50mM sucrose; and at the two highest concentrations of sucrose tested, 100, and 250 mM, mated females and males had higher firing rates than virgins did. The higher initial firing rate for virgin females' at lower concentrations correlates with the higher average of spikes per second at the lower concentrations of sucrose tested. This may indicate that some process is activating response to sugars in virgin females at lower sucrose concentrations and is being repressed in mated females. It has been shown that sex peptide leads to preference of yeast extract in females (Ribeiro and Dickson, 2010; Vargas et al., 2010), so some component of male seminal fluid may lead to the reduction in signaling in the mated female at lower sucrose concentrations so that yeast extract is preferred. Therefore, it appears possible that temporal dynamics play a role in how sucrose is perceived. Yet, more exploration needs to be performed in order to tease apart this process further.

Mated females are attracted to amino acids in yeast extract

Yeast extract is composed of all of the twenty most common amino acids (including 5% glutamate), carbohydrates, B vitamins, and salts (Products, 2011). The yeast extract used in these experiments was composed of 0.2% salt, amino acids, and carbohydrates. Reproducible results from Vargas et al (2010) and Ribeiro and Dickson (2010) were obtained; females do in fact switch their preference from sucrose to yeast

extract after mating. In order to identify which component of yeast extract the mated females are attracted to, each of the major components of yeast extract was tested separately against 5 mM sucrose. The 5 mM sucrose standard was chosen because the dose curve indicated that this concentration was where mated females preferred yeast extract, and male and virgin females preferred sucrose. The yeast extract PER also indicated that detection of 1% and 5% yeast extract at the periphery is similar. The main components of yeast tested were the predominant amino acid glutamate (at 5%), 100 mM trehalose (one of the main storage carbohydrates produced by *S*. *cerevisiae*) (Francois et al., 2012), and 0.2% sodium chloride (the percentage present in the yeast tested). If these components were attractive to the mated female, then her change in feeding preference would have looked similar to that of the full yeast profile; in other words: that individual component would be what the mated female prefers (Figure 17). The mated females showed equal preference to 5% glutamate and 1% yeast extract, and virgin females preferred 1% yeast extract over 5% glutamate.

Mated females showed a high preference to trehalose and glycerol, however so did the virgin females and males. Therefore, it is unlikely these compounds underlie the behavioral switch of mated females. Both mated and virgin females had equal preference to salt and yeast extract; so salt is not what mated females are drawn to. Lastly, the mated females' preference for yeast extract without the presence of amino acids was tested. This showed that mated females preferred the amino acids in the yeast extract, as they were avoiding the trace elements left in the yeast extract. Males and virgin females not attracted to this mixture either, yet there still was a significant difference between virgin

and mated females. The PMR feeding response was heavily reduced in the mated female flies, as they did not prefer yeast extract lacking amino acids. Thus, amino acids appear to be the primary cues for PMR, although some other yeast components may factor in as well.

Discussion

Currently, our knowledge shows us how molecular factors, external factors, and physical factors all come together to play a role in the circuitry of an animal. This information also shows how plastic behavior is; just the act of mating changes so much about an animal neurologically and behaviorally. A multitude of studies have delved into the differences between male and female flies at the molecular, behavior, and neurological level, but relatively few studies have looked at the differences between mated and virgin female flies. Other studies have shown that mated females increase or change their feeding preference, but they do not indicate what she is attracted to (Carvalho et al., 2006; Vargas et al., 2010; Ribeiro and Dickson, 2010). This study is the first to our knowledge, that shows mated females are drawn to amino acids or peptide fragments within yeast or yeast extract. These tests also indicate that mated female flies prefer sucrose in a concentration dependent manner, and neuronal firing dynamics may play a role in the difference in sucrose sensitivity between virgin and mated females.

For years, it has been known that mated females prefer yeast extract to sucrose, yet there was no further research as to why. It was assumed because she needed a high protein source to produce eggs and the components necessary for them (Drummond-Barbosa and Spradling, 2001). By testing a variety of different components of yeast

extract, this study shows that it is the amino acids, and possibly other peptides, that attract the mated female. She may prefer yeast because it has a variety of amino acids that can be used to make proteins for the resumption of oogenesis. Furthermore, there has been research performed on the effects of single amino acids on fertility. Grandison et al. found that methionine increases fecundity in the mated female without affecting her lifespan (2009). Future studies can be performed to look at the effects of yeast extract with missing amino acids to see how preference, fecundity, and lifespan interact. Applications of these studies could be applied to other insect pests in order to create traps and control their populations

Data collected in this study also strongly support the notion that males and virgin females can detect yeast extract. This indicates that differences in behavioral sensitivity are likely not caused by differences in peripheral sensitivity. However, males highly prefer any concentration of sucrose to the 1% yeast extract that was tested, while virgin females highly prefer concentrations of sucrose of 5 mM and above over 1% yeast extract. This verifies that males and virgin females can in fact respond and detect yeast, but they choose not to consume it. This lack of preference may be because they have fewer protein needs than mated females, or that sucrose overrides their need to consume yeast. Sex peptide is involved in this switch from preferring sucrose to yeast extract in females (Ribeiro and Dickson, 2010; Vargas et al., 2010), which explains the dynamics shown here.

Mated *Drosophila* shows a concentration dependent response to sucrose; they preferred higher concentrations of sucrose over 1% yeast extract. This is good to note

because Ribeiro and Dickson used 5% yeast extract in their experiments and a low concentration of sucrose, 20mM (2010). Since 1% yeast extract is much more dilute than 5% yeast extract, it is less potent both in smell and taste properties, and 5% yeast extract may overwhelm the properties of the 20mM sucrose. The mated flies did prefer the yeast extract to lower concentrations of sucrose (1, 5, and 10 mM), but here at 25mM, they began to choose the sucrose over the yeast extract. From this study, Ribeiro, and Dickson's study, it can be inferred that mated females do have a strong preference towards yeast, but at some point, this can be overridden if there is enough sucrose present to overcome this desire.

This dose dependence may depend on the temporal dynamics of the initial firing rate of the sugar neuron when stimulated with sucrose. The spike frequency varied greatly from virgin females, (95 ± 18, n =11), to mated females, (137 ± 12, n =12), to males (119 ± 18, n =11) when stimulated with 100mM sucrose (Figure 15B). This could indicate that virgins are more sensitive to 100mM sucrose, since they sense it with lower firing dynamics than males or mated females. The sucrose-dose curve allowed the temporal dynamics to be parsed out; virgins tended to respond to the lower sucrose concentrations with a higher spike rate, and higher sucrose concentrations with a lower spike rate than the other two groups. Mated females responded to low concentrations of sucrose with low spike rates, and high concentrations with a high spike rate. Male flies showed dynamics that were in between virgin and mated females. These data correlate well with the behavioral two-choice data, where virgins preferred lower concentrations of sucrose, and mated females did not have strong preferences for lower, but for higher

concentrations of sucrose. Preferences of these concentrations may be induced by the neuronal firing rate of each fly group. This is further supported by the fact that the initial firing rate (the first 100msec of contact) of virgins is much higher than that of males or mated females up to 25mM sucrose. At 50mM sucrose and above, the initial firing rate of mated females and males overtakes that of the virgins. The fast initial firing rate for virgins when exposed to low concentrations of sucrose would help them consume needed carbohydrates in the wild, while the low initial firing rate for mated females would make sure to drive them towards yeast, or other protein rich foods. Protein rich foods are needed to support the multitude of behavioral changes mated females will be undergoing (oogenesis, egg laying, etc.). The higher initial firing rate of the mated females toward higher concentrations of sucrose may tell her to "stock up" on carbohydrates that she needs; since mated females need sucrose too. Male fly temporal dynamics seem to be in-between those of mated and virgin flies. This may be because males do not undergo as many physiological or behavioral changes as the female of the species does after mating. A possibility exists that mechanisms underlying reduced sensitivity to yeast extract in males and virgin females may be different from in mated females; or males and virgin females have an increased sensitivity to sucrose compared to mated females.

Figures

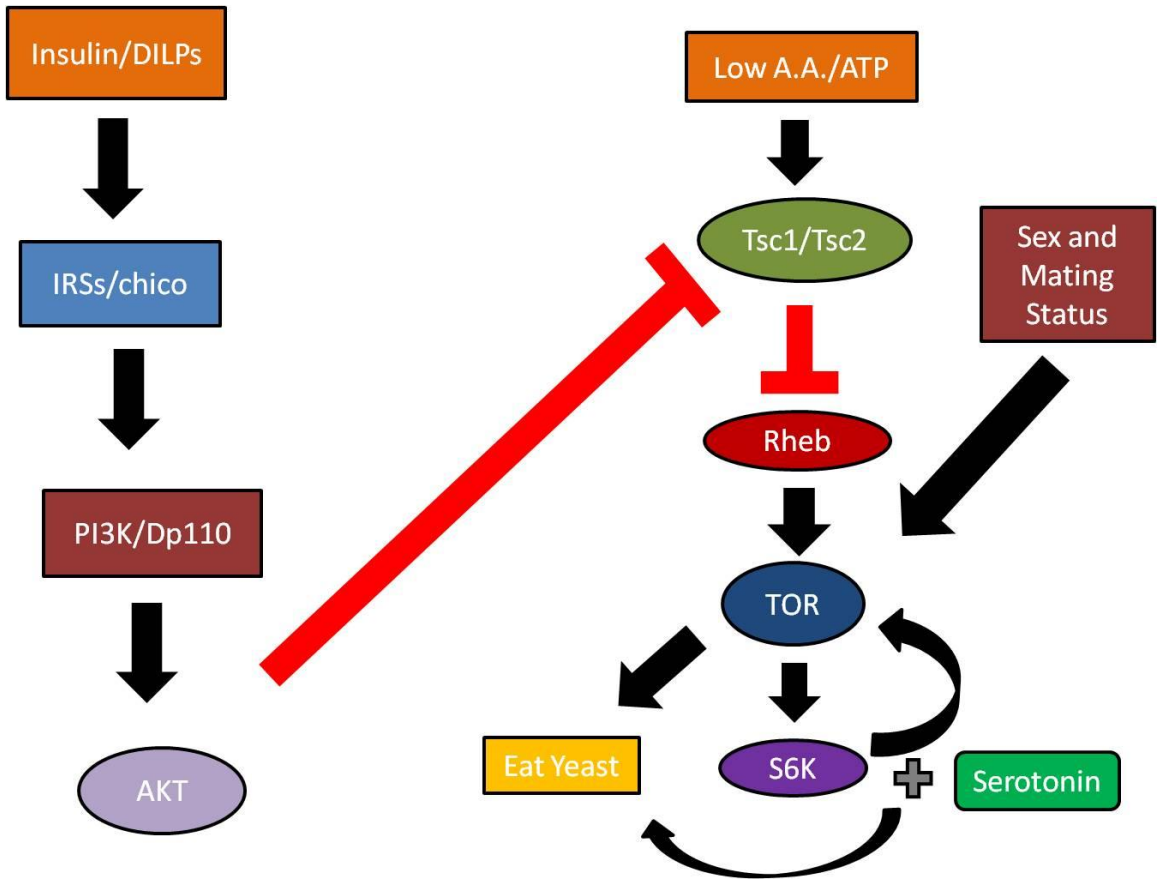
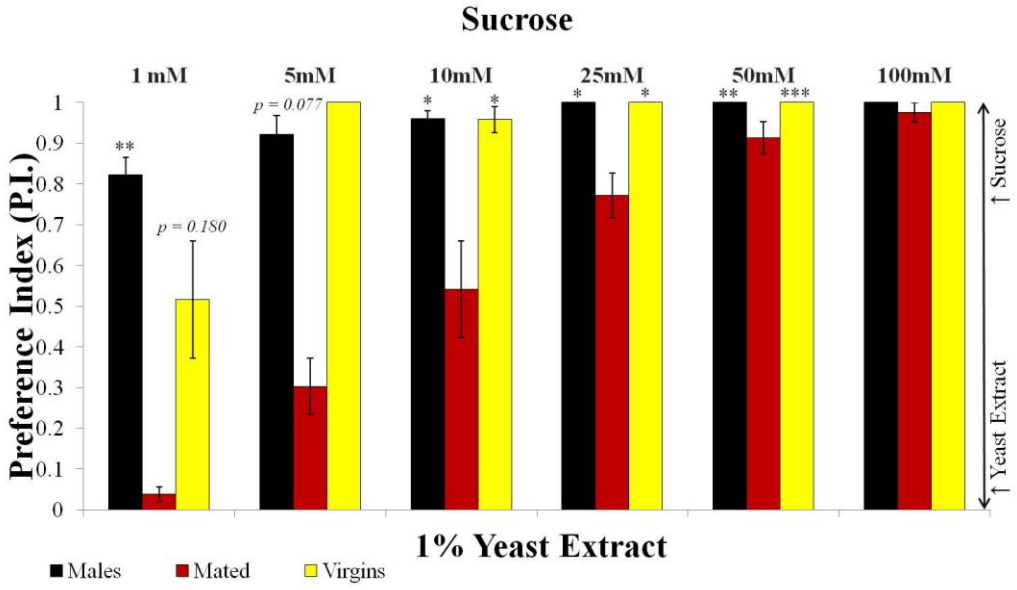
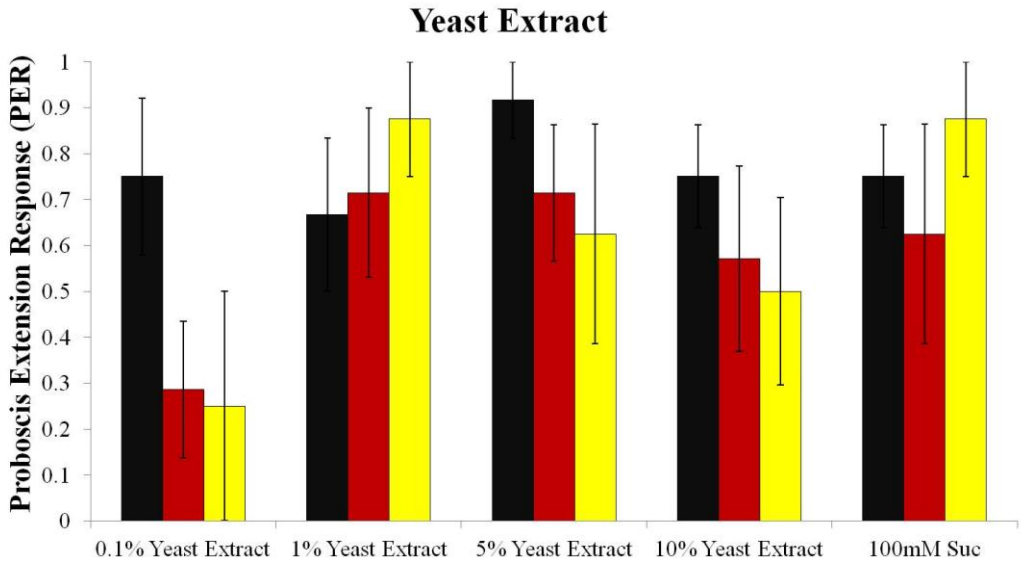


