The Influence of Dietary Factors on Reproduction in the Fruit Fly, *Drosophila melanogaster*

by

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Abstract

Food and nutrition are important for energy balance, reproduction and maintenance of health in all species. *Drosophila melanogaster* feed on yeast and sugar and food availability affects reproduction. In this thesis, I show that mating frequency and fertility are affected by the composition of food in two *D. melanogaster* wild-type strains, *Canton-S* and *Oregon-R*. *Canton-S* flies mate multiple times in the presence of yeast and sugar, while *Oregon-R* only remate in the presence of yeast. However, *Oregon-R* flies have higher fertility counts on all food types compared to *Canton-S.* These effects of food do not appear to depend on smell or taste, because both chemosensory mutants and artificial sweeteners tested fail to block the effects of food on reproduction. Moreover, *Canton-S,* but not *Oregon-R* flies show an interaction between food and group size. I conclude that genetic differences, social context and nutrition interact to regulate reproduction in flies.

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Chapter 1
Environmental Effects on Reproduction

Environmental factors such as nutrient availability, photoperiod and temperature are limiting factors for reproduction in many species (Farner, 1987). In addition to finding a suitable mate, the foraging and acquisition of nutrients seems to be the most crucial of these factors that affect reproduction (Bronson, 1985) and thus it will be the focus of this thesis.

Studies in many invertebrate and vertebrate species indicate the importance of nutrition. For example, a medium lacking nitrogen and/or phosphorous will hinder spore formation in fungal populations, and certain organisms like the unicellular amoeba, will only proliferate when food is available (Agrawal, 2009; Urushihara and Muramoto, 2006). Under conditions of starvation, aggregation occurs in slime moulds and fruiting bodies composed of spore masses and stalks are formed (Urushihara and Muramoto, 2006). In mice, high nutrition levels were found to increase the number of healthy neonates (Vandenbe.Jg et al., 1972). These studies collectively reveal the necessity of food with regards to reproduction.

In most cases of sexual reproduction, an animal must have successfully foraged and acquired food. The ingested nutrients need to be portioned off into necessary facets of the individuals' life, which include energy balance and thermoregulation. (Bronson, 1985).

The fulfillment of these necessary factors are needed for an individual to enter a reproductive state (Bronson, 1985). In the reproductive state, a suitable mate is a requirement for reproductive success. Reproductive success or fitness of an individual is dependent on passing on genes from one generation to the next. Fitness is the ability to survive and reproduce and can be quantified through fecundity and fertility. Fecundity measures the potential reproductive capability of an organism and is usually measured by the number of gametes, while fertility measures the amount of viable offspring (Pyle and Gromko, 1978).

Since reproduction occurs in harmony with dietary, physical, and social variables, it becomes important to dissect out the contribution of such environmental inputs if one wants to understand reproduction. The physical environment can include the annual changing of the seasons, the photoperiod, the varying temperatures and the availability of nutrition. The social cues in the environment on the other hand, can include the other individuals present in the surroundings, where one of the individuals could potentially include a mate.

This thesis will concentrate on the quality of food required for reproduction within a prescribed social context of the fruit fly, *Drosophila melanogaster.* Along with having a relatively short life cycle, a sequenced genome and an extensive library of genetic mutants readily available, the fruit fly offers a great paradigm to study complex animal behaviours. Comparisons will be made to the vertebrate model organism *Mus musculus* throughout the chapter to illustrate how these two apparently distant systems share fundamental biological mechanisms that are conserved across phyla.

Social Environment

For many years, it was believed that females in mating systems of various species were selective and mostly monogamous, while males were promiscuous (Birkhead and Pizzari, 2002). Monogamous mating systems result when females and males of a given species only mate with one partner; polygamous mating systems result when an individual mates with more than one partner (Hosken et al., 2009). Seminal work by Bateman (1948) in *Drosophila melanogaster*, indicated that the fitness of a male increases with every mating partner, where the male has the opportunity to sire more progeny with every mating, while the reproductive success of the female is limited by the number of eggs she can produce (Bateman, 1948). However, studies done in the last 35 years have revealed that females of many animal species including *Drosophila melanogaster* females are in fact polyandrous, they interact with and copulate with multiple partners (Birkhead and Pizzari, 2002). Multiple mating thus must be advantageous not only to males but also to females. There are several theories as to why remating for females might be advantageous. Females may receive indirect genetic benefits through multiple mating (Singh et al., 2002). Indirect genetic benefits include high quality genes for her offspring (Birkhead and Pizzari, 2002). Multiple matings with males may be required in order to replenish sperm stores in order to fertilize more eggs, or protect against male sterility (Chapman et al., 1994). Remating by females allows sperm from multiple males to mix in the female reproductive organs (Pitnick et al., 1999). The mixing of sperm promotes sperm competition, via displacement and incapacitation, which has the advantage of increasing the genetic diversity of the offspring (Chapman et al., 1994), (Fukui and

Gromko, 1989). However, studies have shown that mating for females may have several disadvantages such as lowering female lifespan, as well as decreasing her receptiveness to other males that are encountered later (Chapman et al., 1994). It is thought that accessory gland proteins that are transferred to the male by the female during copulation are responsible for the negative side effects of mating.

The benefit of multiple matings to females is detrimental to male fitness due to sperm competition reducing the number of offpring sired by a particular male. This is thought to create conflict between the sexes. Sexual conflict arises when the behaviour of one sex has a negative impact on the other sex (Singh et al., 2002). In the fruit fly, *Drosophila melanogaster*, proteins in the seminal fluid also known as the accessory gland proteins are thought to mediate this effect in the females (Singh et al., 2002). Due to the negative effects of the proteins, it was widely believed that males obtain greater benefits from mating than females.

A study from our lab by Krupp et al. (2008), studied the social behaviour of fruit flies in a group setting. This study illustrates how communication and interaction between members of a species can affect the behaviour of an individual. In the presence of mutant males, the wild type males increased their mating frequency by 22%. This indicates the effect of the mutant males on the wild type flies causing them to alter their behaviour. This assay which used wild type females housed with 4 wild type males and 2 mutant males was used to determine whether there was an effect when only wild type flies were used in a study by Billeter *et al.* (Submitted). Two well studied wild type strains; *Canton-S* and *Oregon-R* were used for this study. In mixed groups comprising primarily *Canton-S* males or groups that contained primarily *Oregon-R* males, *Oregon-R* females prefer to

mate with *Canton-S* males but did not discriminate between the two types of males when they were present in equal proportions. This suggests that females are able to detect the genetic makeup of the individuals in the social environment and are able to adjust their mating preferences accordingly. Not only were the mating preferences adjusted; there was a specific ratio of progeny that resulted from these matings. In mixed groups of *Canton-S* and *Oregon-R* males, *Oregon-R* females not only sired approximately 75% of progeny from *Canton-S* males, but also remained infertile when mated only with *Oregon-R* males. This indicates that the social context has a considerable effect on an individual's reproductive success and further illustrates that complex social environmental factors influence reproduction.

One of the first studies done by Vandenbergh et al., (1971, 1972) showed that there was an effect of the social environment that can also been seen in mice. The social context has been shown to influence the sexual maturation of both female and male mice. Studies indicate that, in the presence of the opposite sex, both female and male mice displayed an increase in the onset of reproduction. Females housed individually with an adult male mouse got their first oestrus cycles about 6 days before females that were housed without an adult male (Vandenbe.Jg et al., 1972). When no males were present amongst a group of females, the estrous cycle of these females was delayed by approximately 20 days, indicating a strong male stimulation for the early onset of the estrous cycle in females (Vandenbe.Jg et al., 1972). Not only does the presence of males advance sexual maturity in females, it also advances the age of sexual receptivity and fertility (Khan et al., 2008). The age of first mating also occurred earlier in females that were exposed to males when

young (Khan et al., 2008), while the aggressive behaviour of a female was also shown to be higher in females that were between male siblings in the uterus (Vomsaal et al., 1990).

Similarly, the presence of an adult female affected the male reproductive organs by increasing testicular weight and size of the seminal vesicles of a male mouse (Vandenbe.Jg, 1971). On the contrary, the presence of an adult housed with young of the same sex, has an inhibitory effect on their reproductive development. The presence of an adult male with young males revealed an inhibitory effect on male sexual development resulting in decreased testicular weight and seminal vesicle size (Vandenbe.Jg, 1971).

Studies done on mammals as well as flies indicate an effect of social context on individuals. However, these studies also revealed a contribution of nutrition to the development and reproductive output of both mice and flies that cannot be overlooked.

Physical Environment:

Nutrition has been found to affect a variety of characteristics involved in reproduction including offspring size, behaviour and attractiveness of an individual. One of the first studies performed to determine the effect of nutrition on reproduction could be seen in the study described previously by Vandenbe.Jg (1972). The reproductive output of female mice was greatly enhanced when females were being fed high protein diets. It was shown that when female mothers were fed a low protein diet, only 70% of the females produced litters, while mothers fed on intermediate protein diet, 80% produced litters and when fed with high protein diets, all female mice produced litters. Although the dietary component only explained approximately 5% of the variance, it nonetheless had an effect

on the reproductive output of the female (Vandenbe.Jg et al., 1972). Another study was done which showed that although the undernutritioned female mice produced litters, the males of these litters were far less dominant than males born to well fed mothers (Meikle and Westberg, 2001). The male offspring of undernourished mothers were observed to have lower body weight along with smaller seminal vesicles. Moreover, a female in oestrus preferred the odour of males that were born to well fed mothers (Meikle and Westberg, 2001). Contrary to the sons, the daughters of undernourished mothers were not as affected, but their reproductive success was slightly lower than that of daughters born to well fed mothers (Meikle and Westberg, 2001).

As a result of studies described above and others, a link between nutrition and reproduction could be formed. The effects of undernutrition have been reviewed by Hileman et al. (2000), and it has shown to have severe delays on female mice. It has the potential to delay the onset of sexual maturation and in sexually mature females; it can disrupt the oestrus cycle, while generally having negative effects on the reproductive cycle. It has been thought for some time, that puberty was initiated when critical body fats were at appropriate levels which is achieved through adequate nutrition (Hileman et al., 2000). The adipose derived hormone, leptin has been implicated as being a potential signal to the reproductive center of the brain of energy stores and nutritional status (Barash et al., 1996). Levels of leptin have been shown to correlate with the level of body fat in the individual and leptin receptors co-localize with neuropeptides in the brain that control food intake and the reproductive axis (Barash et al., 1996). Animals that lack leptin or the receptors for leptin have been shown to be infertile and do not undergo normal sexual maturation (Barash et al., 1996). When leptin is admininstered to mice

lacking leptin, fertility can be restored in the animal indicating that leptin acts a metabolic signal to the reproductive system, informing the system of the nutritional status of the animal (Barash et al., 1996).

In *Drosophila melanogaster,* the presence of food is known to be an important factor that influences reproductive success. Earlier studies in the fruit fly have shown that larvae require yeast in order to grow while adults need carbohydrates for reproduction (Baumberger, 1917). A study done on females revealed that mated females consumed more food than virgin females suggesting that the food might be incorporated into the production of eggs (Carvalho et al., 2006). It has also been shown that rematings increase with increasing dosage of food and, more specifically, matings increase with increasing dosage of yeast increases (Chapman et al., 1994) (Chapman and Partridge, 1996). This increase in rematings could be due to the fact that flies are consuming more food of higher concentrations (Min and Tatar, 2006) (Carvalho et al., 2006). An increase in matings was not observed on the lower concentrations of food as the flies do not compensate for the low dosage by increasing food intake (Min and Tatar, 2006). Male flies were also affected by the food provided where they exhibited the lowest number of matings on low yeast concentrations (Fricke et al., 2008). The maximum number of progeny also eclosed on intermediate yeast concentrations and could be due to the action of accessory gland proteins, specifically the sex peptide boosting egg laying on higher concentrations of food (Fricke et al., 2008) (Fricke et al., 2009). A recent study also implicated the insulin-like peptide (dilp) genes in having a part in controlling reproduction. It was shown that when the cells expressing these genes were ablated, mating frequency was reduced indicating a role for the metabolic pathway in

reproductive success (Wigby et al., 2010). Moreover the rematings observed in studies done in Krupp et al. (2008) and Billeter et al. (Submitted), were observed in the presence in the food. It was shown in these two studies, when non-nutritious agar was substituted for food for males and females, the mating frequency was significantly decreased (Levine Lab, unpublished data). This indicates that *Drosophila* reproduction is also affected by food. Food is thus a fundamental condition for reproduction.

Recognition of the environment

In animals, the visual, auditory, olfactory and gustatory systems all act to recognize the environment. The social and nutritional cues are mainly assessed by the olfactory and gustatory systems with a relatively minor role played by the visual and auditory systems (Hiroi et al., 2002) (Montell, 2009). The gustatory and olfactory systems together make up the chemosensory system where the chemical information is extracted from the environment.

Perceiving Volatile Cues

The best-known sensory system to recognize volatile signals is the olfactory system. In mammals, the odorants released are detected through the nose (Matsunami and Amrein, 2003). The olfactory system is divided into two parts, the main olfactory system and the accessory olfactory system (Insel and Fernald, 2004). In total, there are approximately 1000 genes in the odourant receptor family found in mice (Matsunami and Amrein, 2003). The accessory olfactory system is the main system via which pheromones or volatile compounds are detected (Insel and Fernald, 2004). The main organ of the

accessory olfactory system is the vomeronasal organ (VNO) (Insel and Fernald, 2004). This organ is dedicated to the sensing of pheromones released by others in the environment and is made up of a basal and apical layer (Matsunami and Amrein, 2003). The V1R or V2R families of G-protein coupled receptors are expressed in each of the layers, where each neuron in the family expresses one receptor (Matsunami and Amrein, 2003).

The odorants that are released are usually secreted through the urine or other body fluids in mice (Lin et al., 2005). The components secreted by the body fluids are used by other mice to determine the sex, strain, social status, and the presence of the estrus cycle (Lin et al., 2005). Evidence from the last few years have identified mitral cells in the main olfactory system that are responsible in recognizing urine (Lin et al., 2005). MTMT, (methylthio)methanothiol, has been identified as the compound present in male urine that contributes to the attractiveness of the urine to estrous females (Lin et al., 2005). Females that were housed on soiled bedding from a male mouse will become sexually mature and get their first estrus cycle at a similar time as females housed with an actual male and much faster than females that were not housed with a male indicating that pheromonal signals and olfaction is likely to be the cause for the acceleration of puberty (Vandenbe.Jg et al., 1972).

There are several pheromones that are released by *Drosophila* that enable flies to assess the quality and status of an individual. The olfactory system in *Drosophila melanogaster* detects volatile compounds and is similar to the chemosensory system of mice. The system is made up of two pairwise head appendages, the hairs present on the 3rd antennal segment and the maxillary palp (Vosshall et al., 1999). The appendages are covered with

numerous sensory hairs, where each hair is thought to have 2 to 4 olfactory sensory neurons (Ebbs and Amrein, 2007). Each olfactory receptor neuron expresses a single odorant receptor and each glomeruls receives information from one class of neurons (Couto et al., 2005).

Odorant receptors are thought to be G-protein coupled receptor and are activated when an odour is sensed (Vosshall et al., 1999). The projections are sent to the glomeruli in the antennal lobe part of the brain through the olfactory receptor neurons where the different odours are then processed (Vosshall et al., 1999) (Couto et al., 2005). There have been 62 odorant receptors identified, where *Or83b* was found to be expressed in all olfactory receptor neurons (Robertson et al., 2003) (Vosshall and Stocker, 2007). *Or83b* has been shown to associate with other odorant receptors in the membrane and target the complex to a specific neuron (Vosshall and Stocker, 2007). *Or67d* was been shown to be a key receptor in recognizing the status of an individual and is used to detect cis-vaccenyl acetate (cVA) (Dickson, 2008). This volatile male pheromone is known to mediate both female and male behaviour (Dickson, 2008). *Or47b* might be a species specific stimulatory cue and it confers sensitivity to fly odours by both males and females (Dickson, 2008). In the reproductive context, the social cues from pheromones like cVA through *Or67d* receptor can act to recognize the reproductive status of a fly.

A new class of olfactory receptors called ionotropic glutamate receptors have recently been discovered (Benton et al., 2009). These receptors are found to be expressed in cells that do not contain G-coupled protein olfactory receptors as they do not co-express with *Or83b* (Benton et al., 2009). The complete functions of these receptors are unknown but

are thought to act as ion channels and recognize olfactory cues in the environment and project neurons into the antennal lobe (Benton et al., 2009).

Perceiving Non-Volatile/Contact Cues

The ability to sense nutritious food is key to the survival of an organism (Scott, 2005). Sweet and umami tastes are generally considered to be nutritious while bitter compounds are perceived to be toxic (Matsunami and Amrein, 2003). Food odours are mostly perceived by the main olfactory system where non-volatile chemicals are processed (Matsunami and Amrein, 2003). As reviewed by Matsunami and Amrein (2003), the chemicals are tasted via clusters of taste cells on the tongue in mice and other vertebrates. The taste cells are located on taste buds and are located in taste papillae of the tongue, palate and pharynx. Taste receptors are thought to be G-protein coupled receptors, however little is known about the identity of specific members of the receptor families. Three members of the T1R taste receptor family have been identified in mice, which detect sweet and umami compounds, while the T2R receptor family detects bitter compounds.

Contrary to mammals, flies taste soluble compounds throughout their body. Along with being able to taste from the labellum/proboscis (mouth parts), the fly has the ability to taste through its wings, legs and the genitalia (Stocker, 1994). All signals obtained by the organs are projected to the subesophageal ganglion (SOG), where all gustatory information is processed (Dunipace et al., 2001). All gustatory organs possess taste bristles which are hair-like sensillae containing two to four chemosensory neurons and one mechanosensory neuron (Stocker, 1994). The *pox-neuro (poxn)* gene was identified

as being crucial to the development of chemosensory bristles; mutants for this gene possess bristles with mechanosensory function and lack chemosensory bristles suggesting that these mutants may lack the ability to taste (Awasaki and Kimura, 1997). Many of the taste neurons are present on the labial palps as it is the primary organ needed to assess the quality of the food before ingestion (Vosshall and Stocker, 2007). There are more taste bristles and neurons on the legs of males as opposed to females, and it is hypothesized, this sexual dimorphism is due to the males using the extra bristles to taste the female pheromones during courtship (Amrein and Thorne, 2005). Another sexual dimorphism present are the microbristles found on the genitalia of females with supposed chemosensory function (Stocker, 1994). The bristles are thought to be near the ovipositor enabling the female to detect appropriate egg-laying sites for her progeny (Stocker, 1994).

Several receptors have been identified in *Drosophila,* which contributes to extracting a variety of gustatory information from the environment. There are over 60 identified gustatory receptors, however, only two receptors that are known to recognize food molecules have known ligands (Clyne et al., 2000) (Dunipace et al., 2001) (Scott et al., 2001). Gr66a has been linked with the avoidance behaviour for bitter substances, while Gr5a is the known receptor for the disscacharide trehalose (Ueno et al., 2001). The *Tre* gene was found to be responsible for the detection of trehalose with the two well studied laboratory strains *Canton-S* and *Oregon-R* (Tanimura et al., 1988). *Canton-S* flies show sensitivity towards trehalose while *Oregon-R* flies displayed low sensitivity (Tanimura et al., 1988). Mutants of the *Gr64a* receptor family have been studied and is suggested that

Gr64a might be sensitive towards sucrose and glucose (Dahanukar et al., 2007) (Jiao et al., 2008).

In *Drosophila melanogaster,* it has been shown that female receptiveness, reproduction and offspring production were dependent on the availability of nutrition, and further more, the lack of nutritious food can possibly have a negative effect and inhibit reproduction (Fricke et al., 2009) (Wheeler, 1996). Along with the food possibly being incoporated into egg production in mated females, it seems that before a female lays eggs, the food is assessed through the ovipositor and usually oogenesis is triggered only when adequate nutrition is present (Carvalho et al., 2006) (Wheeler, 1996). Lack of oogenesis has been linked to Gr5a neurons, where their disruption has the potential to alter the decision to lay eggs (Yang et al., 2008). The results of various studies indicates that there be might a decision making process, on whether to mate and produce offspring that integrates both the presence and quality of food available with the social context an individual find itself in.

Thesis Objectives:

This thesis will investigate the influence of food quality on the reproductive behaviour of the fruit fly, *Drosophila melanogaster.* The majority of this study will focus on two wild type strains, *Canton-S* and *Oregon-R*. I expected to find a difference in reproduction in response to food quality because a previous study showed that the two strains displayed differences in mating behaviour.

The initial component of the thesis will determine whether food is necessary for rematings to occur. If food is found to be necessary, our fly food will be tested one component at a time to determine which of these is necessary and sufficient for the flies. The role of social behaviour will be looked at by comparing group size and strain based sex preferences.

The second part of the thesis will aim to determine whether food is required as energy for females to reproduce or the females assess the food environment for the nutritional content in order to check for appropriate egg laying sites. This will be assessed with the use of artificial sweeteners that contain little to no nutritional value.

Finally, gustatory and olfactory mutants will be used to determine which sensory system is required to link the stimulus perception of food with reproduction.

All experiments and analysis in this thesis were conducted by Samyukta Jagadeesh under the supervision of Dr. Joel Levine.

Chapter 2

Materials and Methods

This chapter describes the materials and methods used throughout this thesis.

Fly Stocks:

The lists of fly stocks used for various experiments in this thesis, along with their descriptions are provided below.