Figure 13 The TOR/S6K pathway. When Rheb, a GTPase, activates TOR, S6K is also activated, and flies start to prefer yeast. If the fly senses a low amount of amino acids or ATP in its system, Tsc1 and Ts2 are activated in order to repress Rheb, and prevent it from activating TOR. However, sex and mating status have an effect on this pathway, as does the presence of serotonin. The insulin signaling pathway in *Drosophila* can also have an effect on the TOR pathway. When insulin or *Drosophila* insulin-like peptides bind to the insulin-receptor substrate (IRS), phosphatidylinositol-3 kinase (PI3K) is activated and goes on to phosphorylate AKT. AKT can then block the TOR/S6K pathway.

A)

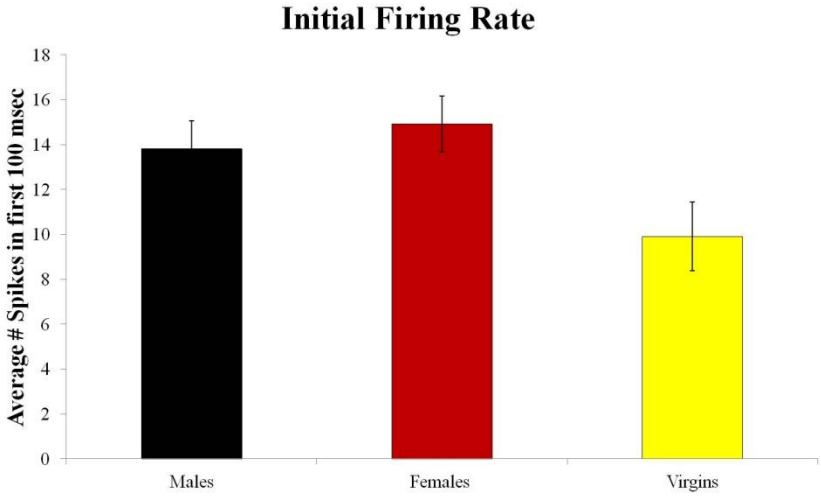




B) Males Mated Virgins

Figure 14 A) Sucrose dose curve; Participation cut off was 35% and above for males and virgins at 1 and 5mM; the cutoff was 50% and above for remaining concentrations. Male n = 3-7; mated n = 3-6; virgin n = 2-8, except at 5mM sucrose, where n = 1.* = $p \leq 0.05$, ** = $p \leq 0.01$, and *** = $p \leq 0.001$. Error bars are S.E.M. **B) Flies can detect yeast extract**. n = 6, 7, and 4 for male, mated, virgin (except for mated, 100mM sucrose, where n =3). Error bars are S.E.M. No significant difference was found by performing Kruskal-Wallis H test.

A)



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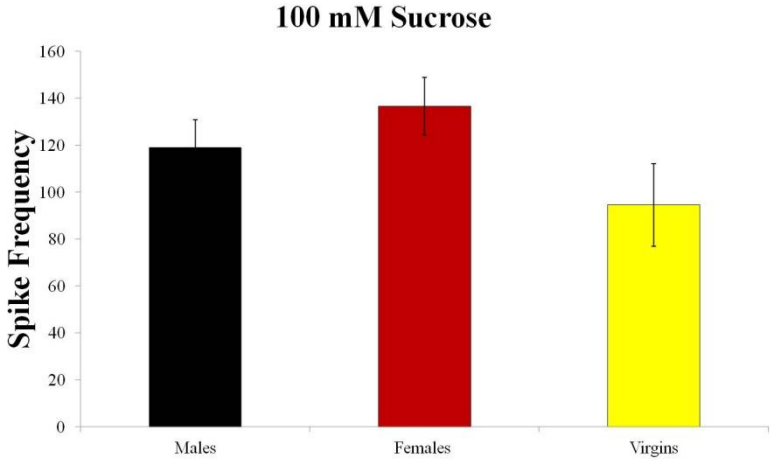
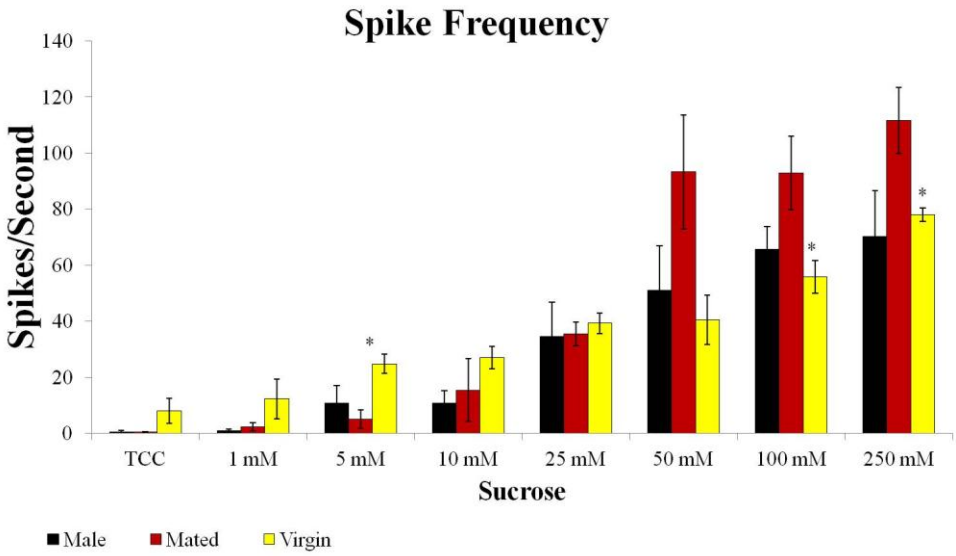


Figure 15 A) Initial firing rate of L-sensilla to 100mM Sucrose. Males 14 ± 1, n =11, mated 15 ± 1, n =12, and virgins 10 ± 2, n =11 spikes per second. Mated females and males have similar firing rates when stimulated with 100mM sucrose, males 119 ± 18, n =11 spikes/sec, mated females 137 ± 12, n =12 spikes/sec, and virgins 95 ± 18, n =11 spikes/sec. (spikes per sec ± S.E.M., n). **B) Spike frequency of L-Sensilla to 100mM sucrose.** Males (119 ± 18, n =11), (Mated) females, (137 ± 12, n =12), virgin females, (95 ± 18, n =11). (spikes per sec ± S.E.M., n). There was no statistical significance as determined by a t-test.

A)



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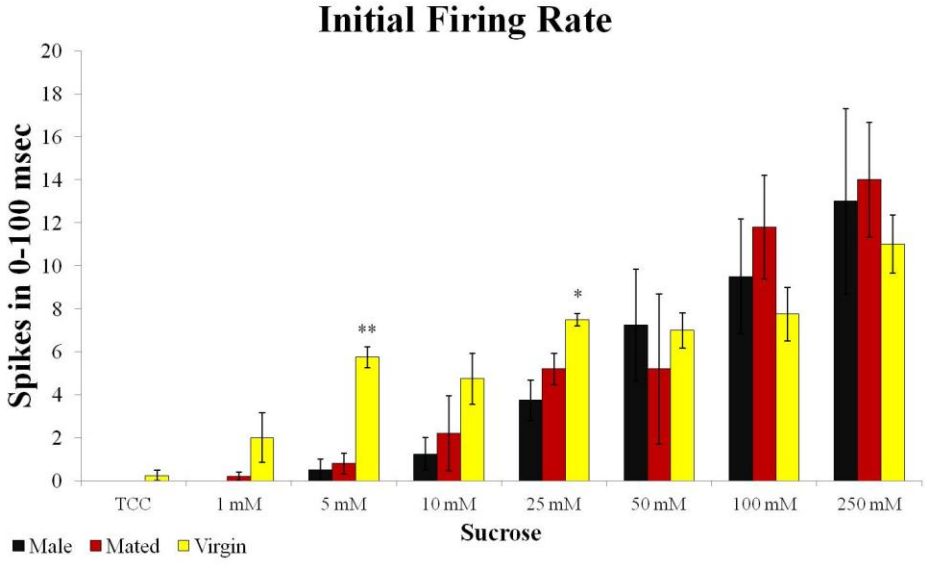


Figure 16. **A) Spike frequency to Sucrose.** TCC = tricholine citrate. Males, n = 4, Mated females, n = 5, except 250mM where n = 5, Virgin females, n = 4 **B) Initial firing rate to sucrose.** TCC = tricholine citrate. Males, n = 4, Mated females, n = 5, except 250mM where n = 5, Virgin females, n = 4. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.005$. Students' t-test.

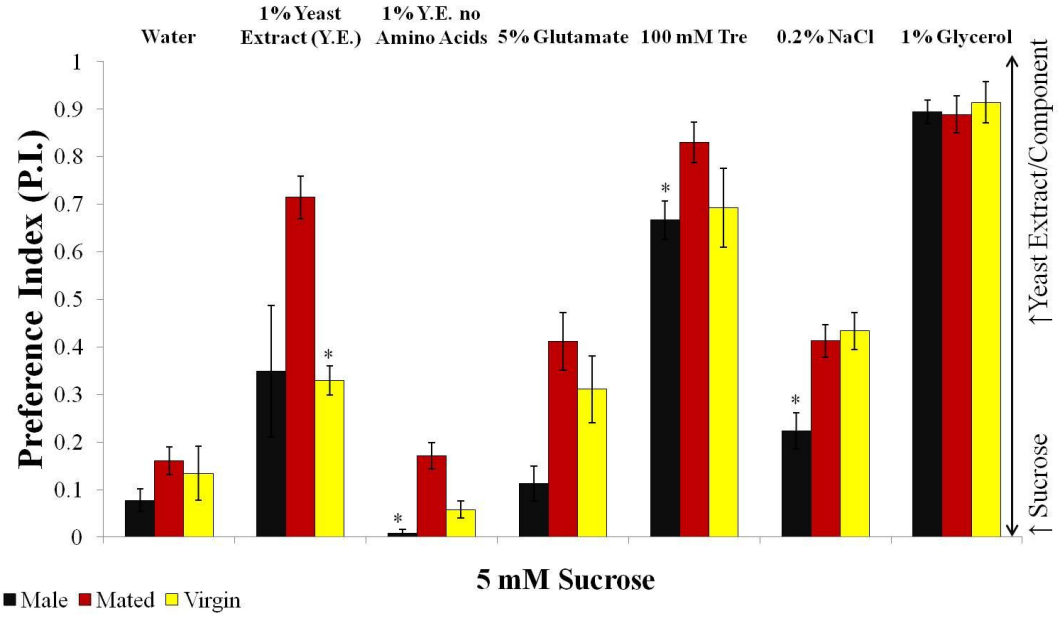


Figure 17 Mated females are attracted to amino acids. Water n = 9-18; 1% Yeast Extract (Y.E.) n = 3-8; 1% Yeast Extract no amino acids (Y.E. no A.A.) n = 3-4; 5% Glutamate (Glu) n = 5-7; 100mM Trehalose (Tre) n = 6-15; 0.2% Sodium Chloride (NaCl) n =4. Participation is ≥ 50%. *= p <0.05. Pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons.