Strain/Genotype	**Description**	**References**
Wild-type strains		
Canton-S	Wild type strain	Levine Lab
Oregon-R	Wild type strain	Levine Lab
Introgression Lines		
CS;OR;OR	*Canton-S* on the X chromosome	Levine Lab; generated by Jean-Christophe Billeter

OR;CS;OR	*Canton-S* on the 2nd chromosome	Levine Lab; generated by Jean-Christophe Billeter
OR;OR;CS	*Canton-S* on the 3rd chromosome	Levine Lab; generated by Jean-Christophe Billeter
Gustatory Mutants		
w^{B}; $Poxn^{\Delta M22\text{-}B5}$ / *CyO*	Mutant with brain and gustatory deficiency	Krstic *et al.,* 2009
ΔxB56,Oregon-R; $Poxn^{\Delta M22\text{-}B5}$	Mutant with gustatory deficiency but normal brain function	Krstic *et al.,* 2009
w^{B}; $Poxn^{\Delta M22\text{-}B5}$*;full-1*	Partial rescue mutant except labellum	Krstic *et al.,* 2009
w^{B}; $Poxn^{\Delta M22\text{-}B5}$*;superA158*	Complete rescue	Krstic *et al.,* 2009
CS;CS; $Gr64a^{2}$	Mutant lacking sensitivity towards glucose and sucrose in a wild type *Canton-S* genetic background	Dahanukar *et al.,* 2007; placed in a *Canton-S* genetic background by Samyukta Jagadeesh

OR;OR;Gr64a^{2}	Mutant lacking sensitivity towards glucose and sucrose in a wild type *Oregon-R* genetic background	Dahanukar *et al.,* 2007; placed in an *Oregon-R* genetic background by Samyukta Jagadeesh
Olfactory Mutants		
CS;CS;Or83b	Mutant with smell deficiency in wild-type *Canton-S* background	Vossahall Lab; placed in a *Canton-S* genetic background by Jean – Christophe Billeter

Rearing Conditions:

All fly stocks were raised in polyproplylene bottles in a 12:12 light:dark cycle at 25°C. The fly stocks were kept on agar-yeast-sugar based food. Method for preparing fly food is provided in Appendix A. The bottles were emptied everyday and flies were collected for various experiments. Fly collections are explained below.

Fly Collections:

Virgin females and males were collected within 8 hours after eclosion for all fly strains used. Virgin females and males were isolated and kept in single sex populations of 20 flies each in polystyrene vials with the standard agar-yeast-sugar based laboratory fly

food. The collected flies in vials were maintained at 25°C in a 12:12 light:dark cycle. The virgin flies were used for the behavioural assays at 5-6 days following eclosion and collection.

Genetics:

Gr64a^{2} (*w-;+; Gr64a*^{2}) mutant flies were placed into either the *Canton-S* or *Oregon-R* genetic background. A detailed crossing scheme is provided in Appendix B. The resulting flies were either *CS;CS;Gr64a*^{2} (*Canton-S* genetic background) or *OR;OR;Gr64a*^{2} (*Oregon-R* genetic background). *Or83b* null mutants were also placed in a *Canton-S* background.

Nutrition:

The different ingredients present in the laboratory fly food were tested individually for remating and progeny assays described below. The different components were tested at concentrations found in our standard fly food. Agar (Bioshop; 12g/L), yeast (Lab Scientific; 35g/L), sucrose (standard Redpath; 44mM), glucose (Lab Scientific;167mM) were each tested independently to determine their contribution to the behavioural assays and progeny analysis. Fructose was also tested (Sigma Aldrich). Dose response curves were tested from 0mM – 333mM to determine the role they play for mating and fertility.

The artificial sweeteners were tested across 3 different concentrations. Aspartame (Fischer Scientific), Sucralose (Sigma Aldrich), Na Saccharin (Sigma Aldrich) at concentrations of 5mM, 44mM and 288mM.

In order to determine which sex is regulating mating frequency, glucose at standard fly food concentration of 167mM was used. The introgression lines were also tested on 167mM of glucose. The *Gr64a*^{2} mutants were tested on 167mM of glucose and 44mM of sucrose. *Pox –neuro* mutants were tested on our standard laboratory fly food as well as on 167mM glucose and *Or83b* null flies were tested using laboratory fly food and 35g/L of yeast.

Behavioural assays:

The 1x1 assay:

A single female and single male of a particular strain were used in these assays. The male and female (age 5-6 days old) were aspirated into a 35x10mm petri dish. The bottom of the petri dish was covered with the appropriate food being tested. The petri dishes were placed at 25°C in a 12:12 light:dark cycle. The number of matings over 24 hours was scored using either the Hitachi CCD camera with the Northern eclipse software (v. 7.0) or the Canon S10 digital camera using the ZoomBrowser EX software. Red light was utilized to visualize the matings during darkness.

The 6x6 assay:

Six females and six males of a particular strain were used. The flies were aspirated into a 60x15mm petri dish containing the food that was being tested. The petri dishes were placed at 25°C in a 12:12 light:dark cycle. The number of matings over 24 hours was scored using either the Hitachi CCD camera with the Northern eclipse software (v. 7.0)

or the Canon S10 digital camera using the ZoomBrowser EX software., while red light was used to visualize the matings during darkness.

Progeny analysis:

Following 24 hours of behavioural experiments, the food present in the petri dishes was removed and transferred onto our standard laboratory fly food present in polystyrene vials. The vials were maintained 25°C in a 12:12 light:dark cycle until eclosion. The subsequent eclosed progeny were then scored to determine which food is ideal for egg laying, larval development and eclosion.

Statistical analysis:

Statistical analysis was performed using the SPSS software (SPSS inc. version 16.0). All within strains analysis was performed using the one-way ANOVA followed by the Tukey-Kramer post hoc test for multiple comparisons. The analysis between strains was performed using the two-way ANOVA followed by the Tukey-Kramer post hoc test

Chapter 3
Food and Reproduction

Introduction:

Why females mate multiple times in the wild has been a widely debated topic for many years and there have been many conflicting results in the literature as reviewed by Singh et al. (2002). *Drosophila melanogaster* females were thought to be monogamous since remating was thought to have a negative impact on the fitness of the female (Bateman, 1948). It was thought that *Drosophila* females remated very infrequently if at all. If a female were to remate, it would generally be every 6-7 days and would be in response to low sperm counts (Pyle and Gromko, 1978). However, over the last 40 years, *Drosophila* females were found to be polyandrous and exhibited multiple matings (Imhof et al., 1998). In the recent studies conducted, where multiple matings were observed, females are given the males periodically over the course of 7 days and measured for remating. In these periodic confinement assays, where the females are housed with males for a certain period of time each day and then removed, *Drosophila* females were found to remate but only a few days after the initial mating (Singh et al., 2002).

A novel assay was developed in our lab where *Drosophila melanogaster* females were housed continuously with males and monitored over a 24-hour time frame. The studies

done recently show that *Drosophila* females mate multiple times within a 24 hour time frame when the females and males are continually housed together, indicating that differences in experimental design significantly affected remating (Krupp et al., 2008) (Billeter *et al.,* Submitted). Based on this finding, this chapter will focus on the necessity of food as a condition for remating over a 24-hour time frame and the type of food that is necessary and sufficient to observe these increased mating frequencies on food. The chapter will also address the importance of the genetic background of the fruit fly and its social context on mating frequency and progeny counts.

The assay was done in petri dishes with males and females. The majority of the experiments performed consisted of one female and one male housed together continuously over 24 hours (1x1 assay). An effect of group size was determined by housing six females with six males together continuously over 24 hours (6x6 assay). To obtain an estimate of the fitness level of each strain, the adult progeny was scored for all experiments as per Materials and Methods (Chapter 2).

Results:

Food is needed to observe rematings in wild type strains of *Drosophila melanogaster*

Food is necessary in order to observe multiple matings and this can be seen in both laboratory *Drosophila melanogaser* wild type strains tested. The mating frequencies for both *Canton-S* and *Oregon-R* were quantified when placed on either standard laboratory

fly food or non-nutritious agar (Figure 1 A-B). In the 1x1 assay as well as the 6x6 assay, both strains displayed significantly increased mating frequencies on fly food, while minimal mating was observed on agar for both strains (Tukey's HSD, $p < 0.001$). Thus, food is a fundamental condition for remating.

Yeast has a significant effect on mating frequency and progeny counts

Once it was determined that food was necessary to observe higher mating frequencies, the fly food was tested for its major components. Breakdown of the fly food was tested on both strains to understand their effect on reproduction. The concentrations tested were those found in the laboratory fly food (Chapter 2: Materials and Methods). Yeast, glucose, sucrose, fructose along with glucose and sucrose combined together were tested to determine their contribution to the mating frequency in a 1x1 context (Figure 1 A-B). The matings observed on standard fly food were higher than the matings on all other types of food tested for both strains. For *Canton-S* flies (Figure 1A), the sugars tested had an effect on the mating frequency while for *Oregon-R* flies (Figure 1B), the mating frequencies observed on the different sugars were not significantly different from the frequencies observed on agar. Contrary to the results observed on the sugars, both *Canton-S* and *Oregon-R* displayed significantly more matings on yeast. The number of matings observed on yeast was significantly more than the matings observed on agar for *Canton-S* **(**Tukey's HSD, $p < 0.05$**)** (Figure 1A) and significantly more than the matings observed on both agar and the sugars for *Oregon-R* (Tukey's HSD, $p < 0.05$) (Figure

1B). The results observed on the sugars and yeast indicates that there is a significant difference between the two strains with regards to the type of food in the environment that stimulates reproduction ($F_{1,473} = 12.229$, $p < 0.01$).

Similar results were observed in the 6x6 assay, where the mating frequencies observed on fly food and yeast were significantly different from the frequencies observed on the different sugars tested (Figure 1 A-B). Once again, there was a significant difference between *Canton-S* and *Oregon-R* ($F_{1,117} = 266.722$, $p < 0.001$). *Canton-S* flies displayed rematings on all foods tested including agar (Figure 1A), while *Oregon-R* flies only remated on yeast (Figure 1B). These two wild type strains differ in the mating frequencies and progeny counts on all foods tested indicating a genetic contribution to food and mating.

To investigate whether the two strains displayed a group size effect, the 1x1 assay was compared to the 6x6 assay within each strain (Figure 1 A-B). *Canton-S* flies display increased matings per female as a result of being in a group on all foods tested ($F_{1,301} = 65.220$, $p < 0.001$) (Figure 1A), while *Oregon-R* flies are not affected by the social context since the number of matings per female in the 1x1 assay is similar to the 6x6 assay on the food sources tested except when tested on standard fly food ($F_{1,246} = 2.933$, $p = 0.088$) (Figure 1B). *Canton-S* flies also exhibit a significant interaction between the group size and food ($F_{5,298} = 5.147$, $p < 0.001$) while *Oregon-R* flies fail to show this interaction ($F_{5,243} = 1.523$, $p = 0.183$). With respect to mating behaviour on different nutritional sources, it can be concluded that the two wild type strains significantly differ from each other in their preferences of food sources and mating frequencies.

An estimate of the fitness level for both strains was determined by counting the adult progeny that eclosed from each experiment (Figure 1 C-D). In general, it resulted that although *Canton-S* flies mate more on average on all foods tested, it is the *Oregon-R* flies that in the end have more adult progeny than the *Canton-S* flies in a 24 hour period. Looking at *Canton-S* flies in the 1x1 assay, they displayed slightly more progeny counts on yeast, as it was significantly different from the progeny counts observed on agar (Tukey's HSD, $p < 0.05$) (Figure 1C). Although, *Oregon-R* flies did not remate on the sugars, they had high numbers of progeny on yeast as well as glucose in the 1x1 assay (Figure 1D). The progeny counts of each female in the 6x6 assay decreased when compared to the 1x1 assay for the *Canton-S* strain ($F_{5,45} = 6.136$, $p < 0.001$) (Figure 1C). However, the results remained similar to the results observed in the 1x1 assay, where *Canton-S* flies in the 6x6 assay had more progeny on yeast on average than the other food sources tested (Figure 1C). Similarly, *Oregon-R* flies also had significantly more progeny on yeast as opposed to the other foods available while also producing significantly more progeny per female in a 6x6 context ($F_{5,46} = 9.719$, $p < 0.001$). Two-way anova releaved a difference b/w the strains in the contribution of nutrition to progeny ($F_{1,101} = 45.274$, $p < 0.001$).

Figure 1

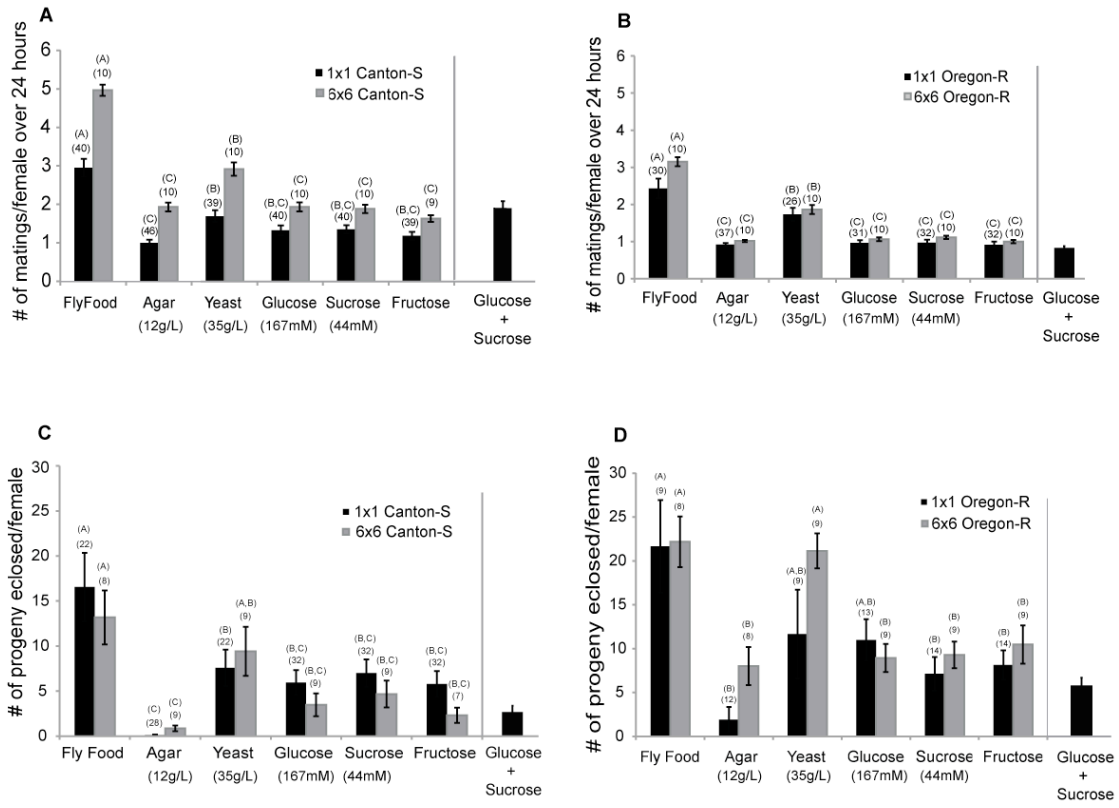

Figure 1 (A-D): Effect of group size on the mean number of matings and offspring on different components of laboratory fly food. The numbers above the bars represent sample sizes while the letters are provided for statistical purposes. Differences within group size should be compared where different letters indicate significance (Tukey's HSD, $p < 0.05$). **(A):** Mean number of matings/female comparing *Canton-S* in a pair (1x1 assay) and group setting (6x6 assay). **(B):** Comparing the mean number of progeny/female from the *Canton-S* strain between pair (1x1assay) and group setting (6x6 assay) **(C):** The mean number of matings/female comparing group sizes for the *Canton-S* strain **(D):** The mean number of progeny/female for the *Oregon-R* strain between different social contexts.

Remating and higher progeny counts on yeast are common to both *Canton-S* and *Oregon-R*

The foods tested previously were only tested at a single concentration found in the standard fly food. Various doses of yeast and the sugars were tested here to determine if different dosages of a particular food type had an effect on reproduction when a wide

range of concentrations were tested. The different components of laboratory fly food were tested in various concentrations in the 1x1 assay for both strains. Yeast was tested at 5 different concentrations, while glucose and sucrose found in fly food was tested over 10 different concentrations (Figure 2 A-F). Despite all the concentrations tested for glucose and sucrose, *Oregon-R* flies only mated multiple times on yeast **(**Tukey's HSD, $p < 0.05$**)** while the matings on glucose **(**Tukey's HSD, $p > 0.05$) and sucrose (Tukey's HSD, $p > 0.05$**)** was not significantly different from the matings on agar (Figure 2 A,C,E). *Canton-S* flies on average remated on glucose **(**Tukey's HSD, $p < 0.05$**)** and sucrose as well as on yeast, however the matings observed on sucrose were not significantly different over any of the concentrations tested ($F_{9,190} = 1.422$, $p = 0.181$) (Figure 2 A,C,E). This resulted in a significant difference between the two strains with respect to matings on glucose ($F_{1,379} = 192.96$, $p < 0.0001$), and sucrose ($F_{9,380} = 82.44$, $p < 0.0001$). As for yeast, no significant difference was observed between *Canton-S* and *Oregon-R* ($F_{1,259} = 1.934$, $p = 0.166$), with both strains mating more on the higher concentrations of yeast tested.

The progeny counts observed on yeast was similar to the trend observed for the matings on yeast for *Oregon-R* flies where they displayed a significantly higher fertility counts for the higher concentrations tested (Tukey's HSD, $p < 0.05$**)** (Figure 2B). *Canton-S* exhibited no significant difference for progeny counts between any of the concentrations tested on yeast (Tukey's HSD, $p < 0.05$**)** (Figure 2B). Similar to the results observed in the 1x1 and 6x6 assays, although *Canton-S* flies mate more, they tend to have less progeny than *Oregon-R* flies indicating a significant difference between the two wild type strains (Tukey's HSD, $p < 0.05$**)**. This trend can once again be noticed on both

glucose and sucrose where *Oregon-R* generally produces more progeny than *Canton-S.* Higher concentrations of glucose and sucrose yielded higher progeny counts for *Canton-S* flies while, increased progeny counts for *Oregon-R* were observed on intermediate concentrations of the sugars (Figure 2 D,F).

Since yeast was the preferred food source for both strains for stimulating reproduction, the sugar component of yeast was tested for its contribution. Trehalose was found to be the main sugar present in yeast (Vandijck et al., 1995). Trehalose was tested over 10 different concentrations to determine its contributions towards increased mating and progeny counts (Figure 2 G-H). It has been previously shown that *Oregon-R* flies display low sensitivity towards trehalose and *Canton-S* flies display high sensitivity towards trehalose (Tanimura et al., 1988). These strain differences were linked to the *Tre* gene present on the X chromosome (Dahanukar *et al.,* 2001). When these two strains were tested, neither strain displayed any significant difference in mating behaviour over any of the concentrations tested (Figure 2G). When the progeny counts were scored, once again *Oregon-R* flies produced more progeny despite mating less on average than *Canton-S*, however, the progeny counts for neither strain was significantly different from agar (Figure 2H). This indicates that it is not the sugar component (trehalose) in the yeast that is responsible for stimulating reproduction.

Figure 2

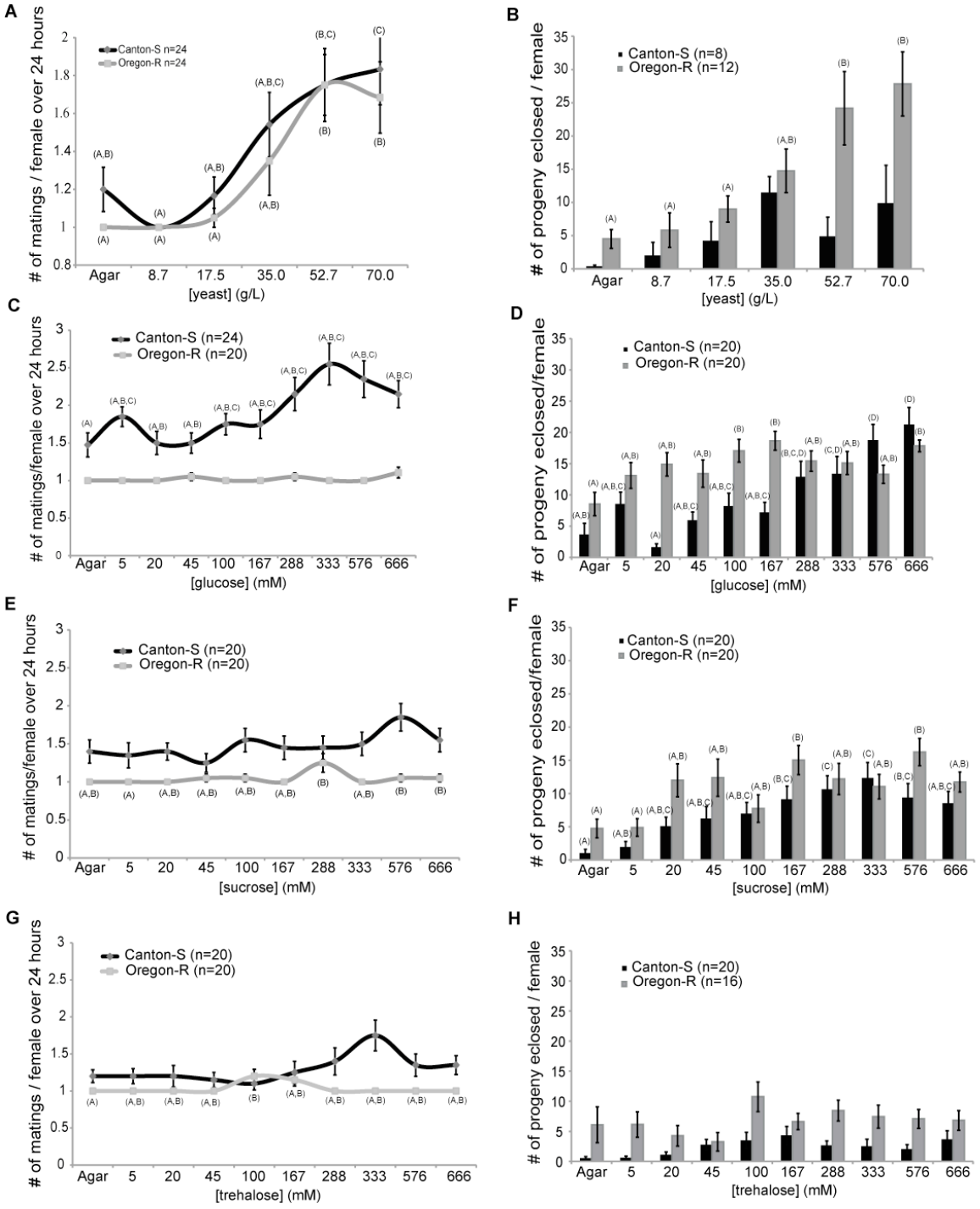

Figure 2 (A-H): Dose response curves and progeny counts on yeast and different sugars tested to determine the optimal concentration for the reproductive behaviour of females of two wild type strains *Canton-S* and *Oregon-R* in a 1x1 assay. The letters are provided for statistical purposes

and differences within strain should be compared where different letters indicate significance ($p < 0.05$). When no statistical letters are present, it indicates no statistical significance ($p > 0.05$) for the particular strain **(A):** Differences in mating behaviour over varying concentrations of yeast **(B):** Comparing the mean number of progeny/female on different concentrations of yeast **(C):** The mean number of matings/female on different concentrations of glucose **(D):** The mean number of progeny/female on different concentrations of glucose for the two strains. **(E):** The mean number of matings/female on differing concentrations of sucrose. **(F):** The progeny counts per female on differing concentrations of sucrose. **(G):** The mean number of matings/female on different concentrations of trehalose. **(H):** Progeny counts per female on differing concentrations of trehalose.