Chapter 4 Oviposition and the Gr5a receptor

Abstract

Oviposition is the process of egg laying. Many insects oviposit, including *Drosophila melanogaster*. Previous studies have shown that *D. melanogaster* avoid ovipositing in quinine and sucrose, but prefer to oviposit in lobeline. Gr66a-expressing neurons in the anterior legs and in the pharynx mediate this attraction to lobeline; the repulsion to sucrose seems to be due to Gr5a-expressing neurons. After further research, this study shows that Gr5a is involved in oviposition between different sugars. Here, further evidence is gathered that Gr5a is involved in the repulsion of egg laying in sucrose. Oviposition preference was determined by counting the eggs flies laid in each agar spot of the two-choice preference assay chambers. The role of Gr5a in oviposition was examined by comparing a Gr5a mutant with flies that ectopically expressed Gr5a through the Gal4/UAS system. Gr5a is found to play a role in oviposition preference when Gr5a dependent sugars are present. In addition, Gr5a appears to play no role in oviposition preference when Gr5a independent sugars are involved. In conclusion, Gr5a plays an important role in oviposition choice.

Introduction

The process of mating entails the transfer of sex peptide and other accessory proteins from the male to the female. These proteins induce oviposition. Ovulin, one of these accessory proteins, induces ovulation and egg laying behavior for about twenty-four hours after mating. After this time has passed, the accessory protein Sex Peptide (SP) is mainly responsible for maintaining oviposition activity. Before she has mated, the virgin female lays few eggs; without the signals she receives from the male, oocyte (egg)

development is arrested. Some mature eggs develop, and these are laid, but for the most part, the females' eggs are arrested. After mating, oocyte progression continues to maturation and the female lays mature eggs, which are fertilized as they pass through the uterus. Sperm that is stored in seminal receptacles fertilizes the mature eggs as they pass by the openings. From one mating, a mated female can lay between 300-400 eggs. (Schnakenberg et al., 2012).

Oviposition involves searching for a suitable oviposition site, ovipositing, cleaning the ovipositor, resting, and repeating the process as needed (Yang et al., 2008). When they are given a choice, mated females prefer to lay their eggs on lobeline in lieu of sucrose; however they avoid lobeline in two-choice feeding assays (Yang et al., 2008; Joseph and Heberlein, 2012). This avoidance of sucrose is concentration dependent; at very low concentrations of sucrose, 0.5 and 1 mM, mated females will have no preference between sucrose and lobeline (Yang et al., 2008). In addition, this avoidance of sucrose is context dependent; mated females will choose to lay eggs in sucrose if the other option is a region of much higher concentrated sucrose (2008). Females also chose to lay eggs on plain agar over sucrose, and have almost equal preference to lobeline and plain agar sites (2008). Flies have an almost equal preference of oviposition sites of plain agarose and lobeline (2008). Yang et al. went on to show that oviposition avoidance on sucrose containing media is dependent on Gr5a neurons (2008). Interestingly, mated female flies will oviposit on sucrose medium if they have no other option or if there is another medium more repulsive in the vicinity (2008). From this, it can be inferred that

oviposition choice is extremely plastic. Sucrose is an attractive tastant, but an aversive oviposition site, while lobeline is an aversive tastant but an attractive oviposition site.

How can lobeline be attractive for oviposition and aversive for feeding? How can sucrose be attractive for feeding, but aversive for oviposition? Both behaviors require the use of the proboscis, adding to the mystery. Recently, it was discovered that *Gr66a*-expressing neurons in the anterior legs send signals to the SOG; from there the signal moves to the mushroom body (MB), which tells the fly that lobeline is an aversive tastant (Joseph and Heberlein, 2012) . Internal *Gr66a*-expressing neurons in the pharynx have been found to send signals to the SOG; they then travel to the MB and relay that lobeline is a good oviposition substrate (Joseph and Heberlein, 2012).

Yang et. al. have shown that *Gr5a*-expressing neurons are needed in order to avoid laying eggs on sucrose media (Yang et al., 2008). *ILP7*-neurons have also been found to be important for females to select oviposition sites (Yang et al., 2008). These ILP7-neurons may relay the acceptability of egg laying sites to the reproductive tract and on to the ovipositor motor program (Yang et al., 2008). It is possible that these Gr5a, Gr66a, and ILP7-expressing neurons integrate in the mushroom body to send signals to the ovipositor that lobeline is a good substrate while sucrose is a less suitable substrate for ovipositing. Gr5a-Gal4 neurons detect sucrose and are responsible for the low number of eggs laid on high concentrations (Yang et al., 2008), while Gr66a-Gal4 neurons help flies choose to lay eggs on a lobeline substrate (Joseph and Heberlein, 2012). The Yang group tested oviposition preferences of sucrose verses lobeline, and different concentrations of sucrose verses sucrose, while Joseph and Heberlein tested preference

on lobeline verses agar (Yang et al., 2008; Joseph and Heberlein, 2012). In order to understand how flies choose where to lay their eggs, more experiments with different combinations of tastants need to be performed. This study shows the preliminary results of some of those needed experiments.

Materials and Methods

Fly stocks

Flies were raised on a standard dextrose-cornmeal-agar diet at 24-25°C at ≥ 50% humidity on a 12:12hr light: dark cycle. The flies chosen for the control in the initial screenings were wCS, (white-CS). Flies screened in the mutant panel were ΔGr64f/ΔGr64f, ΔGr64a/ΔGr64a, ΔGr64e, ΔGr43a, ΔEP5/ΔEP5 (P-element deletion of Gr5a), ΔGr61a/ΔGr61a, and ΔGr5a; ΔGr64a/ΔGr5a; ΔGr64a. Later screenings used EP(X)0496/FM7i (as a control), ΔEP5/ΔEP5 (ΔGr5a), and ΔEP5/ΔEP5; Gr5a-Gal4-6; UAS-Gr5a-3 (ΔGr5a rescue). The following cross was performed to create ΔGr5a rescue flies:

ΔEP5/FM7 virgins **X** +/Y; Gr5a-Gal4-6; UAS-Gr5a-3 → ΔEP5/Y; Gr5a-Gal4-6; UAS-Gr5a-3

ΔEP5/FM7 virgins **X**ΔEP5/Y; Gr5a-Gal4-6; UAS-Gr5a-3 → ΔEP5/ΔEP5; Gr5a-Gal4-6; UAS-Gr5a-3 ♀ & ♂

After flies were obtained, they were kept at 24-25°C at ≥ 50% humidity. All flies were raised as mentioned above.

Two-Choice Preference Tests

Fifteen to twenty 0- to 1-day old flies were collected and maintained on fresh fly food for 3-4 days. Flies were sorted into male and female groups. Virgins were transferred to fresh vials. On the subsequent day, they were transferred to starvation vials', containing wet Kim wipes, for 24 ± 2 hours. Flies were assayed the following day. Assays were run

using tight-fit Petri dishes (Falcon 35-1006). Solutions containing the stimuli and either 0.25 mg/mL indigo carmine (Sigma I8130) or 0.5 mg/mL sulforhodamine B (Sigma 230162) were prepared before the start of the experiment in 0.75% agarose and spotted in the Petri dishes. Starved flies were mildly anesthetized with carbon dioxide, transferred to the dishes (Figure 5A), and placed in a humidified box at 25°C for 2 hours in the dark. Then they were frozen at -80°C for twenty minutes, moved to -20°C, and their abdomens scored within 72 hours (Figure 5B). Data analysis includes trials where at least 50% of the flies participated, unless otherwise stated. Experiments were performed between 2 and 6 PM. Preference index values were calculated using the formula:

$$P.I. = (Nr + (Np)/2) / (Nr + Nb + Np)$$

where Nr is the number of red flies, Np is the number of purple flies and Nb is the number of blue flies.

Tastants

All compounds were obtained from Sigma-Aldrich.

Egg Laying

The number of eggs laid in each stimulus (blue or red) was counted per Petri dish from the two-choice feeding preference assays. Only plates with 50% or more participation were used, unless otherwise noted.

Statistics

Statistical testing was performed with IBM SPSS Statistics 20 software, or Excel (Student's t-test). Outliers were included in the analysis.

Results

ΔGr5a shows an oviposition phenotype

Oviposition assays were performed on other sugar mutants to investigate their oviposition phenotypes, if any. A two-choice feeding preference assay was set up so flies could choose oviposition sites while they ate. This is in contrast to other studies, where oviposition preference was assayed alone. However, this setup is akin to that in the wild; flies are more likely to oviposit near or in food sources. This egg-laying assay is more akin to how natural environments are and gives a more accurate reading of their preference.

Mated females prefer yeast extract over sugars, and usually avoid ovipositing in sucrose (Ribeiro and Dickson, 2010; Vargas et al., 2010; Yang et al., 2008). The question remained: where would flies chose to lay their eggs when given a choice between yeast extract (an attractive substance) and sucrose (attractive in general and aversive for egg laying)? Four of the other sugar receptor mutants were tested to investigate their oviposition phenotype, ΔGr61a, ΔGr64a, ΔGr64e, ΔGr64f, alongside the double mutant ΔGr5a; ΔGr64a, ΔGr43a, and ΔGr5a (Figure 18A and B). The double mutant have largely no sugar response in labellar hairs, since the two main sugar receptors are missing (Dahanukar et al., 2007). The number of eggs that most mutant groups laid was similar, ranging from ΔGr64e laying the lowest overall with 758 eggs, and ΔGr64a and ΔGr61a both laying the highest at 1,033 eggs. The double mutant laid the fewest eggs out of all the mutant lines, with 384 eggs.

For comparison, the wild-type control, wCS, laid 150 eggs, 92% of which were in 100mM sucrose and 7% in yeast extract. Most of the mutants chose to lay the majority of their eggs in 100mM sucrose, (70%-99%), with the remainder of the eggs being oviposited in the yeast extract. This seems odd, because it has been shown that flies avoid sucrose; however, yeast extract may be an aversive oviposition site. The only Gr that showed a drastic switch in preference was ΔGr5a, with 88% of their eggs being laid in yeast extract, and 12% being laid in 100mM sucrose, akin to previous studies. This further implicates Gr5a as being a major player in oviposition site selection. *Gr5a* mutants had a feeding preference for 100 mM sucrose, but an oviposition preference for 1% yeast extract. The other mutants showed an oviposition and feeding preference to 100 mM sucrose. This further supports Gr5a being involved with egg laying.

Virgin female oviposition preferences are similar to mated females

Virgin female flies do not lay many eggs, but as they grow older, the number of eggs they lay increase over time (Figure 19B). Some mutant virgin females take a longer time to lay eggs than others do. ΔGr64f, ΔGr5a, and ΔGr61a virgin females aged five to six days were the only genotypes to lay eggs (Figure 19A); virgin females aged nine to ten days that laid eggs included the wild type, ΔGr64f, ΔGr64a, ΔGr43a, and ΔGr5a (Figure 19B). Interestingly, virgin female ΔGr5a flies laid the majority of their eggs in yeast extract, the same as their mated counterparts, which was also consistent over time; the other mutants laid most of their eggs in sucrose. Virgin females preferred 100mM sucrose to yeast extract, whereas there was a decrease in preference across mated

genotypes (although the majority of them still preferred sucrose). Feeding and oviposition preference remain consistent over time and mating status.

Gr5a plays a role in oviposition

When the parental strain of ΔGr5a, EP(X)0496, was tested, it was shown that most eggs were laid in sucrose, and the flies also preferred sucrose to yeast extract (Figure 20). Gr5a's role in oviposition was thus tested further, as the mutant phenotype was not a result of the genetic background. The feeding and oviposition preference of the ΔGr5a was then observed when given a choice between 100 mM trehalose and 100 mM sucrose, as trehalose is a ligand for Gr5a (Dahanukar et al., 2001; Chyb et al., 2003) (Figure 21). The EP(X)0496 background strain had a feeding and oviposition preference for 100 mM sucrose (56%), while the ΔGr5a had a feeding preference for 100 mM Sucrose, and an oviposition preference for 100 mM trehalose (94%). This is in contrast to what Yang et al. found (Yang et al., 2008). They silenced the *Gr5a* neurons by hyperpolarizing them, and saw that flies had no preference for egg laying sites, either 100 mM sucrose or 500 μM lobeline (Yang et al., 2008). This may be because Yang et al. tested lobeline against sucrose and here sucrose versus trehalose was tested, another sweet tasting substance. Another possibility, Yang et. al. silenced the Gr5a neurons, while this study uses a Gr5a mutant in which other sweet Grs are still expressed. Either way, ΔGr5a flies seem to be avoiding sucrose to the fullest extent possible. In addition, ΔGr5a and EP(X)0496 laid a similar number of eggs (322 and 312, respectively, in trehalose and sucrose), which indicates that ΔGr5a does not affect egg formation or deposition, only oviposition preference.

To investigate whether Gr5a causes this oviposition phenotype, further compounds and conditions were tested. A rescue line was created by genetically manipulating the flies, so a fly without the Gr5a coding region, as well as exogenous expression of the Gr5a receptor through the Gal4-UAS system could be tested. The feeding and oviposition preference of flies to trehalose or sucrose was investigated at first. Both males and females of all genotypes preferred sucrose to trehalose (Figure 22A). As for oviposition choice, EP(X)0496 laid the majority of their eggs in sucrose, while ΔGr5a, Gr5a-Gal4; UAS-Gr5a, and ΔGr5a/ΔGr5a; Gr5a-Gal4/+; UAS-Gr5a/+ preferred to lay their eggs in trehalose. ΔGr5a/ΔGr5a; Gr5a-Gal4/+; UAS-Gr5a/+ failed to rescue ΔGr5a (Figure 22B); this may be due to the rescue being heterozygous for Gr5a expression, the limited number of trials here, or both. It is also possible that the Gr5a-Gal4 driver does not rescue all the neurons that are necessary for this behavior.

Next, sucrose was paired up against maltose. Both sucrose and maltose are Gr64a dependent sugars, so this test served as a control. Since maltose and sucrose are both Gr64a sugars, the hypothesis was that flies would prefer each site equally both for food and oviposition sites (Figure 23), which they did.

Preference to glucose or sucrose was then tested (Figure 24). All of the genotypes tested preferred ingesting sucrose over glucose; this was hypothesized, since sucrose is sweeter and more attractive than glucose. Again, the ΔGr5a flies avoided sucrose and instead chose glucose as the site to lay their eggs; the ΔGr5a/ΔGr5a; Gr5a-Gal4/+; UAS-Gr5a/+ also preferred glucose. However, again the rescue did not fully restore the phenotype. Feeding and oviposition preferences were then tested for sucrose against m-a-

glucoside, another Gr5a dependent sugar (Figure 25). All sexes and genotypes preferred sucrose. Here, ΔGr5a also preferred to avoid sucrose and lay most of their eggs in m-a-glucoside. ΔGr5a/ΔGr5a;Gr5a-Gal4/+;UAS-Gr5a/+ resulted in a partial rescue; it also preferred to lay eggs in m-a-glucoside, yet their preferences were not as extreme as that of ΔGr5a, as it was approaching that of EP(X)0496. The last sugar pair, sucrose verses melezitose (a Gr5a dependent sugar), resulted with a feeding preference towards sucrose (Figure 26A). Again, ΔGr5a avoided laying eggs in sucrose, and chose overwhelmingly to lay them in melezitose; ΔGr5a/ΔGr5a; Gr5a-Gal4/+; UAS-Gr5a/+ again resulted in a partial rescue (Figure 26B).

Other investigators have shown that flies prefer to lay their eggs in lobeline (Yang et al., 2008; Joseph and Heberlein, 2012). Yet, no other sugar has been compared against lobeline. Here, trehalose was paired against lobeline to investigate where they would lay their eggs (Figure 28). Trehalose was picked, as it is one of the main sugars in yeast extract, which mated females prefer. Here, EP(X)0496 preferred to oviposit in trehalose, while ΔGr5a flies oviposited mainly in lobeline. Oviposition preference was rescued slightly. Similar results were obtained when sucrose was paired against lobeline (Figure 27). Here, however the rescued phenotype was higher than with any other pairing.

Discussion

Why would flies choose to lay their eggs in bitter substances such as lobeline, and avoid sweet substances such as sucrose? There is a variety of possibilities for this. One is that *Drosophila* larva cannot sense sweet substances at the level that the adult can. Studies have shown that larva lack the Gr-Gal4 expression of sweet gustatory receptors

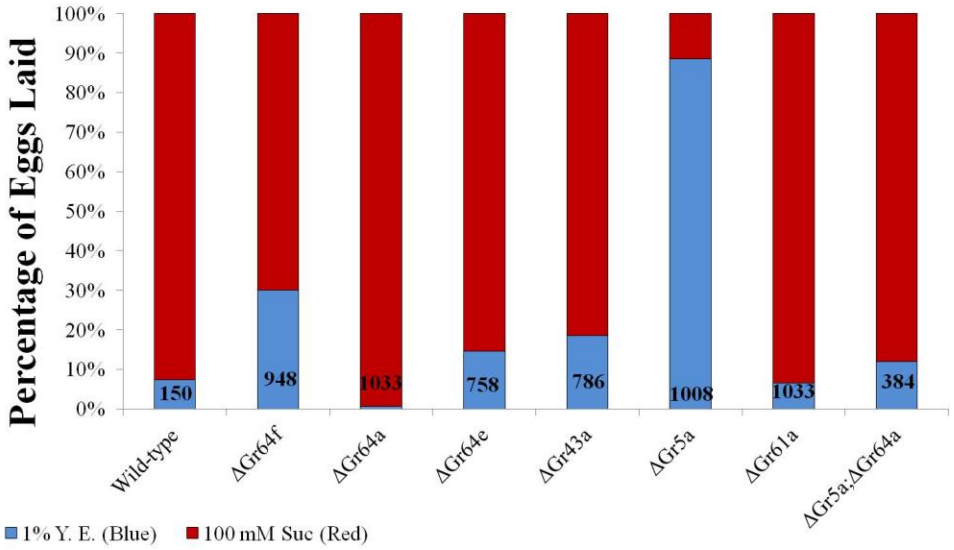
Gr5a, Gr61a, and Gr64a-f (Kwon et al., 2011). However, there is expression of Gr43a, which detects fructose; and as they mentioned, there may be other Grs, IRs, or other receptors that detect sugars (Kwon et al., 2011; Miyamoto et al., 2012). Larva must have some way of detecting sugars, since reports indicate that they can detect and prefer certain types of sugars (Schipanski et al., 2008). Larvae prefer glucose at 2 M and trehalose at 200 mM after a few minute exposure (2008). Surprisingly, larva have an innate preference for 20 mM, 200 mM, and 2M sucrose and 20 mM, 200 mM, and 2 M fructose (2008). Concentrations of sucrose and fructose also have an effect on feeding. High levels of sucrose and fructose appear to inhibit feeding, as food consumption decreases at 2 M and 4 M (2008). This may be because larvae are eating machines and need to eat constantly. Perhaps when the concentration is high enough, they have gained enough calories, and thus feeding is inhibited. On the other hand, low levels of sucrose (200 mM) and fructose (20 mM) appear to activate feeding (2008).

Seeing as how females prefer to lay eggs in bitter substances, larva appear to lack the canonical sugar receptors, and high levels of sucrose and fructose inhibit feeding, it makes sense that sucrose would be avoided as an oviposition substrate. Adult flies prefer sweet substances to bitter substances. By laying eggs in bitter conditions, the mothers could be avoiding the potential for other flies and insects to reduce the food source for their offspring. Oviposition preference for lobeline could also deter predators from consuming eggs and larvae. However, bitter compounds are generally aversive, and larva do express many bitter Grs (Kwon et al., 2011).

When egg-laying preference was assayed by ΔGr5a/ΔGr5a; Gr5a-Gal4/+; UAS-Gr5a/+, only a partial rescue occurred, with a phenotype usually between that of EP(X)0496 and ΔGr5a. This may be because the production of the Gr5a protein by the Gal4/UAS system is not enough to confer wild-type status. Gr5a-Gal4; UAS-Gr5a expression occurs in the proboscis and the tarsi of adult flies (Chyb et al., 2003). Expression of Gal4/UAS-Gr5a can also be found in multidendritic neurons in the abdominal wall of the fly (Park and Kwon, 2011). These neurons project to the abdominal ganglion, which has been shown to regulate respiration, heartbeat, posture of the abdomen, as well as having roles in genital and ovipositor function (Park and Kwon, 2011; Nässel, 1996). Neurons that are involved with ovulation and mating behaviors project from the reproductive area to the abdominal ganglion (Häsemeyer et al., 2009; Yang et al., 2009). Gr5a projection neurons may interact with the neurons that have recently been found that play a role in ovulation and mating, i.e. there may be some cross talk between the neurons on the abdominal wall and the neurons located in the female's reproductive system. If the Gr5a-Gal4/UAS process was not expressed in all of these neurons, it would explain why only a partial rescue of the egg laying phenotype occurred. Lastly, there may not be enough Gr5a protein to confer a normal response, as modeled by Tanimura (Tanimura et al., 1988). However, Gr5a appears to play a role in oviposition preference when Gr5a dependent sugars are present, but not when Gr5a independent sugars are present.

Figures

A)



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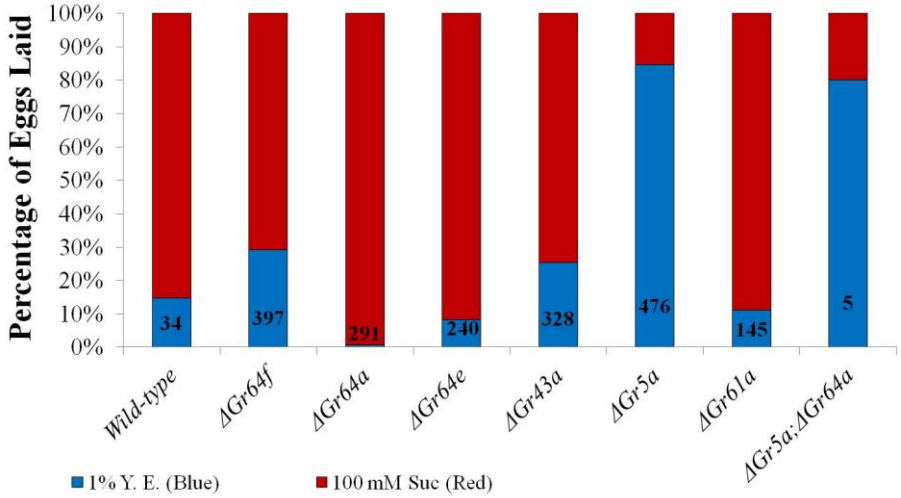
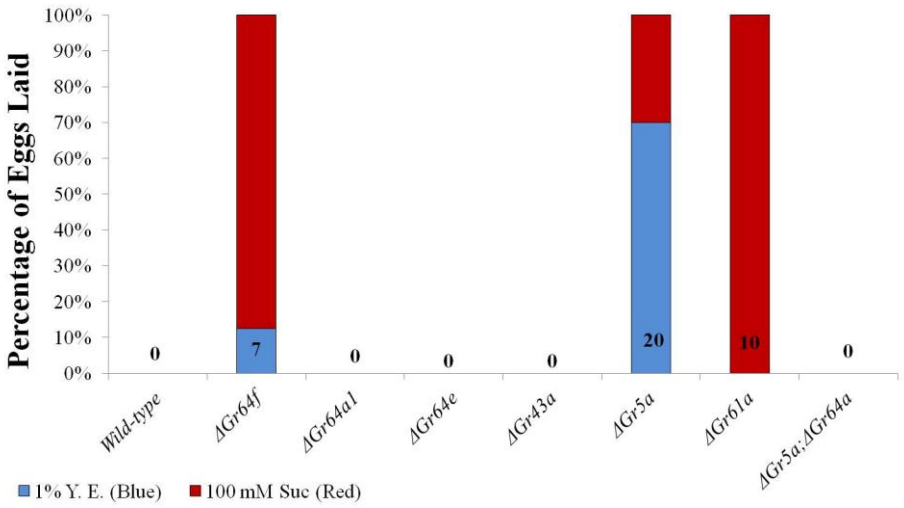


Figure 18 A) ΔGr5a mated females have an oviposition phenotype at 5 days, and B) 10 days. A) The number in bold at the bottom of each bar is the number of eggs laid by each genotype. Blue indicates that the flies chose to lay their eggs in the yeast extract, while red indicates that the flies chose to lay their eggs in 100 mM sucrose. n = 8 -15. B) The number in bold at the bottom of each bar is the number of eggs laid by each genotype. Blue indicates that the flies chose to lay their eggs in the yeast extract, while red indicates that the flies chose to lay their eggs in 100 mM sucrose. n = 8 - 10; except for the double mutant where $n = 2$.