Females are modulating remating and egg laying

The differences observed between *Canton-S* and *Oregon-R* for mating frequencies and progeny counts were used to determine which sex is modulating reproductive activity in response to food factors. Since the differences in behaviour were readily observed on glucose, this food substrate at a fly food concentration of 167mM was used for testing (Figure 2 C-D). *Canton-S* females were paired with *Oregon-R* males and vice versa in a 1x1 assay while same strain couples were used as controls. If females were to regulate reproductive behaviour, experiments with *Canton-S* females should show higher mating and fertility counts than experiments with *Oregon-R* females. If the males were found to be controlling behaviour, experiments with *Canton-S* males should show similar results to the *Canton-S* controls, while experiments with *Oregon-R* males should show similar mating and fertility counts to the *Oregon-R* control. When matings for the different combinations were tested, it indicated that there was no significant difference on agar (Figure 3A). However on glucose, *Canton-S* females with *Oregon-R* males mate significantly more than either the *Canton-S* or the *Oregon-R* controls (Tukey's HSD, $p < 0.05$). *Oregon-R* females with *Canton-S* males have mating frequencies that are not

significantly different from the controls (Tukey's HSD, $p > 0.05$) (Figure 3A). Since the *Canton-S* males with *Oregon-R* females did not display the same mating frequencies as the frequencies observed with *Canton-S* females with *Oregon-R* males, the females seem to be the major determinants of the mating frequency (Figure 3A).

It is once again shown that *Oregon-R* flies have more progeny than *Canton-S* flies (Figure 3B) and it seems that females have majority control over egg laying and progeny counts (Figure 3B). *Oregon-R* females with *Canton-S* males had significantly more progeny than any other combination on both agar and glucose (Figure 3B). The progeny counts for *Canton-S* females with *Oregon-R* males were similar on average to the counts observed for the *Canton-S* controls. The change in females contributes to approximately 13% of the variance observed in the progeny counts and is thus extremely significant ($F_{3,137} = 7.82$, $p < 0.001$). This indicates that the variation in progeny numbers is largely under female control.

Figure 3

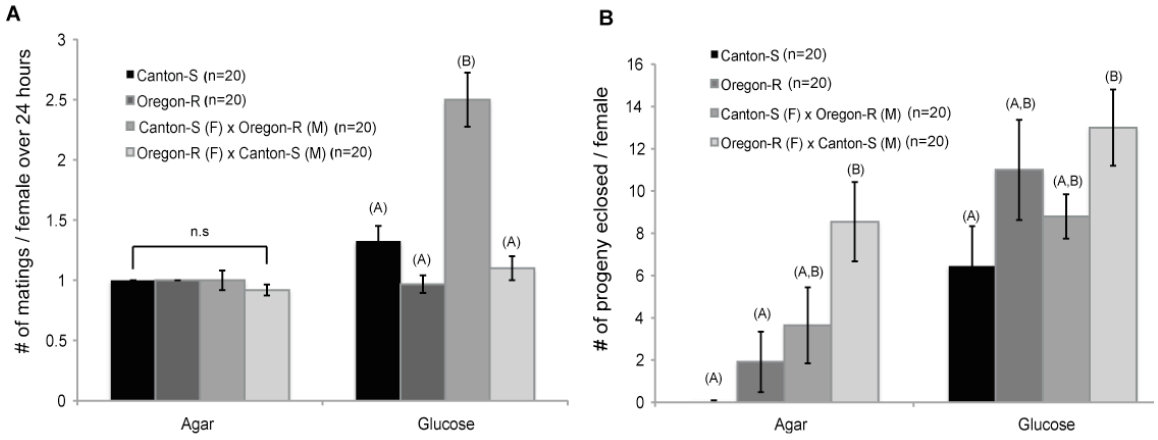

Figure 3 (A-B): Sex dependence of remating and egg laying behaviour on agar and glucose as food substrates in a 1x1 assay. The letters above the bars are provided for statistical analysis. The

set of flies tested should be compared between each other on a particular food where significance is indicated with different letters ($p < 0.05$) and n.s. represents no significance on the particular food ($p > 0.05$). **(A):** The mean number of matings per female over 24 hours on agar and glucose (167mM) **(B):** The mean number of progeny that eclosed from the matings per female on agar and glucose.

The X and 2nd chromosomes are contributing towards differences in fertility counts

Once it was established that there was strain difference between *Canton-S* and *Oregon-R*, the gene responsible for the difference observed on glucose could potentially be identified. In order to begin mapping a candidate gene, the effect needs to be narrowed down to a chromosome. Once again, glucose at a concentration of 167mM was used as the food substrate since it gave a significant difference between the two strains for reproduction (Figure 2 C-D). When tested, the mating frequency could not be narrowed down to a chromosome on glucose since none of the flies tested were significantly different from each other (Tukey's HSD, $p > 0.05$**)** (Figure 4A). However, when *CS;OR;OR* and *OR;CS;OR* were analyzed for progeny, these flies were not significantly different from the *Canton-S* control but were significant different from the *Oregon-R* control progeny (Figure 4B). *OR;OR;CS* flies were not significantly different from the *Oregon-R* controls (Figure 4B). This indicates that the X and 2nd chromosome in the fly are responsible for the differences observed in *Canton-S* and *Oregon-R* on glucose and there is a potential interaction between these two chromosomes that gives rise to the stain differences in fertility on glucose.

Figure 4

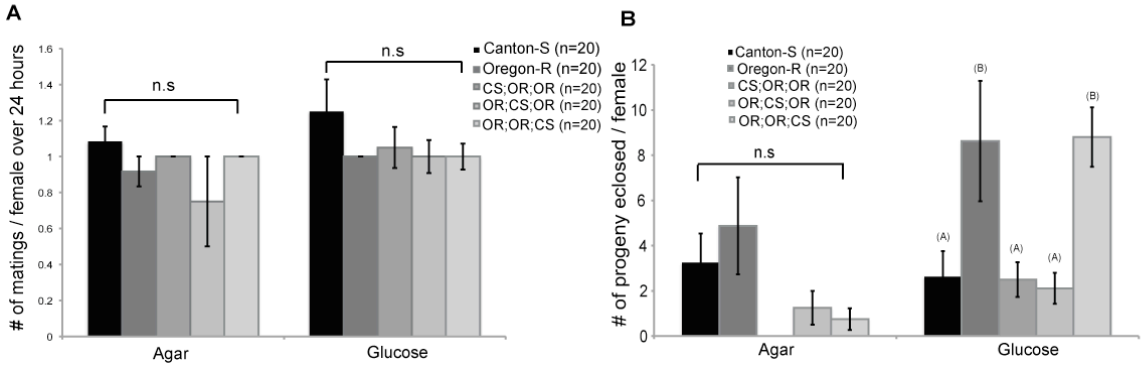

Figure 4 (A-B): The mating and egg laying behaviour of the introgression lines on agar and glucose (167mM) in a 1x1 assay. The letters above the bars are provided for statistical analysis. Each food group should be compared separately where different letters indicate significance ($p < 0.05$) and n.s. represent no significance on the particular food ($p > 0.05$). **(A):** The number of matings per female over 24 hours on agar and glucose **(B):** The mean number of progeny from the matings per female on agar and glucose.

Conclusion:

These results indicate that for both *Canton-S* and *Oregon-R,* food in the environment is a requirement for remating. Both strains display multiple matings within 24 hours on our standard laboratory fly food and are consistent with the data shown in previous studies (Krupp et al., 2008) and Billeter et al. (Submitted). Although the two strains remate, they exhibit differences on the type of food that is required for rematings indicating that there is a genetic basis to the mating behaviour. *Oregon-R* flies will only remate on yeast while *Canton-S* flies remate on yeast as well as on the sugars tested (Figure 1 and Figure 2). Further confirmation that remating is under genetic control is the fact that *Canton-S* females will always remate more on average than the *Oregon-R* females in pairs as well as in groups (Figure 1 A-B). The effect of group size is evident in the *Canton-S* strain where there are more matings per female as a result of being in a larger group. This result

was not that pronounced in *Oregon-R* flies where the group effect was only seen on standard fly food (Figure 1 A-B). Although, the mating frequencies observed for introgression lines, did not give conclusive results, it could possibly be an artifact of the food type being tested on. More experiments need to be run with other food types to conclude a genetic contribution of a chromosome towards mating frequency. This indicates that remating is under genetic control as well as under the control of the social environment given a specific food type.

Progeny analysis revealed that although the mating frequency of *Canton-S* females is higher, *Oregon-R* flies tend to have more progeny on almost all foods tested (Figure 1 and Figure 2). Within the introgression lines, the progeny effect seen by the two strains could be narrowed down to the X and the 2nd chromosome. Within each strain, on most foods tested the higher progeny counts were observed on higher concentrations of food indicating that the level and quality of food present is important for increasing progeny counts for the next generation.

Chapter 4

Perception of Sweetness and Reproduction

Introduction:

It was determined that food is necessary to observe rematings in both *Canton-S* and *Oregon-R* wild type strains (Chapter 3). Flies have the ability to smell and taste their environment through the olfactory and gustatory neurons and gather chemical information about their surroundings (Scott, 2005). This suggests that the necessary food could either be perceived via the sensory systems as either nutritious or non-nutritious or the food could be metabolized in the fly and thus assessed for its caloric value. This chapter will focus on the olfactory and gustatory mutants available in *Drosophila melanogaster* to determine their effect on reproduction in the fly and if the perception of food is important. On the other hand, to test whether the calories present in the food are required, artificial sweeteners with no calories will be tested for their effect on reproduction.

The assay was done in petri dishes with one male and one female (1x1 assay) over 24 hours. The gustatory mutant, *Gr64a* was used to test for sugar perception since it confers sensitivity to a wide range of sugars including glucose and sucrose (Jiao et al., 2007). To test for sensitivity of all external taste bristles that are responsible for tasting, *pox-neuro* flies were used where gustatory deficiencies were found on various parts of the fly's body (Krstic et al., 2009) (Boll and Noll, 2002). *Or83b* null flies were used as olfactory

mutants to assess the importance of smell impaired mutants on mating frequency and progeny counts. The artificial sweeteners tested were substitutes for sucrose and were tested at three different concentrations (Chapter 2). The progeny for all experiments was determined as per Materials and Methods (Chapter 2).