A)



B)

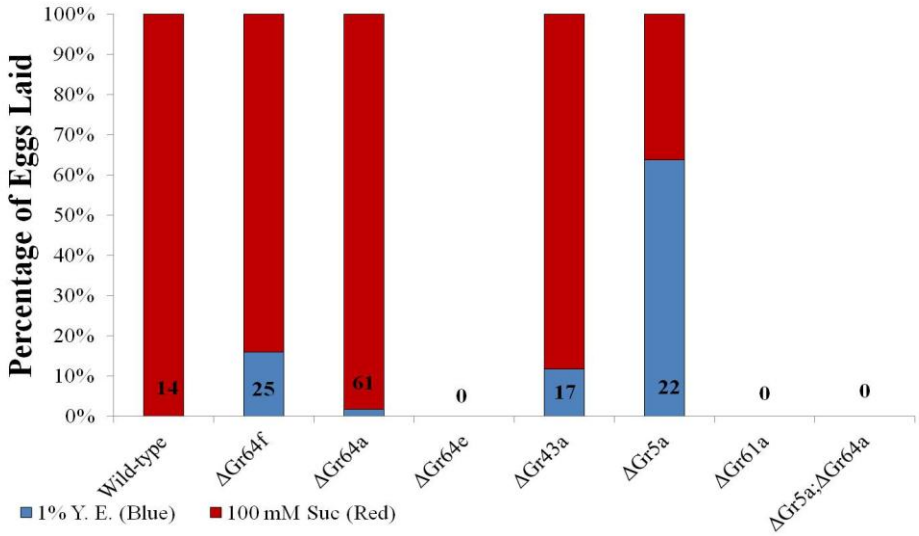
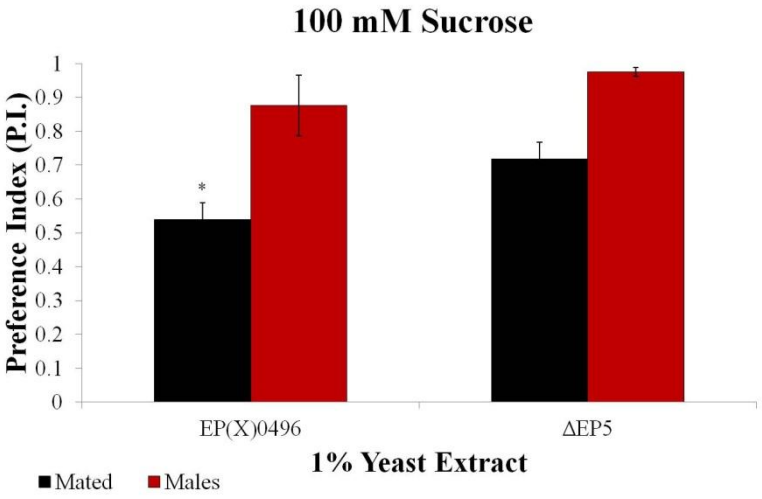


Figure 19 A) Egg laying preference in wild type and mutant virgin females aged 5 days. The number in bold at the bottom of each bar is the number of eggs laid by each genotype. Blue indicates that the flies chose to lay their eggs in the yeast extract, while red indicates that the flies chose to lay their eggs in 100 mM sucrose. n = 4 -10 ; *ΔGr64e* except ΔGr64e, where *n = 2* ; **B) Egg laying preference in wild-type and mutant virgin females aged 10 days.** The number in bold at the bottom of each bar is the number of eggs laid by each genotype. Blue indicates that the flies chose to lay their eggs in the yeast extract, while red indicates that the flies chose to lay their eggs in 100 mM sucrose. n = 4 – 6, except for *ΔGr64f* , *ΔGr64e*, *ΔGr5a*, and *ΔGr61a,* where *n = 2*.

A)



B)

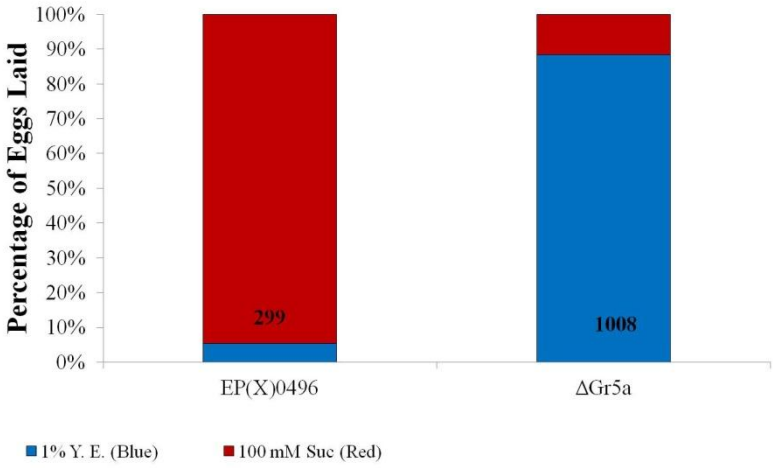
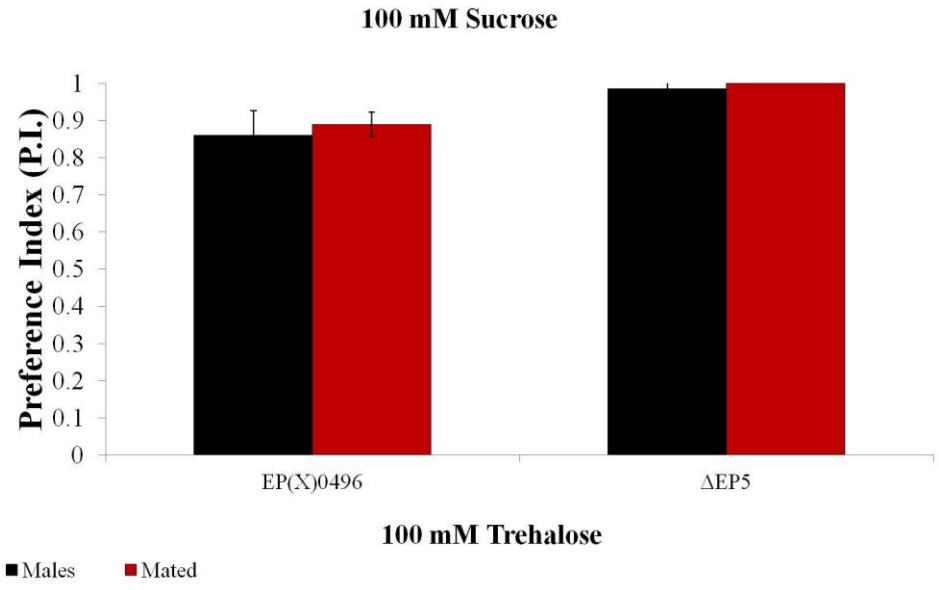


Figure 20 A) EP(X)0496 and ΔGr5a feeding preference. There was a statistical difference between mated females feeding preference, $p = \leq 0.05$. Males are shown as reference. EP(X)0496 n = 6; dGr5a n = 6. **B) EP(X) and ΔGr5a egg laying preference**. The number in bold at the bottom of each bar is the collective number of eggs laid across the trials. Blue indicates that the flies chose to lay their eggs in the yeast extract, while red indicates that the flies chose to lay their eggs in 100 mM sucrose. EP(X)0496 n = 6; dGr5a n = 6.

A)



B)

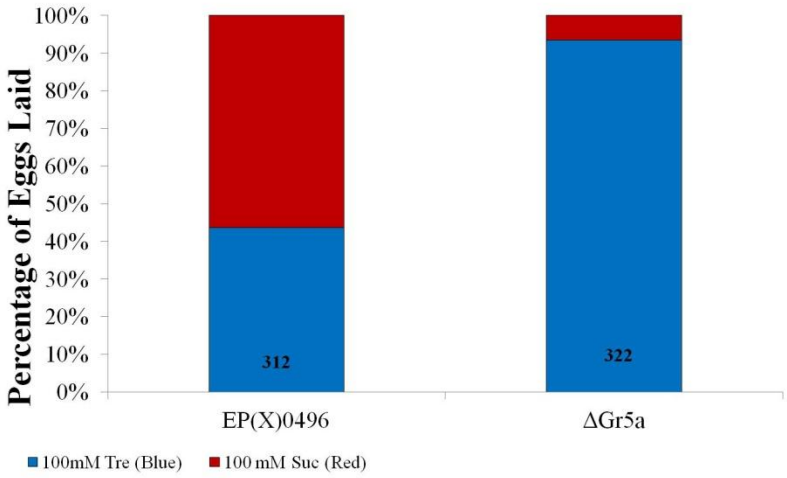
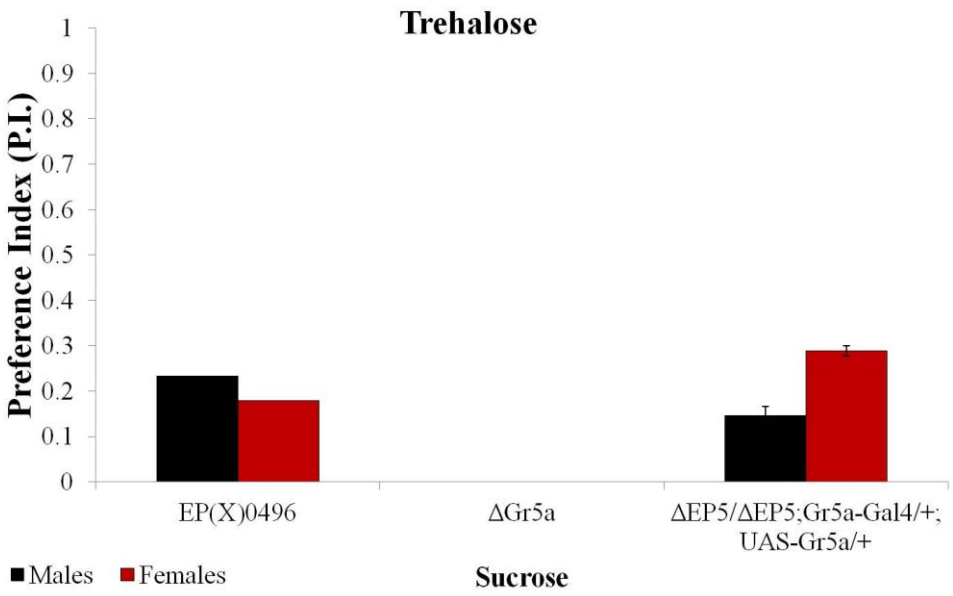


Figure 21. A) EP(X)0496 and Δ*Gr5a* Sucrose vs. Trehalose Feeding and B) Oviposition Preference. A) A Wilcoxon Signed-Rank test was run to determine if there were differences in preference between the two groups of females and two groups of males. There was a statistically significant difference in both groups ($p < 0.05$). N = 6 for all. B) Preference represented in percentage of eggs laid per tastant. The number in bold at the bottom of each bar is the number of eggs laid by each genotype. Blue indicates that the flies chose to lay their eggs in the yeast extract, while red indicates that the flies chose to lay their eggs in 100 mM sucrose. EP(X)0496 mated n = 6; *ΔGr5a* mated n = 6.

A)



B)

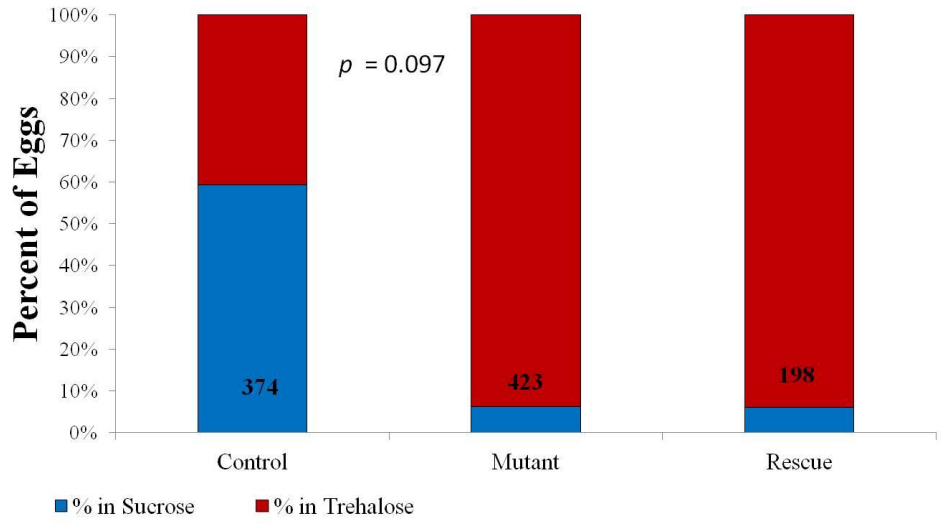
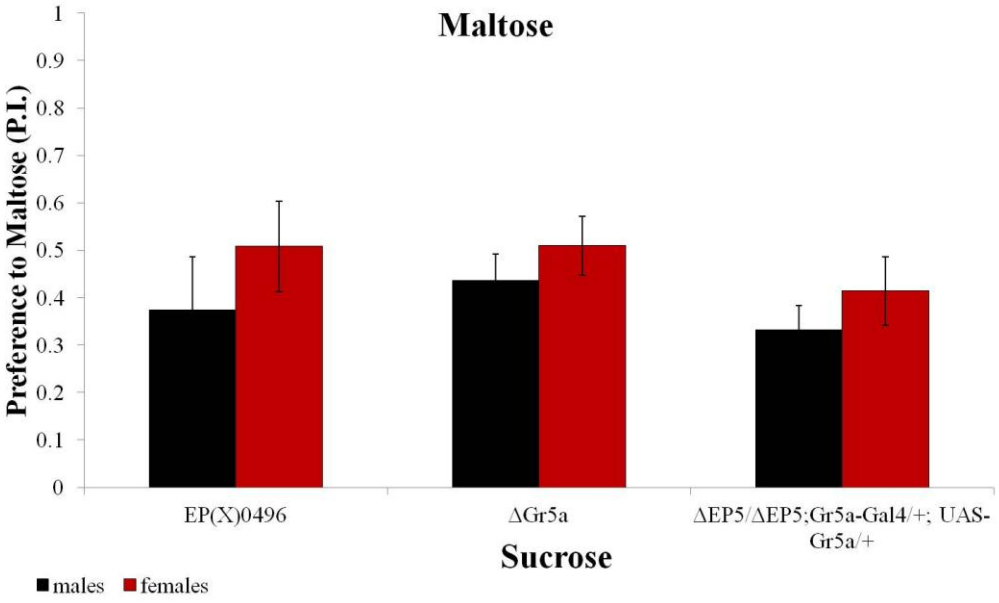


Figure 22 A) Feeding Preference to Trehalose; N = EP(X)0496, ΔGr5a, Gr5a-Gal4; UAS-Gr5a, ΔEP5/ΔEP5; Gr5a-Gal4/+; UAS-Gr5a/+, males followed by females. n = 1, 1, 3, 2; n = 1, 1, 3, 2. There was no statistical difference. Kruskal-Wallis H test. **B) Oviposition Preference to Trehalose.** Preference represented in percentage of eggs laid per genotype. These are the same flies as in A. The number in bold at the bottom of each bar is the collective number of eggs laid across the trials. Blue indicates that the flies chose to lay their eggs in the sucrose, while red indicates that the flies chose to lay their eggs in trehalose. There was no statistical difference, as established by a Welch ANOVA.

A)



B)

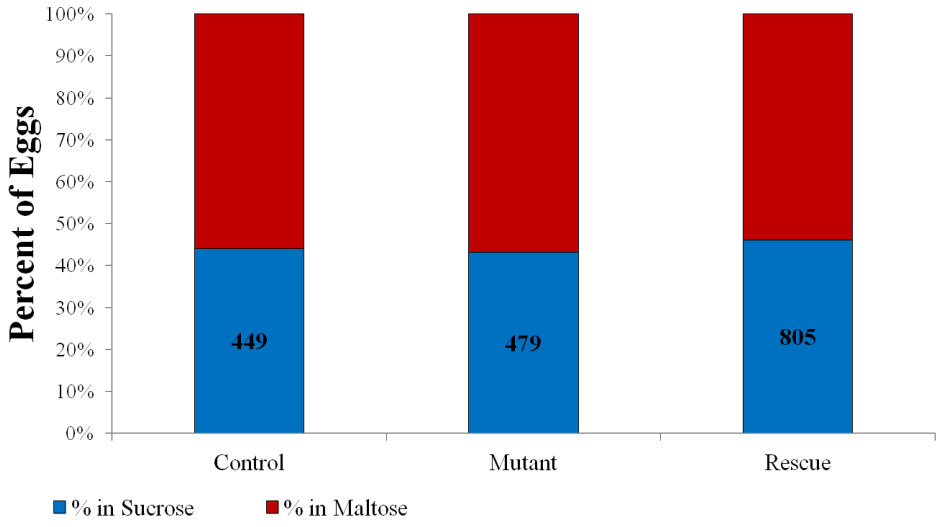
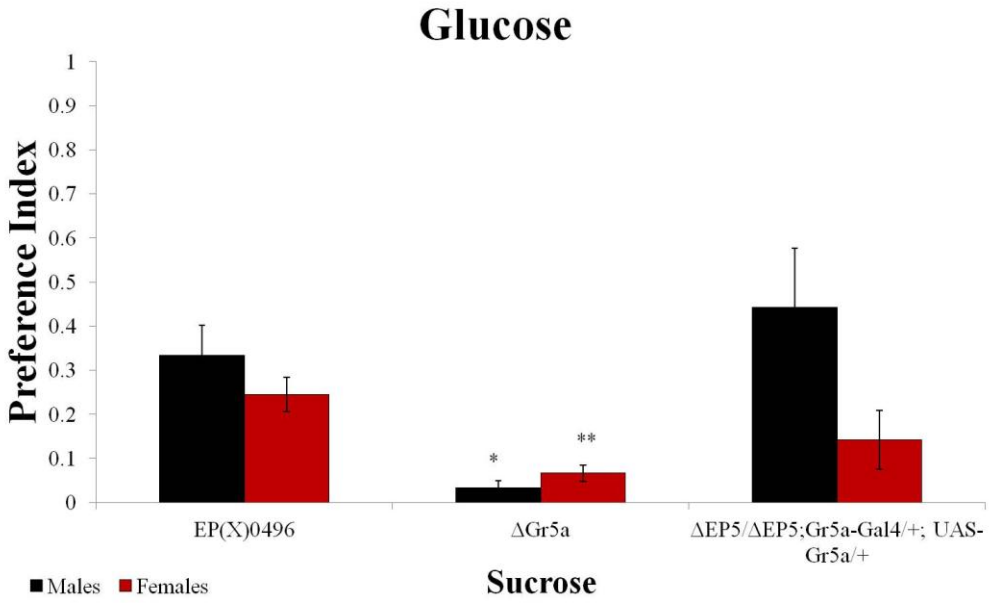


Figure 23 Maltose Preference. A) Feeding preference to Maltose. N = EP(X)0496, ΔGr5a, Gr5a-Gal4; UAS-Gr5a, ΔEP5/ΔEP5; Gr5a-Gal4/+; UAS-Gr5a/+; N = 6, 12, and 12 for males, and n = 10, 12, and 12 for females. There is no statistical significance between groups as determined by a One-way ANOVA. B) Oviposition preference to Maltose. N is the same as in B. No significant differences were found, Kruskal-Wallis H test.

A)



B)

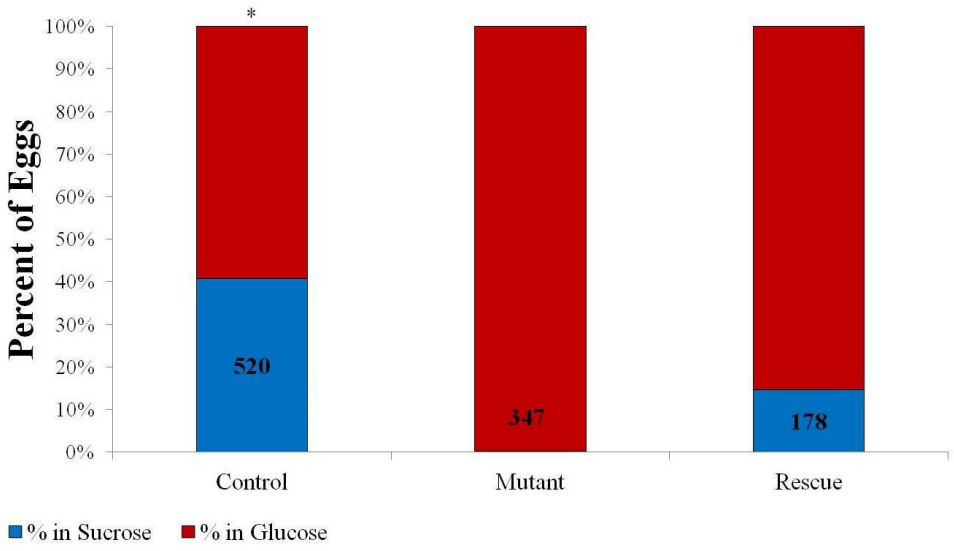
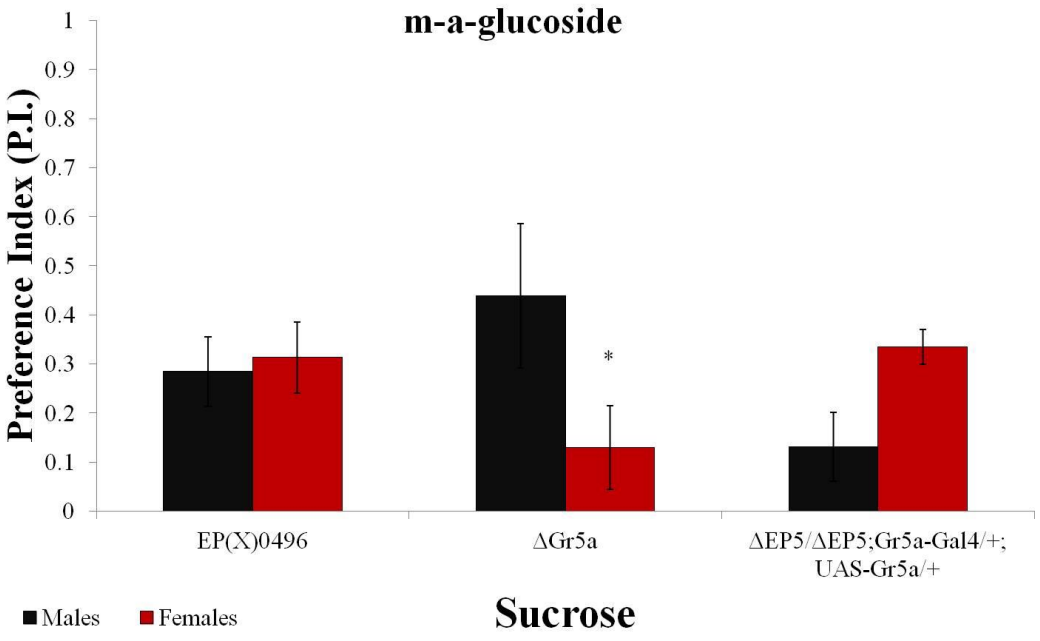


Figure 24 A) Feeding and B) Oviposition Preference to Glucose.