Results

Gustatory mutant has no effect on mating and fertility

In order to investigate the role of the gustatory receptors in reproduction, *Gr64a*^{2} mutant was used. This mutant lacks sensitivity towards glucose and sucrose. This mutant was placed in one of two genetic backgrounds, either *Canton-S* (*CS;CS;Gr64a*) or *Oregon-R* (*OR;OR;Gr64a*) for testing along with using *Canton-S* and *Oregon-R* flies as controls. Neither set of flies exhibited any significant difference when compared with the control flies for mating frequency or progeny counts (Figure 5 A-B). *CS;CS;Gr64a* flies do show a decrease when compared to *Canton-S* on glucose, however, this decrease is not statistically significant and thus there is no significant difference between the mutants and the *Canton-S* controls on the two sugars tested ($F_{1,48} = 5.47$, $p = 0.0635$) (Figure 5A). A similar result was observed for the *Oregon-R* flies where there was neither a increase nor decrease in mating frequency between the two sets of flies tested (Figure 5B) and therefore, there was no significant difference between the controls and the gustatory mutants in mating behaviour on glucose and sucrose ($F_{2,50} = 0.13$, $p = 0.8741$).

The progeny counts reflected the mating frequency and were not significantly increased nor decreased between the controls and the gustatory mutants (Figure 5 C-D). Once again, *Oregon-R* flies had slightly more progeny than the *Canton-S* flies but within each strain and its mutants, there was no significant difference between *Canton-S* and *CS;CS;Gr64a* ($F_{2,48}$ = 1.03, p = 0.3653) or *Oregon-R* and *OR;OR;Gr64a* ($F_{2,50}$ = 0.56, p = 0.5772). The results observed for mating and progeny counts indicate that the lack of a gustatory receptor for certain substrates will not hinder mating frequency or fertility counts on that food source.

Figure 5

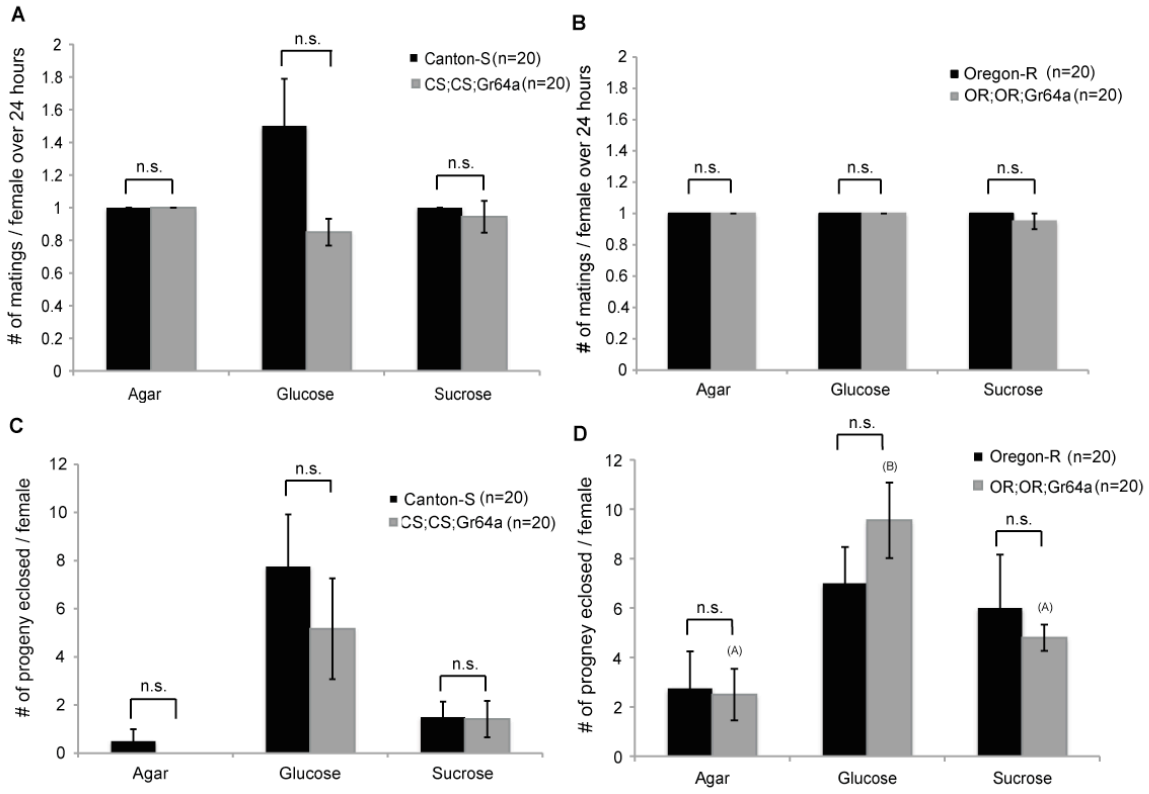

Figure 5 (A-D): The mating and egg laying behaviour of *Gr64a* gustatory mutants on different sugars in a 1x1 assay. The letters above the bars are provided for statistical purposes and each set of flies should be compared across the different food substrates. Different letters indicate significance (p < 0.05). When no statistical letters are present, the set of flies did not give significantly different letters across the food groups (p < 0.05). **(A):** The mating behaviour of

Gr64a mutants in a *Canton-S* genetic background over 24 hours **(B):** The mean number of matings over 24 hours for *OR;OR;Gr64a* (*Gr64a* mutant in the *Oregon-R* background) **(C):** The mean number of progeny per female for *Gr64a* mutants in a *Canton-S* genetic background **(D):** The mean number of progeny per female for *OR;OR;Gr64a.*

Pox-neuro mutants and *Or83b*$^{-}$ mutants have little effect on reproduction

Since flies lacking *Gr64a* did not show significant changes in mating, *pox-neuro* mutant flies were tested for mating behaviour and progneny counts (Figure 6 A-B). A range of mutant flies were tested where they consisted of having a brain and gustatory defect (w^{B};$Poxn^{\Delta M22\text{-}B5}$/*CyO*), gustatory defect with normal brain function (*ΔxB56,Oregon-R*;$Poxn^{\Delta M22\text{-}B5}$), partial rescue expect labellum (w^{B}; $Poxn^{\Delta M22\text{-}B5}$;*full-1*), and complete resuce (w^{B}; $Poxn^{\Delta M22\text{-}B5}$;*superA158*) (Krstic et al., 2009). All flies tested remated on standard fly food and as expected exhibited no rematings on agar (Figure 6A). The rematings on standard fly food were not significantly different between the genotypes despite the gustatory deficiencies found (Tukey's HSD, $p > 0.05$) (Figure 6A). The lack of significant differences between the different *pox-neuro* flies was observed for progeny counts as well when tested on standard fly food (Figure 6B). Flies with the gustatory deficiencies and flies with complete rescue were tested on glucose to determine if the lack of gustatory bristles blocked reproduction when placed on sugar alone. Both mating frequency and fertility levels remained the same between the two sets of flies tested (Figure 6 A-B) and were not significantly different from each other on glucose (Tukey's HSD, $p > 0.05$).

In order to determine if the olfactory system played a role in reproduction, *Or83b* null flies were tested (Figure 6 C-D). Smell impaired *Or83b* flies did not decrease the levels of mating frequency (Figure 6C). As there were no differences between the *Canton-S* controls and the *Or83b* mutants on yeast or fly food, it suggested that the sense of smell is not contributing to the behaviours observed. The lack of smell also had little effect on progeny counts (Figure 6D). There were no significant differences between the control and mutant flies. These results suggest that the lack of smell does not hinder reproduction.

Figure 6

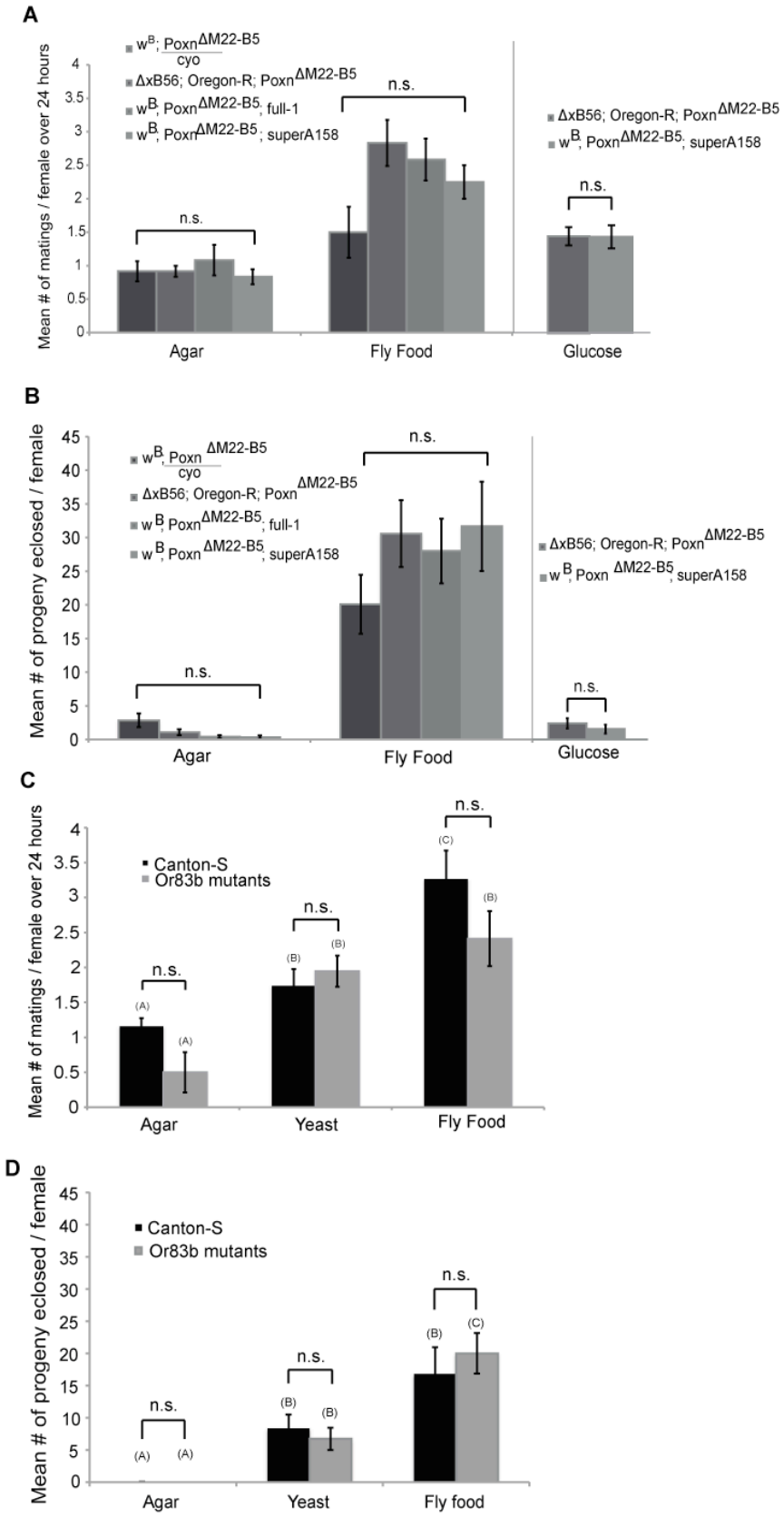

Figure 6 (A-D): The effect of chemosensory mutants on the reproductive behaviour of *Drosophila melanogaster* in a 1x1 assay. The letters above the bars are provided for statistical purposes where different letters indicate significance ($p < 0.05$). n.s. represents no significance between groups of flies tested ($p > 0.05$) **(A-B):** *Pox-neuro* mutants were tested for mating frequency over 24 hours and their subsequent progeny counts scored on agar, standard fly food and glucose. (w^B; $Poxn^{\Delta M22-B5}$ / cyo) indicates flies that have brain and gustatory defect, (ΔxB56;Oregon-R;$Poxn^{\Delta M22-B5}$) have gustatory defect with normal brain function, (w^B; $Poxn^{\Delta M22-B5}$;full-1) have partial rescue except for labellum and (w^B; $Poxn^{\Delta M22-B5}$;superA158) have complete rescue of gustatory function. **(C-D):** *Or83b* null flies (*Canton-S* genetic background) were tested for mating frequency over 24 hours and the progeny counts scored per female.

Canton-S and *Oregon-R* do not remate or lay eggs on artificial sweeteners

Testing with artificial sweeteners provided further confirmation that the gustatory system is not solely responsible for making the decision to remate or have progeny. The sweeteners tested merely provide the "sweet" taste but provide no calories when ingested. Aspartame, sucralose and sodium saccharin were tested over three different concentrations. There were significant differences between *Canton-S* and *Oregon-R* on the three artificial sweeteners tested for certain doses (Figure 7 A,C,E) (Tukey's HSD, $p < 0.05$). However, there was no significant difference within each strain over any of the doses tested for any sweeteners (Figure 7 A,C,E). This indicates that the mating behaviour is dependent on more than simply tasting the sweet substrate.

The progeny analysis followed the same trend as the results observed for the mating behaviour (Figure 7 B,D,F). The strain difference is once again evident in that although *Oregon-R* females mate less on average than *Canton-S* females, they tend to have more progeny on average as this can be seen for almost all of the doses of sweeteners tested. Similar to the mating behaviour, the progeny counts observed across the doses tested were not significantly different from the non-nutritious agar on any sweetener tested indicating that it is not enough for the progeny to be able to just taste the sweet substrate.

Figure 7

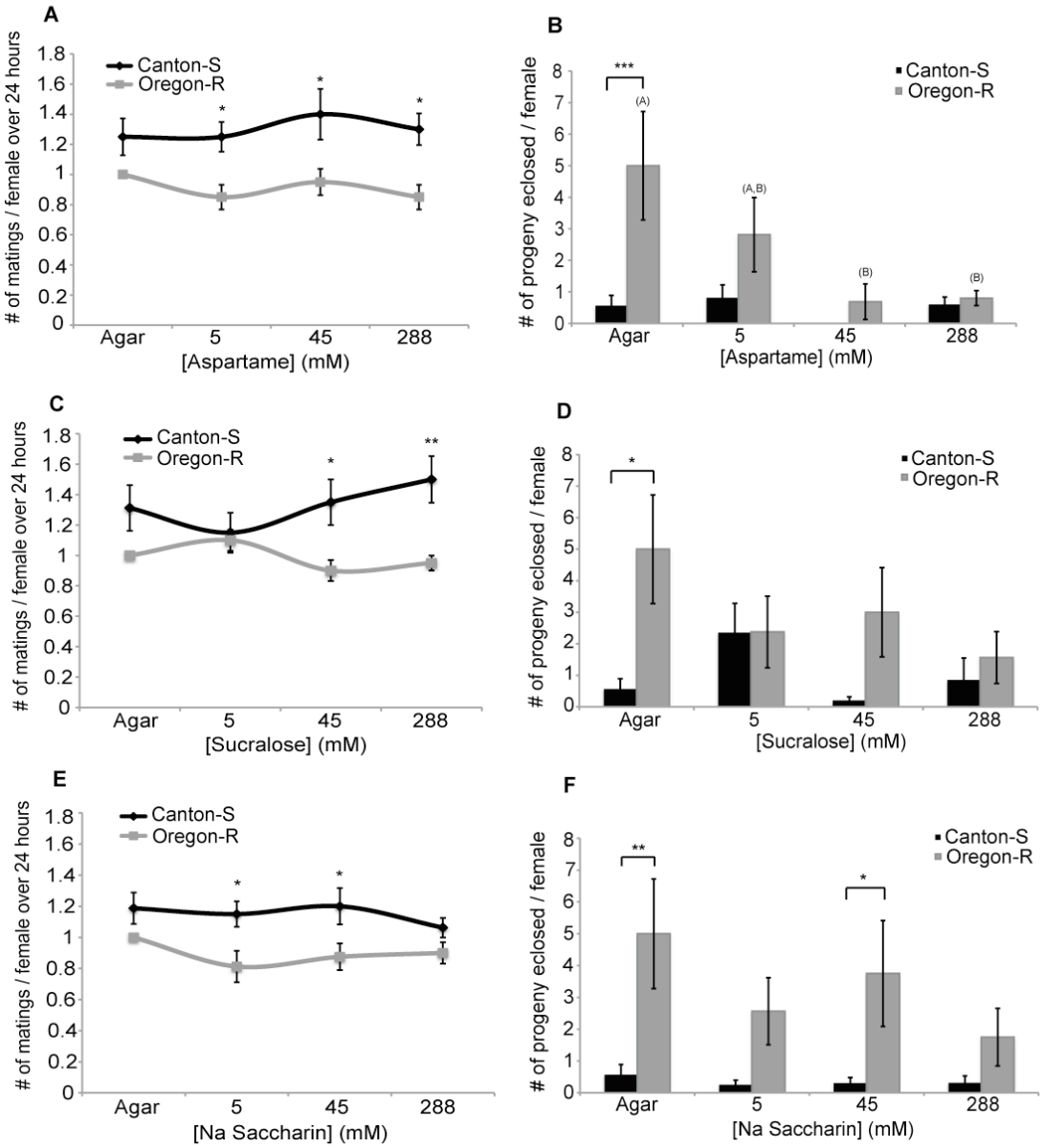

Figure 7 (A-F): Reproductive behaviour of two wild type strains on varying concentrations of three different artificial sweeteners in a 1x1 assay. The letters above the bars are provided for statistical analysis and should be compared within each strain across the different concentrations of sweeteners. Different letters represent statistical significance ($p < 0.05$) while no letters represent no significance ($p > 0.05$). (*) indicates $p < 0.05$, (**) indicates $p < 0.01$ and (***) indicates a $p < 0.001$ between the two strains for a particular sweetener dose. **(A):** Mean number of matings per female on varying concentrations of aspartame **(B):** Mean number of progeny per female on aspartame **(C):** Mean number of matings per female on sucralose **(D):** Mean number of progeny eclosed per female on sucralose **(E):** Mean number of matings on Na Saccharin **(F):** Mean total progeny per female on Na Saccharin

Conclusions:

It was shown here that gustatory and olfactory mutants have little to no effect on the mating frequency and progeny counts of females. The sweet taste of the artificial sweeteners was also not enough for reproduction in the wild type flies tested as there was no significant differences between the frequencies observed on agar and the sweeteners. This indicates that the perception of sweetness is not enough for reproduction suggesting that there must be a different mechanism by which the flies are assessing the food.

Chapter 5
Discussion

There have been many reasons suggested over the years as to why most species mate multiple with many partners over their lifetime instead of remaining monogamous. Remating might occur to renew or replenish sperm supply while protecting against male sterility (Chapman et al., 1994). Females of some species even gain nutrients from the male when mating occurs suggesting that it might be beneficial to remate when food is scarce (Chapman et al., 1994). When females remate, the sperm is stored in specialized reproductive organs in the female and this storage of sperm could promote sperm competition and lead to an increase in genetic diversity if the female mates multiple times (Chapman et al., 1994).

It has been shown in many studies that both males and females of *Drosophila melanogaster* mate mutiple times over their lifetime (Harshman et al., 1988) (Chapman and Partridge, 1996) (Imhof et al., 1998). Although the findings in this thesis generally agree with this finding, the details appear to be assay dependent. In earlier studies, remating was measured by providing the female with different males periodically over couple of days and observing mating (Pyle and Gromko, 1978). If females mate with more than one male that was provided to them, it was concluded that *D. melanogater* females remate. Based on this assay, this remating observed occurs days after the first mating. It has been suggested that this assay mimics the happenings in the wild since females might only remate once their sperm stores are depleted and remating does not

increase with density (Gromko et al., 1984) (Gromko and Gerhart, 1984). However, other studies found that remating frequencies increased with population density and with the level of genetic diversity in the environment (Ochando et al., 1996) (Imhof et al., 1998). My results indicate that females actually mate multiple times over a period of 24 hours on our laboratory fly food. Not only do flies tested remate, *Canton-S* and *Oregon-R,* the wild type strains of *D. melanogaster* remate at different levels over 24 hours in both the pair assay as well as with the 6x6 assay in the presence of standard fly food. This result is consistent with the data observed in previous studies (Krupp et al., 2008) and Billeter *et al.* (Submitted). These results suggest that there is an effect of the size of the group on an individual, which results in the females increasing their matings over 24 hours. The observations found this thesis is contradictory to the literature and could be due to different experimental designs used to measure remating, timing and nutrition provided for the flies.

Since it was concluded that flies mate multiple times in 24 hours in the presence of fly food, the different components of the food were tested to determine which component gives rise to this remating behaviour. Breaking down the components of the food gave further confirmation that reproduction was strain dependent. *Canton-S* flies remated on all food types while *Oregon-R* only remated in the presence of yeast. *Canton-S* females also remated at higher frequencies on all doses of different food types tested. The opposite was observed for fertility counts. *Oregon-R* flies had more progeny in a 24-hour time frame than *Canton-S* flies. Apart from the fact the same strain of flies weren't used and differences in experimental design, the general conclusion was similar to other results that have been found.

Previous studies were mostly conducted in the presence/absence of yeast where higher remating frequencies and fertility counts resulted when higher doses of yeast were tested and in some cases death was observed in the lower doses tested which could possibly be due to malnutrition (Gromko and Gerhart, 1984) (Harshman et al., 1988) (Chapman and Partridge, 1996). Trehalose makes up about 15-20% of the sugar content in yeast and is the main sugar present in yeast (Vandijck et al., 1995). *Oregon-R* flies are spontaneous mutants for the *Tre* gene that codes for the *Gr5a* receptor that recognizes trehalose and was therefore not surprising that these flies did not display significant differences for mating and fertility when tested with trehalose (Tanimura et al., 1988). *Canton-S* flies are able to sense trehalose but similar to *Oregon-R,* these flies did not show significant differences in reproduction on trehalose. Trehalose is made up of two glucose molecules and is the main sugar present in the hemolymph of flies (Chyb et al., 2003). However, trehalose had no effect on mating frequency and fertility in this assay for either *Canton-S* or *Oregon-R*. Since the sugar present in the yeast played no role in reproduction, it could either be the smell of yeast that is an attractant to flies or it might be the protein present.