Figure 24 A) N = EP(X)0496, ΔGr5a, Gr5a-Gal4; UAS-Gr5a, ΔEP5/ΔEP5; Gr5a-Gal4/+; UAS-Gr5a/+, males followed by females. n = 5, 7, 6, 2; n = 7, 7, 6, 2. There is a statistical difference between male EP(X)0496 and ΔGr5a, and ΔGr5a/ΔGr5a; Gr5a-Gal4/+; UAS-Gr5a/+, $p = 0.020$, and 0.05 respectively. There is a statistical difference between female EP(X)0496 and ΔGr5a, $p = 0.004$. Males were analyzed with a Kruskal-Wallis H test, females with a One Way ANOVA, followed by the Tukey's post-hoc test. * = $p \leq 0.05$, ** = $p \leq 0.01$.

B) Preference represented in percentage of eggs laid per genotype. These are the same flies as in A. The number in bold at the bottom of each bar is the collective number of eggs laid across the trials. Blue indicates that the flies chose to lay their eggs in the sucrose, while red indicates that the flies chose to lay their eggs in 100 mM glucose. Pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. Amount of eggs laid in sucrose was statistically significant different between the different genotypes, p = 0.001. *Post-hoc* analysis revealed statistically significantly differences for eggs laid between ΔGr5a and Gr5a-Gal4; UAS-Gr5a (p = 0.048) and the genotypes ΔGr5a and EP(X)0496, (p = 0.000). N is the same as in A. * = $p \leq 0.05$

A)



B)

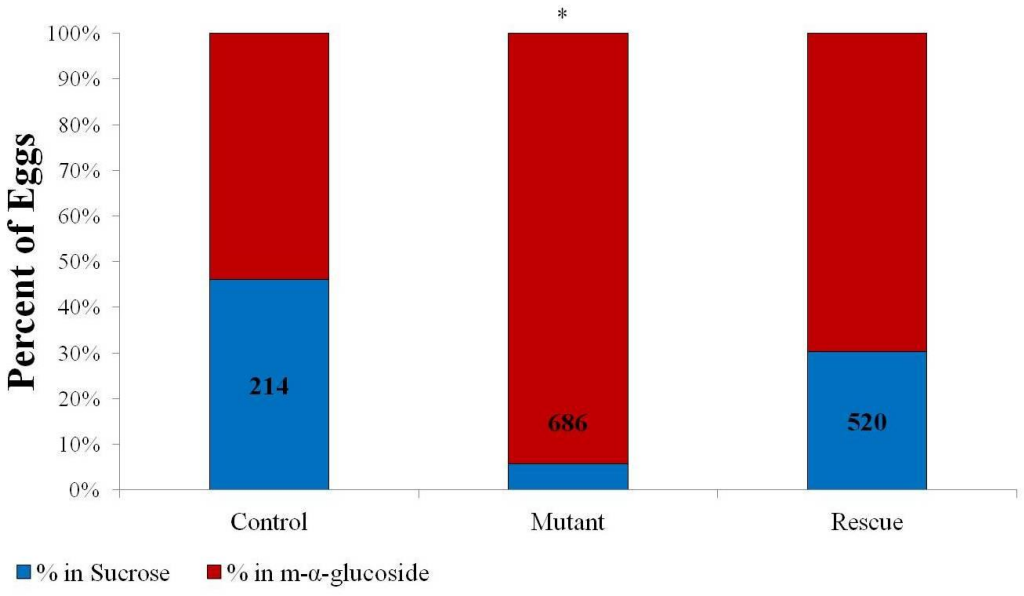


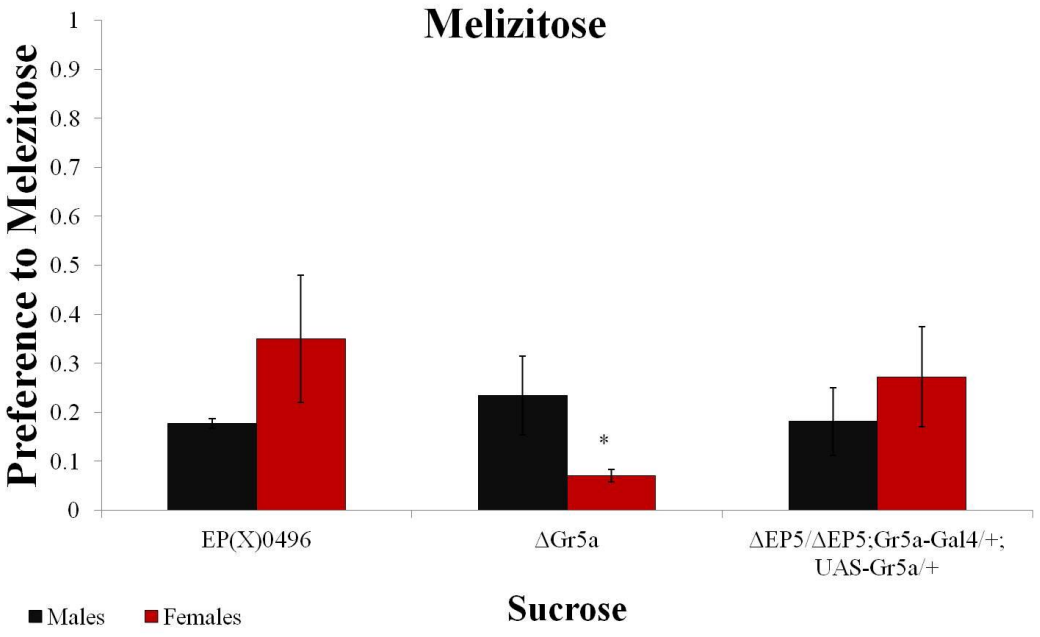
Figure 25 A) Feeding and B) oviposition preference to m-a-glucoside.

Figure 25

A) N = EP(X)0496, ΔGr5a, Gr5a-Gal4; UAS-Gr5a, ΔEP5/ΔEP5; Gr5a-Gal4/+; UAS-Gr5a/+, males followed by females. n = 7, 9, 13, 5; n = 6, 10, 12, 7. There was a statistically significant difference between ΔGr5a and ΔEP5/ΔEP5; Gr5a-Gal4/+; UAS-Gr5a females, as determined by a Kruskal-Wallis H test. * = $p \leq 0.05$.

B) The number in bold at the bottom of each bar is the number of eggs laid by each genotype. Blue indicates that the flies chose to lay their eggs in the sucrose, while red indicates that the flies chose to lay their eggs in 100 mM m-a-glucoside. A Kruskal-Wallis test was run to determine if there were differences for eggs laid in m-a-glucoside between the four genotypes. Pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. Amount of eggs laid in m-a-glucoside was statistically significant different between the different genotypes, p = 0.001. *Post-hoc* analysis revealed statistically significantly differences for eggs laid between the dGr5a and Gr5a-Gal4/UAS-Gr5a groups (p = 0.001) and the groups Gr5a-Gal4/UAS-Gr5a groups and ΔGr5a/ΔGr5a; Gr-5a-Gal4/+ ($p = 0.050$).

A)



B)

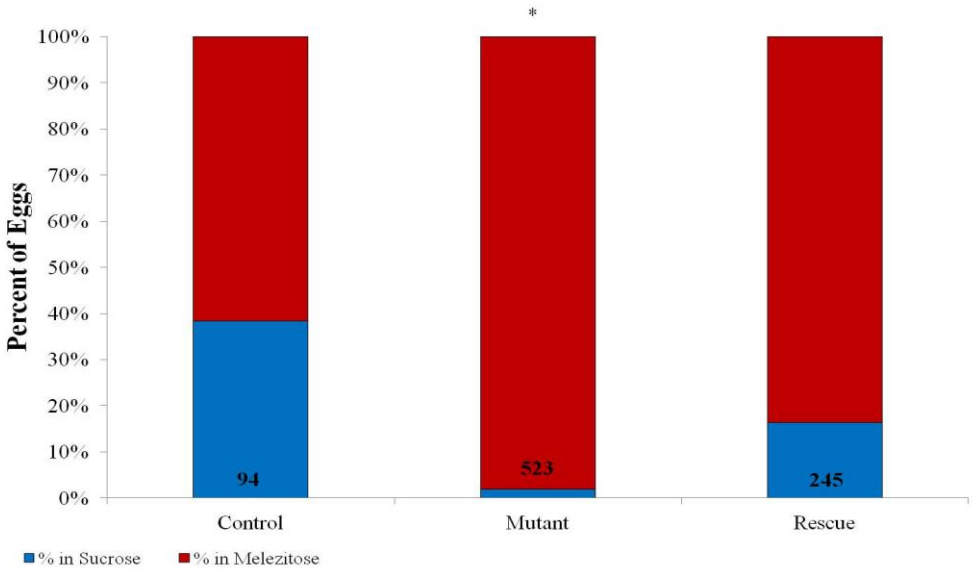


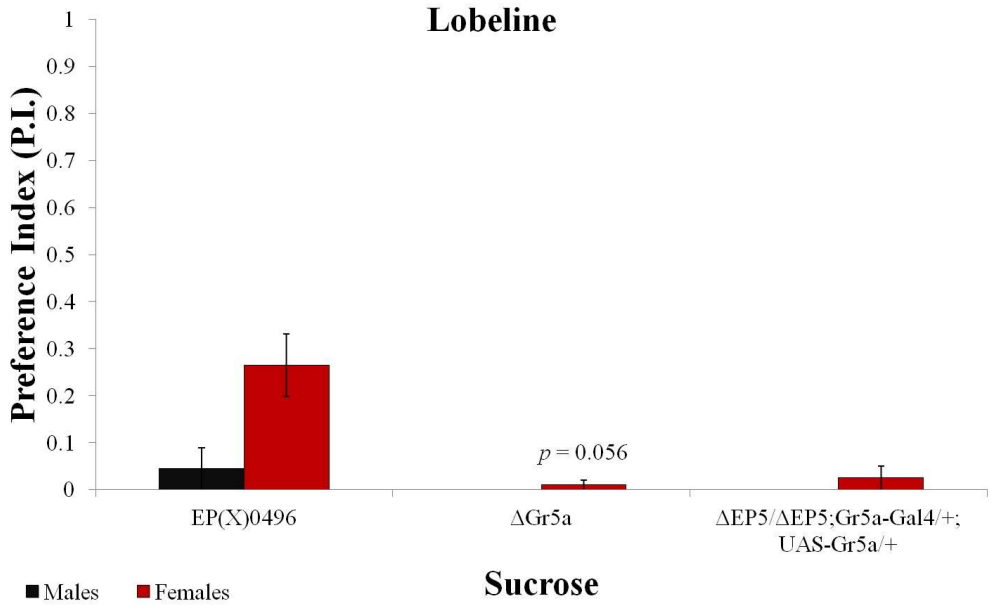
Figure 26 A) Feeding and B) oviposition preference in melezitose

Figure 26

A) Feeding. Zero indicates preference for sucrose, one indicates preference for melezitose, and 0.5 indicates no preference. N = EP(X)0496, ΔGr5a, Gr5a-Gal4; UAS-Gr5a, ΔEP5/ΔEP5; Gr5a-Gal4/+; UAS-Gr5a/+, males followed by females. n = 2, 7, 9, 3; n = 4, 8, 8, 3.There is a statistical difference female EP(X)0496 and ΔGr5a flies. * = $p \leq 0.05$. Kruskal-Wallis H test.

B) Preference represented in percentage of eggs laid per tastant. These are the same flies as in A. The number in bold at the bottom of each bar is the collective number of eggs laid across the trials. Blue indicates that the flies chose to lay their eggs in the sucrose, while red indicates that the flies chose to lay their eggs in 100 mM melezitose.

A)



B)

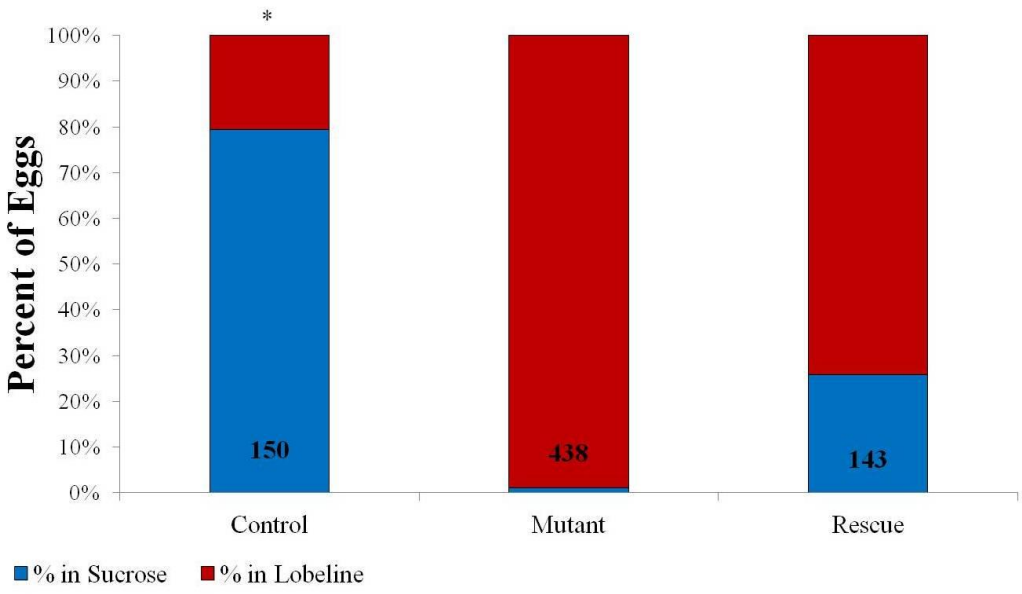


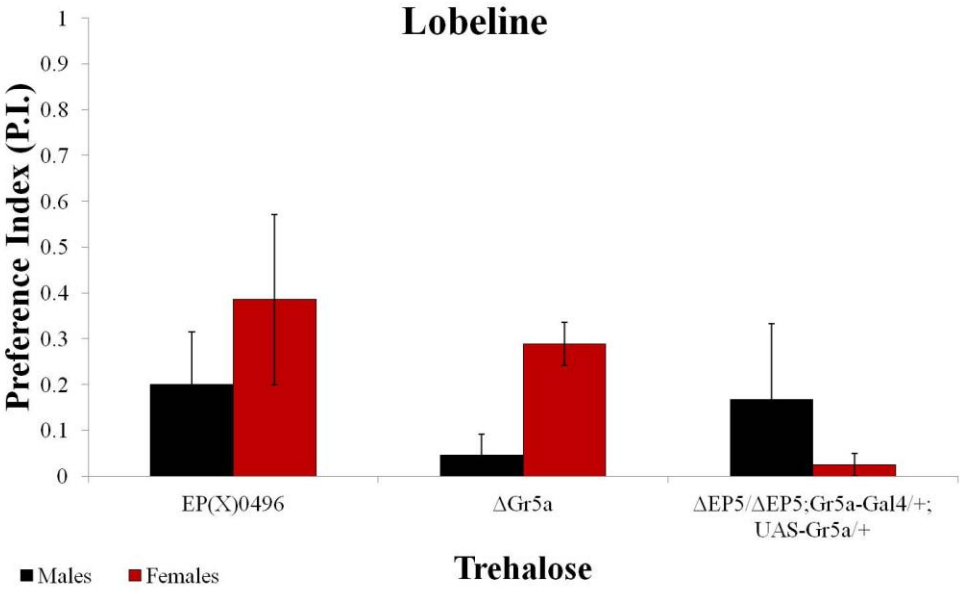
Figure 27 Sucrose vs. Lobeline A) feeding and B) oviposition

Figure 27

A) Feeding. N = EP(X)0496, ΔGr5a, Gr5a-Gal4; UAS-Gr5a, ΔEP5/ΔEP5; Gr5a-Gal4/+; UAS-Gr5a/+, males followed by females. n = 2, 3, 2, 2; n = 4, 3, 2, 2. There is no statistical difference between the genotypes. $p = 0.056$ between EP(X)0496 and ΔGr5a. Kruskal-Wallis H test.

B) Preference represented in percentage of eggs laid per tastant. These are the same flies as in A. The number in bold at the bottom of each bar is the collective number of eggs laid across the trials. Blue indicates that the flies chose to lay their eggs in the sucrose, while red indicates that the flies chose to lay their eggs in lobeline. There is a statistical difference between the amount of eggs laid in sucrose between ΔGr5a, and Gr5a-Gal4; UAS-Gr5a, ΔEP5/ΔEP5; Gr5a-Gal4/+; UAS-Gr5a/+. $p = 0.001$. Welch ANOVA and Gammes-Howel post-hoc.

A)



B)

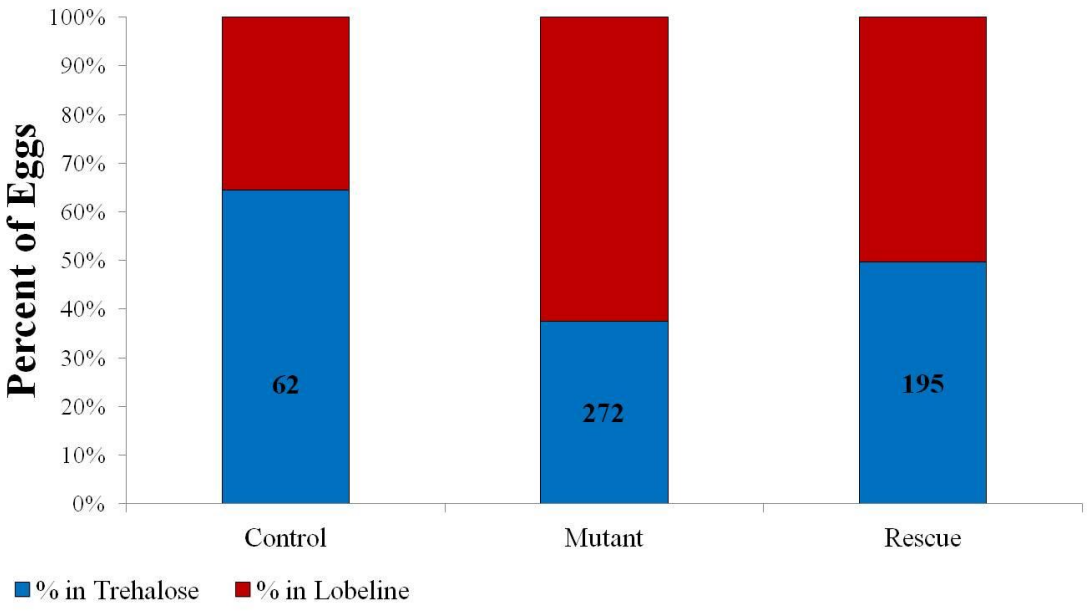


Figure 28 Trehalose vs. Lobeline. A) Feeding B) Oviposition Preference

Figure 28

A) N = EP(X)0496, ΔGr5a, Gr5a-Gal4; UAS-Gr5a, ΔEP5/ΔEP5; Gr5a-Gal4/+; UAS-Gr5a/+, males followed by females. n = 1, 4, 3, 2; n = 1, 2, 3, 2. There were no statistical differences. Kruskal-Wallis H test.

B) Preference represented in percentage of eggs laid per tastant. These are the same flies as in A. The number in bold at the bottom of each bar is the collective number of eggs laid across the trials. Blue indicates that the flies chose to lay their eggs in the trehalose, while red indicates that the flies chose to lay their eggs in lobeline. There were no statistical differences. Kruskal-Wallis H test.

Chapter 5 Conclusions

In conclusion, Gr61a is involved with diet regulation, mated females prefer yeast/yeast extract because of the amino acid content, and mated females will avoid laying their eggs in sucrose. This partial characterization of the Gr61a receptor increases the knowledge of how choice is regulated in a fly's diet. Regulation of what food to ingest is important in times of stress, plentiful food sources, and other situations the fly may come across.

This is the first time that it has been shown that mated females are drawn to yeast extract because of its amino acid content. This may be because females now have to build many more proteins in order to produce eggs for the next generation to continue. This increase in preference could come about because of increased expression of receptors in the GRNs or ORNs that respond to amino acids. Perhaps regulation of these amino acid sensing receptors is up-regulated in mated females so that they can find these resources for food. Alternatively, these receptors are always present at a standard concentration, but are blocked or inhibited pre-mating; post-mating results might result in a freeing of these receptors so that they can be activated in order to change their choice of nutrition.

This change in choice could be the result of the resumption of oogenesis, as the process requires amino acids. Drummond-Barbosa and Spradling have shown that insulin-like peptide signaling is necessary for follicle cell proliferation and vitellogenesis (Drummond-Barbosa and Spradling, 2001). This pair has also showed that presence of rich food sources, such as yeast, enhances egg production (2001). Yeast is a multi-component food source composed of salt, glutamate, sugars such as trehalose, b-vitamins, glycerol, and amino acids. Amino acids are the building blocks of life, as they are needed

to make proteins involved in every mechanism and function in an organism. Ribeiro and Dickson have shown that yeast preference is not dependent on egg production, as sterile mated females prefer yeast extract as well (Ribeiro and Dickson, 2010). Therefore, mating must trigger a switch in the female that activates her preference for protein; this aids in egg development, which is dependent on the insulin-like receptor pathway. Ribeiro and Dickson also discovered that activation of the SPR in the *ppk*+ neurons in the females reproductive tract are necessary to switch preference towards yeast; also, manipulation of the InR signaling pathway does not affect food choice (2010). The SP/SPR pathway seems to regulate feeding preference, while the InR pathway regulates egg production. However, it is possible that the insulin pathway plays some role in feeding preference, or there is minor cross talk between the two pathways. The increase in yeast preference is relatable to the increased need of protein in pregnant women's diets.

It is recommended that non-pregnant women have 46 grams of protein a day, men and pregnant women ~56 grams, and 66 grams for nursing mothers (Nierenberg, 2011). This extra protein intake required by pregnant and nursing women is to support the developing offspring, as well as to aid in milk production (Nierenberg, 2011). By studying the dynamics of choices *Drosophilae's* diet and how they affect offspring, potential parallels can be drawn to mammals, and eventually humans.

By choosing to avoid sucrose as an oviposition site and laying eggs in other sweet or bitter substances, the female fly may be increasing the chance of her future offspring's survival. Predators would avoid bitter tasting substances because the bitter taste usually means a toxic or harmful substance. Seeing as there are no sweet receptors (known as of

yet) that are expressed in larvae, but many bitter receptors (Weiss et al., 2011) this may be an evolutionary adaptation that aids larvae. Since the mothers are avoiding sucrose and laying in bitter, larvae may have needed to express more bitter receptors in order to sense their surroundings. Larvae could also develop better on bitter substances. On the other hand, the larvae may have many bitter receptors so that it can travel down the concentration gradient of bitters, to one that is of lower bitter concentration that may still be provide a sufficient food source to help them develop. This would allow the larvae to avoid toxic concentrations of bitter substances.

By studying the effects of diet, sex, and mating status on feeding and oviposition sites, the basic mechanisms of choice can be elucidated. Altering a fly's diet allows researchers to examine how changes in diet affect food choices. This area of research could potentially help explain the choices that humans make while on different diets. By manipulating these diets over time, as humans tend to often do, long term effects of the diets on health, nutrition, and fecundity of the individual can be learned.

Similarly, investigating what mated females are attracted to, compared to virgin females and males, allows us to see how different stages of life affect our nutritional choices. Investigating the pathways involved in carbohydrate or protein choice among flies of different mating statuses, has the potential to help us understand mechanisms of choice in pregnant women.

Lastly, studying oviposition preference has more immediate applications. As mentioned earlier, sex peptide reduces the sexual receptivity of females and increases their egg laying rate (Chen et al., 1988). *D. melanogaster* sex peptide also reduces sexual

receptivity and activates egg laying in *D. simulans*, *D. mauritiana*, *D. sechellia*, and *D. suzukii* (Chen et al., 1988; Schmidt et al., 1993). It is of most interest that *D. melanogaster* sex peptide activates similar PMR behaviors in *D. suzukii*, as this species is a pest that causes billions of dollars of damage to berry crops in the Western United States (Bruck et al., 2011). *D. suzukii* is not to distantly related from *D. melanogaster*, and it also contains two peptides that are similar to *D. melanogaster* sex peptide (Schmidt et al., 1993). Seeing as how *D. suzukii* has sex peptide like and a sex peptide like receptor, further investigation of choice in *D. melanogaster* could translate to similar mechanisms in *D. suzukii*, which would help curtail the damage of *D. suzukii* on U.S. crops.

Research into mated female nutrition and oviposition choice could also help with the control of another pest species, *Aedes aegypti*. *A. aegypti* has a receptor that is similar in structure and function to that of *D. melanogaster's* sex peptide receptor. Sex peptide from *D. melanogaster* activates the similar SPR in *A. aegypti in vitro* (Yapici et al., 2008). Since both *Drosophila* and *Aedes* have a similar receptor that is activated by *D. melanogaster* sex peptide and DUP99B, further examination of choices that flies make in feeding and oviposition sites could help control *A. aegypti's* transmission of yellow fever and dengue (Yapici et al., 2008; Jasinskiene et al., 1998).

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