Or83b is broadly expressed in all olfactory receptor neurons and is needed to associate with members of the olfactory family and target them to the antenatal lobe (Couto et al., 2005) (Vosshall and Stocker, 2007). Flies deficient for the *Or83b* gene cannot smell. Yeast has been shown to be the food of larvae and for this reason it might be an attractant to the adult fly as favourable sites for oviposition (Baumberger, 1917). Flies that cannot smell the yeast due to null mutation in the *Or83b* gene, are also attracted to yeast as a site for mating and for egg laying. These results indicate that when it comes to assessing food and nutrition in the environment, the portion of the olfactory system that requires *Or83b*

as a co-receptor plays little to no role in blocking reproduction. However, *Or83b* mutants have fully functional taste bristles and ionotropic receptors that could be aiding in identifying the environment.

Gr64a mutants that were not able to taste glucose and sucrose were tested and showed that this receptor is not needed for the reproductive behaviour observed in *Canton-S* and *Oregon-R*. *Gr64a* in general is needed for sensing a wide range of sugars, as individuals deficient for this gene fail to fire action potentials when stimulated with these sugars (Jiao et al., 2007) (Jiao et al., 2008). *Pox-neuro* flies have their chemosensory bristles transformed into bristles that are similar to mechanosensory bristles and lack the sense of taste. Flies that lacked taste bristles in various parts of the body remated and had fertility counts on both standard fly food and glucose and the reproductive behaviour was similar to the control flies tested that were rescued for the taste bristles. Since there was no difference in mating or fertility on fly food, the lack of tasting yeast or the sugars in the food has minimal effect on reproduction. The result that these two genes are not needed for reproduction suggests that there might be alternate mechanism, by which the fly is able to extract information from the environment about the supply of food available. Although the taste mutants still maintain the ability to smell, the experiments with smell impaired *Or83b* flies suggest that smell has little effect on reproduction. However, an experiment that could provide further confirmation that chemosensation might not be the mechanism by which flies extract information for reproduction could be by testing flies that are both smell and taste deficient.

Results obtained from experiments with artificial sweeteners indicated that the perception of the environment might not be important for reproduction. Wild type flies with fully

functional olfactory and gustatory systems were tested on these sweeteners that were substitutes for sucrose. Even though these flies were able to smell and taste, there was no differences between matings observed on agar and those on the sweeteners for either strain. When the mating frequencies for sucrose and the artificial sweeteners were compared, there were no significant differences in the mating behaviour of flies that were given sucrose or one of the artificial sweeteners. However, there were significant differences when progeny counts were compared between flies that were given sucrose vs. the artificial sweeteners. Higher fertility counts were observed on the higher concentrations of sucrose, while there was no difference between the doses of sweeteners tested. Fertility on the artificial sweeteners did not differ from each other significantly for each strain, but on higher doses of aspartame, fertility was decreased compared to agar. Few studies have looked at the responses of flies when treated with artificial sweeteners and shown that flies respond to all sweeteners tested including aspartame, sucralose and sodium saccharin (Breslin et al., 2008). Sucralose was found to give highest responses when tested in flies (Breslin et al., 2008). It was found that flies did respond to the sweeteners, but the concentrations tested (1mM-5mM) were far below the concentrations tested (5mM-288mM) in this thesis (Breslin et al., 2008). Artificial sweeteners have been tested on primates, mice, and ants and it has been found that flies behave more like humans with regards to tasting sweet substances than any other organism tested (Breslin et al., 2008). Since the doses tested for each artificial sweetener were not different for reproduction from each other or from agar, even at lower concentrations, tasting "sweetness" might not be enough for reproduction and especially for fertility. This indicates that flies might be either assessing the food by ingesting the food for energy or

the females might be ingesting the food for the purposes of egg production, which suggests that there could be a potential role for the metabolic pathway involved in reproduction.

It is thought that adult flies require carbohydrates for reproduction while larvae require yeast for development. (Amrein and Thorne, 2005). Most studies done regarding reproduction and nutrition have used sex peptide mutants. Sex peptide is present in the accessory gland proteins of the male and is thought to exhibit various post-mating responses when transferred to the female upon mating, while sex peptide mutants failed to transfer sex peptide to the female resulting in a reduction of post-mating responses (Carvalho et al., 2006). Numerous studies have linked post-mating responses like decrease in female receptivity and increased egg production to seminal fluids and more specifically to the sex peptide that get transferred to the female by the male. Sex peptide increases feeding in mated females, however this increased feeding only takes place on the higher dosages of yeast (Carvalho et al., 2006) (Min and Tatar, 2006). Although the sex peptide lowers female receptivity and remating on all food levels, increased fecundity and egg laying was only seen on higher quality foods indicating that sex peptide only increased egg production at higher food levels (Fricke et al., 2009). This suggests that food is the limiting factor for reproduction in flies. Several studies have implicated the involvement of various pathways in the fly that could potentially contribute to the assessment of the food environment that leads to reproduction. Median neurosecretory cells (MNCs) express drosophila insulin-like peptides (dilps) and are a component of the insulin pathway in flies. Remating was significantly reduced in flies that had their MNCs ablated and this reduction was due to the loss of dilps and not due to a reduction in the

neurons (Wigby et al., 2010), suggesting a possible metabolic input into the decision making of whether or not to mate. The neuronal Target of rapamycin (TOR) pathway might be another pathway that aids in the decision making process. Ribosomal S6 kinase (S6K) is a target of the TOR pathway and together makes up TOR/S6K pathway. The pathway is activated by amino acids and found that mated females consume more yeast than virgin females (Vargas et al., 2010). Since seminal fluid releases amino acids into the female and mated females that are mutants for the sex peptide receptor (SPR) behaved like virgin females and did not increase their yeast consumption, it suggests that the amino acids from the seminal fluid might be acting on the TOR/S6K pathway and blocking feeding and reproduction.

The chemosensory mutants tested and the results from this thesis indicated that neither taste nor smell appear to be affecting reproduction directly. Although a significant portion of the olfactory pathway can be eliminated via the need for *Or83b* as a co-receptor, there are ionotropic receptors that were recently found that could potentially be aiding the olfactory system in smelling compounds (Benton et al., 2009). Since trehalose nor the smell present in yeast had any effect on reproduction in either strain, a potential hypothesis could be that the protein in the yeast is breaking down into its amino acid constituents. The amino acids or the energy obtained from the amino acids might be used for egg production and could be affecting either the TOR/S6K pathway or the insulin pathway responsible for metabolism.

Taken together these studies show that remating occurs frequently in wild type strains of *Drosophila melanogater*. However, different strains display different levels of remating indicating a genetic contribution to mating frequency. Moreover, different strains display

varied mating and progeny frequencies when tested on various types of food. It was found that yeast contributes to the majority of mating in both *Canton-S* and *Oregon-R*, with higher mating frequencies and progeny counts observed on higher dosages of yeast provided. The two strains were also found to differ in their interactions with the group for mating as well as progeny counts with *Canton-S* females mating more on average as a result of being in a bigger group. The chemosensory mutants tested did not hinder reproduction, however this result cannot conclude that chemosensation of food is not important for reproduction. Therefore, from the results of this thesis one can conclude that the genotype, the social environment of the fly and the nutritional composition of the food are all interacting to stimulate reproduction in the fruit fly.

The results from my thesis indicate that the perception of the environment may not directly affect reproduction. For future work, I hypothesize a role of the metabolic pathways in reproduction where various pathways of the fly's internal metabolism could be interacting. In order to test this hypothesis, one could manipulate nutritent availability and various pathways such as the adipokinetic hormone (AKH) and the *Drosophila* insulin like peptide (dilp) pathways. These two pathways are counter regulatory pathways that control carbohydrate metabolism in the fly. If various parts of either pathway are manipulated, circulating carbohydrate levels can be altered leading to changes in the metabolic status of the fly. This modification of metabolic status could lead to phenotypic changes in behaviour. On the other hand, manipulating the TOR/S6K pathway would allow for the understanding of the role of amino acids in affecting reproduction. Modifying protein expression levels in this pathway leads to altered perception of available amino acids. Through these studies, it can potentially be shown

there is a decision making process in the neural circuitry that assess the metabolic status as well as the environment before copulation takes places. Although this thesis only addresses food stimulus on reproductive behaviour, similar mechanisms may be in use for other complex behaviours as well. Behaviours such as foraging for food and feeding, initiating aggressive behaviour toward others and learning and memory could be affected by the social and physical environment as well as the internal status of the fly.

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Appendix A: Fly Food Making Protocol

Ingredients:

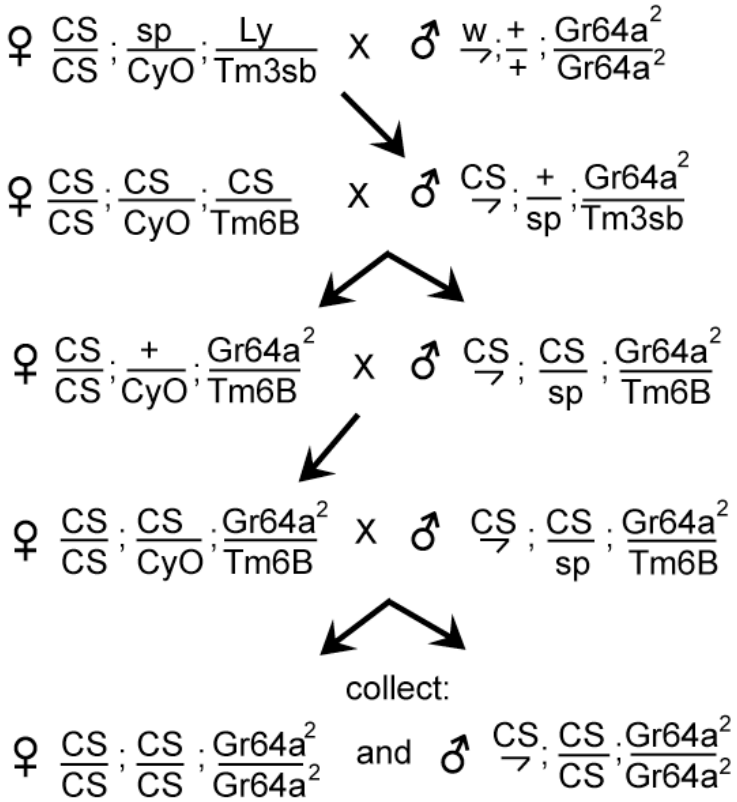
- 1L water
- 10 g/L Agar
- 15 g/L Sucrose
- 30 g/L Glucose
- 15 g/L Cornmeal
- 10 g/L Wheat germ
- 10 g/L Soy Flour
- 30 g/L Molasses
- 35 g/L Yeast
- 5 mL Propionic Acid
- 2 g/L Tegosept
- 10 mL 95% Ethanol

Protocol:

Set hot plate to 395°C and set water to boil with stir bar. Add agar, yeast, glucose, sucrose, cornmeal, wheat germ, soy flour and molassess to the boiling water. Mix and monitor temperature frequently. Once temperature reaches 95°C, turn temperature down to 120°C for 10 minutes and continue mixing. After 10 minutes, turn hot plate off but continue mixing. Prepare tegosept solution while the fly food is cooling. Add tegosept to 95% ethanol and mix and store in 4°C fridge. Once fly food temperature reaches 48°C, add propionic acid and tegosept solution to the fly food and mix for 2 minutes. After 2 minutes, the food is ready to be used.

Appendix B: Genetic Crosses

Placing *Gr64a*^{2} mutant flies in a Canton-S genetic background:



Placing $Gr64a^2$ in an *Oregon-R* genetic background: