

ABSTRACT

CHOHAN, KULTARAN SINGH. The Genetic Architecture of Ethanol Response in the *Drosophila melanogaster* Genetic Reference Panel. (Under the direction of Trudy F.C. Mackay).

Behaviors are complex traits that are produced by the nervous system in response to internal and external environmental cues and enable animals to interact with and adapt to their environments. Complex traits are influenced by numerous genes, the environment, and gene by environment interactions which make it difficult to tease apart and identify the genetic and environmental components underlying such traits. *Drosophila melanogaster* provides a genetically tractable model system for elucidating the genetic basis for specific behaviors because it enables the precise control of the genetic background, and environmental conditions and chemosensory behavioral responses can be quantified accurately.

Animals encounter a wide array of odors in their natural environments and have evolved sophisticated chemosensory systems to detect and interpret such information. The ability to recognize and respond to chemical stimuli is critical to the fitness of most animals not only in terms of locating food sources and potential mates, but also in detecting and avoiding predators and toxic compounds. Chemosensory behavioral responses involve perception of information from the environment, assessing its appropriate biological significance by integrating and processing that information in the brain, and directing motor outputs that translate into behavioral responses.

Drosophila encounters and is attracted to odors produced by rotten fruits, its primary food source, including ethanol which is produced by fermentation of yeast

present in such fruits. Ethanol is used by *Drosophila* as a chemosensory cue to detect and locate transient food sources, oviposition sites, and potential mates. However, ethanol is also a toxin and when consumed at high concentrations has fitness costs associated with it. This creates an interesting scenario where on the one hand an ecologically relevant compound could be used to detect and locate food sources but on the other hand consuming such a compound can reduce fitness.

The focus of this dissertation is to understand the genetic architecture that underlies chemosensory behavioral responses to ethanol. To achieve this goal, I exploited the natural variation that exists in olfactory and gustatory behavioral responses to ethanol in the *Drosophila melanogaster* Genetic Reference Panel (DGRP), a wild-derived inbred population of inbred lines with full genome sequences, to identify naturally occurring polymorphisms that are associated with phenotypic variation in these behavioral traits.

The results indicate that chemosensory behavioral responses to ethanol depend on the context in which and sensory modality through which ethanol cues are detected by flies. I observed considerable phenotypic and genotypic variation among the DGRP lines for chemosensory behavioral responses to ethanol and significant sexual dimorphism in these behavioral responses. This approach helped identify genetic variants that explain a relatively large portion of the phenotypic and genetic variance associated with olfactory and gustatory behavioral responses to ethanol. The results of this study indicate that relatively few single nucleotide polymorphisms (SNPs) segregating within the DGRP account for a large portion of the phenotypic and genetic variation in chemosensory behavioral responses to

ethanol. I identified and functionally tested novel genes, including chemosensory genes, which have not been previously implicated in chemosensory behavioral responses to ethanol. There was little correlation or overlap between genes affecting variation in olfactory behavioral response to ethanol and variation in the behavioral response to ethanol consumption. Further, the results indicate that input from different chemosensory organs and genes interact dynamically to produce appropriate behavioral responses to ethanol cues. Finally, knowledge of causal variants identified through this study enables us for the first time to assess the nature of selective forces acting on these traits through future studies in laboratory evolution settings where we can track changes in allele frequencies of the causal variants over time under different fitness conditions.

The Genetic Architecture of Ethanol Response in the *Drosophila melanogaster*
Genetic Reference Panel

by
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DEDICATION

To my loving mom, brother, sister-in-law, and in memory of my loving dad.

BIOGRAPHY

I was born in Zambia, Africa, where I spent the early part of my life. I moved to Nigeria when I was five. Living in Africa as a little kid, experiencing nature, interacting with different cultures, is something I can never forget. I moved to India when I was nine and spent most of my early adulthood there. I got a law degree from India and decided I wanted to broaden my horizons by coming to the US for further studies. I went to Washington University, St. Louis, MO., and got a LL.M. in intellectual property and technology law. It was during law school that I decided that I wanted to pursue higher studies in biology. I completed a MS in biology from University of North Carolina Greensboro under the mentorship of Drs. Adamson and LaJeunesse. My interest in quantitative genetics spawned, from a class at UNCG, on quantitative and population genetics. My interest in quantitative genetics is what brought me to NCSU and my present work under the guidance of Dr. Trudy Mackay and Dr. Robert Anholt.

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CHAPTER ONE

INTRODUCTION

Behaviors are complex traits that are produced by the nervous system in response to internal and external environmental cues and enable animals to interact with and adapt to their environments. An animal's ability to interact and adapt to its environment is one of the most important factors in determining its fitness.

Behavioral traits provide the mechanisms through which evolutionary forces, such as natural selection, can act. Behavioral responses are influenced by many factors which include: the developmental history of the animal, its genetic makeup, its nervous system, its physiological state, and its physical and social environment.

Behaviors, as complex traits, are influenced by numerous genes, the environment, and gene by environment interactions making it difficult to tease apart and identify

the genetic and environmental components underlying such traits. *Drosophila*

melanogaster provides a genetically tractable model system for elucidating the genetic basis for specific behaviors because it enables precise control of the genetic

background and environmental conditions. *D. melanogaster* has been an excellent

model system for studying the genetic architecture of chemosensory behaviors as

flies exhibit robust responses to odors and tastants which can be studied under

controlled conditions using relatively simple quantitative assays (Benton 2008; Su et

al. 2009), and flies are amenable to genetic, neuroanatomical, electrophysiological

and behavioral manipulation. In addition, unlimited numbers of flies with the same

genetic makeup can be grown under precisely controlled environmental conditions.

All of these factors, along with the availability of complete genome sequence

(Adams et al. 2000), make *D. melanogaster* an ideal organism for studies aimed at

dissecting and understanding the genetic architecture that underlies chemosensory behavior.

Rotten fruits are the primary food source of *Drosophila* and the odors emitted by such fruits enable the flies to detect and locate them. Ethanol is one such odorant, produced by fermentation of yeast in rotting fruits, and is probably used by *Drosophila* as a chemosensory cue to detect and locate transient food sources, oviposition sites, and potential mates (McKenzie and Parsons 1972; Dudley 2002). Olfactory and gustatory responses to different odorants have been well characterized in *Drosophila*, but there have been relatively few studies that have been done in ecologically relevant settings under controlled conditions or that have looked at the interaction between the olfactory and gustatory sensory systems to the same chemical stimuli. Most of these studies have been done on few inbred strains available in the laboratory and none have been done on a genomic scale.

The goal of my research is to study the genetic architecture that underlies olfactory and gustatory behavioral responses to ethanol, an ecologically relevant chemical encountered by the flies in and around their food source, in a large population of wild-derived inbred lines of *Drosophila*. To achieve this goal, I am exploiting natural variation that exists in olfactory and gustatory behavioral responses to ethanol in the *Drosophila melanogaster* Genetic Reference Panel (DGRP), a wild-derived inbred population of inbred lines with full genome sequences (Mackay et al. 2012), to identify naturally occurring polymorphisms that are associated with phenotypic variation in these behavioral traits. I will also use this approach to identify transcripts

that are associated with variation in chemosensory behavioral responses to ethanol. Together, these results will help us learn about the genetic architecture that underlies olfactory and gustatory behavioral responses to ethanol and, more generally, provide insights into the general principles that broadly apply to behavioral traits.

In Chapter One, I provide a detailed overview of the chemosensory system of *D. melanogaster*, which includes a detailed description of odorant receptors, gustatory receptors, and odorant binding proteins. This provides an essential framework to help analyze the chemosensory behavioral responses to ethanol in flies. In Chapter Two, I discuss chemosensory behavioral responses in flies, the different behavioral assays used to measure olfactory and gustatory behavioral responses to ethanol, the principles governing the use and interpretation of results from behavioral assays, and the quantitative genetic analysis of natural variation that exists in the DGRP lines used to measure these chemosensory behavioral responses. In Chapter Three, I describe the use of a Genome Wide Association (GWA) mapping approach to study the genetic architecture underlying behavioral traits and the results from the GWA analyses conducted to identify DNA polymorphisms associated with chemosensory behavioral responses to ethanol. In Chapter Four, I provide results from the analyses used to identify transcript abundance associated with chemosensory behavioral responses to ethanol. In Chapter Five, I provide conclusions on the insights gained on the genetic architecture that underlies chemosensory behavioral responses to ethanol and future directions.

Chemosensory Systems

Animals encounter a wide array of odors in their natural environments and have evolved sophisticated chemosensory systems to detect and interpret such information. Information from the chemosensory systems is conveyed via the peripheral nervous system to the central nervous system, where it is processed by the brain and appropriate behavioral responses are produced based on the animal's physiological needs and/or past experiences. This ability to recognize and respond to chemical stimuli is critical to the fitness of most animals not only in terms of allowing an animal to locate food sources and identify mates, but also in detecting and avoiding predators and toxic compounds.

A major breakthrough in chemosensory research was the discovery of odorant receptor genes in rodents (Buck and Axel 1991), *C. elegans* (Sengupta et al. 1996; Troemel 1999), and *D. melanogaster* (Clyne et al. 1999b; Vosshall et al. 1999).

Such studies enabled the detailed characterization of the functional organization of the olfactory system, particularly at the level of olfactory receptor neurons and projections of neurons with specific odorant receptors to specific glomeruli in the antennal lobes. Similar bioinformatics approaches that were used to identify olfactory receptors were later used to identify gustatory receptors which subsequently enabled the characterization of the gustatory system (Clyne et al. 2000; Dunipace et al. 2001; Scott et al. 2001; Thorne et al. 2004; Wang et al. 2004; Weiss et al. 2011). These studies, along with evidence from odorant receptor

expression patterns, have revealed similarities in the convergent organization of neurons expressing the same odorant receptors on output neurons in the antennal lobe and olfactory bulb of insects and mammals respectively (Hildebrand and Shepherd 1997; Strausfeld and Hildebrand 1999). The relative simplicity of fewer chemosensory neurons in *Drosophila* (Smith 2007) has facilitated studies that have helped characterize in detail both the olfactory and gustatory system.

Olfactory System in Adult Flies

Olfactory Organs

Flies detect volatile odors using olfactory organs that are located on the head. The olfactory organs include the third antennal segments and the maxillary palps. The third antennal segment contains ~1,200 olfactory receptor neurons (ORNs) housed in specialized hairs, called sensilla, which are divided into three morphologically distinct classes: basiconic (large and small), coeloconic, and trichoid sensilla (Figure 1.1(b)). Each maxillary palp contains ~120 ORNs that are housed only in the basiconic sensilla (Stocker 1994; Shanbhag et al. 2000; Su et al. 2009). The sensilla are hollow structures that contain between one and four ORNs bathed in sensillum lymph secreted by surrounding support cells (Hallem and Carlson 2006). Through pores present in the cuticular wall, odorants enter the sensilla and dissolve in the sensillar lymph where they activate the ORNs. The ORNs are bipolar and from the apical side extend a chemosensory dendrite into the shaft of the sensillum where it can be activated by specific odorants. From the basal end, ORNs project a

single axon to an olfactory glomerulus in the antennal lobe (Stocker 1994; Hildebrand and Shepherd 1997). The antennal lobe contains ~43 identifiable spherical structures of neuropil called “glomeruli” (Laissue et al. 1999) where synapses between first-order and second-order olfactory neurons occur (Figure 1.1(c)). ORNs that express the same odorant receptors are projected bilaterally onto one or few glomeruli (Stocker 1994). Projection neurons typically innervate only a single glomerulus and (Marin et al. 2002) connect the antennal lobe with higher olfactory centers. Unlike the antennal ORNs which project their axon directly to the antennal lobe, the axons of the maxillary palp ORNs fasciculate with gustatory neurons in the labial nerve and project through the suboesophageal ganglion (SOG) to reach the antennal lobe (Vosshall and Stocker 2007).

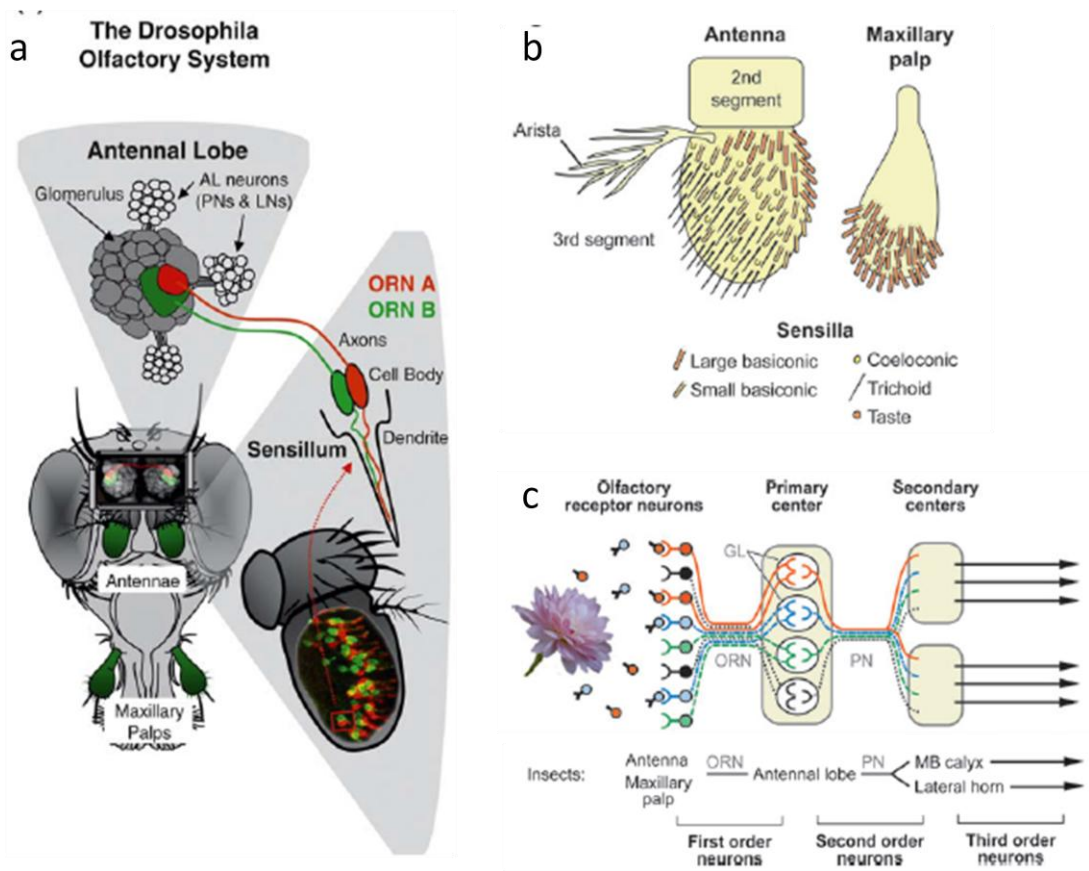


Figure 1.1: Adult olfactory system in *Drosophila*. (a) Olfactory receptor neurons (ORNs) are localized in the third antennal segments and the maxillary palps which contain different types of sensilla. ORNs project to distinct glomeruli in the antennal lobe. Figure adapted from Brochtrup and Hummel (2011). (b) Distribution of different types of sensilla on the 3rd segment of the antenna and the maxillary palp. Figure adapted from Benton et al. (2006). (c) Odorants stimulate distinct subsets of ORNs, which converge on glomeruli in the antennal lobe and are then relayed to higher brain centers. Figure adapted from Tanaka et al. (2004).

Large-scale single unit electrophysiological studies have been possible since the sensillar lymph, secreted by the support cells, surrounding each sensillum keep them electrically insulated from their neighbors enabling single-unit electrophysiology studies for given sensillum (Clyne et al. 1997). Since each ORN in a sensillum has a characteristic spike amplitude, it is possible to infer activity of individual neurons and various studies, using the single unit electrophysiology technique, have been able to define the ligand receptive range for every maxillary palp ORN (de Bruyne et al. 1999) and a majority of the antennal ORNs (de Bruyne et al. 2001; Hallem and Carlson 2006). These studies have identified 18 classes of ORNs in the antenna, housed in three types of large basiconic sensilla and five types of small basiconic sensilla. The antennal sensilla have been designated ab1 through ab8, where the ab1 sensillum contains four ORNs and the rest contain two ORNs. On the other hand, six different functional classes of ORNs have been identified in three types of basiconic sensilla in the maxillary palp. These sensilla have been designated pb1 through pb3. Each sensillum contains two ORNs, such that pb1 contains pb1A and pb1B, pb2 contains pb2A and pb2B, and pb3 contains pb3A and pb3B (de Bruyne et al. 1999; de Bruyne et al. 2001; Elmore et al. 2003).

Odorant Receptors

A major breakthrough that has significantly helped advance chemosensory research was the discovery of odorant receptor genes in rats (Buck and Axel 1991). Odorant receptors (ORs) were identified based on the assumption that they belonged to the

G-protein-coupled receptor (GPCR) superfamily, that controls G-protein-coupled cyclic AMP transduction pathways involved in olfactory transduction, and would be expressed specifically in the olfactory epithelium (Buck and Axel 1991). *Drosophila* Ors were identified through a combination of molecular (Vosshall et al. 1999) and bioinformatic approaches (Clyne et al. 1999b; Gao and Chess 1999; Vosshall et al. 1999) that searched for a family of seven transmembrane domain proteins selectively expressed in ORNs. The availability of the *Drosophila* whole genome sequence enabled a bioinformatics search to identify 60 *Or* genes that encode 62 OR proteins, two of which are the product of alternative RNA splicing (Robertson et al. 2003). The fly OR proteins are a family with seven membrane spanning domains that show no homology to the worm and vertebrate GPCRs (Buck and Axel 1991; Troemel 1999) and exhibit a membrane topology which is inverted relative to that of conical GPCRs (Figure 1.2), such that the N-terminus faces the cytosol (Benton et al. 2006). Recent studies indicate that insect ORs are odorant-gated ion channels (Sato et al. 2008; Wicher et al. 2008) which are mechanistically different from metabotropic olfactory receptors present in vertebrates. Most ORNs express a unique OR along with the ubiquitous OR83b receptor (Larsson et al. 2004; Benton et al. 2006). The common OR83b receptor forms a heterodimer with neuron specific ORs and this complex forms an odorant activated cation channel (Sato et al. 2008; Wicher et al. 2008). OR83b is also essential for the transport and insertion of specific odorant receptors into the dendritic membrane (Benton et al. 2006).

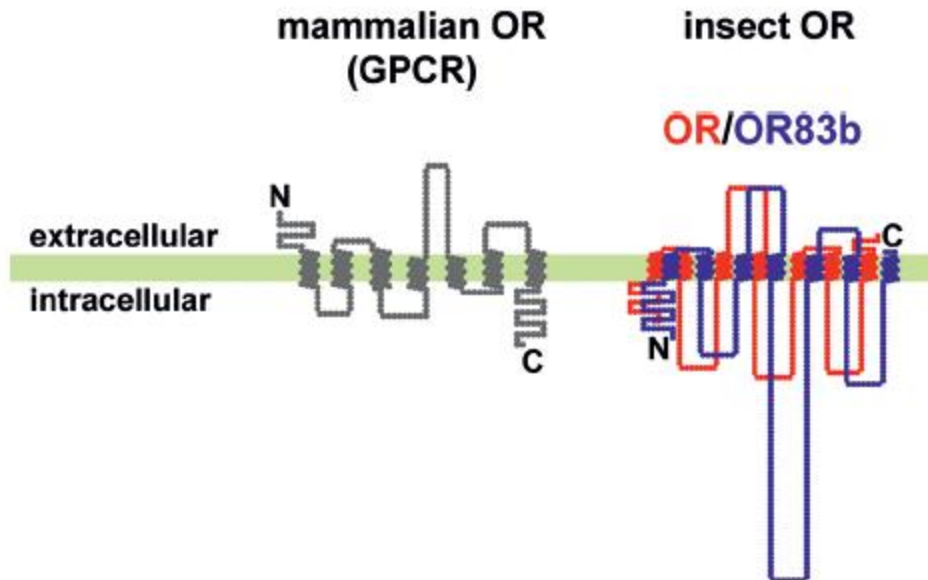


Figure 1.2: Insect OR forms a heteromeric complex with the ubiquitous OR83b and has a membrane topology which is inverted relative to mammalian ORs. Figure adapted from Benton (2006).

Fly *Ors* evolved independently of chemosensory receptors in worms and vertebrates (Robertson et al. 2003). In *Drosophila*, the *Or* gene family is distributed across all three major chromosomes and many of these genes occur in small clusters of two or three genes. While some of these clusters appear to have originated through recent duplication events, most are distantly related (Robertson et al. 2003). The presence of relatively low over all amino acid homology (~20%) across the *Or* genes and the absence of large clusters of related *Or* genes suggest that this gene family has a very ancient origin (Robertson et al. 2003).

Recently, a new family of chemosensory receptors, called ionotropic receptors (IRs), was identified in *Drosophila* through a bioinformatics screen for genes expressed in the fly antennae (Benton et al. 2009). While the *Ors* are predicted to contain seven transmembrane domains, the *Irs* are related to ionotropic glutamate receptors (iGluR) and are predicted to contain three transmembrane domains and a pore loop (Su et al. 2009). This family comprises 61 predicted genes and two pseudogenes, which are expressed in coeloconic sensilla and are believed to be involved in the detection of ammonia, amines, water vapor, and alcohols (Spletter and Luo 2009). The *Ir* genes are extremely divergent with an overall amino acid sequence identity of 10%-70%. Comparative genomic analysis of ionotropic receptor repertoires indicate that at least 14 ionotropic receptors from the antennal subfamily are conserved across insects. Two closely related *Irs*, *Ir8a* and *Ir25a*, have the most similar primary sequence to iGluR, show homologous genes across Protostomia, and a broad distribution of their transcripts in antennal IR expressing ORNs. These features distinguish them from a divergent subfamily of 45 *Irs* which are largely specific to drosophilids and include some *Irs* that are implicated in taste detection (Benton et al. 2009; Croset et al. 2010).

Expression of Odorant Receptor and Ionotropic Receptor Genes

Expression of *Or* Genes

Both insect and mammalian olfactory systems include populations of first-order and second-order neurons, that within the insect antennal lobe and the mammalian

olfactory bulb form spherical structures of dense neuropil (called “glomeruli”), and different subsets of ORNs innervate different glomeruli (Hildebrand and Shepherd 1997; Gao et al. 2000; Feinstein and Mombaerts 2004). The identification and characterization of expression patterns of *Or* genes has helped in understanding the general principles that underlie the organization of the olfactory systems.

The expression of *Or* genes has been examined using different techniques, including *in situ* hybridization, immunohistochemistry, and reporter gene analysis.

These studies have revealed that in *Drosophila*, each *Or* gene is expressed in a subpopulation of ORNs and each ORN expresses only one or a small number of *Or* genes together with the ubiquitous *Or83b*. This suggests that the ORs are the primary determinants of the functional properties of the ORN (Vosshall et al. 1999; Larsson et al. 2004; Fishilevich and Vosshall 2005; Benton et al. 2006). This is in contrast to *C. elegans*, where individual ORNs express a large number of odorant receptors (Troemel 1999), implying that the olfactory behavioral responses are determined by the ORN and not the receptor since there are multiple receptors that can activate it (Troemel 1999).

In situ hybridization has revealed that in adult flies seven *Or* genes are expressed in the maxillary palp, 31 *Or* genes are expressed in the basiconic and trichoid sensilla, and one *Or* gene (*Or35a*) is expressed in coeloconic sensilla (Figure 1.3) (Vosshall et al. 2000; Dobritsa et al. 2003; Elmore et al. 2003). Reporter gene analysis based on the *GAL4-UAS* binary expression system (Brand and Perrimon 1993) has been used to study ORN projections in *Drosophila* by driving GFP expression under the

control of specific *Or* promoters. These studies have shown that ORNs which express the same *Or* gene converge onto a single or a small number of glomeruli in the antenna lobes (Vosshall et al. 2000). The basic principle of a single neuron expressing a single receptor and projecting to a common glomerulus which underlies the molecular organization of the olfactory system appears to be conserved from insects to mammals. ORNs in most mammals appear to express a single or a small number of *Ors* (Malnic et al. 1999) and the ORNs expressing the same *Or* gene converge onto the same glomeruli in the olfactory bulb (Mombaerts et al. 1996). The odor information received by each glomerulus depends on the ligands that activate the ORs that converge on that glomerulus and the activation of different combination of glomeruli in turn affect odor.

Expression of Ionotropic receptor Genes

Ir genes were recently discovered through a bioinformatics screen for genes expressed in the fly antennae (Benton et al. 2009). The reason why such a screen was conducted was because previous comprehensive studies conducted on expression of *Ors* in *Drosophila* suggested the existence of other types of insect chemosensory receptors. These observations, along with electrophysiological analysis, revealed the existence of coeloconic ORNs which were tuned to acids, ammonia, and humidity even though the ORNs present in coeloconic sensilla do not express *Or83b* or members of the OR or gustatory receptor (GR) gene family (with exception of *Or35a-Or83b*) (Yao et al. 2005), suggesting the existence of other chemosensory receptors. Among the 61 predicted *Ir* genes identified in the screen, 15 showed expression in the antenna (these include *Ir8a*, *Ir21a*, *Ir25a*, *Ir31a*, *Ir40a*, *Ir64a*, *Ir75a*, *Ir75b*, *Ir75c*, *Ir75d*, *Ir76a*, *Ir76b*, *Ir84a*, *Ir92a*, and *Ir93a*) while two showed expression in the proboscis (these include *Ir25a* and *Ir76a*) (Benton et al. 2009). As mentioned above, *Ir* genes are not expressed in basiconic or trichoid sensilla and do not co-express with *Or83b*. One exception to this is a subpopulation of coeloconic ORNs that express both *Ir76b* and *Or35a-Or83b* (Couto et al. 2005; Yao et al. 2005; Benton et al. 2009). The expression of the different *Irs* reveal that they are present in the coeloconic sensilla, a feather-like projection called the arista which is part of the mechanosensory/acoustic system and may play a role in heat sensing, and an internal three-chambered pocket called the sacculus which contains coeloconic sensilla (Benton et al. 2009).

Double and triple RNA *in situ* hybridization revealed that individual neurons express between one and three *Ir* genes and are organized into specific clusters of two or three neurons. Based on the stereotyped combination of *Ir* genes, the clusters containing two or three neurons can be organized into four distinct clusters (Benton et al. 2009). These four clusters of IR expressing neurons in the antenna are consistent with the presence of four types of coeloconic sensilla (ac1-ac4) which have distinct yet partially overlapping sensory specificities that were identified through electrophysiological recordings (Yao et al. 2005).

Receptor-to-Neuron Map

Studies conducted to determine the receptor-to-neuron map for both the antenna and the maxillary palp reveal that, in terms of sequence similarity and genomic location, *Or* genes that are expressed in the same sensillum are no more closely related to each other than to the rest of the *Or* genes. Similarly, receptors that exhibit similar patterns of spatial distribution do not show similarities in sequence or genomic location. Additionally, ORs show high redundancy in their responses to an odorant since studies on ligand specificities of individual ORs reveal that multiple receptors can be activated by the same ligand (Dobritsa et al. 2003; Hallem et al. 2004a).

Two odorant receptors, OR22a and OR22b, have been characterized in detail and have been shown to coexpress specifically in the ab3A antennal neuron present in the basiconic sensillum (Dobritsa et al. 2003). A deletion mutant ($\Delta halo$) that lacks

both endogenous receptor genes, *Or22a* and *Or22b*, shows loss of odorant responses in the ab3A neuron (Dobritsa et al. 2003). Analysis conducted using this *in vivo* expression system (Figure 1.4) revealed that the OR dictates the odor response spectrum of the ORN and is the primary determinant of other ORN response properties, such as the spontaneous firing rate, the mode of olfactory signaling (i.e. whether it is excitatory or inhibitory), and the rate of response termination (i.e. abrupt or prolonged) (Hallem et al. 2004a).

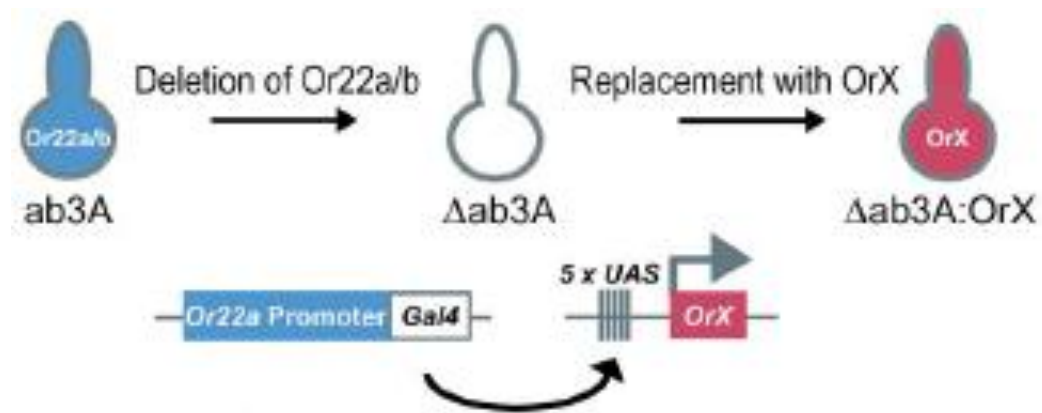


Figure 1.4: *In vivo* expression system using empty neuron. Specific *Ors* can be introduced into the mutant ab3A antennal neuron lacking its endogenous receptor genes, *Or22a* and *Or22b*, using the *GAL4-UAS* binary expression system. Figure adapted from Hallem et al. (2004).

However, this still leaves open the question of how, from a repertoire of 60 *Or* genes, do individual ORNs select which receptor gene to express? Two models have been proposed to answer this question, the deterministic model and the

stochastic model (Shykind et al. 2004). Under the deterministic model, different receptor genes contain different combinations of *cis*-acting elements and an individual gene is selected for expression in those ORNs where corresponding transcription factors exist. Under the stochastic model, individual receptor genes are selected by an unknown process that can act on only one gene at a time. Evidence supporting the deterministic model is emerging as the mechanism under which an ORN selects a particular receptor gene to express (Ray et al. 2008). Comparison of upstream regions of five orthologous maxillary palp *Or* genes between two species of *Drosophila*, *D. melanogaster* and *D. pseudoobscura* which diverged approximately 46 million years ago (Bergman et al. 2002), led to the identification of conserved gene-specific elements (*cis*-regulatory elements) for each of the five *Or* genes. Further analysis of these conserved elements across ten other *Drosophila* species identified the most conserved elements. Functional analysis of these conserved elements revealed that they were sufficient to recapitulate endogenous receptor gene expression and that the maxillary palp neurons used a combinatorial code of unique and shared *cis*-acting elements, where the positive regulatory elements directed expression of a particular *Or* in subsets of maxillary palps and negative regulatory elements restrict its expression to a single ORN class (Figure 1.5) (Ray et al. 2008).

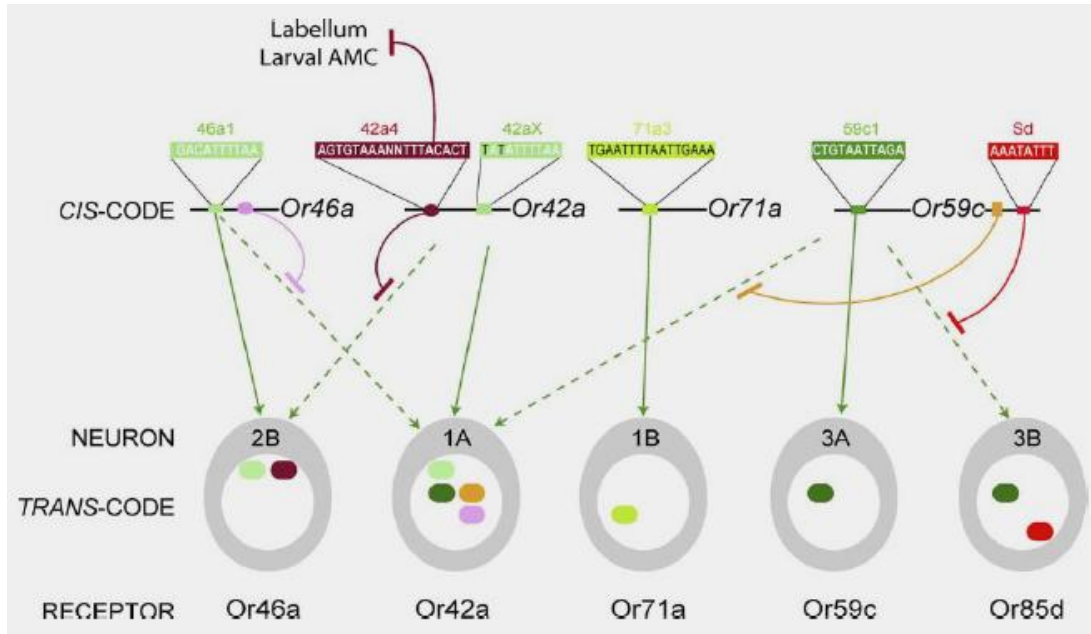


Figure 1.5: Model for combinatorial coding of odor receptor gene choice in the maxillary palp. Conserved gene specific regulatory elements are color coded. Red, pink, and orange are negative regulatory elements and green elements are positive elements. Gray balls represent different maxillary palp ORNs. Figure adapted from Ray et al. (2009).

Various transcription factors play an important role in directing *Or* gene expression in specific ORNs. POU domain transcription factors *acj6* and *pdm3* are required for *Or* gene expression (Clyne et al. 1999a; Clyne et al. 1999b; Komiyama et al. 2004; Tichy et al. 2008). *Acj6* is expressed in all maxillary palp neurons and is required for expression of a subset of *Or* genes in the palps and the antenna (Clyne et al. 1999a; Clyne et al. 1999b; Komiyama et al. 2004). The *pdm3* transcription factor is expressed in a subset of the ORN classes and is required for the expression of a

very limited subset of *Or* genes in the maxillary palp and in the antenna (Tichy et al. 2008). The TEA domain transcription factor *scalloped*, in *scalloped* mutant flies, has been shown to refine expression of *Or59c* to the pb3A neuron in the maxillary palp by repressing the expression of *Or59c* in the pb3B neuron (Ray et al. 2008).

Receptor-to-Glomerulus Map

As mentioned earlier, the detection of smell generally occurs through peripheral systems and discrimination occurs through central systems. Odors are detected at the periphery by ORs that activate specific ORNs that express them. The ORNs project their axons centrally to glomeruli in the brain and, once activated by ORs, fire action potentials which result in spatially defined patterns of glomerular activity in the brain (Galizia et al. 1999; Mori et al. 1999). Studies in mammals and insects show that each ORN expresses just a single OR and each glomerulus generally receives input from a single class of ORNs (Hildebrand and Shepherd 1997). Thus, all ORNs expressing a give OR target one or sometimes two glomeruli (Gao et al. 2000; Vosshall et al. 2000).

The *Drosophila* antennal lobe contains between 40-50 morphologically identifiable glomeruli whose size, shapes, and positions are strongly conserved between different animals (Laissue et al. 1999). Genetic tracing studies have revealed a nearly complete map of projections from the peripheral olfactory organs to the glomeruli. These studies show that 23 glomeruli are innervated by antennal basiconic ORNs, eight are innervated by antennal trichoid ORNs, a further eight are

innervated by antennal coeloconic ORNs, and six glomeruli are innervated by maxillary palp basiconic ORNs leaving four glomeruli unassigned (Figure 1.6) (Laissue et al. 1999; Couto et al. 2005; Fishilevich and Vosshall 2005). These results reveal that projections from different sensilla types tend to cluster in the antennal lobe, with antennal basiconic neurons at the medial edge, antennal trichoid neurons at the lateral edge, palp basiconic neurons at the anterior middle portion, and coeloconic neurons at the ventral middle region (Couto et al. 2005). Also, two large lateral glomeruli innervated by the trichoid sensilla are sexually dimorphic (Kondoh et al. 2003) and *fruitless*-positive (Manoli et al. 2005), making them good candidates for being involved in the processing of pheromonal cues. Furthermore, ORNs expressing *Or67d* (along with the Lush odorant binding protein) that mediate recognition of the courtship pheromone 11-*cis*-vaccenyl acetate project to one of these glomeruli (Ha and Smith 2006).

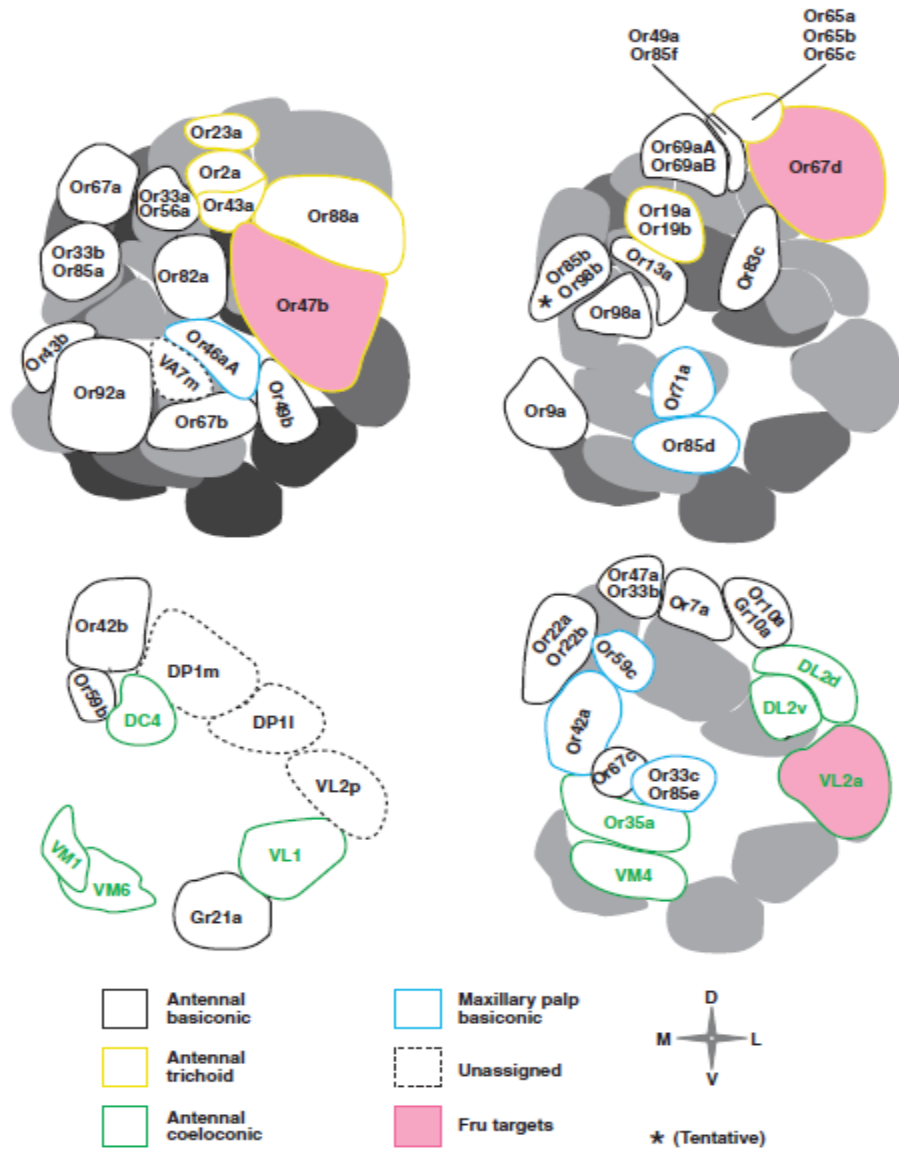


Figure 1.6: Molecular neuroanatomy of the adult *Drosophila* antennal lobe with the molecular and functional identity of the glomeruli. Glomeruli receiving projections from ORNs expressing a given OR or GR are shown. Antennal basiconic, trichoid, and coeloconic projections are shown in black, yellow, and green respectively. Maxillary palp projections are in cyan, and unmapped glomeruli are indicated

Figure 1.6: Continued

through black dotted lines. Glomeruli innervated by *fruitless* positive neurons are indicated in pink. Figure adapted from Vosshall et al. (2007).

While some odorants, such as pheromones, are narrowly tuned and are detected by a single receptor, many other odorants activate multiple receptors. At higher concentrations, odorants can activate multiple receptors, while at lower concentrations, odorants generally activate fewer receptors. This provides a mechanism through which the receptor repertoire can extend a dynamic range where intensity coding would depend on both the strength of activation of individual receptors and the total number of receptors activated. The combination of receptors activated by a single odorant would allow for a more precise assessment of the concentration of the odorant, especially if different receptors have different activation thresholds for the odorant (Hallem and Carlson 2006). The receptor repertoire can also produce a complex temporal representation of an odor stimulus by showing differences in their response kinetics to the same odor based on the quality and quantity of the odor. Some give strong initial responses that terminate quickly, while others show tonic responses that continue well beyond the end of the odor stimulus (Hallem and Carlson 2006). The molecular complexity of an odor stimulus (including its concentration) also appears to be an important factor in odor discrimination. Odorants from the same chemical class or with similar structures might be difficult to discriminate (Hallem and Carlson 2006). All these factors result in the activation of

different combinations of ORs and through them different combinations of glomeruli in the antennal lobe which in turn affects odor discrimination and behavioral responses.

Flies show robust behavioral responses to food odors which contain many individual odorants. The activation of multiple ORs by food odors activates multiple glomeruli.

Drosophila shows robust attraction to low concentrations of apple cider vinegar which induces activation of six glomeruli in the fly antennal lobe. Out of these six glomeruli, two glomeruli DM1 and VA2 innervated by *Or42b* and *Or92a* expressing ORNs individually affect and can account for the attractive behavioral response in the flies (Semmelhack and Wang 2009). However, a higher concentration of apple cider vinegar excites an additional glomerulus, DM5, innervated by *Or85a* expressing ORNs which results in a behavioral switch from attraction to avoidance at high concentrations of the odor (Semmelhack and Wang 2009). These results suggest that activation of an individual glomerulus can be sufficient to produce innate behavioral responses and that the further recruitment of glomeruli (for example at high concentrations of an odor) can modify such behavioral responses.

Functional Characterization of Receptors

Odorant Receptors

Detailed studies have been conducted through which the ligand specificity of individual ORs have been determined. The first OR to be functionally characterized was the *C. elegans* receptor *odr-10* and diacetyl was identified as its ligand

(Sengupta et al. 1996). The first insect OR to be functionally characterized was the *D. melanogaster* antennal receptor Or43a. Through the overexpression of Or43a in the antenna, as well as heterologous expression in *X. laevis* oocytes, the odorants cyclohexanone, cyclohexanol, benzaldehyde, and benzyl alcohol were identified as ligands for Or43a (Stortkuhl and Kettler 2001; Wetzel et al. 2001). The deletion mutant ($\Delta halo$) (Figure 1.4) that lacks both endogenous receptor genes, *Or22a* and *Or22b*, provides an *in vivo* expression system that has been used as an important tool in the detailed functional analysis of *Drosophila* ORs. Using the *GAL4-UAS* binary expression system, individual ORs have been ectopically expressed under the *Or22a* promoter and electrophysiological responses to a large battery of different odorants have been measured (Hallem et al. 2004a; Hallem and Carlson 2006). This system has also been used to characterize odorant receptor specificities of larval ORs (Kreher et al. 2005) and ORs in other insect species. In *Anopheles gambiae*, it was used to measure electrophysiological responses of specific receptors to a large battery of different odorants (Carey et al. 2010) and also helped in identifying the female specific receptor *AgOr1* which is involved in recognition of the odorant 4-methylphenol emitted by their human hosts (Hallem et al. 2004b). This system also identified odorant receptor OR22a as associated with electrophysiological responses to ethanol in flies (Hallem and Carlson 2006). As mentioned earlier, OR83b is a ubiquitous receptor that is coexpressed with receptor genes in all 120 maxillary palp neurons and about 70-80% of antennal ORNs and is also highly conserved across insect species. Based on studies using

Or83b mutants, it has been shown that OR83b alone does not recognize and respond to different odorants. However, when coexpressed with other ORs, it plays an important role in enabling these ORs to recognize and respond to different odorants (Larsson et al. 2004). The OR83b receptor forms a heterodimer with neuron specific ORs and this complex forms an odorant activated cation channel (Sato et al. 2008; Wicher et al. 2008) which is required for odorant recognition and plays a role in signal transduction. Additionally, *Or83b* mutants show defects in dendritic localization of odorant receptors indicating that OR83b also plays an essential role in the transport and insertion of specific ORs into the dendritic membrane (Larsson et al. 2004). Although initial reports of *Or83b* expression suggested that it is expressed in only 70-80% of all antennal ORNs (Larsson et al. 2004), further functional experiments have shown that *Or83b* is required for the trafficking and functioning of all basiconic and trichoid odorant receptors in adults and all the larvae (Figure 1.3) (Benton et al. 2006). The only class of ORNs that do not express or require *Or83b* are the CO₂ sensitive ORN expressing GR21a and GR63a (Suh et al. 2004; Benton et al. 2006; Faucher et al. 2006) or the ORNs expressing IRs in the coeloconic sensilla (Benton et al. 2009). GR21a and GR63a are coexpressed in the antenna and involved in the detection and mediation of avoidance behavior to CO₂ in *Drosophila* (Scott et al. 2001; Suh et al. 2004; Jones et al. 2007).

Carbon dioxide (CO₂) mediates avoidance behavior in *Drosophila* at concentrations lower than 2% through the activation of *Gr21a* and *Gr63a* neurons in the antennae

(Suh et al. 2004; Faucher et al. 2006; Jones et al. 2007). However, flies with inactivated *Gr21a/Gr63a* neurons still show avoidance behavior to CO₂ at concentrations higher than 5% indicating that another population of antennal neurons mediates avoidance to high CO₂ concentrations. Recently, a pair of dorsal glomeruli, termed DC4, was found to be activated by ~5% CO₂ (Ai et al. 2010). DC4 is stimulated by protons from carbonic acid, produced by high concentrations of CO₂, in the sensillar lymph and is innervated by *Ir64a* expressing neurons which also innervated another glomerulus, DP1m. *Ir64a* expressing neurons, which project to the DC4 glomerulus, are necessary for acid sensation and sufficient to produce avoidance behavior in flies with *Ir64a* mutants showing a significant decrease in avoidance to acids. Interestingly, while pure 5% acetic acid produced an avoidance behavior in flies, apple cider vinegar, which contains 5% acetic acid, induces attractive behavior in flies and in both cases the DC4 glomerulus is activated. These results suggest that vinegar contains attractants capable of overcoming DC4 mediated avoidance behavior by activating other olfactory receptors, such as *Or42a* and *Or92a* mentioned above (Semmelhack and Wang 2009; Ai et al. 2010).

Ionotropic Receptors

Ionotropic receptors are a family of iGluR related proteins that lack most of the known glutamate-binding residues, suggesting that they recognize distinct ligands. IRs are expressed in sensory neurons that respond to distinct odors but do not express odorant receptors or gustatory receptors. IRs are directly involved in odor

detection as their expression is concentrated in olfactory cilia and their misexpression in olfactory neurons is sufficient to confer novel odor responsiveness (Benton et al. 2009).

IR8a and IR25a receptors show heterogeneous expression in distinct but partially overlapping populations of neurons in the *Drosophila* antenna. Furthermore, their broad expression and localization to dendritic cilia indicated that these receptors might have a widespread role in odorant detection in IR neurons. This was confirmed through electrophysiological recording of odor evoked neuronal responses in individual coeloconic sensilla in *Ir8a* and *Ir25a* mutants. A panel of seven odors (sensilla specific agonists) believed to be recognized by different IRs were tested and the *Ir8a* and *Ir25a* mutants showed loss of multiple distinct ligand-evoked responses which was subsequently rescued through the expression of corresponding cDNA transgenes using the *Ir8a* or *Ir25a* promoters. These results suggest that these two receptors function as coreceptors that act with different subsets of odor specific IRs (Abuin et al. 2011).

IR8a and IR84a are coexpressed in ac4 sensilla and are required for the response to phenylacetaldehyde. In *Ir8a* mutants, IR84a no longer properly localizes to the sensory cilium of its neurons but when IR8a expression is restored the localization defect of IR84a is rescued. Similar observations were found in IR64a, which also is coexpressed with IR8a. These results suggest the requirement of both the common receptor IR8a along with neuronal specific IRs in promoting proper cilia targeting and odor-evoked responses (Abuin et al. 2011). The codependency of IR8a and IR84a

for cilia localization and odor-evoked responses suggest that these proteins might form a complex similar to the structure of many types of iGluR and this was confirmed through optical imaging of fluorescent protein-tagged receptors (Abuin et al. 2011).

IR25a is essential for ac4-specific electrophysiological responses to phenylethyl amine and based on analysis of IR expression map, IR76a and its coreceptors IR76b were identified as candidate receptors for this odor. Further analysis revealed a unique case where coexpression of three receptors IR25a, IR76a, and IR76b was required to form a functional olfactory receptor for the odorant phenylethyl amine (Abuin et al. 2011).

Olfactory Processing in Higher Brain Regions

Each ORN expresses a single odorant receptor from a family of *Or* genes and the odorant receptor defines the odor sensitivity of the neuron. A single odorant can activate multiple ORNs while inhibiting others and a single ORN can respond to multiple odorants. Additionally, odorants can vary in their temporal dynamics such that a single odorant can elicit a quick terminal response in some ORNs but a prolonged response in others and an individual ORN might show sustained responses to some odorants but an abruptly terminal response to others. The combinatorial activation of odor receptors and ORNs by odorants provides a mechanism through which the coding capacity of *Drosophila* olfactory system, that

relies only on ~60 genes (de Bruyne et al. 2001), can expand its dynamic range. Similar odor coding mechanisms are found in vertebrates (Malnic et al. 1999). Information received by ORNs is transferred to the antennal lobe in a one-to-one fashion, such that single classes of ORNs expressing the same ORs project their axons to one or two glomeruli (Wilson and Mainen 2006). Since a glomerulus is generally innervated by a single type of ORN, activation of a glomerulus reflects activation of a specific OR. Combinatorial activation of ORs by odorants generates a pattern of peripheral activity that is translated into a temporal and spatially dynamic activation pattern of glomeruli in the antennal lobes (Figure 1.7(a)) (Su et al. 2009). In the antennal lobe, the two major target neurons of the ORNs are local interneurons (LNs) which provide “horizontal” connections among glomeruli, and cholinergic projection neurons (PNs) which link individual glomeruli “vertically” with the mushroom body and the lateral horn of the protocerebrum in the brain (the two higher olfactory centers) (Stocker 1994; Wong et al. 2002). The intricate synaptic connections within the glomeruli provide an opportunity for olfactory information to be modified in the antennal lobe before it is passed on to the mushroom body and lateral horn. The LNs are mostly GABAergic (Wilson and Laurent 2005) and receive excitatory input from ORNs and PNs and establish inhibitory synapses with PNs. A second class of cholinergic excitatory LNs may play a role in interglomerular excitation of PNs (Figure 1.7(b)) (Shang et al. 2007). This intricate LN network might play an important role in synchronizing PN activity within a given glomerulus or between PNs innervating different glomeruli (Ng et al. 2002). This has the potential

to widen the range of odor sensitivities in the PNs and to generate a modified glomerulus-specific odor image, reflected both by temporal and by spatial PN activity, before it is transferred to higher olfactory centers (Laurent 1996; Lei et al. 2004). The PNs project their axons to both the calyx in the mushroom body and the lateral horn. The majority of PNs send their dendrites to only one glomerulus and the PNs innervating the same glomerulus show strikingly similar axonal topography. However, PNs from different glomeruli show very different patterns of projections in the protocerebrum (Wong et al. 2002). Projections of the PNs reveal a spatial representation of glomerular activity in higher brain centers, but axons projecting to the protocerebrum are diffuse and extensive, which is in sharp contrast to the tight convergence of axons to specific glomeruli in the antennal lobe (Wong et al. 2002).

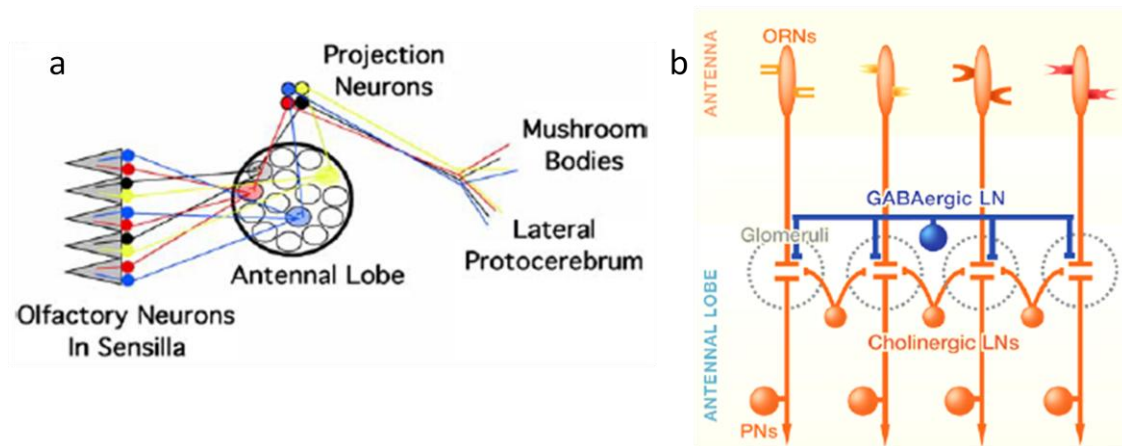


Figure 1.7: Olfactory processing in higher brain regions. (a) ORNs located in sensilla expressing the same OR converge to the same glomerulus in the antennal lobe and activity in the glomeruli is transferred to higher brain centers via projection

Figure 1.7: Continued

neurons. Figure adapted from Smith (2007). (b) GABA releasing LNs mediate interglomerular inhibition and excitatory cholinergic LNs mediate interglomerular excitation. Figure adapted from Su et al. (2009).

Gustatory System in Adult Flies

Gustatory Organs

The gustatory system receives environmental cues through direct contact with nonvolatile compounds. It plays an important role in evaluating nutrient rich foods, toxic compounds, oviposition sites, and potential mates. Although insects and mammals display similar appetitive responses to sugars and aversive responses to bitter compounds, their gustatory systems are organized differently. While in vertebrates the gustatory organ is restricted to the head, in flies it is distributed over their entire body. *Drosophila*, like many other insects, detects tastants through hairlike projections, called taste sensilla, in the proboscis, pharynx, legs, wings, and female vaginal plate (Stocker 1994; Singh 1997). The main taste tissue in *Drosophila* is the proboscis which is composed of two labial palps located at its distal end and contains both external and internal sensilla that house gustatory receptor neurons (GRNs). There are two morphological types of taste sensilla, taste hairs or bristles located on the surface of the fly body, and taste pegs located on the inner labial palps. Each palp is covered with 31 external sensilla or taste bristles which contain between two and four GRNs, one mechanosensory neuron, and several

types of accessory cells (Stocker 1994; Amrein and Thorne 2005). In addition, each labellum has ~30 taste pegs housing one GRN each (Amrein and Thorne 2005). These are the primary gustatory neurons that allow flies to evaluate food quality before ingesting it. The pharynx contains three bilaterally symmetric internal taste organs, the labral sense organ (LSO), the ventral cibarial sense organ (VCSO), and the dorsal cibarial sense organ (DCSO) (Figure 1.8) (Gendre et al. 2004). The LSO contains nine sensilla, three of which are gustatory, for a total of ten GRNs, the VCSO contains three sensilla housing a total of about eight GRNs, and the DCSO contains two sensilla with three GRNs each (Gendre et al. 2004). Thus, each side of the proboscis has a total of 69 taste sensilla.

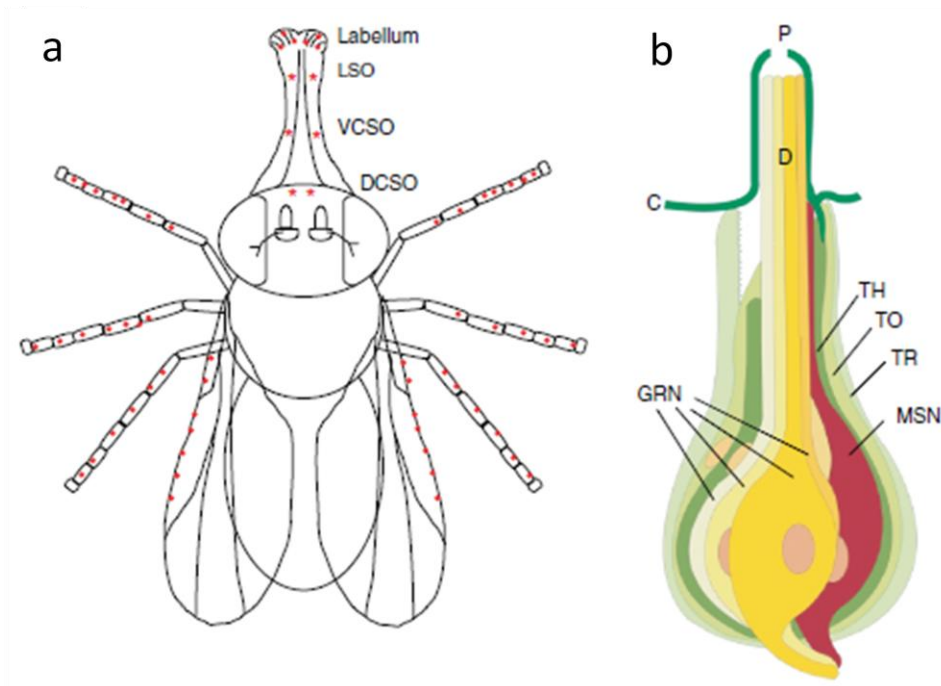


Figure 1.8: Organization of *Drosophila* gustatory system. (a) Distribution of taste sensilla throughout the fly's body. (b) Structure of a taste bristle. It contains two to four GRNs and one mechanosensory neuron (MSN). There is a pore (p) at the tip through which soluble chemicals come in contact with the dendrite (D). Space between the dendrite and the bristle is filled with a secretion from surrounding support cells, which include the thecogen (TH), the tormogen (TO), and the trichogen (TR) cell. Figure adapted from Amrein and Thorne (2005).

Based on their size, distribution, and number of GRNs, the external sensilla have been classified into three types, namely long (L-type) sensilla each having four GRNs, intermediate (I-type) sensilla having two GRNs each, and short (S-type) sensilla having four GRNs each and each individual sensillum of a class is identified

by a subscript, e.g. L₁ (Amrein and Thorne 2005). Electrophysiological analysis of the tuning of the s-type and l-type sensilla on the labellum suggest that each contains one neuron tuned to sugar (S cell), one neuron that is activated by water (W cell), one neuron activated by low salts (L1 cell), and one neuron activated by high salts (L2 cell) (Amrein and Thorne 2005). The l-type sensilla has two GRNs, where the first GRN responds to sugar and low concentrations of salt, therein combining both S and L1 cell activity, while the second GRN detects a range of bitter compounds and high salt concentrations revealing L2 cell properties (Hiroi et al. 2004).

The taste sensilla contain a single pore through which nonvolatile compounds enter and encounter multiple cell types before they stimulate the appropriate GRNs. Each GRN is a bipolar cell with a single dendrite extending to the tip of the sensilla and one axon that projects to the subesophageal ganglion, where taste information is initially processed. The internal sensilla, in the pharyngeal part of the proboscis, enable flies to evaluate compounds at the time of ingestion without consuming them. These sensilla, along with sensilla on wings and legs, contribute to survival by allowing flies to evaluate the nutrient content or toxicity of a compound before introducing it into their digestive system. Taste bristles also play a role in pheromone detection. There is a difference in the number of taste sensilla on the forelegs of males compared to females (~50 in males versus ~37 in females) and this sexual dimorphism is due to male specific sensilla that are used to detect nonvolatile pheromones that promote courtship and mating with females (Bray and

Amrein 2003). There are 30 and 32 taste sensilla on each second and third leg, respectively, with no sexual dimorphism in number or variation and each of the leg taste sensilla house between two and four GRNs. There are a total 40 taste sensilla on the wing margins which house four GRNs each. The female vaginal plate has ~10 sensilla believed to be involved in identifying oviposition sites and modulating egg-laying behavior, but they are still poorly characterized (Yang et al. 2008).

Gustatory Receptors

The same bioinformatic approaches used to identify olfactory receptors in *Drosophila* were subsequently used to identify gustatory receptors (GRs). This putative taste receptor gene family was identified by looking for DNA sequences encoding G-protein coupled seven-transmembrane receptors that were different from other multi-transmembrane proteins (Clyne et al. 2000). A thorough search of the *D. melanogaster* genome identified a family of 68 receptors encoded by 60 *Gr* genes (Robertson et al. 2003). GRs are distantly related to ORs and as a class are even more divergent than ORs. Most of the GRs commonly share only between 8-12% overall amino acid identity. The *Gr* genes are dispersed throughout the genome with about two-thirds of them in clusters of up to six genes. The clustered genes share a much higher sequence similarity to each other of up to 70% relative to the remaining *Gr* genes suggesting that these *Grs* probably arose through recent gene duplication and transposition events (Robertson et al. 2003).

The expression of some *Gr* genes has been confirmed using RT-PCR (Clyne et al. 2000), however only a small number of *Gr* genes are expressed at levels sufficient to be detected by RNA *in situ* hybridization (Scott et al. 2001). Additionally, taste neurons are widely spread throughout the body of the fly on many appendages which are not amenable to *in situ* hybridization methods (Dunipace et al. 2001). Therefore, for most of the GRs that have been studied, the tissue and cell type specific expression patterns have been visualized using the well-established *GAL4-UAS* binary expression system (Brand and Perrimon 1993). In this system, *Gr* gene promoters are used to drive expression of the yeast transcription factor *GAL4* which binds to a promoter known as the upstream activating sequence (*UAS*). The *UAS* promoter then activates transcription, with high specificity, of green fluorescent protein (GFP) or β -galactosidase reporter genes which are cloned downstream of the *UAS* promoter. Expression studies of *Gr* genes using RNA *in situ* hybridization and the *GAL4-UAS* system have revealed a complex expression profile for the *Gr* gene family as a whole and show them to be expressed in distinct subpopulations of GRNs (Dunipace et al. 2001; Scott et al. 2001; Thorne et al. 2004; Wang et al. 2004). The expression of most *Gr* genes is restricted to a few neurons in one or two taste organs, but some *Grs* (e.g. *Gr22e*) are widely expressed (Dunipace et al. 2001; Scott et al. 2001). Interestingly, three *Gr* genes, *Gr10a*, *Gr21a*, and *Gr63a*, are expressed in the fly antenna suggesting that they might be involved in olfactory functions (Scott et al. 2001). However, unlike the ORNs where a single ORN expresses a single olfactory receptor, many GRNs express multiple receptors.

Two major genes *Gr5a* and *Gr66a* are expressed in a very large number of non-overlapping GRNs. The GR5a receptor is expressed in approximately one-fourth to one-half of all proboscis neurons, including sensilla in the taste bristles and taste pegs, and detects the sugar trehalose (Chyb et al. 2003; Thorne et al. 2004; Wang et al. 2004). Though the GR5a receptor specifically detects trehalose, cells containing the GR5a receptor also recognize sucrose and glucose suggesting that these cells may express additional receptors. The GR66a receptor is expressed in approximately one-fourth of the cells in the proboscis, including taste bristle neurons, which do not contain the GR5a receptor, and the cells containing GR66a receptors recognize aversive compounds (Chyb et al. 2003; Thorne et al. 2004; Wang et al. 2004). Since GR5a marks cells that respond to sugars and GR66a marks cells that respond to aversive compounds, GRNs have been characterized according to the GRs they express. Neurons expressing the GR5a receptor detect trehalose, sucrose, glucose, and possibly other sugars (Wang et al. 2004) and the GRNs expressing the GR66a receptor are neurons that detect aversive cues which probably help in the behavioral discrimination of toxins (Thorne et al. 2004; Wang et al. 2004).

Further genetic analysis revealed that multiple GRs, GR61a and GR64a through GR64f, co-express with the GR5a receptor and are required for responses to sugars and sugar neurons are present in all labellar sensilla (Dahanukar et al. 2007; Jiao et al. 2007; Slone et al. 2007; Jiao et al. 2008). Similar analysis has shown that *Gr32a*, *Gr33a*, *Gr39a.a*, *Gr66a*, and *Gr89a* are expressed in all bitter neurons which, unlike

the sugar neurons which are universally expressed in all labellar sensilla, are limited to the small (S) sensilla (except S₄ or S₈) and intermediate (I) sensilla and are absent from the long (L) sensilla (Weiss et al. 2011).

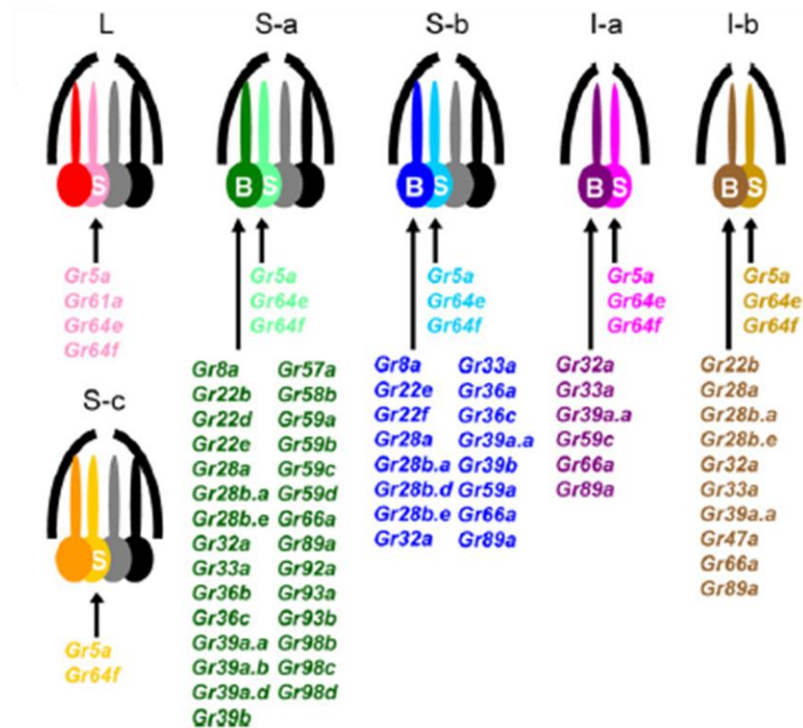


Figure 1.9: Expression of *Drosophila* gustatory receptors in different taste sensilla.

Figure adapted from Weiss et al. (2011).

Functional Characterization of Gustatory Receptors

The *GAL4-UAS* system has been used for analyzing neural pathways that drive behaviors. Toxins, such as diphtheria or tetanus toxin, can be expressed in specific neural populations using the *GAL4-UAS* system. Cell specific expression of these

toxins results in genetic ablation (diphtheria toxin) or inactivation of neurons (tetanus toxin). Studies where *Gr5a* neurons are ablated resulted in flies with taste defects in behavioral responses to different sugars (Wang et al. 2004). Capsaicin, an active component of chili peppers, is an irritant for mammals but is not perceived by *Drosophila*. When the mammalian capsaicin receptor was expressed under the control of the *Gr5a* promoter, administration of capsaicin resulted in activation of *Gr5a* neurons which induced acceptance behavior indicating that the *Gr5a* neurons are hardwired for acceptance behavior in flies (Marella et al. 2006). While the GR5a receptor specifically detects trehalose, neurons expressing GR5a receptors are also involved in detection of other sugars (Dahanukar et al. 2001; Ueno et al. 2001; Chyb et al. 2003), such as sucrose, maltose, and glucose, implying the existence of additional sugar receptors other than GR5a (Dahanukar et al. 2007; Jiao et al. 2007; Slone et al. 2007; Jiao et al. 2008). Seven additional gustatory genes, *Gr61a* and *Gr64a* through *Gr64f*, are expressed with *Gr5a* in sugar sensing neurons and detect a broad diversity of sweet ligands (Dahanukar et al. 2007; Jiao et al. 2007; Slone et al. 2007; Jiao et al. 2008). The GR64a receptor is required for detection of fructose, stachyose, maltose, maltotriose, and sucrose, and *Gr5a/Gr64a* double mutants show a lack of response to all these sugars (Dahanukar et al. 2007). This poses an interesting question as to why do sugar sensing neurons express additional receptors, especially since *Gr5a/Gr64a* double mutants appear to be sufficient to eliminate responses to sugars? The expression of additional receptors, the GR64b-f group, in sugar sensing neurons suggests that GR5a and GR64a may function in

conjunction with these receptors to detect specific sugar stimuli. This was confirmed by a study that showed GR64f is required in combination with GR5a for behavioral responses to trehalose. Further, GR64f is also required in combination with GR64a to rescue defects in sensitivities to sucrose, maltose, and glucose. Taken together, these results suggest that *Drosophila* sugar receptors function as multimers and that GR64f is required broadly as a coreceptor for the detection of sugars (Jiao et al. 2008).

Just as *Gr5a* expressing neurons are hardwired to mediate acceptance behavior to different sugars in *Drosophila*, bitter neurons that express *Gr66a* are hardwired to mediate avoidance behavior to bitter compounds (Marella et al. 2006). While the GR66a receptor appears to be a subunit of a caffeine receptor, as a mutation in GR66a eliminates caffeine avoidance behavior and caffeine induced action potentials in the GRNs, misexpression of GR66a by itself is not sufficient to produce a caffeine response suggesting that additional gustatory receptors might be required for caffeine detection. The GR93a receptor is expressed in the same GRNs as GR66a which suggests its possible role in detection of bitter compounds (Lee et al. 2009). Accordingly, both *Gr93a* mutants and *Gr66a* mutants are unable to detect caffeine, but show normal behavioral responses to other bitter compounds and normal preference to sugars. However, the misexpression of both GR66a and GR93a in GR5a expressing GRNs was not sufficient to recapitulate behavioral and electrophysiological responses to caffeine. These results suggest that, in addition to

GR66a and GR93a, other receptors or factors are required to produce the requisite avoidance behavior to caffeine (Lee et al. 2009).

Another receptor that is coexpressed with GR66a is GR32a. While GR32a is coexpressed with GR66a in all labellar neurons, in the tarsi they are expressed in distinct and non-overlapping sets of GRNs (Miyamoto and Amrein 2008). The GR32a expressing GRNs are associated with courtship behavior and GR32a is necessary for suppressing male-to-male courtship and courtship of mated female flies. From this it appears that GR32a receptors discriminate between the two sexes and female mating status by detecting inhibitory male pheromones that are released by male flies and are received by females after copulation (Miyamoto and Amrein 2008). In addition to GR32a, another putative pheromone receptor GR68a has been identified (Bray and Amrein 2003). GR68a is expressed in neurons of about 20 male-specific gustatory bristles in the forelegs and a reduction in the expression of GR68a protein levels leads to significant reduction in male courtship performance. These results suggest its role in detecting attractant pheromones transmitted by female flies (Bray and Amrein 2003).

GR33a is a receptor that has been implicated in sensing nonvolatile repellent chemicals that include both tastants and pheromones. This gustatory receptor is expressed widely in GRNs that respond to aversive chemicals and *Gr33a* mutant flies showed defects in behavioral and electrophysiological responses to a wide range of bitter compounds, including caffeine, quinine, denatonium, berberine, lobeline, papaverine, and strychnine (Moon et al. 2009). Since GR33a is required

for responding to aversive compounds it is possible that it is co-required with other gustatory receptors to produce such aversive responses. In the case of response to caffeine, it appears that three gustatory receptors, GR66a, GR93a, and GR33a, are required; however, misexpression of all three gustatory receptors in *Gr5a*-expressing GRNs did not produce a response to caffeine again suggesting the need/role for other receptors or factors in producing the requisite avoidance behavior to caffeine. In addition, *Gr33a* mutant male flies also display increased male-to-male courtship, suggesting that they function in detecting inhibitory male pheromones, in addition to the above mentioned detection of aversive compounds.

A systematic functional analysis on how bitter taste is encoded by the major taste organ of *Drosophila*, the labellum, by physiologically testing 16 bitter compounds against all 31 taste hairs and through expression analysis of all 68 gustatory taste receptors, revealed a receptor-to-neuron-to-response map (Figure 1.10) (Weiss et al. 2011). Different bitter compounds were tested over a range of concentrations and elicited different degrees of aversive behavioral responses. Denatonium benzoate produced the strongest aversive response that was more than two orders of magnitude greater than the compound escin, which elicited the weakest avoidance. Examination of electrophysiological responses of the labellar sensilla showed that individual tastants elicited responses from subsets of sensilla and individual sensilla were activated by subsets of tastants, revealing extensive heterogeneity among the labellar sensilla and the bitter neurons within them (Weiss et al. 2011). The L sensilla, along with S₄ and S₈, did not respond to any bitter

compound tested. The remaining S type sensilla were broadly tuned, responding to 9-15 of the 16 compounds tested. The I type sensilla were more narrowly tuned and responded to 3-7 compounds. Based on the responsiveness to the different compounds tested, the labellar sensilla can be divided into five functional classes: two classes of broadly tuned sensilla (S-a and S-b), two classes of narrowly tuned sensilla (I-a and I-b), and a class that does not respond to any of the tested bitter compounds (L, and S-c). Additionally, different compounds produced different electrophysiological responses. Some compounds elicited responses with delays of different lengths in the interval between the times at which the tastant was introduced and the onset of spike discharge and other compounds elicited shorter delays in spike onset that differed among sensilla (Weiss et al. 2011).

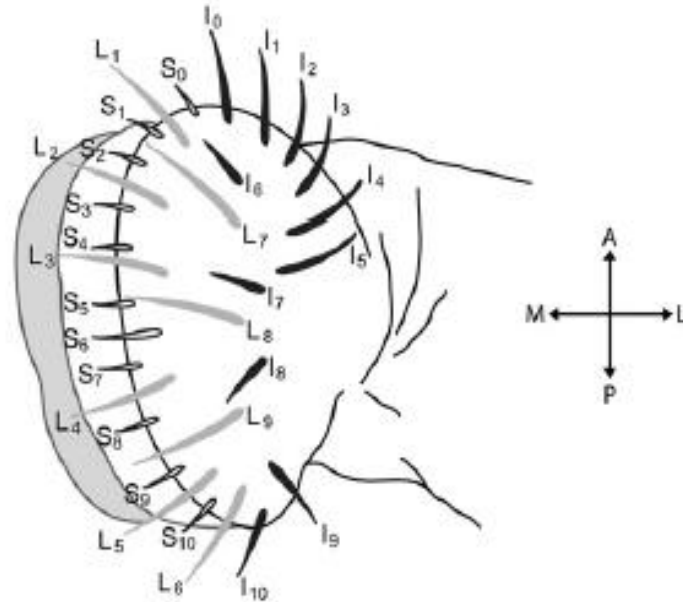


Figure 1.10: Depiction of the *Drosophila* labellum. It comprises of two labellar palps, each of which has 31 sensilla categorized and numbered based on their position and morphology. Figure adapted from Weiss et al. (2011).

Based on the classification of the labellar sensilla and using expression analysis of all 68 gustatory taste receptors, it is possible to make predictions about the functions of certain receptors. For example, GR59c is expressed in I-a sensilla and is absent in I-b sensilla. The I-a sensilla responds strongly to berberine chloride, denatonium benzoate, and lobeline hydrochloride, but the I-b sensilla does not respond to these compounds. These observations suggest that GR59c might respond to these compounds and the response of GR59c to these compounds was confirmed through its misexpression in different sensilla (Weiss et al. 2011).

In addition to the sugar neuron marked by *Gr5a* and the bitter neurons marked by *Gr66a*, an additional population of taste neurons that detect CO₂ in solution was identified using a combination of anatomical, calcium imaging, and behavioral approaches (Fischler et al. 2007). These neurons, designated as E409, were identified using a *Gal4* enhancer trap line. They are expressed in the taste pegs within the labellum and mark taste cells that do not contain *Gr5a* or *Gr66a*. The E409 neurons show a dose-dependent activation to CO₂ in solution with significant activation at low concentrations of CO₂. Like the sugar neurons, the E409 neurons mediate acceptance behavior towards CO₂, but this preference is not as strong as that mediated by sugar neurons, suggesting that while CO₂ does not provide any nutrition on its own it may enhance taste preference in conjunction with other food components (Fischler et al. 2007). Interestingly, recent studies have identified olfactory neurons in *Drosophila* that are activated by gas-phase CO₂ and express two gustatory receptors, *Gr21a* and *Gr63*. Both mediate avoidance behavior towards CO₂ vapors and are expressed in the abC1 neurons in the antenna that project to the V glomeruli in the antennal lobes (Suh et al. 2004; Jones et al. 2007). This is an example of where a single compound can act as a gustatory and/or olfactory cue and can elicit different behavioral responses depending on the context in which it is detected and the neurons that it activates.

Gustatory Projections

The GRNs of *Drosophila* project to the suboesophageal ganglion (SOG) which is located slightly behind and ventral to the brain. The SOG contains no apparent morphologically structural divisions such as the glomeruli present in the antennal lobe. The *Gr5a* sugar neurons, *Gr66a* bitter neurons, and the E409 neurons define three classes of GRNs that mediate the detection of different tastants and direct appropriate behavioral responses to such tastants. These three classes of GRNs send axons to distinct areas within the SOG. *Gr5a* neurons project ipsilaterally to the lateral region of the SOG, *Gr66a* neurons project to a ring-like web in the medial SOG that is slightly anterior to targets of *Gr5a* (Thorne et al. 2004; Wang et al. 2004), and E409 axons terminate in snake-like projections in the anterior SOG that do not cross the midline and are spatially segregated from *Gr5a* and *Gr66a* projections (Fischler et al. 2007).

A recent study, which tried to identify specific neurons that contribute to taste behaviors, identified E49 neurons which when silenced generated a specific motor defect in the proboscis extension reflex of the flies in response to a tastant (Gordon and Scott 2009). The proboscis extension reflex is a behavioral assay that measures preference of a compound by applying tastants to gustatory sensilla on the legs or labellum. Attractive stimuli induce extension of the proboscis, while noxious stimuli inhibit it. The E49 mutant flies display specific defects in the proboscis extension reflex such that the mutant flies do not lift the rostrum, which is the basal portion of the proboscis, out of the head while other movements remain

intact. The expression of different exogenous, ligand-gated ion channels, such as the VR1 mammalian cation channel from the TRP family which is also known as the capsaicin receptor, in the E49 neurons and subsequent activation of E49 motor neurons was sufficient to generate this rostrum lifting subprogram (Gordon and Scott 2009). There is no simple monosynaptic connection between sensory neurons and E49 motor neurons, suggesting the existence of higher order neurons in this taste circuit which remain unidentified (Gordon and Scott 2009).

Receptor expression, functional, and behavioral studies show clear differences between the fly gustatory and olfactory systems, reflecting differences in the manner through which environmental cues of smell and taste are detected and processed. The distinct target regions represented by *Gr5a*, E409, and *Gr66a* suggest that taste is primarily involved in mediating either attractive or aversive behavioral responses.

Olfactory and Gustatory System in Larvae

Drosophila larvae undergo complete metamorphosis to form an adult and larvae and adults display distinct lifestyles. Larvae live directly on their food source negating the need for long-range locomotion. This is in contrast to the adult fly which has to forage over considerable distances to locate nutrients, mates, and oviposition sites. This difference in lifestyles is paralleled by differences in the organization of the underlying neural circuits. Larvae respond to many volatiles (Cobb and Domain 2000; Fishilevich et al. 2005) but have an olfactory system which is much simpler, in terms of cell numbers, than that of adult flies (Fishilevich et al. 2005; Kreher et al.

2005; Ramaekers et al. 2005). Larvae have three major sense organs on the head, namely the dorsal organ, terminal organ, and ventral organ, as well as three pharyngeal organs (Gendre et al. 2004). These sense organs contain multiple sensilla housing between one to several sensory neurons. Electrophysiological and ablation studies revealed that the dorsal organ is innervated by 21 ORNs (Heimbeck et al. 1999; Kreher et al. 2005). Some of the sensilla of the dorsal organ and the terminal and ventral organs are innervated by GRNs (Vosshall and Stocker 2007). Larvae have a dramatically reduced number of chemosensory neurons compared to the adult flies. Adult flies have ~1,300 ORNs and ~300 GRNs (Stocker 1994; Matsunami and Amrein 2003), while the larvae have only 21 ORNs and ~80 GRNs (Python and Stocker 2002). The larva GRNs outnumber the ORNs, which probably reflects the fact that they are embedded in their food source and mostly need short-range chemical perception (Vosshall and Stocker 2007). While the olfactory and gustatory systems within a larva share peripheral organs, they clearly segregate into distinct pathways within the CNS. The larval olfactory system resembles the organization of the adult olfactory system such that axons from individual ORNs project to corresponding glomeruli in the antennal lobe (Figure 1.11). Axons from 21 ORNs project to 21 glomeruli (Fishilevich et al. 2005; Kreher et al. 2005; Ramaekers et al. 2005) and interact with LNs and PNs, and the PNs project to the mushroom body calyx. The reduced number and 1:1 ratio of ORNs to glomeruli in larvae depict a lower dimension of complexity and lack of redundancy that is seen in adult flies (Vosshall and Stocker 2007).

The larval gustatory system is less well studied and differences between the adults and larvae are less obvious. Four major target subregions have been identified in the larval SOG and they receive projections based on the location of the GRNs in the peripheral organs (Colomb et al. 2007). In the larvae, *Gr66a* neurons project ipsilaterally which is in contrast to the adult *Gr66a* neurons which project bilaterally to the medial SOG (Scott et al. 2001).

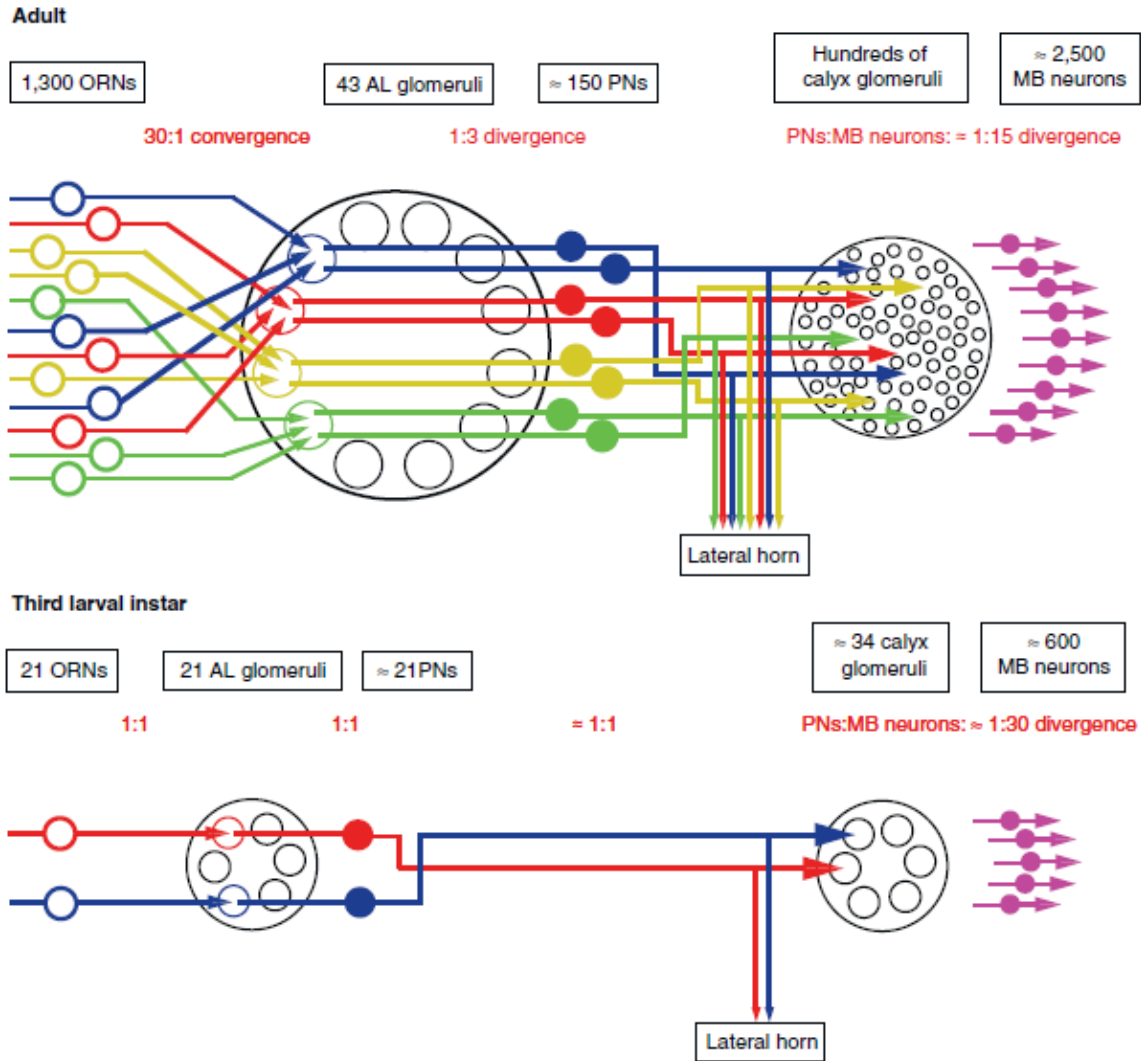


Figure 1.11: Comparative circuitry of adult and larval olfactory systems. It depicts a lower dimension of complexity in the larval olfactory system compared to the adults.

Figure adapted from Ramaekers et al. (2005)

Odorant Binding Proteins

In *Drosophila*, chemosensory behavioral responses are mediated by multigene families of chemoreceptors and odorant binding proteins (OBPs). The *Drosophila*

genome encodes a large family of *Odorant binding protein (Obp)* genes (Galindo and Smith 2001; Graham and Davies 2002; Hekmat-Scafe et al. 2002). OBPs contain six cysteines with conserved spacing between cysteines 2 and 3 (three residues) and cysteines 5 and 6 (eight residues) which are the defining feature of the family (Vogt et al. 1991). OBPs are found in both vertebrate and invertebrate olfactory systems, but are not related as revealed through X-ray crystal structure data (Bianchet et al. 1996). There are 51 genes in the *Drosophila Obp* gene family which are found in large clusters within the genome and the genes within a cluster show strikingly different expression patterns (Galindo and Smith 2001). OBPs are secreted in the perilymph by support cells in the sensilla and their proposed functions include: solubilizing odorants in the sensillar lymph, transportation of odorants through the lymph to the odorant receptors, removal of deleterious compounds from the lymph, and deactivation of odorants following receptor activation (Hallem et al. 2006). Thus, OBPs play a very important role in mediating chemosensory behavioral responses through their interaction with odorants and chemosensory receptors. Additionally, OBPs might be involved in other functions, other than their role chemosensation, since *Obp* mRNA has been detected in other non-chemosensory tissues, such as the accessory glands (Chintapalli et al. 2007). Evidence for the role of OBPs in odorant recognition comes from the finding that the LUSH OBP is essential to mediate response to the courtship pheromone 11-*cis*-vaccenyl acetate (cVA) (Xu et al. 2005; Laughlin et al. 2008). LUSH is expressed specifically in antennal trichoid sensilla and, in *lush* mutants, activation of *Or67d*

neurons located in T1 trichoid sensilla by cVA is abolished (Xu et al. 2005). cVA is a male-specific pheromone present on the cuticle, it is secreted by the ejaculatory duct, and is transferred to females upon mating (Smith 2007). Additionally, cVA can induce aggressive behavior (Wang and Anderson 2010) and suppress male courtship towards females as well as males (Kurtovic et al. 2007). Expression of recombinant LUSH protein directly in the sensillar lymph of *lush* mutants restores the response to cVA. Also, since the spontaneous activity of *Or67d* neurons in *lush* mutants is reduced, LUSH might play a role in directly activating *Or67d* expressing neurons (Xu et al. 2005). Through a genetic screen for cVA insensitive mutants a third protein, in addition to LUSH and *Or67d*, required for cVA reception has been identified (Jin et al. 2008). This protein, sensory neuron membrane protein (SNMP), is specific to trichoid sensilla and is a homologue of the silk moth SNMP (Rogers et al. 1997). In addition to the detection of cVA, *lush* mutants were initially identified as displaying abnormal olfactory behavioral responses to short chain alcohols and benzaldehyde (Kim et al. 1998).

In *Drosophila sechellia*, two OBPs have also been identified as crucial mediators of preference to its host plant, *Morinda citrifolia*. *Obp57d* and *Obp57e* are responsible for differences in behavioral responses to hexanoic acid and octanoic acid between two *Drosophila* species, *D. melanogaster* and *D. sechellia* (Matsuo et al. 2007).

Drosophila sechellia, which is a specialist, shows a preference for and resistance to the ripe fruit of *M. citrifolia*. However, its closely related species, *D. melanogaster* which is a generalist, avoids the fruit as it dies when it comes in contact with it. A

4bp insertion in the *Obp57e* gene in *Drosophila sechellia* accounts for differences in expression of *Obp57e* in the tarsi of *D. melanogaster* and *D. sechellia* and causes loss of avoidance to hexanoic acid and octanoic acid produced by *M. citrifolia*. This supports the use of a specific OBP in host plant specialization of this fruit by *D. sechellia* (Matsuo et al. 2007).

Furthermore, polymorphisms in the *Obp99a-d* group are associated with phenotypic variation in responses to benzaldehyde (Wang et al. 2007) and acetophenone (Wang et al. 2010). In a recent study, the systematic dissection of the functions of 17 OBPs was conducted through RNAi-mediated suppression of *Obp* gene expression and measurements of behavioral responses to 16 ecologically relevant odorants (Swarup et al. 2011). Flies in which the expression of a specific OBP was suppressed often showed sex dependent altered behavioral responses to more than one, but not all, odorants. Similarly, responses to a specific odorant were frequently affected by suppression of expression of multiple, but not all, OBPs, thereby implicating the role of OBPs in olfactory mediated behavioral responses and showing that OBP-dependent odorant recognition is combinatorial (Swarup et al. 2010).

Conclusions

D. melanogaster is an excellent model system that has all the attributes required for studying the genetic basis of chemosensory behavioral responses. They exhibit robust behavioral responses to odors and tastants, can be studied under controlled

conditions using relatively simple quantitative assays, are amenable to genetic, neuroanatomical, electrophysiological and behavioral manipulation, and their olfactory and gustatory systems have been well characterized. While most of this information has been gained through single mutational analysis, using an approach which exploits naturally occurring allelic variation within the DGRP offers the opportunity to identify naturally occurring polymorphisms associated with variation in chemosensory behaviors. This along with whole-genome transcript data will enable us to identify genes, including chemosensory genes, associated with variation in these behaviors and provide information about the genetic architecture underlying olfactory and gustatory behavioral responses to ethanol.

My Research Focus

Chemosensory behaviors can be studied using two complementary approaches: (1) a single gene mutational approach and/or (2) an approach which exploits naturally occurring allelic variation to identify genes affecting variation in such behaviors (Anholt and Mackay 2004). Most of the information detailed in this Chapter has come from single gene mutation studies. Single gene mutation studies have been invaluable in functionally characterizing chemosensory genes (Sengupta et al. 1996; Dunipace et al. 2001; Stortkuhl and Kettler 2001; Hallem et al. 2004a; Thorne et al. 2004; Wang et al. 2004; Xu et al. 2005; Hallem and Carlson 2006; Matsuo et al. 2007; Abuin et al. 2011; Weiss et al. 2011). However, since behaviors are complex traits influenced by numerous genes, the environment, and gene by environmental

interactions (Falconer and Mackay 1996), single gene mutations studies are not appropriate to understand the genetic architecture underlying such behaviors. Single gene mutation studies make it difficult to understand the combinatorial effects of mutations on the trait as they do not detect all the relevant loci that might affect the trait and the loci identified through mutagenesis may not be relevant to natural variation in the trait. Additionally, relatively few studies on chemosensory behaviors have been done in ecologically relevant settings, under controlled conditions or have looked at the interaction between the olfactory and gustatory systems to the same chemical stimulus. Furthermore, only a few studies have looked at the affect of natural polymorphisms on variation in chemosensory behaviors (Wang et al. 2007; Rollmann et al. 2010; Wang et al. 2010).

In Chapter Two, I quantify naturally occurring phenotypic variation in chemosensory behavioral responses to ethanol within the DGRP. I describe the approach of using natural occurring variation as a way to identify genes affecting variation in chemosensory behavioral responses to an ecologically relevant stimulus. In Chapter Three, I describe the results from the GWA analyses conducted to identify DNA polymorphisms associated with chemosensory behavioral responses to ethanol. In Chapter Four, I provide results from the analyses used to identify transcript abundance associated with chemosensory behavioral responses to ethanol.

In this study, I addressed several fundamental questions that we are able to answer only because of the power afforded by *Drosophila* genetics and the use of a

tremendous resource like the DGRP. I asked if the genetic architecture underlying olfactory and gustatory behavioral responses to ethanol is simple or complex? Are both these traits correlated with each other? Do we observe sexual dimorphism in chemosensory behavioral responses to ethanol? If so, what are the possible reasons for such sexual dimorphic behaviors? Can we gain any insights into possible selective pressures acting on chemosensory behavioral responses to ethanol? If so, how can we use this information to design future experiments to assess the strength and nature of selection occurring on these traits? Can we identify chemosensory genes associated with olfactory and gustatory behavioral responses to ethanol and functionally validate them? Does the olfactory and gustatory system interact in modulating appropriate behavioral responses to ethanol? And, are there common biological processes underlying olfactory and gustatory behavioral responses to ethanol? Through this study, I have addressed and at least partially answered these important questions.

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CHAPTER TWO

QUANTITATIVE GENETIC ANALYSIS OF NATURAL VARIATION IN CHEMOSENSORY BEHAVIORAL RESPONSES TO ETHANOL

Chemosensory Behavioral Responses in Flies

Animals have evolved sophisticated chemosensory systems that help mediate interactions with their environments. Behaviors are complex traits that are driven by epistatic networks of multiple segregating genes with pleiotropic effects (Mackay 2001; Flint and Mackay 2009; Mackay et al. 2009). Additionally, behaviors are highly sensitive to environmental variation which can cause variation in behavioral phenotypes among genetically identical individuals. In order to understand the genetic architecture that underlies a specific behavioral response, one must identify all the genes that contribute to the behavioral response and determine how these genes interact in functional ensembles (Anholt and Mackay 2004).

Researchers have developed several simple, robust, and quantitative behavioral assays to study olfactory and gustatory behavioral responses in *Drosophila* (Devaud 2003). These assays have been developed in the *Drosophila* model system as: (a) a way to study the genetic and neural bases of smell and taste which can be extended to other organisms (Vosshall 2001; Kim and Carlson 2002), (b) a method to study the differences in olfactory and gustatory behavioral responses to ecologically relevant odors and their role in producing genetic isolation and evolutionary divergence among different *Drosophila* species (R'Kha et al. 1991; Coyne et al. 1994), and (c) it provides a way of studying fly chemosensory behavioral responses under precisely controlled conditions in the laboratory which in turn enables the study of the mechanisms that regulate and control complex behaviors (Anholt et al. 1996; Carlson 1996; Sambandan et al. 2006).

Quantifying Complex Traits

The starting point for studying the genetic architecture that underlies complex behaviors, such as olfactory and gustatory responses to chemical stimuli, involves the development of appropriate quantitative assays. Ideally, such assays should be simple enough to enable large and rapid screens (Anholt and Mackay 2004).

Behavior Assays:

The different behavioral assays used to measure and quantify *Drosophila* gustatory or olfactory response can be grouped into two classes: (i) choice assays that involve a choice made by the animal and requires orientation behavior and (ii) assays that only include responses to a single odorant or taste stimulus (Devaud 2003).

Assays measuring feeding preference and T-maze or Y-maze tests, which measure odor preference, are amongst the most widely used choice assays. In a feeding preference assay, the flies are given a choice between a food source which contains a chemical stimulus (which is being tested) and the same food source without the chemical stimulus (the control) to measure attractive or aversive behavioral responses of flies. The two choices contain different color dyes which enables determining the food choice consumed through visual inspection of the fly's abdomen and indicates whether a chemical acts as an attractant or an aversive stimulus (Figure 2.1A). Here the preference for the chemical stimulus is determined by calculating the proportion of flies that consumed the food source. This assay was used to demonstrate that *Gr5a* mutants exhibit defects in detecting trehalose (Dahanukar et al. 2001). The T-maze and Y-maze tests are designed to measure

odor preference. Flies are introduced into the choice point and make a choice between two compartments containing the test odorant or a control, or in some cases containing different odors (Figure 2.1B, C). Here, the preference for the odorant is evaluated by calculating the proportion of flies that made the choice to walk into a specific odor compartment. T-maze assays have been used quite extensively to measure odor preference in flies. For example, a T-maze was used to identify a *Drosophila* mutant that exhibited defective behavioral responses to benzaldehyde (Helfand and Carlson 1989). In addition to the T-maze and Y-maze assays, another assay called the trap assay (Figure 2.1F) has been used to measure odor preference (Zhu et al. 2003; Larsson et al. 2004). Here, the flies are introduced into a container containing two traps. One trap contains the odorant being tested and the other trap acts as the control. If the flies have a preference to the chemical stimulus, they enter the trap containing this chemical. The preference for the chemical is determined by calculating the proportion of flies that are trapped.

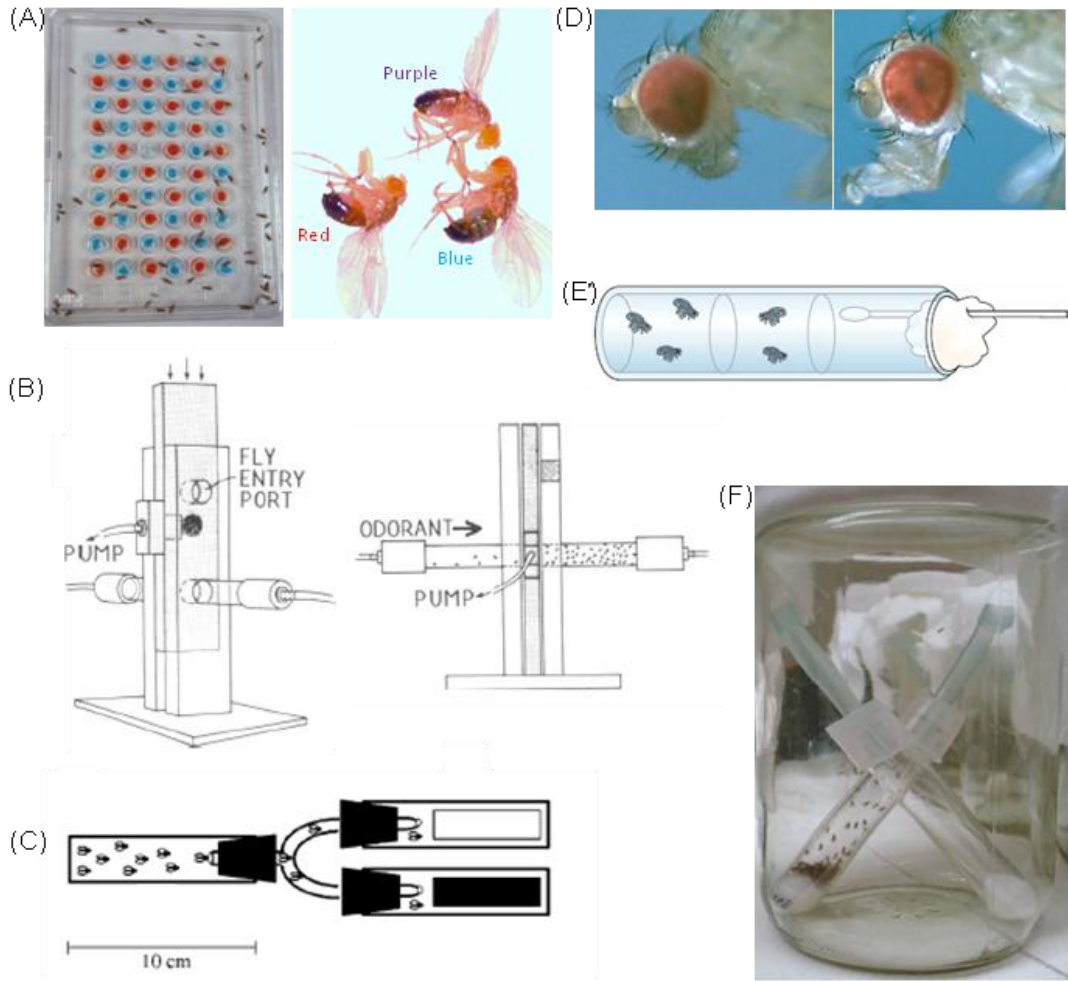


Figure 2.1: Chemosensory behavior assays. (A) Feeding preference test. Flies are given the choice to feed on two different food sources with different color dyes. The food choice made by the flies is evaluated by examining their abdomens. (B) T maze. Flies are introduced through the entry port into a chamber in the central, vertical sliding plate. The plate is pushed down and the flies can move into the left or right collecting tube which contains different odorants. Figure adapted from (Helfand and Carlson 1989). (C) Y maze assay. Flies are introduced into a start tube that connects to two separate tubes containing filter paper soaked with either

Figure 2.1: Continued

the odorant or solvent. Figure adapted from (Martin et al. 2001). (D) Proboscis extension reflex. This does not measure food intake, but a gustatory reflex associated with feeding behavior. The fly tarsi or labellum is stimulated by applying a drop of sugar solution which provokes extension of the proboscis. Figure adapted from (Amrein and Thorne 2005). (E) Olfactory avoidance assay. Single-sex groups of five flies are placed into a horizontal tube with marked compartments. A repellent odor is introduced at one end of the tube using a cotton swab and avoidance response is recorded by calculating the number of flies that migrate to the opposite end of the tube. Figure adapted from (Anholt and Mackay 2004). (F) Olfactory trap assay. Flies are introduced into a bottle containing two traps. One trap contains an odor source and the other contains water. Preference for the chemical stimulus is assessed based on the proportion of flies in the trap containing the chemical stimulus.

There are other assays that are more straightforward and involve a simple response to a single stimulation source. The proboscis extension reflex (Vaysse and Medioni 1973) is a taste assay which measures the flies reflex response to a sugar solution, or a sugar solution mixed with a bitter compound, that is applied to the tarsus or labellum (Figure 2.1D). This assay has been used to identify taste specificities of individual gustatory receptors and separating responses from sugar and bitter taste neurons (Marella et al. 2006; Dahanukar et al. 2007; Fischler et al. 2007).

Additional assays have been designed to measure olfactory behavioral responses in flies. One such assay is the “dipstick” assay which is used to measure avoidance behavior of flies to specific odorants (Figure 2.1E). The response of the flies is quantified by counting the number of flies in the compartment farthest from the odor source at successive intervals (Anholt et al. 1996). This assay was used to identify a large number of *smell impaired (smi)* mutants in a forward genetic screen (Anholt et al. 1996; Sambandan et al. 2006).

Interpretation of Results from Behavioral Assays:

Behavioral assays should be conducted under careful control and great care should be taken while interpreting the results measured from such assays. Complex behaviors are modular; for example, olfactory and gustatory behaviors require the detection of a chemical stimulus, motivation (for example hunger), orientation, locomotion, and the appropriate behavioral response (which can be moving towards an attractant or avoiding an aversive compound). Phenotypic variation in any one of these components will affect olfactory and gustatory behavioral responses. Many assays measure only one aspect of a behavioral response and this, by itself, might not always be sufficient for the complete evaluation of a particular phenotype.

Aspects of a behavioral response that are not directly measured by a specific behavioral assay might nonetheless affect the animal’s performance. For example, flies exhibiting aberrant avoidance responses to repellent odorants could be impaired not only in olfaction but other components necessary to produce the

appropriate behavioral response, and this necessitates the need to use multiple assays to evaluate different aspects of a behavior (Anholt and Mackay 2004). Differences in genetic background can have profound effects on behavioral phenotypes. Therefore, care should be taken to ensure that the genetic background of animals being tested is the same and thereby exclude any confounding effect of the genetic background on the behavioral trait (Anholt and Mackay 2004). Another significant source of phenotypic variation in behavioral traits is the effect of sex (Anholt and Mackay 2004). Sexual dimorphism is prevalent and found in chemosensory behaviors. Variation in sexual dimorphism can occur if a mutation or quantitative trait locus (QTL) affects only one sex (i.e. has sex-specific effects), affects both sexes but to different degrees (i.e. has sex-biased effects), or affects both sexes but in opposite directions (i.e. has sex-antagonistic effects) (Anholt and Mackay 2004). Because of this, male and female behavioral traits should be evaluated separately.

As mentioned earlier, behavioral traits are highly sensitive to environmental variation and such sensitivity can result in a range of behavioral phenotypes among genetically identical individuals. Behavioral assays must be designed keeping in mind the effect of common environmental variation on expression of behaviors. Individuals raised in the same common environment (for example, reared in the same vial, at the same temperature, under the same humidity and barometric pressure, measured on the same day and by the same handler) will have phenotypes more similar to each other than individuals reared in different common

environments (Anholt and Mackay 2004). Furthermore, behavioral responses display circadian variations. For example, most behaviors involve locomotion and spontaneous locomotor activity varies throughout the day (Konopka and Benzer 1971; Allada et al. 2001; Ashmore and Sehgal 2003). Similarly, the sensitivity of the antennae (Krishnan et al. 1999) and feeding behaviors exhibit circadian variations (Chatterjee and Hardin 2010). Additionally, behavioral responses show age-dependent variation and therefore the age of the animals needs to be controlled when studying behaviors. Finally, based on the amount of phenotypic variation (which is composed of genetic and environmental variation) appropriate sample size need to be used. For behavioral traits exhibiting relatively large phenotypic variation, environmental variation can be accounted for by strict randomization and statistically accurate estimates of genotypic effects can be obtained by increasing the number of individuals whose behavioral responses are measured (Anholt and Mackay 2004).

Another important factor to be kept in mind when measuring olfactory and gustatory behavioral responses is that these responses can change dramatically based on the concentration of the stimulus used. A fly's behavioral response can shift from showing an attractive response to exhibiting an aversive response, and vice versa, with changes in concentrations of the stimulus being tested (de Bruyne et al. 1999; Devaud 2003).

Genetic Architecture Underlying Chemosensory Behaviors

Chemosensory behavioral responses involve perception of information from the environment, assessing its appropriate biological significance by integrating and processing that information in the brain, and directing motor outputs that translate into behavioral responses. Understanding the genetic architecture that enables the nervous system to perceive, process, and direct appropriate behavioral responses to environmental cues involves: (a) identifying genes that guide olfactory and gustatory behaviors, (b) assessing the effects of alleles, of these genes, on phenotypic variation, (c) determining whether causal allelic polymorphisms are in coding or regulatory regions, (d) determining whether allelic polymorphisms in regulatory regions are *cis* or *trans* acting, and (e) assessing how genes interact by constructing networks of interacting genes. Studies on the genetic architecture of behavioral traits, such as aberrant olfactory behavior, alcohol sensitivity, aggression, and sleep, have led to the identification of numerous genes, with diverse molecular functions, which interact within genetic networks (Anholt et al. 1996; Anholt et al. 2003; Edwards and Mackay 2009; Harbison et al. 2009; Morozova et al. 2009). These findings suggest that behavioral traits are a product of highly dynamic epistatic networks of pleiotropic genes that are influenced by sex, developmental history, and genotype by environmental interactions (Anholt 2004; Austin et al. 2004; Venken and Bellen 2005; Mackay et al. 2009)

Single gene mutants have provided valuable information in chemosensory behavior studies and several null mutants with large effects have been generated through

chemically induced mutations. One such mutant, the *acj6* mutant, was defective in its response to benzaldehyde (Helfand and Carlson 1989) and encodes a POU-domain transcription factor that plays a role in olfactory receptor choice of olfactory receptor neurons (ORNs) and in ORN axon guidance (Clyne et al. 1999). However, mutations with large effects often produce homozygous lethality or sterility.

Additionally, a single gene mutational analysis approach is not appropriate to understand the genetic architecture underlying chemosensory behaviors because such studies make it difficult to understand the combinatorial effects of mutations on the trait, since they do not detect all the relevant loci that might affect the trait, and the loci identified through mutagenesis may not be relevant to natural variation.

A complementary strategy for studying and understanding the genetic basis of natural variation in chemosensory behaviors (and other complex traits) is to use a genome wide association (GWA) mapping approach. Such an approach is not limited to looking at the effects of single genes but rather has the ability to simultaneously identify multiple genes that affect such complex traits. The *Drosophila* model system is well suited for using the GWA mapping approach to determine the molecular genetic basis of natural variation in chemosensory behavioral responses. In addition to all the advantages of using this model system for studying complex traits mentioned in Chapter One, linkage disequilibrium (LD) decays rapidly in genomic regions with normal recombination in *Drosophila* (Long et al. 1998; Carbone et al. 2006; Mackay et al. 2012) and will help in not only

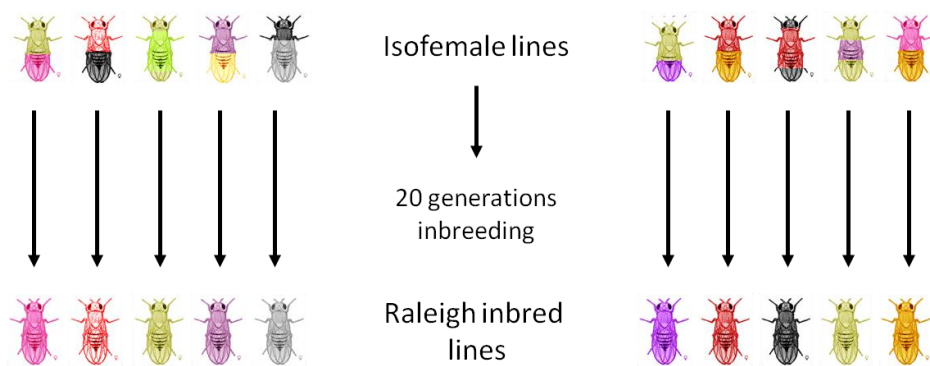
identifying genes but also the causal polymorphisms that are associated with chemosensory behavioral responses.

The *Drosophila melanogaster* Genetic Reference Panel

In order to use a GWA approach to identify natural occurring polymorphisms that are associated with phenotypic variation for a chemosensory trait, we need a population of flies which exhibit natural phenotypic variation. In addition, we need markers at the genome level to identify polymorphisms that are associated with variation in the trait. The *Drosophila melanogaster* Genetic Reference Panel (DGRP) is a population of 192 wild-derived inbred lines, out of which 168 lines have been fully sequenced to date (Mackay et al. 2012). The DGRP was constructed by collecting mated females from the Raleigh, USA population, followed by 20 generations of full-sib inbreeding of their progeny (Figure 2.2). There is minimal genetic variation within these lines, while genetic variation between the lines is preserved and reflective of the population from which the flies were collected. This enables the precise quantification of behavioral phenotypes by repeated measurements of individuals with the same genotype and allows us to exploit natural occurring phenotypic variation between the lines to identify polymorphisms that affect behavioral phenotypes through GWA mapping studies. The number of markers required to do a GWA study depends on the pattern of LD, and since LD decays rapidly over short physical distances in *Drosophila* (Long et al. 1998; Carbone et al. 2006; Mackay et al. 2012), information of all DNA sequence variants is required. The DGRP lines

have been fully sequenced and all single nucleotide polymorphisms (SNPs) have been identified (Mackay et al. 2012). For the first time, this offers us a tremendous resource to study the genetic architecture underlying chemosensory behavioral responses.

Drosophila melanogaster Genetic Reference Panel



Minimizes V_G within lines while preserving V_G among the wild-derived inbred lines

Figure 2.2: *Drosophila melanogaster* Genetic Reference Panel. This is a population of 192 wild-derived inbred lines that was constructed by collecting mated females flies from Raleigh, USA population, followed by 20 generations of full-sib inbreeding of their progeny. Out of the 192 wild-derived inbred lines, 168 have been fully sequenced to date. Due to 20 generations of full-sib inbreeding there is minimal genetic variation within a line while genetic variation between the lines is preserved (Mackay et al. 2012).

Ecologically Relevant Behavioral Responses of *Drosophila* to Ethanol

Drosophila encounters and is attracted to odors produced by rotten fruits, its primary food source, including ethanol which is produced by fermentation of yeast present in such fruits. Ethanol is probably used by *Drosophila* as a chemosensory cue to detect and locate transient food sources, oviposition sites, and potential mates (McKenzie and Parsons 1972; Dudley 2002). Ethanol is present in fermenting fruits at concentrations that can range anywhere from 0.02% to 6% (Gibson, May et al. 1981) depending on the type of fruit and its state of ripeness. However, ethanol is also a toxin and when consumed at high concentrations has fitness costs associated with it (Bokor and Pecsénye 2000). This creates an interesting scenario where on the one hand an ecologically relevant compound could be used to detect and locate food sources but on the other hand consuming such a compound can reduce fitness. There is evidence to suggest that a single compound can mediate different behavioral responses depending on the sensory neurons it activates. In *Drosophila*, CO₂ mediates avoidance behavior when detected as an olfactory cue but, at low concentrations, when detected as a gustatory cue it mediates an attractant response, which could be used by the flies to detect yeast that it feeds on (Fischler et al. 2007).

Natural Phenotypic Variation in Olfactory and Gustatory Behavioral Responses to Ethanol

Dose-Dependent Response to Ethanol:

As mentioned earlier, olfactory and gustatory responses can change dramatically depending on the concentration of the stimulus (Devaud 2003). Additionally, to perform a GWA study to identify polymorphisms associated with phenotypic variation in olfactory and gustatory responses to ethanol, we need to be able to detect extensive natural phenotypic variation for these traits in the experimental population of flies (the DGRP). In order to identify the appropriate concentration of ethanol to be used in the experiment, I measured dose-dependent olfactory and gustatory responses to ethanol.

As the genetic background of an organism plays an important role in determining its behavioral phenotype, it is important that the dose-dependent behavioral responses to ethanol are measured in a population of flies that can mimic the natural phenotypic variation in olfactory and gustatory responses to ethanol within the DGRP. In order to achieve this, I measured responses to ethanol in a synthetic outbred population of flies called the “Flyland” population. This population was generated by crossing 40 DGRP lines in a round robin design to create an outbred population (Figure 2.3).

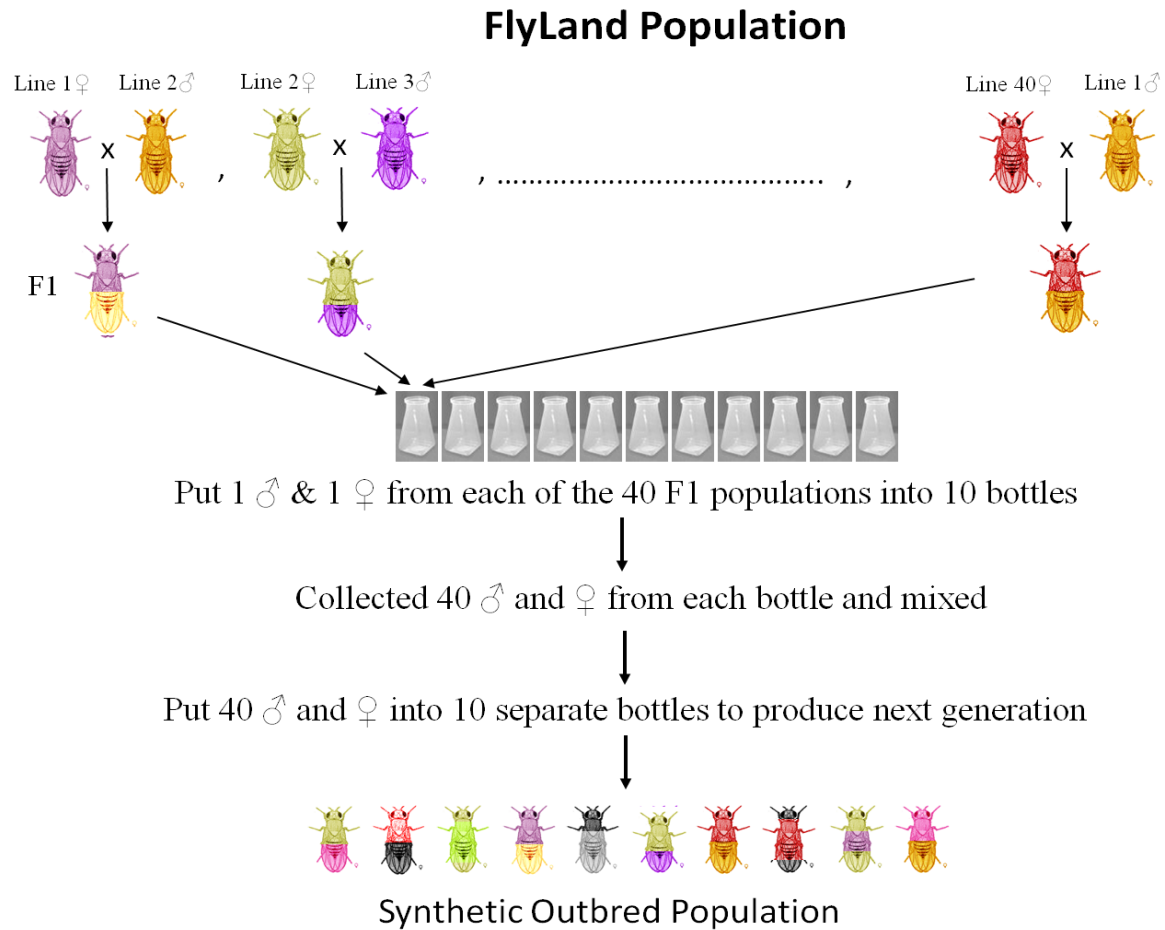


Figure 2.3: Flyland Population. This is a synthetic outbred population of flies created from a subset of 40 lines taken from the DGRP. This population was derived by crossing the 40 lines in a round robin design (Line 1 ♀ × Line 2 ♂, Line 2 ♀ × Line 3 ♂,....., Line 40 ♀ × Line 1 ♂) to create an F1 population. One male and one female from each of the 40 F1 progeny were placed into 10 separate bottles. They were allowed to mate and lay eggs for two days and then discarded. In the following generation, 14 days after the cultures were initiated, 40 male and 40 female flies were randomly collected from each of the 10 bottles and mixed, and

Figure 2.3: Continued

then 40 male and 40 female flies were randomly seeded into 10 separate bottles to produce the next generation.

MATERIALS AND METHOD

***Drosophila* Rearing**

Flies were reared on standard cornmeal molasses agar medium at 25°C, 70% relative humidity, 12 hour light/dark cycle, and controlled density, unless specified otherwise.

Flyland Population

The Flyland population was derived by crossing 40 wild-derived inbred lines, that were a subset of the 168 lines that form the DGRP, in a round robin design (Line 1 ♀ × Line 2 ♂, Line 2 ♀ × Line 3 ♂,....., Line 40 ♀ × Line 1 ♂) to create an F1 population. One male and one female from each of the 40 F1 progeny were placed into 10 separate bottles. They were allowed to mate and lay eggs for two days and then discarded. In the following generation, 14 days after the cultures were initiated, 40 males and 40 females were randomly collected from each of the 10 bottles and mixed, and then 40 males and 40 females were randomly seeded into 10 separate bottles to produce the next generation (Figure 2.3). This process has since been continued and the population was at ~75 generations at the time they were used in

this experiment to determine their olfactory and gustatory dose-dependent response to ethanol.

Olfactory Behavioral Assay

This assay measures the behavioral response to ethanol when detected as a volatile cue by adult flies. Flies were aspirated into a bottle containing two tubes (traps) with a pipette tip inserted in each tube providing a narrow opening (Figure 2.1(F)). One tube contained water and the other different concentrations of ethanol. These tubes offer two choices to the flies and once a fly makes a choice by entering a tube, it is trapped. For each assay, I used ~70, 3-7 day old with 10 replicates per sex / genotype (line) using a randomized block design. N = ~700 / sex / genotype. The assay was conducted for 24 hours and a preference index was calculated by subtracting the number of flies in the control tube (containing water) from the number of flies in the ethanol tube.

Preference Index =

$$\frac{\text{No. of flies [Ethanol Trap]} - \text{No. of flies [Control Trap]}}{\text{Total No. of flies [Bottle]}}$$

Scores below zero indicate that the flies avoid the tube containing ethanol, scores at zero indicate the flies show no preference, and scores above zero show that the flies are attracted to ethanol.

To minimize any day effect, the preference score for each replicate was normalized using the preference score of the *Canton-SB* laboratory strain. This was done by

subtracting the preference score of each replicate of a DGRP line from the preference score of the *Canton-SB* laboratory strain obtained on that particular day and subsequently adding the average preference scores for the *Canton-SB* laboratory strain back to the preference score of each DGRP line.

Gustatory Behavioral Assay

This assay measures the gustatory behavioral response of adult flies to consumption of ethanol in their food source. I starved the flies for 20 hours, to motivate feeding behavior, and then transferred them to a 60 well plate and ran the following assay for 2 hours (Figure 2.1(A)). The flies are given a choice between sucrose (2mM) mixed with different concentrations of ethanol versus sucrose (2mM). Both the choices contain separate colored dyes, 0.125 mg/ml FD&C Blue 1 or 0.5 mg/ml Acid Red 40, to determine which food choice the flies consumed by looking at the color of their abdomens. The dye was swapped between choices and the assay was conducted in the dark to minimize any dye effects. For each assay, I used ~60, 3-7 day old flies with 8 replicates per sex (4 replicates using blue and red dye each) / genotype (line) using a randomized block design. N = ~480 / sex / genotype. A preference index was calculated by counting the number of flies that consume the food choice containing ethanol plus half the number of flies that consume both food choices (these flies had purple abdomens) divided by the total number of flies.

Preference Index =

$$\frac{[N_{\text{blue}} + 0.5N_{\text{purple}}]}{[N_{\text{red}} + N_{\text{purple}} + N_{\text{blue}}]}$$

N refers to number of flies.

Scores of 0.5 indicate that the flies show no preference for the presence or absence of ethanol in the food, scores above 0.5 indicate that the flies prefer to consume the food choice containing ethanol, and scores below 0.5 indicate that the flies prefer to avoid consuming the food choice containing ethanol.

Any day effects on the preference score were minimized using the same method used for the olfactory behavioral assay.

Quantitative genetic analyses

ANOVA models were used to estimate the genetic and environmental variance components for both the olfactory and gustatory behavioral traits.

Factorial, mixed model ANOVAs of form $Y = \mu + S + L + S \times L + \varepsilon$ were used to partition variation of the quantitative trait phenotypes between sexes (fixed), DGRP lines (random), the $S \times L$ interaction (random), and the error variance (ε). Total genetic variance between the lines for both traits was obtained using $\sigma_G^2 = \sigma_L^2 + \sigma_{LS}^2$, where σ_L^2 is the variance between the lines and σ_{LS}^2 is the variance component for the $L \times S$ interaction. The total phenotypic variance was estimated using $\sigma_p^2 = \sigma_G^2 + \sigma_E^2$, where σ_E^2 is the environmental variance. Broad sense heritability (H^2) was estimated as $H^2 = (\sigma_L^2 + \sigma_{LS}^2) / (\sigma_L^2 + \sigma_{LS}^2 + \sigma_E^2)$, where σ_L^2 and σ_{LS}^2 are variance components mentioned above and σ_E^2 is the within line variance component. Each

sex was analyzed separately for both traits using reduced ANOVAs. Broad sense heritabilities were analyzed for the sexes separately as $H^2 = (\sigma_L^2) / (\sigma_L^2 + \sigma_E^2)$, where σ_L^2 is the variance component between the lines for males or females. Cross sex genetic correlations (r_{MF}) for the traits for which the sex \times line interaction term was significant were estimated as $r_{MF} = \sigma_L^2 / (\sigma_L^2 + \sigma_{SL}^2)$ from the analysis pooled across sexes. Cross trait genetic correlations (r_G) (between the two behavioral traits) was estimated as $r_G = \text{cov}_{ij} / \sigma_i \sigma_j$, where cov_{ij} is the covariance of line means between trait i (i.e. olfactory behavioral) and trait j (i.e. gustatory behavior), and σ_i and σ_j are the square roots of the variance components between the lines for the two traits.

Evaluation of Olfactory and Gustatory behavioral Assays

An *Or83b* mutant was used to evaluate whether the olfactory behavioral assays measures olfactory behavioral responses in flies. For this experiment, I used the null mutant *w ; w Orco*² which was obtained from the Bloomington stock center (Larsson et al. 2004). I back-crossed the *Or83b* mutant stock to the *Canton-SB* laboratory strain for five generations to minimize any genetic background effects. I compared the chemosensory behavioral responses of the back-crossed mutant stock to *Canton-SB* (control). Similarly, a *Pox neuro (Poxn)* mutant was used to evaluate the whether the gustatory choice assay measures gustatory behavioral responses in flies. The *Minos*-element mutant, *Mi{ET1}Poxn*^{MB00113}, and its co-isogenic control was obtained from the *Drosophila* Gene Disruption Project (Bellen et al. 2011). Statistically significant differences between the preference index of the

mutant and control flies were evaluated using one-way analysis of variance (ANOVA) for each sex separately.

RESULTS

Dose Dependent Olfactory and Gustatory Behavioral Responses to Ethanol

To determine the appropriate concentration of ethanol that should be used to detect the maximum phenotypic variation in olfactory and gustatory behavioral responses to ethanol in the DGRP lines, I measured dose dependent olfactory and gustatory responses of the Flyland flies.

I measured the behavioral response of flies when they detect ethanol as an olfactory cue using the olfactory trap assay. The flies were given a choice between a control tube containing water and a tube containing a specific concentration of ethanol. I tested the olfactory behavioral response of the flies to different concentrations of ethanol (0.002M, 0.02M, 0.2M, 2M, and 20M). From the results, when given a choice between water and ethanol, both male and female flies show significant dose dependent attractive responses to ethanol with half maximal effects at 10^{-2} M ethanol (Figure 2.4). While the flies exhibit attractive behavioral response to ethanol when they detect it as an olfactory cue at concentrations that range from 0.002M (~0.01%) to 2M (~10%), they exhibit indifference at 20M (~100%) ethanol. This is an example

where a behavioral response can change with variations in concentration of the stimulus.

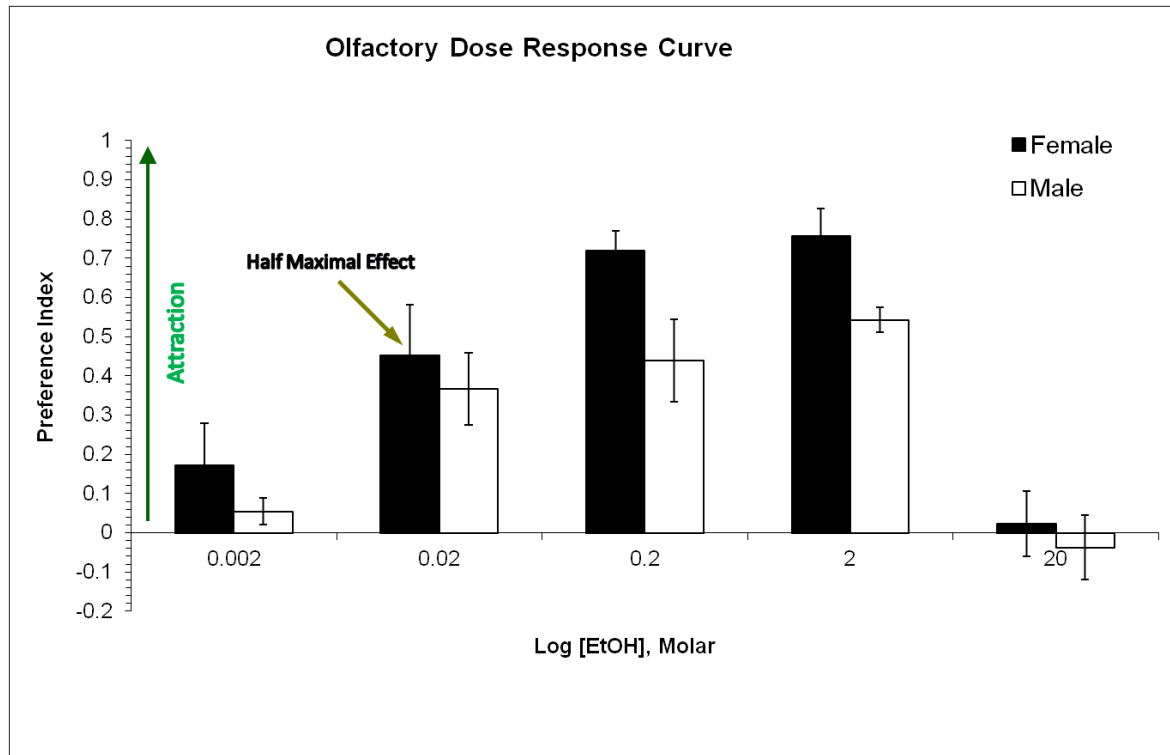


Figure 2.4: Dose dependent olfactory behavioral response to ethanol. Both male and female flies show a dose dependent attractive behavioral response to ethanol when given a choice between ethanol and water. The preference of ethanol over water is lost when ethanol concentrations reach 20M (~100%).

The behavioral response of flies for ethanol consumption was measured using the gustatory choice assay. Here flies were given a choice between sucrose versus sucrose containing a specific concentration of ethanol. The gustatory behavioral

response of the flies was tested at different concentration of ethanol (10^{-8} M, 10^{-6} M, 10^{-4} M, 10^{-2} M, and 1M). The flies did not show any significant statistical difference in the behavioral responses of males and females. Contrary to the olfactory behavioral response where the flies showed a dose dependent attractive response to ethanol, the flies exhibited a dose dependent aversive response to ethanol consumption when they detect it in their immediate proximity with half maximal effect at 10^{-4} M ethanol (Figure 2.5).

These results indicate that when *Drosophila* encounters ethanol as a volatile it is attracted to it in a dose dependent manner (Figure 2.4). When it detects ethanol in its immediate proximity, present in the food source, it exhibits a dose dependent aversive response to ethanol consumption (Figure 2.5). Thus, from the results it appears that ethanol is an attractant as an olfactory cue, but a powerful repellent as a gustatory cue. The flies were able to detect ethanol at nanomolar concentrations and at no concentration showed a preference to the food choice containing ethanol. Additionally, these results suggest that these behavioral responses might have evolved as a mechanism to balance the need of finding transient food sources, oviposition sites, and mates using olfactory cues while at the same time minimizing fitness costs associated with consuming a toxic compound producing such cues.

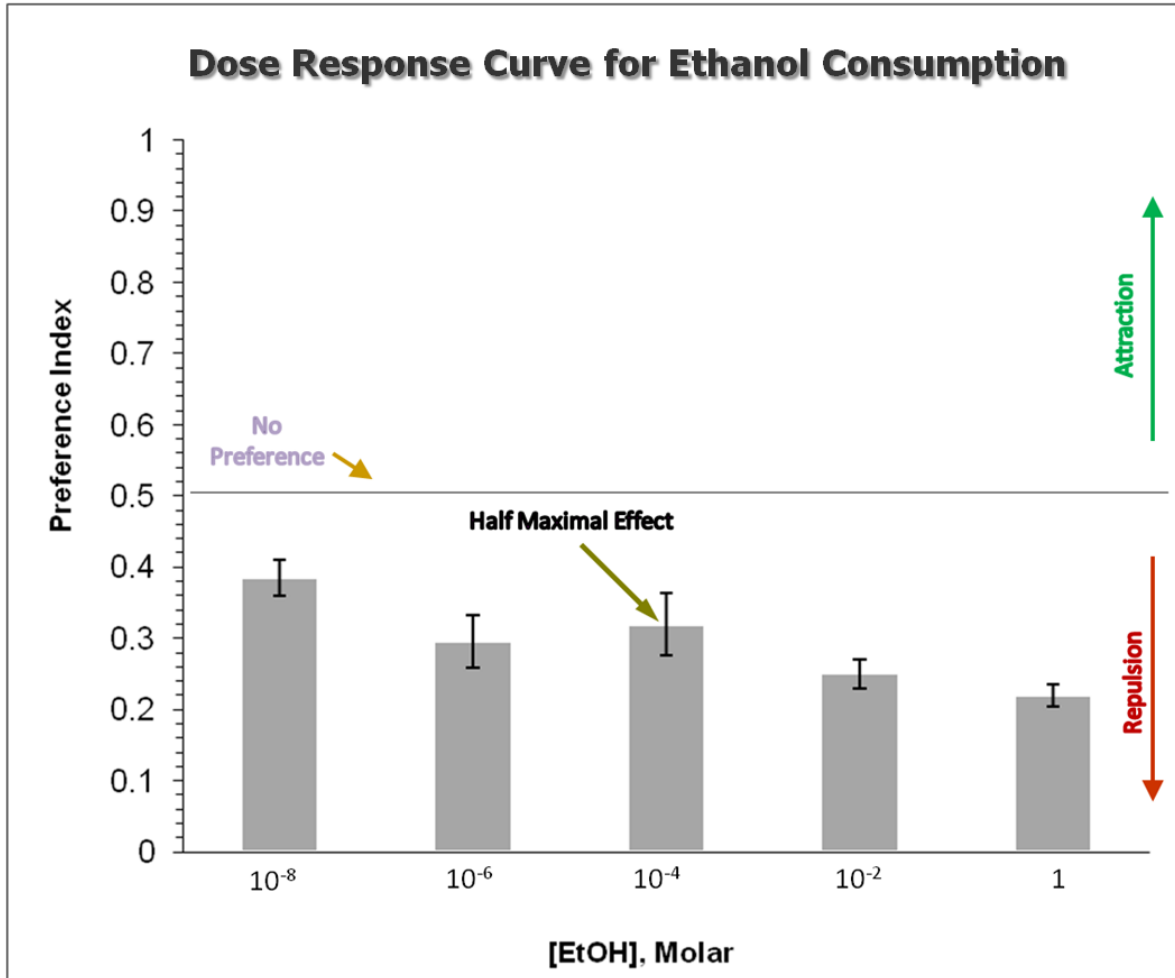


Figure 2.5: Dose dependent response for ethanol consumption. Flies were able to detect ethanol at nanomolar concentrations and exhibited a dose dependent aversive behavioral response to ethanol consumption when given a choice between a food source containing sucrose versus a food source containing sucrose and ethanol.

Behavioral Response of *Or83b* and *Poxn* Mutants

To ascertain whether the olfactory and gustatory behavioral assays used in this study are measuring olfactory and gustatory behavioral responses to ethanol, I tested *Or83b* null mutants using the olfactory trap assay and *Poxn Minos*-element mutants using the gustatory choice assay.

As mentioned in Chapter One, most olfactory receptor neurons express a unique odorant receptor along with the ubiquitous OR83b receptor (Larsson et al. 2004; Benton et al. 2006). The OR83b receptor forms a heterodimer with neuron specific odorant receptors and this complex forms an odorant activated cation channel (Sato et al. 2008; Wicher et al. 2008). Additionally, OR83b is essential for the transport and insertion of specific odorant receptors into the dendritic membrane (Benton et al. 2006). Thus, OR83b plays an important role in odor recognition and *Or83b* null mutants are impaired in their ability to recognize different odorants (Larsson et al. 2004). On the other hand, the *Poxn* gene of *Drosophila* is a member of the Pax gene family and encodes transcription factors with a DNA-binding paired domain (Bopp et al. 1989). In *Poxn* mutants most of the chemosensory bristles are transformed into mechanosensory bristles (Awasaki and Kimura 1997) and their gustatory perception is abolished (Boll and Noll 2002).

If the olfactory trap assay is measuring the olfactory behavioral response of the flies, we expect the assay to detect impaired olfactory behavioral response to ethanol in *Or83b* mutants relative to control flies. Both male and female *Or83b* mutants displayed significantly lower attraction to ethanol at 0.1% and 1% relative to their

controls (Figure 2.6), indicating that the olfactory trap assay was measuring the olfactory behavioral response of the flies to ethanol. Interestingly, though *Or83b* mutants showed impaired olfactory behavioral response to ethanol relative to the controls, they were still attracted to ethanol (Figure 2.6). This indicates that other chemosensory receptors, like IRs which are not coexpressed with *Or83b*, might be involved in ethanol detection and preference behavior. Similarly, the role of gustatory receptors in detection of ethanol as an olfactory cue cannot be completely ruled out, especially since gustatory receptors have been previously implicated in the detection of olfactory cues such as CO₂ (Jones et al. 2007).

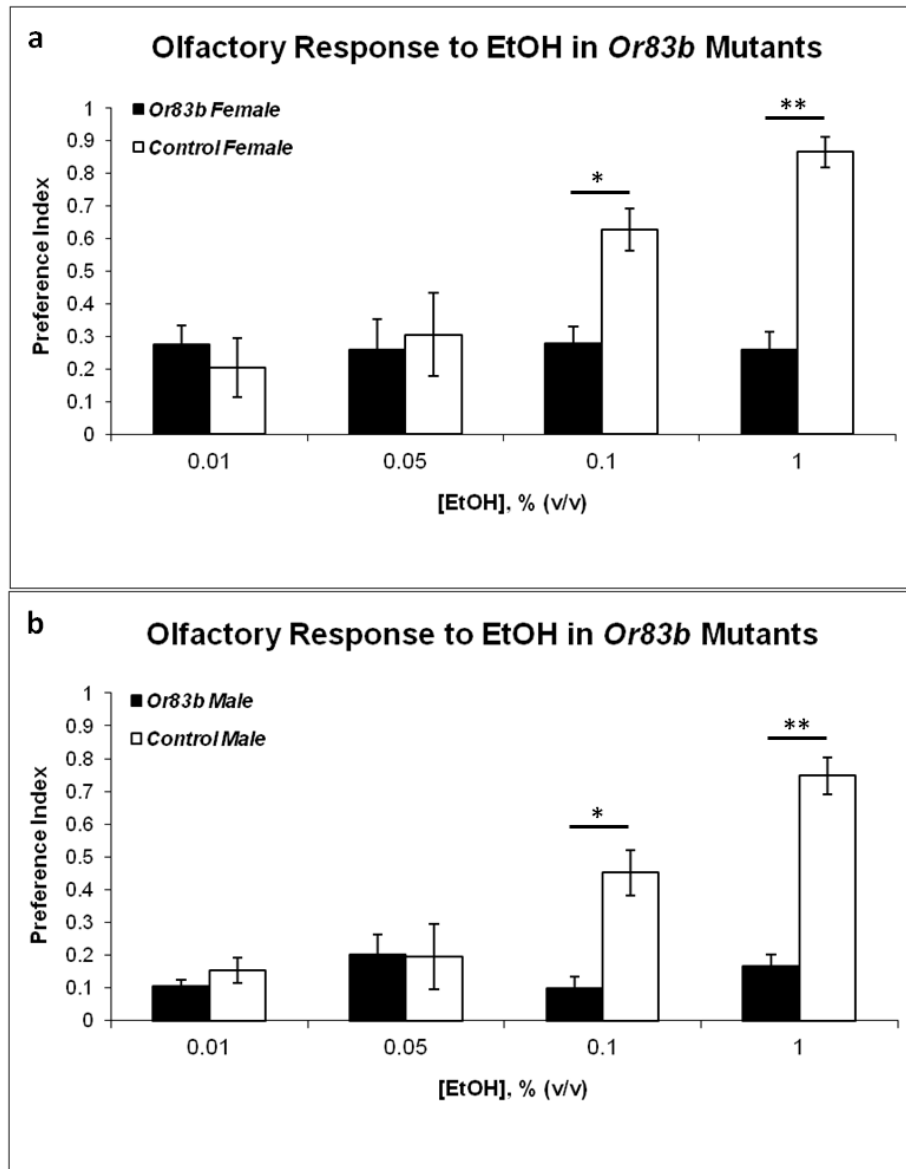


Figure 2.6: Olfactory behavioral response to ethanol in *Or83b* mutants. (a) Female *Or83b* mutants show significantly lower attraction to ethanol at 0.1% and 1% relative to their controls. (b) Similarly, male *Or83b* mutants show significantly lower attraction to ethanol at 0.1% and 1% relative to their controls (Statistically significant

Figure 2.6: Continued

differences calculated using one-way ANOVA for sexes separately. $p < 0.01$:* and $p < 0.001$: **).

I measured the behavioral response of *Poxn* mutants to test whether the gustatory choice assay measures gustatory behavioral response for ethanol consumption. If the assay measures the gustatory behavioral response of the flies, then the *Poxn* mutants should exhibit an impaired behavioral response in their preference for ethanol consumption relative to the controls since these mutants are impaired in their gustatory perception of chemical cues (Boll and Noll 2002).

Both male and female *Poxn* mutants displayed significantly reduced aversion (i.e. high preference scores) to consumption of the food choice containing 10^{-4} M ethanol relative to their controls (Figure 2.7). The results indicate that *Poxn* mutants are impaired in their ability to detect soluble ethanol relative to their controls and that the gustatory choice assay measures the gustatory behavioral response of the flies for ethanol consumption. However, interestingly even with their impaired gustatory perception, both male and female *Poxn* mutants exhibited some aversion to ethanol consumption (Figure 2.7, a score of 0.5 depicts no preference to either the food choice containing ethanol or without ethanol, whereas a score below 0.5 depicts an aversive response to ethanol consumption with complete avoidance of the food choice containing ethanol at a preference score of 0). This suggests that the mutant flies are able to detect ethanol, albeit not as strongly as the controls. This indicates

that chemosensory receptors other than gustatory receptors such as IRs, which have been found to be expressed in the proboscis (Benton et al. 2009), might be involved in the detection of ethanol. Similarly, ORs might also be involved in modulating the behavioral response of the flies to the consumption of ethanol in the gustatory choice assay since the flies can also detect ethanol mixed in the food source as a volatile cue. From these experiments, it appears that the olfactory trap assay is measuring the behavioral response of the flies when they detect ethanol as a volatile from a distance primarily through their olfactory system. On the other hand, the gustatory choice assay measures the gustatory behavioral response of ethanol consumption in flies when they detect it in their immediate proximity, both as a gustatory and an olfactory cue. Thus, while the gustatory choice assay appears to be primarily quantifying the gustatory preference of the flies for ethanol consumption, we cannot rule out the role of the olfactory system in modulating the gustatory behavioral response of the flies.

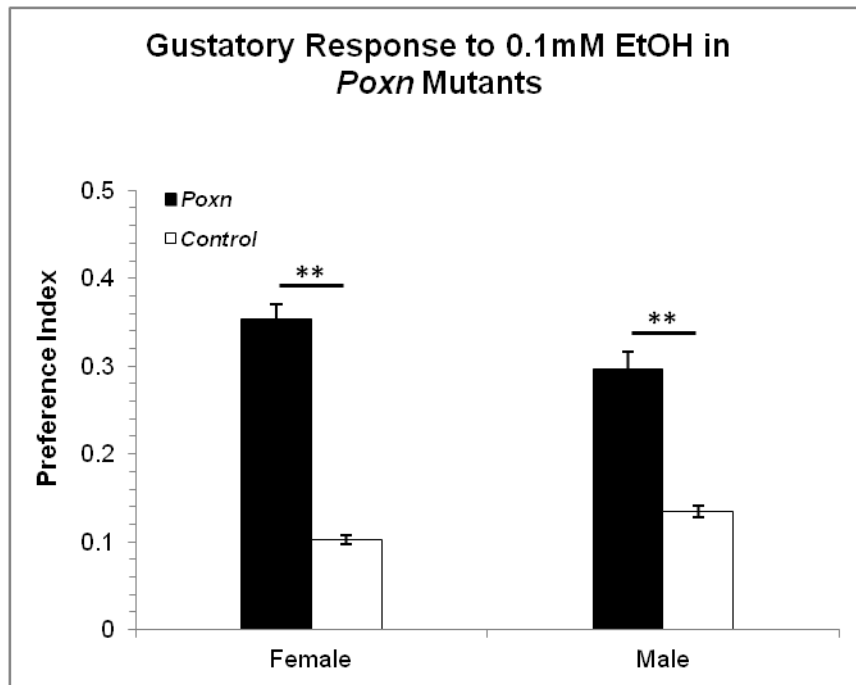


Figure 2.7: Gustatory behavioral response to ethanol in *Poxn* mutants. Both male and female *Poxn* mutants show significant higher preference scores (reduced aversion) in their gustatory behavioral response compared to their controls. (Statistically significant differences calculated using one-way ANOVA for the sexes separately. $p < 0.001$: **).

Natural Phenotypic Variation in Olfactory Behavioral Response to Ethanol in the DGRP

I measured the olfactory behavioral responses of the DGRP (except line RAL_591 which died out before it could be phenotyped) in the olfactory trap assay at 10mM ethanol to assess natural occurring variation that exist in the DGRP for this trait (Figure 2.8). I found extensive phenotypic variation (Figure 2.9, Table 2.1). On a

preference scale of 0 to 1, where 0 indicates no preference and 1 indicates complete preference for ethanol, the male preference to ethanol ranged from 0.05 to 0.98 and female preference ranged from 0.21 to 0.99 (Table 2.2).

There is sexual dimorphism in the behavioral response of males and females (Figures 2.10 and 2.11). The reaction norms illustrate sexual dimorphism within the DGRP with some crossing over of the reaction norms between the sexes (Figure 2.10). When averaged across all the lines, females exhibited significantly higher preference for ethanol compared to males: average female preference score was 0.62 compared to 0.55 for males ($p < 0.0001$). Out of 167 DGRP lines, females from 107 lines showed higher preference to ethanol compared to the males within that line (Figure 2.11).

Table 2.1: Analysis of variance of olfactory behavioral response to ethanol.

DF: degrees of freedom; MA: Type III Mean Squares; σ^2 : variance component.

Analysis conducted without RAL_591.

Trait	Analysis	Source of Variation	DF	MS	F	p-value	σ^2	Heritability
Olfactory Response to Ethanol	Sexes Pooled	Sex	1	4.102	43.08	<.0001	Fixed	$H^2 = 0.34$
		Line	166	0.71	7.45	<.0001	0.0307	
		Sex x Line	166	0.01	1.46	0.0002	0.003	
		Error	3006	0.07			0.066	
	Females	Line	166	0.38	5.7	<.0001	0.0313	$H^2 = 0.32$
		Error	1503	0.07			0.067	
	Males	Line	166	0.425	6.68	<.0001	0.0362	$H^2 = 0.36$
		Error	1503	0.064			0.064	

The broad sense heritability was high suggesting that a significant portion of the phenotypic variation is due to genetic variation among the DGRP lines (Table 2.1). For this trait, the DGRP lines showed a relatively strong phenotypic (Pearson phenotypic correlation $r_p = 0.76$) and genetic cross sex correlation ($r_{MF} = 0.91$) (Figure 2.12). This strong genetic correlation suggests that there should be overlap in the underlying genetic architecture of olfactory behavioral response to ethanol among males and females.

Table 2.2: Mean phenotypic values of chemosensory behavioral responses to**ethanol.** Mean phenotypic values for olfactory response (Trap) and gustatory

response (Gus), separately for females (*F) and males (*M) in the DGRP lines.

RAL_591 not included.

Line	Trap*F	Trap*M	Gus*F	Gus*M	Line	Trap*F	Trap*M	Gus*F	Gus*M
RAL_21	0.29	0.47	0.11	0.12	RAL_237	0.81	0.36	0.31	0.35
RAL_26	0.74	0.79	0.23	0.07	RAL_239	0.77	0.69	0.35	0.35
RAL_28	0.64	0.87	0.38	0.24	RAL_256	0.33	0.29	0.25	0.28
RAL_38	0.92	0.88	0.38	0.33	RAL_280	0.81	0.63	0.10	0.37
RAL_40	0.79	0.62	0.13	0.15	RAL_287	0.59	0.39	0.38	0.25
RAL_41	0.54	0.58	0.33	0.19	RAL_301	0.32	0.08	0.19	0.29
RAL_42	0.91	0.83	0.06	0.04	RAL_303	0.21	0.31	0.17	0.31
RAL_45	0.48	0.42	0.16	0.20	RAL_304	0.50	0.27	0.34	0.38
RAL_49	0.62	0.47	0.28	0.26	RAL_306	0.46	0.26	0.27	0.27
RAL_57	0.71	0.55	0.28	0.27	RAL_307	0.41	0.36	0.28	0.23
RAL_59	0.71	0.74	0.24	0.11	RAL_309	0.55	0.47	0.25	0.17
RAL_69	0.34	0.52	0.28	0.21	RAL_310	0.76	0.85	0.08	0.09
RAL_73	0.89	0.85	0.35	0.30	RAL_313	0.51	0.42	0.21	0.20
RAL_75	0.96	0.71	0.37	0.35	RAL_315	0.31	0.35	0.23	0.32
RAL_83	0.71	0.47	0.16	0.19	RAL_317	0.36	0.45	0.18	0.15
RAL_85	0.66	0.76	0.17	0.22	RAL_318	0.83	0.68	0.10	0.09
RAL_88	0.84	0.56	0.24	0.20	RAL_320	0.57	0.38	0.46	0.27
RAL_91	0.68	0.52	0.32	0.25	RAL_321	0.64	0.77	0.19	0.17
RAL_93	0.79	0.77	0.36	0.32	RAL_324	0.26	0.23	0.16	0.22
RAL_101	0.39	0.32	0.17	0.37	RAL_325	0.62	0.39	0.17	0.26
RAL_105	0.84	0.76	0.22	0.12	RAL_332	0.54	0.58	0.45	0.28
RAL_109	0.53	0.35	0.30	0.20	RAL_335	0.52	0.49	0.40	0.39
RAL_129	0.78	0.76	0.11	0.13	RAL_338	0.65	0.61	0.42	0.25
RAL_136	0.29	0.41	0.31	0.22	RAL_350	0.92	0.76	0.37	0.28
RAL_138	0.66	0.71	0.18	0.26	RAL_352	0.92	0.93	0.32	0.26
RAL_142	0.75	0.77	0.24	0.28	RAL_356	0.71	0.46	0.25	0.17
RAL_149	0.76	0.78	0.05	0.08	RAL_357	0.40	0.30	0.28	0.33
RAL_153	0.84	0.87	0.29	0.19	RAL_358	0.46	0.54	0.37	0.29
RAL_158	0.72	0.73	0.28	0.25	RAL_359	0.73	0.56	0.21	0.16
RAL_161	0.49	0.38	0.30	0.13	RAL_360	0.33	0.08	0.16	0.22
RAL_176	0.81	0.69	0.42	0.28	RAL_362	0.40	0.26	0.35	0.34
RAL_177	0.66	0.78	0.23	0.15	RAL_365	0.42	0.42	0.33	0.24
RAL_181	0.77	0.33	0.03	0.16	RAL_367	1.00	0.49	0.27	0.31
RAL_195	0.44	0.22	0.13	0.14	RAL_370	0.80	0.76	0.08	0.21
RAL_208	0.24	0.39	0.11	0.23	RAL_371	0.72	0.83	0.34	0.13
RAL_217	0.78	0.44	0.20	0.19	RAL_373	0.60	0.45	0.20	0.28
RAL_227	0.59	0.44	0.33	0.11	RAL_374	0.44	0.55	0.33	0.20
RAL_228	0.74	0.51	0.07	0.16	RAL_375	0.53	0.56	0.38	0.36
RAL_229	0.78	0.62	0.12	0.27	RAL_377	0.56	0.37	0.19	0.23
RAL_233	0.99	0.67	0.30	0.22	RAL_379	0.56	0.46	0.23	0.07
RAL_235	0.55	0.52	0.27	0.41	RAL_380	0.50	0.54	0.14	0.17

Table 2.2: Continued

Line	Trap*F	Trap*M	Gus*F	Gus*M	Line	Trap*F	Trap*M	Gus*F	Gus*M
RAL_381	0.24	0.35	0.27	0.37	RAL_732	0.52	0.52	0.30	0.19
RAL_383	0.50	0.38	0.10	0.37	RAL_737	0.79	0.56	0.12	0.14
RAL_386	0.76	0.62	0.05	0.06	RAL_738	0.75	0.77	0.13	0.18
RAL_391	0.33	0.08	0.28	0.17	RAL_757	0.96	0.97	0.04	0.05
RAL_392	0.50	0.28	0.08	0.11	RAL_761	0.67	0.57	0.21	0.32
RAL_399	0.77	0.65	0.17	0.15	RAL_765	0.21	0.27	0.25	0.18
RAL_405	0.57	0.25	0.17	0.19	RAL_774	0.60	0.56	0.15	0.21
RAL_406	0.87	0.81	0.17	0.14	RAL_776	0.54	0.62	0.17	0.16
RAL_409	0.64	0.57	0.08	0.14	RAL_783	0.74	0.69	0.18	0.26
RAL_426	0.49	0.50	0.02	0.18	RAL_786	0.58	0.32	0.41	0.27
RAL_427	0.49	0.46	0.20	0.21	RAL_787	0.58	0.51	0.20	0.28
RAL_437	0.33	0.15	0.31	0.30	RAL_790	0.63	0.73	0.25	0.20
RAL_439	0.88	0.77	0.10	0.10	RAL_796	0.58	0.66	0.01	0.19
RAL_440	0.79	0.70	0.22	0.13	RAL_799	0.42	0.40	0.37	0.31
RAL_441	0.71	0.45	0.07	0.43	RAL_801	0.73	0.72	0.32	0.19
RAL_443	0.64	0.72	0.30	0.35	RAL_802	0.98	0.83	0.23	0.21
RAL_461	0.95	0.94	0.17	0.17	RAL_804	0.42	0.65	0.11	0.06
RAL_486	0.48	0.55	0.37	0.15	RAL_805	0.93	0.82	0.26	0.19
RAL_491	0.35	0.18	0.23	0.26	RAL_808	0.37	0.44	0.35	0.17
RAL_492	0.77	0.78	0.22	0.21	RAL_810	0.97	0.73	0.10	0.21
RAL_502	0.53	0.54	0.14	0.15	RAL_812	0.87	0.73	0.21	0.29
RAL_508	0.79	0.81	0.03	0.26	RAL_818	0.67	0.75	0.35	0.12
RAL_509	0.85	0.88	0.20	0.22	RAL_820	0.26	0.23	0.30	0.22
RAL_513	0.30	0.44	0.27	0.21	RAL_822	0.75	0.56	0.28	0.24
RAL_517	0.46	0.47	0.20	0.24	RAL_832	0.88	0.57	0.22	0.13
RAL_531	0.83	0.73	0.08	0.27	RAL_837	0.68	0.71	0.41	0.21
RAL_535	0.69	0.70	0.34	0.06	RAL_852	0.50	0.51	0.33	0.23
RAL_555	0.26	0.05	0.38	0.33	RAL_855	0.88	0.66	0.20	0.17
RAL_563	0.70	0.37	0.25	0.39	RAL_857	0.53	0.62	0.23	0.16
RAL_589	0.38	0.16	0.31	0.30	RAL_859	0.56	0.44	0.26	0.35
RAL_595	0.68	0.45	0.10	0.12	RAL_861	0.50	0.55	0.21	0.22
RAL_639	0.36	0.15	0.28	0.20	RAL_879	0.71	0.69	0.15	0.20
RAL_642	0.80	0.64	0.10	0.15	RAL_882	0.50	0.26	0.22	0.22
RAL_646	0.52	0.45	0.19	0.15	RAL_884	0.58	0.78	0.11	0.12
RAL_703	0.91	0.72	0.14	0.23	RAL_887	0.75	0.70	0.21	0.01
RAL_705	0.63	0.65	0.25	0.14	RAL_890	0.78	0.84	0.16	0.14
RAL_707	0.33	0.40	0.37	0.35	RAL_892	0.62	0.61	0.11	0.04
RAL_712	0.58	0.58	0.25	0.21	RAL_894	0.89	0.98	0.32	0.28
RAL_714	0.41	0.23	0.21	0.26	RAL_897	0.73	0.67	0.21	0.14
RAL_716	0.69	0.75	0.18	0.08	RAL_907	0.45	0.40	0.04	0.07
RAL_721	0.54	0.69	0.32	0.24	RAL_908	0.71	0.47	0.05	0.10
RAL_727	0.90	0.87	0.27	0.31	RAL_911	0.66	0.55	0.08	0.14
RAL_730	0.42	0.24	0.28	0.25					

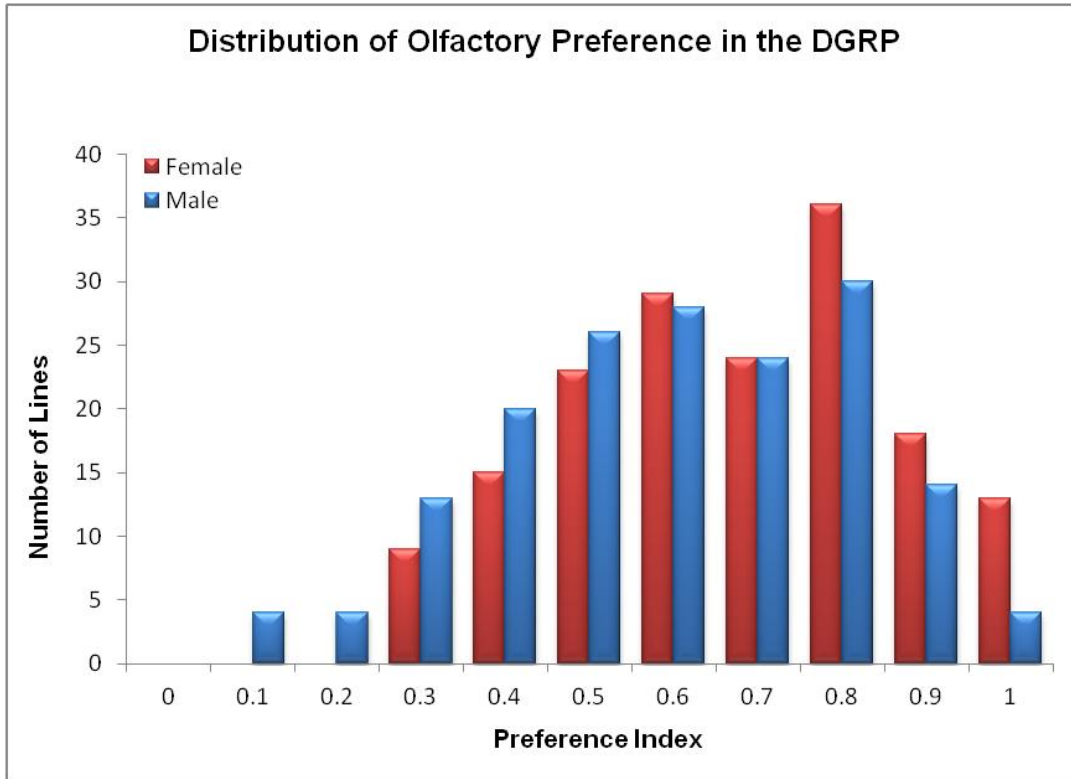


Figure 2.8: Distribution of mean preference scores for olfactory behavioral response to ethanol in the DGRP. Females shown in red bars and male shown in blue bars. Does not include line RAL_591.

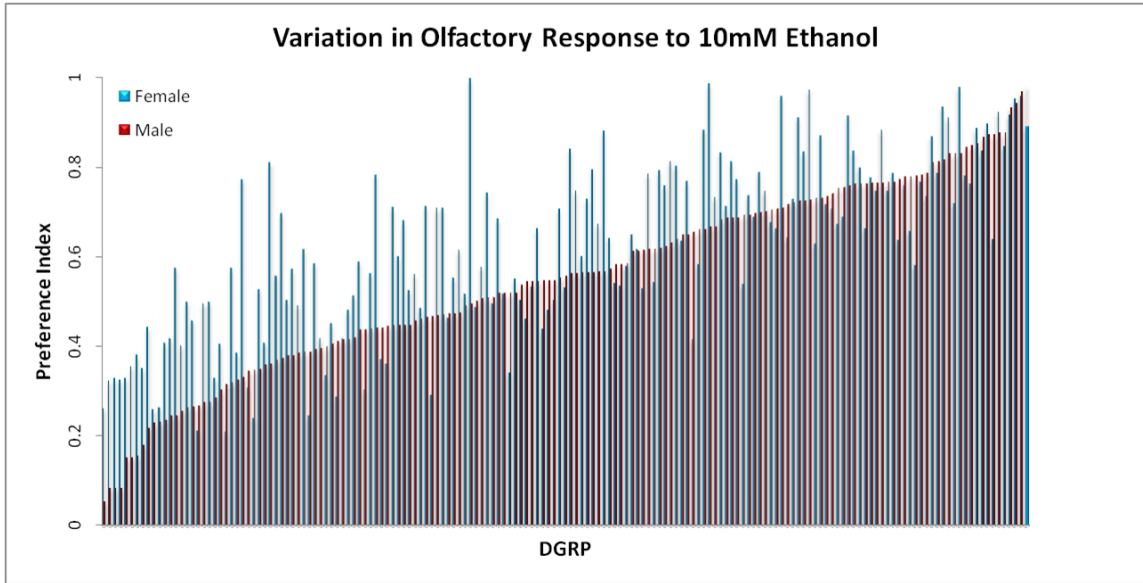


Figure 2.9: Natural phenotypic variation in olfactory behavioral response to 10mM ethanol among the DGRP lines. Line means are ranked from smallest to the largest based on male mean preference index. Does not include line RAL_591.

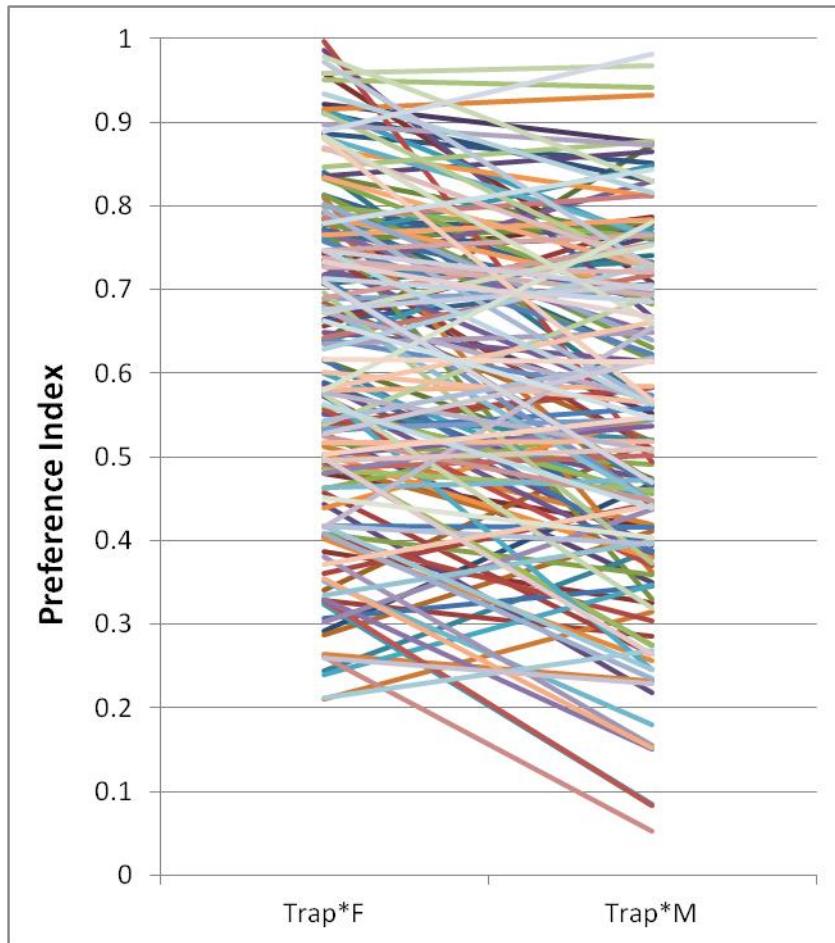


Figure 2.10: Reaction Norm for olfactory behavioral responses to 10mM ethanol among the DGRP. There is some crossing over of reaction norms between the sexes.

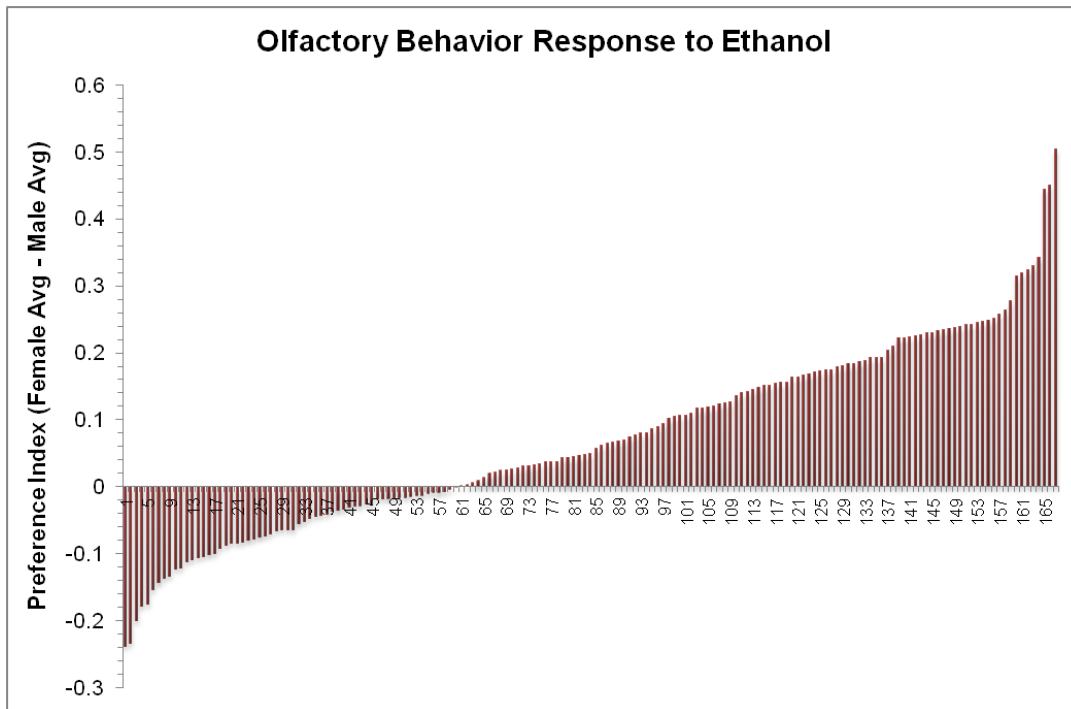


Figure 2.11: Female versus male olfactory behavioral response to ethanol.

Preference index is calculated by subtracting average male scores from average female scores within a DGRP line. 107 DGRP lines exhibit higher female score relative to males within the same line.

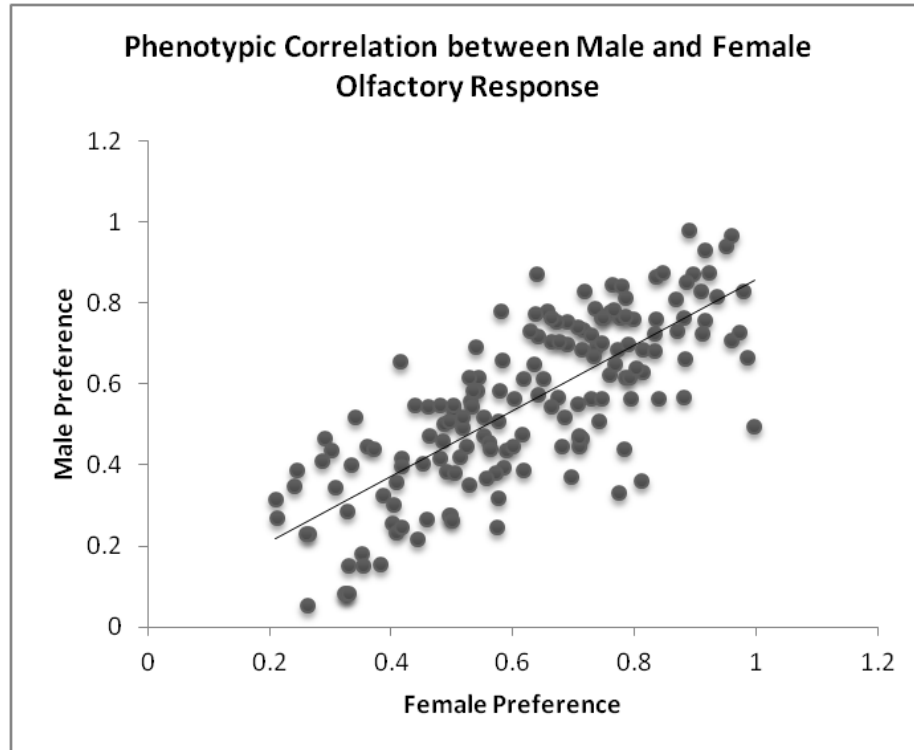


Figure 2.12: Phenotypic correlation between male and female olfactory behavioral response to ethanol. Pearson phenotypic correlation $r_p = 0.76$. Cross sex genetic correlation $r_{MF} = 0.91$.

Natural Phenotypic Variation in Behavioral Response to Ethanol Consumption

I measured the behavioral response of the DGRP (except line RAL_591 which died out before it could be phenotyped) to ethanol consumption using the gustatory choice assay at 10^{-4} M ethanol to assess natural occurring variation that exist in the DGRP for this trait (Figure 2.13). I found extensive phenotypic variation for this trait (Figure 2.14) with significant differences among the DGRP lines (Table 2.3). There was sexual dimorphism in the behavioral response of males and females (Figures

2.15 and 2.16). The reaction norms illustrate sexual dimorphism within the DGRP with extensive crossing over of the reaction norms between the sexes (Figure 2.15), which appears to be more complex than that observed in their olfactory behavioral response to ethanol (Figure 2.10).

On a preference scale of 0 to 1, where 0 indicates complete avoidance to the food choice containing ethanol, 0.5 indicates no preference to the presence or absence of ethanol in the food, and 1 indicates complete preference to the food choice containing ethanol. The male preference to the food choice containing ethanol ranged from 0.01 to 0.43 and female preference ranged from 0.01 to 0.46 (Table 2.2). It is important to note that none of the DGRP lines had a preference score above 0.5, meaning that neither males nor females from the DGRP showed a preference for the food choice containing ethanol. When averaged across all the lines, females exhibited a slightly lower aversive response to ethanol consumption (i.e. higher preference score) compared to males: average female preference score was 0.23 compared to 0.22 for males ($p < 0.01$). Out of 167 DGRP lines, females from 93 lines showed lower aversion to ethanol consumption compared to the males within the same line (Figure 2.16).

Table 2.3: Analysis of variance of behavioral response to ethanol consumption.

DF: degrees of freedom; MA: Type III Mean Squares; σ^2 : variance component.

Analysis conducted without RAL_591.

Trait	Analysis	Source of Variation	DF	MS	F	p-value	σ^2	Heritability
Gustatory Response to Ethanol	Sexes Pooled	Sex	1	0.094	2.27	0.1335	Fixed	$H^2 = 0.61$
		Line	166	0.103	2.5	<.0001	0.0039	
		Sex x Line	166	0.041	7.64	<.0001	0.0045	
		Error	2338	0.0054			0.0053	
	Females	Line	166	0.085	18.33	<.0001	0.01	$H^2 = 0.67$
		Error	1169	0.005			0.005	
	Males	Line	166	0.06	9.7	<.0001	0.007	$H^2 = 0.54$
		Error	1169	0.006			0.006	

The broad sense heritability of this trait is high suggesting that a significant portion of the phenotypic variation is due to genetic variation between the DGRP lines (Table 2.3). The males and females exhibit positive cross sex phenotypic (Pearson phenotypic correlation $r_p = 0.43$) and genetic correlations ($r_{MF} = 0.47$) (Figure 2.17) but this correlation is lower compared to the cross sex genetic and phenotypic correlation observed for the olfactory response to ethanol (Figure 2.12). The genetic correlation suggests that there might be some overlap in the underlying genetic

architecture of gustatory behavioral response to ethanol between males and females.

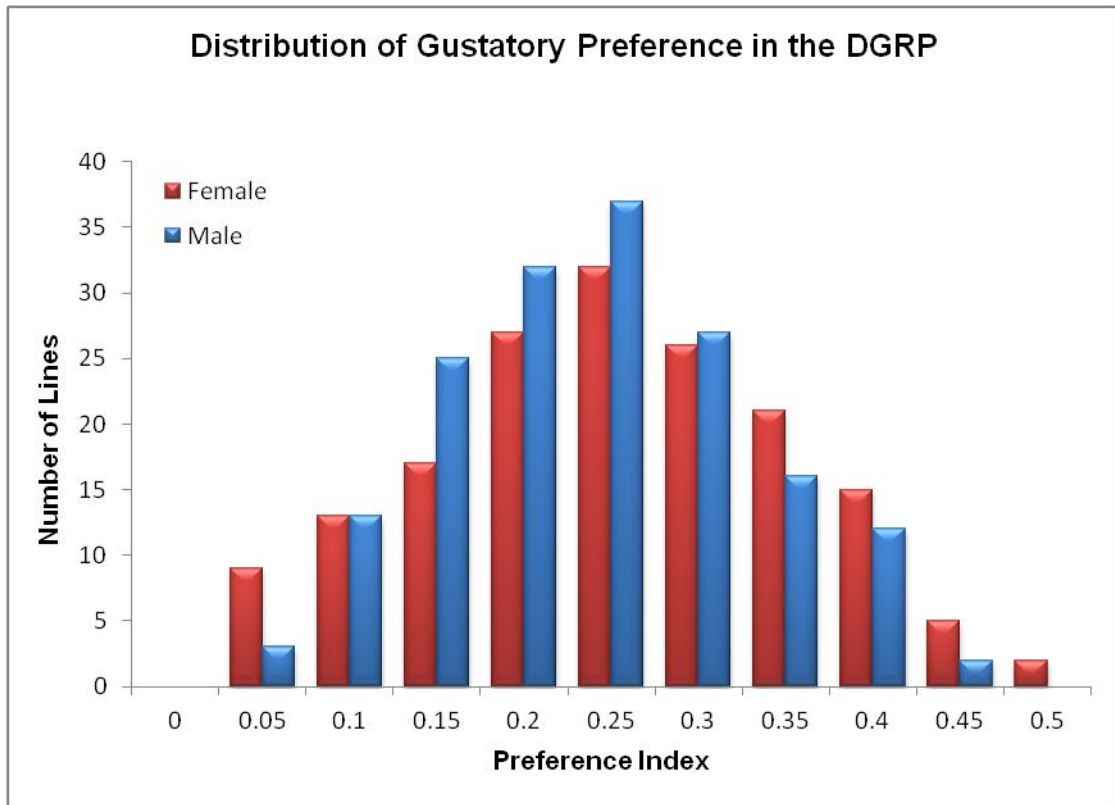


Figure 2.13: Distribution of mean preference scores for behavioral response to ethanol consumption in the DGRP. Females shown in red bars and male shown in blue bars. Does not include line RAL_591.

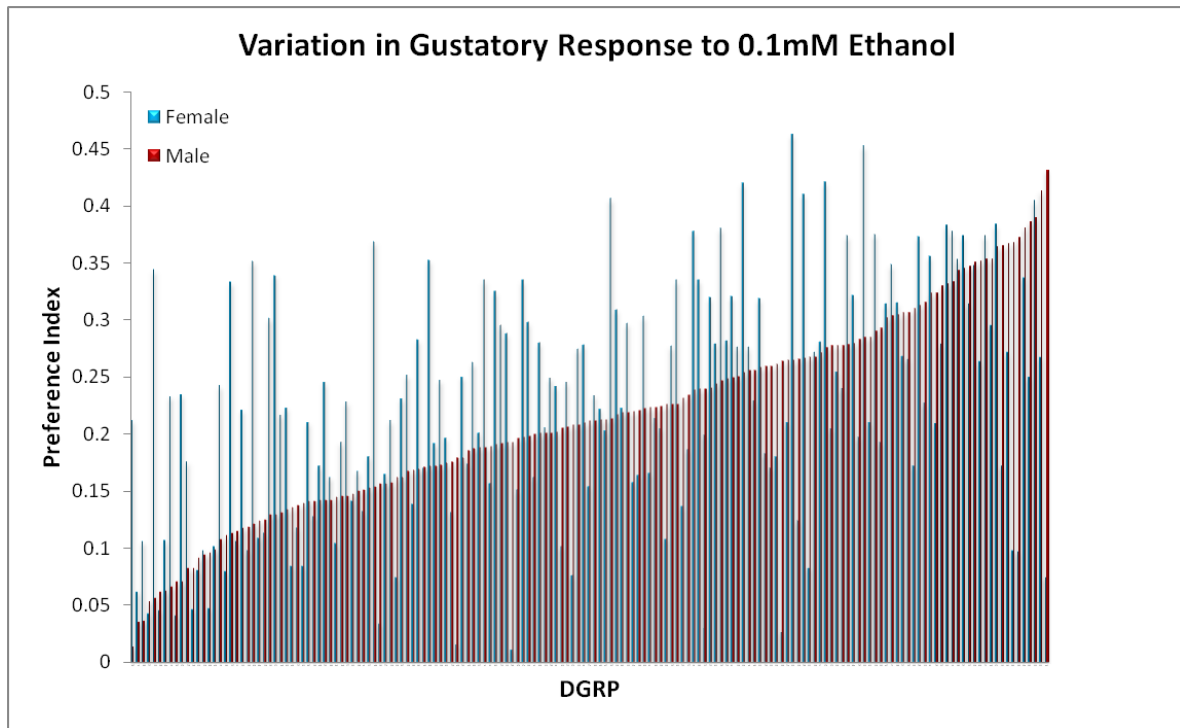


Figure 2.14: Natural phenotypic variation in behavioral response to 0.1mM ethanol consumption among the DGRP. Line means are ranked from smallest to largest based on male mean preference index. Does not include line RAL_591.

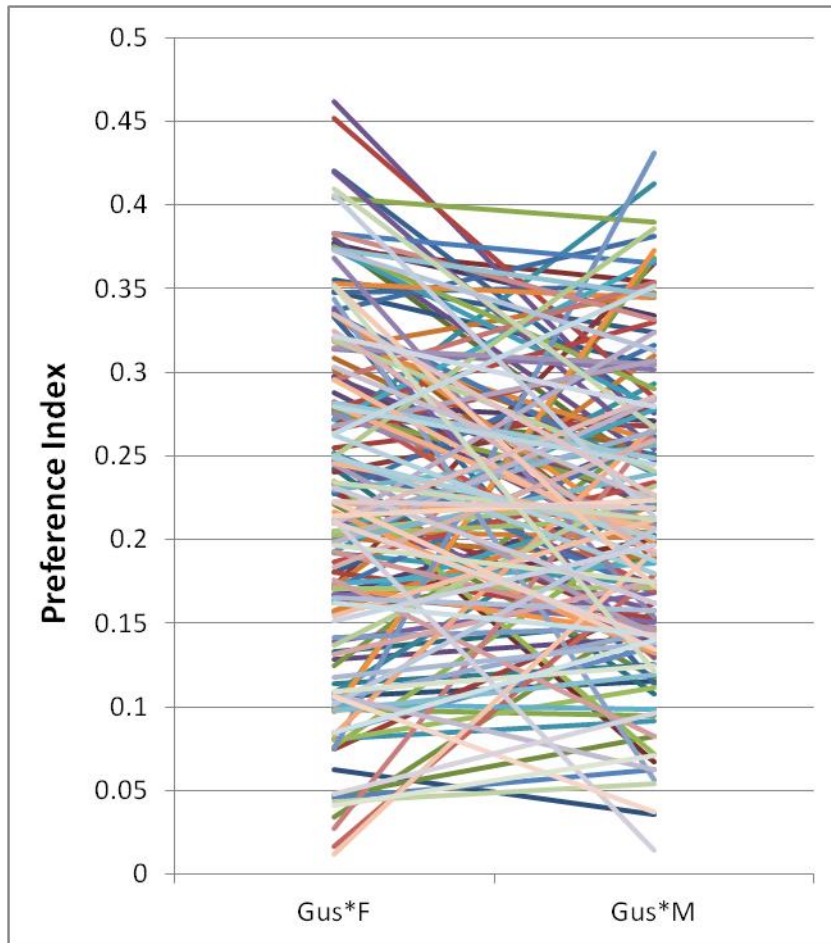


Figure 2.15: Reaction Norm for behavioral response to 0.1mM ethanol consumption among the DGRP. There is extensive crossing over of reaction norms among the sexes.

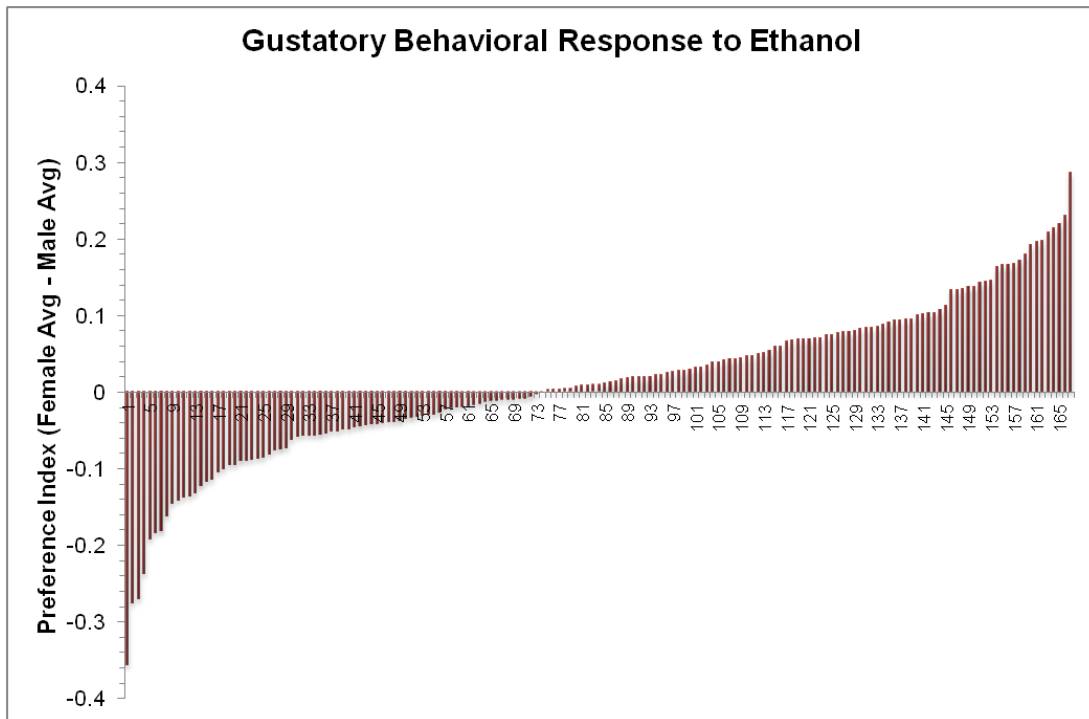


Figure 2.16: Female versus male behavioral response to ethanol consumption. Preference index is calculated by subtracting average male scores from average female scores within a DGRP line. 93 DGRP lines exhibit higher female score relative to males within the same line.

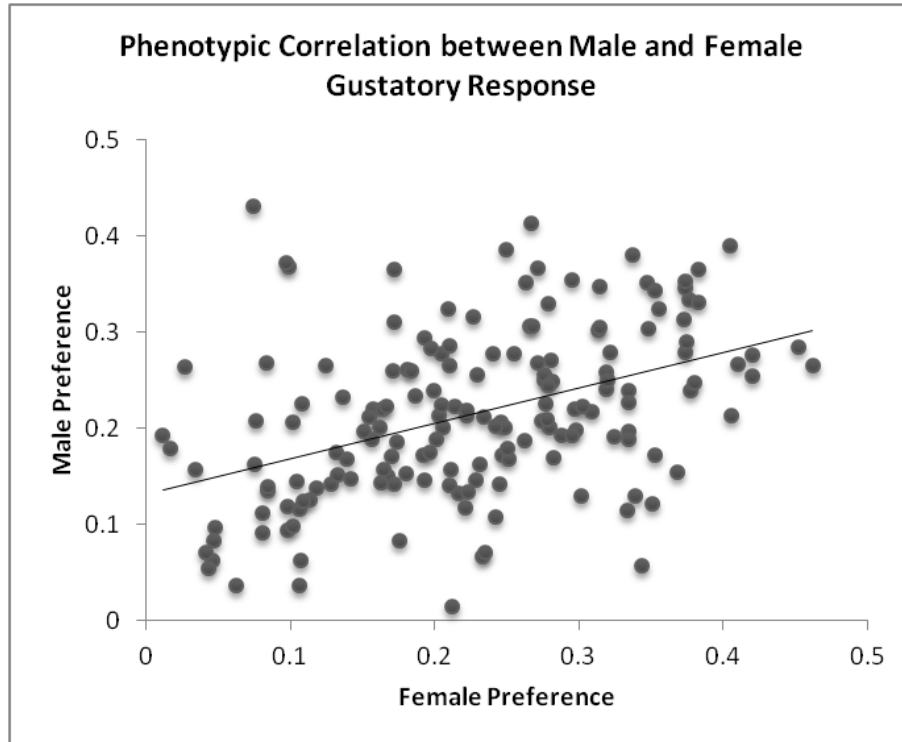


Figure 2.17: Phenotypic correlation between male and female behavioral response to ethanol consumption. Pearson phenotypic correlation $r_p = 0.43$. Cross-sex genetic correlation $r_{MF} = 0.47$.

Genetic Correlation between Olfactory and Gustatory Behavioral Responses to Ethanol

Among the DGRP lines, the cross trait genetic correlation between the olfactory and gustatory behavioral responses to ethanol is relatively low (Figure 2.18). This could be due to the differences in the neuronal pathways that underlie these two different behavioral traits.

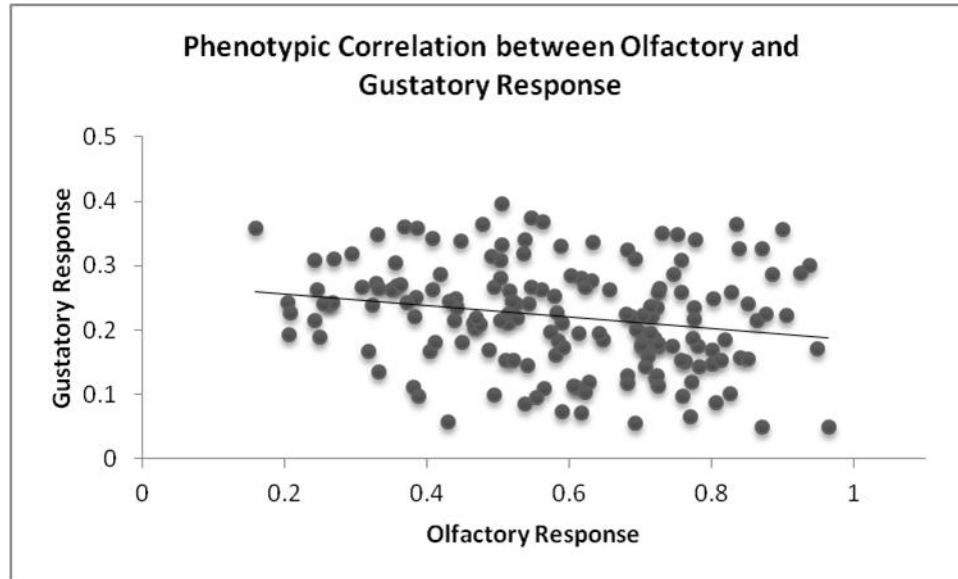


Figure 2.18: Phenotypic correlation between olfactory and gustatory behavioral response to ethanol. Pearson phenotypic correlation $r_p = -0.21$. Cross trait genetic correlation $r_G = -0.29$.

DISCUSSION

This is one of the first studies to quantify natural variation in olfactory and gustatory behavioral response to an ecologically relevant compound, ethanol, in a large wild-derived inbred population of flies (DGRP).

Behavioral Assays

I have used the olfactory trap and gustatory choice assay to quantify olfactory and gustatory behavioral responses to ethanol in the DGRP lines. The results of the

behavioral experiments using the *Or83b* and *Poxn* mutants (Figures 2.6 and 2.7) suggest that the olfactory trap assay quantifies the behavioral response of the flies when they detect ethanol from a distance, most probably by their olfactory system. The gustatory choice assay quantifies the gustatory response of the flies to consumption of soluble ethanol when detected in close proximity, both as a gustatory cue and olfactory cue. While the preference for ethanol consumption in flies appears to be primarily driven by the gustatory system it is possible that the olfactory system plays an important role in modulating the gustatory behavioral response of the flies.

Natural Variation in Olfactory and Gustatory Behavioral Responses to Ethanol

I quantified olfactory and gustatory behavioral responses to ethanol in 167 DGRP lines using an olfactory trap assay and a gustatory choice assay, respectively. There was extensive phenotypic variation among the lines for both the traits. The broad-sense heritability (H^2) of olfactory and gustatory behaviors was 0.34 (Table 2.1) and 0.61 (Table 2.3) respectively, indicating that a significant portion of the phenotypic variation is due to genetic variation among the lines (Falconer and Mackay 1996). This gave us confidence that, using a GWA mapping approach, we could identify loci affecting variation in olfactory and gustatory behavioral responses to ethanol (described in Chapter Three) and also identify transcripts for which variation in transcript abundance is associated with variation in these traits (described in Chapter Four).

We observed significant sexually dimorphism in the behavioral responses of the flies for both the traits indicating that the sexes within a DGRP line behave differently, either in direction or magnitude, in their response to ethanol (Figures 2.10 and 2.15). For preference scores measuring olfactory response to ethanol averaged across all the lines, the females exhibited significantly higher preference for ethanol compared to males. Out of 167 DGRP lines, 107 lines exhibited higher female preference to ethanol compared to males within the same line. One plausible explanation for the stronger preference of females for ethanol could be due to additional selective pressures, if ethanol is used as an oviposition cue to locate oviposition sites among ripening fruits. The males exhibited a broader range of phenotypic variation than females. Among the males, there was ~20-fold range of olfactory preference to ethanol in the DGRP lines: from an average of 0.05 to 0.98. On the other hand, among the female there was ~5-fold range of olfactory preference in the DGRP lines: from an average of 0.21 to 1.

For preference scores measuring gustatory behavioral response to ethanol consumption averaged across all the lines, the females exhibited slightly lower aversive response to ethanol (i.e. higher preference score) compared to males. Out of the 167 DGRP lines, 93 lines showed lower female aversion to ethanol consumption compared to males within the same line. Unlike the olfactory behavioral response to ethanol where males exhibit a broader range of phenotypic variation than females, the males and females exhibit similar range of phenotypic variation in their gustatory behavioral response to ethanol consumption. A plausible

explanation for a similar range of aversive behavioral responses in males and females could be due to similar fitness costs associated with consuming a toxic compound (Bokor and Pecsénye 2000). Among males, there was ~31-fold range of gustatory preference for ethanol consumption in the DGRP lines: from an average of 0.014 to 0.431. On the other hand, among females there was ~39-fold range of gustatory preference in the DGRP lines: from an average of 0.012 to 0.462.

Correlation between the Chemosensory Traits

We found relatively low phenotypic (Pearson phenotypic correlation $r_p = -0.21$) and cross trait genetic correlation ($r_{MF} = -0.29$) between olfactory and gustatory behavioral responses to ethanol (Figure 2.18). This suggests that most of the loci affecting natural variation in these traits, as measured by the olfactory and gustatory behavioral assays, will not overlap. However, it will be interesting to see if both the olfactory and gustatory systems are implicated in chemosensory behavioral responses to ethanol.

Conclusions

Ethanol is an ecologically relevant stimulus which flies encounter in and around rotting fruits, their primary food source. The results show that flies are attracted to ethanol when they detect it as an olfactory cue. However, when flies detect ethanol in rotten fruits both as an olfactory and gustatory cue they try to minimize its consumption. This aversive behavioral response to ethanol has probably evolved as

a mechanism to minimize toxic effects associated with ethanol consumption. This may indicate that different behavioral responses might have evolved as a mechanism to balance the need of finding food sources using olfactory cues while at the same time minimizing fitness costs associated with consuming a toxic compound.

The results show that there is considerable phenotypic and genotypic variation among the DGRP lines for both olfactory and gustatory behavioral responses to ethanol. This along with information on single nucleotide polymorphisms (SNPs) present in the DGRP will enable us to identify polymorphisms that underlie the genetic architecture of both these chemosensory behavioral traits.

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CHAPTER THREE

GENOME WIDE ASSOCIATION ANALYSES OF CHEMOSENSORY BEHAVIORAL REPOSSES TO ETHANOL

The ability to recognize and respond to chemical cues in the environment is critical to the fitness of most animals. It enables animals to find and evaluate food and oviposition sites, identify potential mates, and detect and avoid toxic compounds or predators. Chemosensory systems allow insects to detect and discriminate a diverse array of chemicals. Chemosensory behavioral responses, like other behaviors, are influenced by the developmental history of the animal, its genetic makeup, its nervous system, its physiological state, and its physical and social environment. As a complex trait, chemosensory behavior is influenced by numerous genes, the environment, and gene by environmental interactions. Understanding the genetic architecture that underlies such traits involves identifying the genes (quantitative trait loci [QTLs]) regulating these traits, the subset of QTLs affecting naturally occurring variation in the trait, the homozygous, heterozygous, epistatic, and pleiotropic effects of QTL alleles, the molecular basis of allelic variation, and the evolutionary forces responsible for maintaining genetic variation for quantitative traits in nature (Mackay 2004).

Drosophila feeds on rotten fruits and locates such food sources by detecting the odors produced by them using its chemosensory systems. Ethanol is a component of decaying fruit that is produced by yeast fermentation and serves as an odor cue. From the results of the olfactory and gustatory behavioral assays (described in Chapter Two), we observed that flies were attracted to ethanol vapor in a dose dependent manner. However, when ethanol was detected as a soluble in its food source, flies exhibited a dose dependent aversive response. Thus, ethanol is an

attractant as an olfactory cue and maybe used by the flies to locate food sources and oviposition sites. However, when ethanol is detected primarily as a gustatory cue, flies minimize its consumption probably due to the fitness costs associated with the consumption of a toxic compound (Bokor and Pecsénye 2000). Flies have evolved mechanism to coordinating feeding and oviposition site selection with reproductive behaviors (Grosjean et al. 2011). Similarly, there are possible selective pressures on flies to maintain preference for ethanol as an olfactory cue, as it enables them to find transient food sources, oviposition sites, and mates, while at the same time limit their consumption of ethanol.

With exception to a few studies that have exploited naturally allelic variation as an approach to identify genes affecting chemosensory behaviors (Wang et al. 2007; Arya et al. 2010; Rollmann et al. 2010; Wang et al. 2010), most studies that have identified different genes involved in the recognition of volatile and soluble chemicals have used a single gene mutational approach utilizing few inbred strains or mutants. Additional, only two chemosensory genes, olfactory receptor *Or22a* (Hallem and Carlson 2006) and odorant binding protein *lush* (Kim et al. 1998), have been identified to be involved in the detection of ethanol.

Recently, a population of 168 wild-derived inbred lines of *Drosophila melanogaster*, derived from Raleigh, North Carolina, known as the *Drosophila melanogaster* Genetic Reference Panel (DGRP) has been fully sequenced (Mackay et al. 2012). This population, for the first time, affords an opportunity to perform Genome Wide Association (GWA) analyses for chemosensory traits where all genetic variants

within the population are known. Since the DGRP lines are inbred, there is minimal residual heterozygosity and the genetic variance among the lines is increased to at least double that of the outbred population from which it was derived (Falconer and Mackay 1996) which increases the power of GWA mapping. Additionally, precise quantification of behavioral phenotypes by repeated measurements of large numbers of genetically identical individuals increases the power to reduce statistical noise due to environmental variation. The low level of local linkage disequilibrium (LD) and available information on all molecular variants in the DGRP are favorable for identifying candidate causal polymorphisms. Additionally, the genetic tools available in the *Drosophila* model system, including analyses of mutants, allow validation of candidate genes and identification of causal single nucleotide polymorphisms (SNPs).

As described in Chapter Two, I measured the olfactory and gustatory behavioral responses to ethanol in the DGRP lines using two different behavioral assays. I found extensive phenotypic and genetic variation in both olfactory and gustatory behavioral responses. There was also sexual dimorphism in the behavioral responses of males and females for both traits. These results, along with information on all single nucleotide polymorphisms (SNPs) present in the DGRP, provide us the opportunity to use a GWA mapping approach to gain insights into the genetic architecture underlying chemosensory behavioral responses to ethanol.

MATERIALS AND METHODS

***Drosophila* Rearing**

We used 167 inbred lines of the DGRP (Mackay et al. 2012). Flies were reared on standard cornmeal molasses agar medium at 25°C, 70% relative humidity, 12 hour light/dark cycle, and controlled density, unless specified otherwise.

Genome Wide Association Analysis

We tested olfactory and gustatory behavioral responses to ethanol for association with 2,481,491 SNPs previously identified by whole genome sequencing of the DGRP lines (Mackay et al. 2012). All analyses were performed on line means. All segregating sites within lines were treated as missing data. SNPs used in the analysis were filtered based on the following criteria: (1) the minor allele had to be present in at least four DGRP lines; (2) SNPs were excluded if coverage from whole genome sequencing was less than 2 or greater than 30 (Mackay et al. 2012); (3) SNPs with more than two segregating alleles within the DGRP lines were excluded from the analysis and; (4) SNPs had to be genotyped in at least sixty of the DGRP lines.

Each SNP was tested for association with olfactory and gustatory behavioral responses to ethanol using ANOVA of the form $Y = \mu + M + S + M \times S + L(M) + \epsilon$, where M is the effect of polymorphic site, S is the fixed effect of sex, L is the random effect of the DGRP line, and ϵ is the error variance. Reduced analyses of the form

$Y = \mu + M + \varepsilon$ were also performed for males and females separately. A p -value of $<10^{-5}$ was used as a cut-off to nominate SNPs for further study. The main effect (a) of each SNP was estimated as one-half the difference in trait mean between marker classes (polarized by allele frequency, such that the effect is the difference between the major and minor alleles) (Falconer and Mackay 1996). For analyses pooled across sexes, the interaction effect between SNP and sex was calculated as the difference between the female and male effects.

Gene-Centric Forward Selection Models

To determine what fraction of total genetic variation in olfactory and gustatory behavioral responses to ethanol is accounted for by markers, we used multiple regression models, because single marker analysis can lead to biased estimates of allelic effects when multiple markers affect the trait and the markers are correlated (Mackay et al. 2012). Gene-centered forward regression was used to calculate multiple regression models. Only significant SNPs ($p < 10^{-5}$) that were within 2 kb of an annotated gene were used. The most significant marker identified from the GWA analyses was fit into the model first, and subsequent markers, up to a maximum of 12 markers, were added until the maximum r^2 was attained. Missing marker data for nominated SNPs were imputed based on nearest marker information for the final model. Models were fitted for each trait and performed separately on males, females, and average of both sexes. Once a final model was selected, an ANOVA of the form $Y = \mu + H + L(H) + \varepsilon$ was performed, where H is the effect of haplotype,

and $L(H)$ is the effect of line nested within haplotype. The fraction of total genetic variation accounted for by the multiple regression model is $\sigma_H^2/(\sigma_H^2 + \sigma_L^2)$, where σ_H^2 is the among-haplotype variance component of significant SNPs and σ_L^2 is the among-line variance component.

Mutant Validation

For eight of the candidate olfactory behavioral response genes and 10 of the candidate genes associated with ethanol consumption identified in the GWA study, I tested *P*-element, *Minos*-element mutants and their co-isogenic controls lines for effect on olfactory and gustatory behavioral responses to ethanol consumption. *P*-element, *Minos*-element, and their co-isogenic controls were obtained from the Berkeley *Drosophila* Gene Disruption Project (Bellen et al. 2004), the *Drosophila* Gene Disruption Project (Bellen et al. 2011), and the Exelixis collection (Thibault et al. 2004). I also tested 13 RNAi lines for functional validation of candidate genes from both traits. These lines that express RNAi corresponding to candidate gene transcripts under UAS promoters inserted in the neutral phiC31 integration site along with the progenitor control line ($y, w^{1118}; P\{attP, y^+, w^{3'}\}$) were obtained from the Vienna *Drosophila* RNAi Center (<http://www.vdrc.at>) (Dietzl et al. 2007). Each of these lines and the progenitor control was crossed to a ubiquitous tubulin-GAL4 driver line ($y^1 w; P\{tubP-GAL4\}LL7/TM3, Sb^1$) to suppress the expression of the target candidate gene. F1 offspring was used for both behavioral experiments.

Specific alleles tested for 11 candidate genes identified from the GWA study for olfactory behavioral response to ethanol were *PBac{WH}Glu-Rl^{f05411}* (*Glu-Rl*), *PBac{PB}kat-60L^{1c01236}* (*kat-60L1*), *Mi{ET1}CG32082^{MB08199}* (*CG32082*), *Mi{ET1}CG3290^{MB04669}* (*CG3290*), *Mi{ET1}CG34381^{MB02243}* (*CG34381*), *Mi{ET1}Ir10a^{MB03273}* (*Ir10a*), *Mi{ET1}Takr99D^{MB09356}* (*Takr99D*), *Mi{ET1}wb^{MB06467}* (*wb*), *P{KK108760}* (*CG10340*), *P{KK102461}* (*Dic1*), and *P{KK111731}* (*Or63a*).

Specific alleles tested for 20 candidate genes identified from the GWA study for gustatory behavioral response to ethanol consumption were *PBac{WH}Ccn^{f03521}* (*Ccn*), *PBac{WH}CG42260^{f00046}* (*CG42260*), *Mi{ET1}bab1^{MB01972}* (*bab1*), *Mi{ET1}CG34411^{MB01485}* (*CG34411*), *Mi{ET1}dpr15^{MB07991}* (*dpr15*), *Mi{ET1}dpr9^{MB07019}* (*dpr9*), *Mi{ET1}Gr36b^{MB05141}* (*Gr36b*), *Mi{ET1}Ir67b^{MB10077}* (*Ir67b*), *P{KK103520}* (*CG4168*), *P{KK112020}* (*CR31084*), *P{KK111131}* (*Obp57a*), *P{KK105942}* (*Or22b*), *P{KK110129}* (*Or59b*), *P{KK105421}* (*Or59c*), *P{KK106458}* (*Or83a*), *P{KK103474}* (*Or88a*), *P{KK112302}* (*Pbprp3*), *P{KK105240}* (*Wnt5*), *mb^{BG01127}*, and *Sesn^{BG01215}*,

The behavioral assays used to measure phenotypic variation in olfactory and gustatory behavioral responses to ethanol (described in Chapter Two) were used to assess behavioral responses of the mutant flies. Significant behavioral differences between the mutant lines and their respective controls were assessed by Dunnett's test.

Bioinformatics

Statistical analyses were performed using SAS software (SAS, Cary, NC, USA).

Functional gene annotations are based on FlyBase (Drysdale and Crosby 2005;

Wilson et al. 2008; Tweedie et al. 2009). Gene ontology enrichment analysis used

the DAVID program (Huang da et al. 2009). Tissue specific gene expression data

was obtained from FlyAtlas (Chintapalli et al. 2007).

RESULTS

SNPs Associated with Variation in Chemosensory Behavioral Responses to

Ethanol

SNPs associated with olfactory preference for ethanol

We performed GWA analysis using 2,481,491 SNPs previously identified by sequencing the DGRP lines (Mackay et al. 2012) to identify genes that contain alleles associated with variation in olfactory and gustatory behavioral responses to ethanol. We performed single marker analysis for males and females separately and pooled across sexes.

We found 80 SNPs associated with phenotypic variation in olfactory behavioral response to ethanol at $p < 10^{-5}$ (Figure 3.1), out of which 44 SNPs were present within genes. When we considered the sexes separately, 34 SNPs were significant

for females and 24 SNPs were significant for males. We did not observe any long range LD between the significant SNPs (Figure 3.1).

We found a majority of the SNPs associated with variation in olfactory response to ethanol to be at intermediate frequency (Figure 3.2). The SNP effects for females, males, and sexes pooled taken together were more positive than negative (such that the preference for ethanol was stronger in lines with the major allele relative to the lines with the minor allele). Among females, males, and sexes pooled it was only in females that the number of SNPs showing negative effects (where lines with the minor allele exhibited stronger preference for ethanol compared to the lines with the major allele) outnumbered the SNPs showing positive effects. In the females, 15 SNPs showed positive effects and 19 SNPs showed negative effects (Figure 3.3(a)). In the males, 20 SNPs showed positive effects and 4 SNPs showed negative effects (Figure 3.3(b)). In sexes pooled, 25 SNPs showed positive effects and 7 SNPs showed negative effects (Figure 3.3(c)).

As observed from the quantitative genetic analyses in Chapter Two, there was significant genetic variation in sexual dimorphism for olfactory preference to ethanol. From the GWA analysis, we identified 21 SNPs with significant SNP by sex interaction terms at $p < 10^{-5}$. Both the major and minor alleles were associated with increased olfactory preference to ethanol in females relative to males (Figure 3.4). Additionally, while the minor allele conferred greater preference for ethanol in females relative to the major allele (except one minor allele), the minor allele conferred lower preference in males (except for the same minor allele that does not

increase preference in females, compared to the major allele (Figure 3.4). Thus, all the SNPs exhibiting SNP by sex interactions for olfactory preference to ethanol exhibited sex-biased or sex-specific effects.

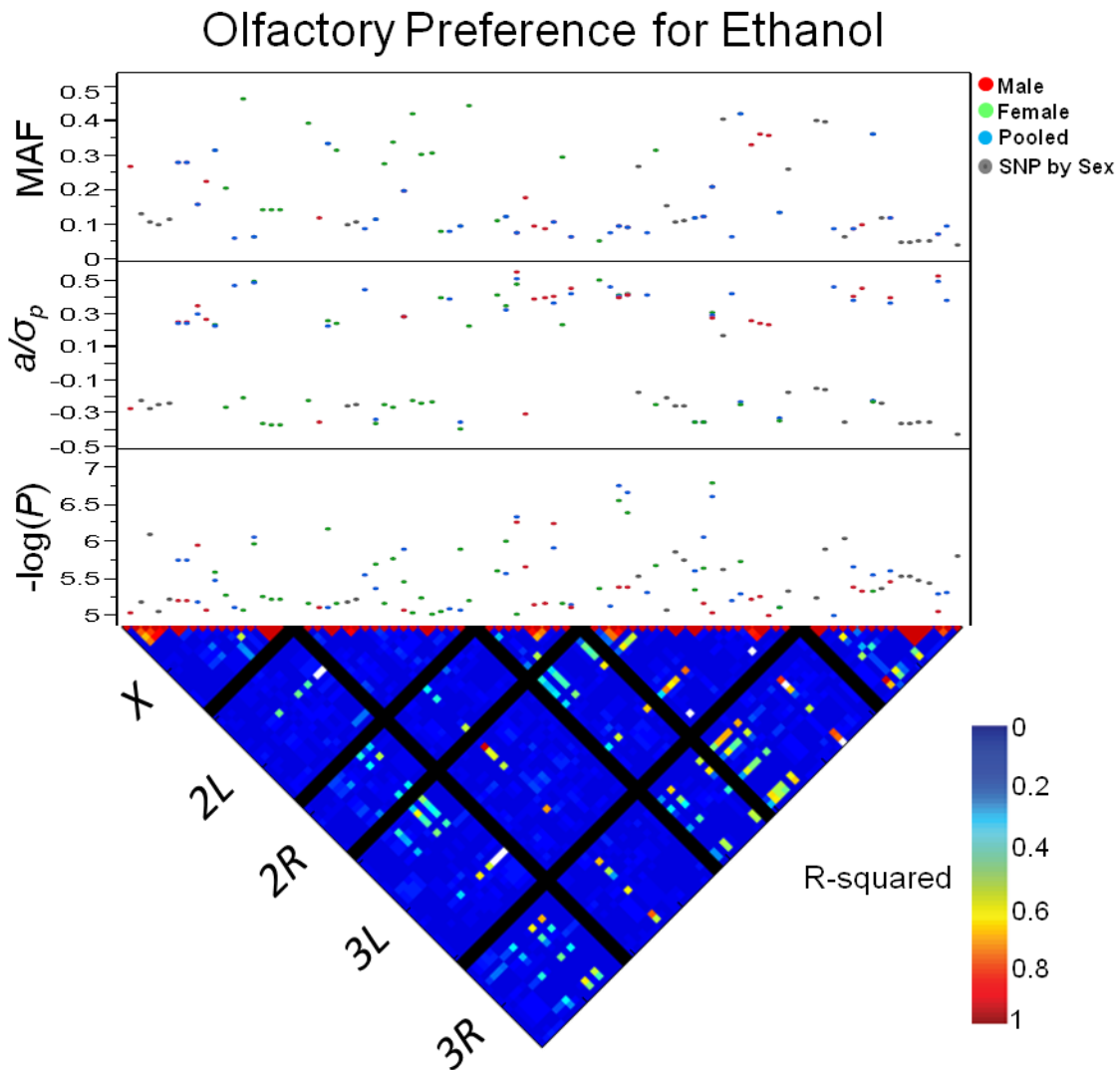


Figure 3.1: Genome wide association analyses for variation in olfactory behavioral response to ethanol. All SNPs from single marker analysis with a nominal $p < 10^{-5}$

Figure 3.1: Continued

are shown. Significant associations for males are depicted by red dots; females by green dots; sexes pooled by blue dots; and SNP by sex interactions by grey dots. The lower triangle shows the degree of LD between the SNPs as measured by r^2 , with the five major chromosome arms demarcated by black lines. The heat map indicates the magnitude of LD with blue signifying absence of LD and red signifying complete LD. The upper panel shows the significance threshold ($-\log_{10} p$ -value), the middle panel shows the effect size in phenotypic standard deviation units (a/σ_p), and the lower panel shows the minor allele frequency (MAF).

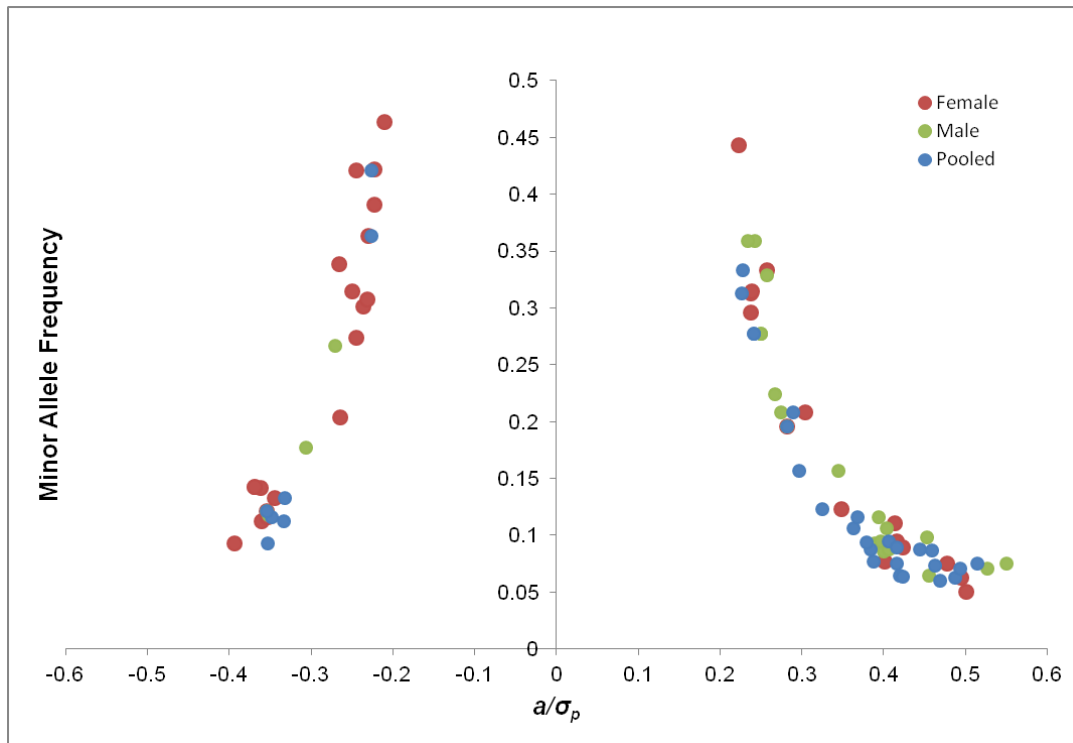


Figure 3.2: Minor allele frequency versus effect size. All main SNP effects of females, males, and sexes pooled for olfactory behavioral response to ethanol are shown. (Effect size is calculated as one-half the difference in trait mean between the major and minor allele).

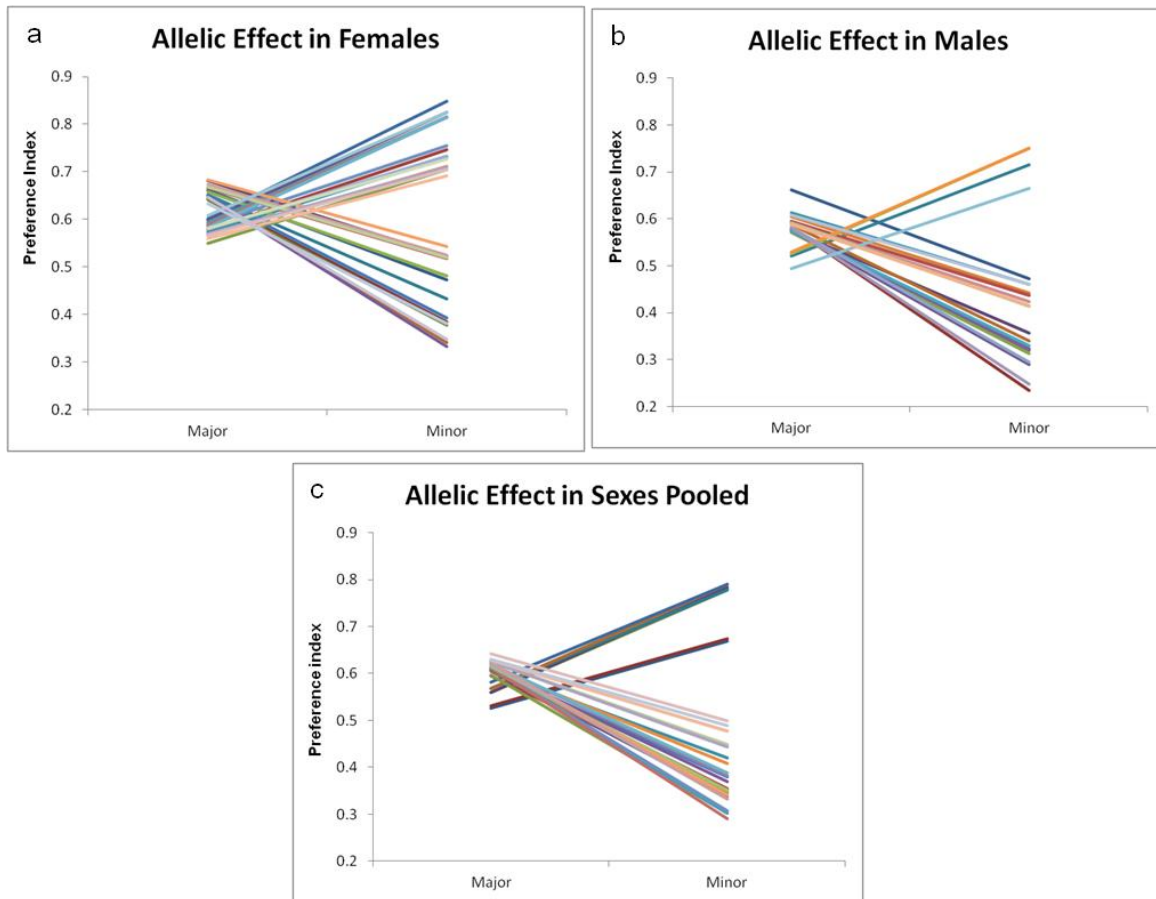


Figure 3.3: Effect of major and minor allele for significant SNPs (females, males, and averaged across the sexes) associated with olfactory preference for ethanol.

(a) Shows allelic effect in females. 15 major alleles show higher preference scores compared to their minor allele and 19 minor alleles show higher preference scores relative to their major allele. (b) Shows allelic effect in males. 20 major alleles show higher preference scores compared to their minor allele and 4 minor alleles show higher preference scores relative to their major allele. (c) Shows allelic effect in sexes pooled. 25 major alleles show higher preference scores compared to their

Figure 3.3: Continued

minor allele and 7 minor alleles show higher preference scores relative to their major allele.

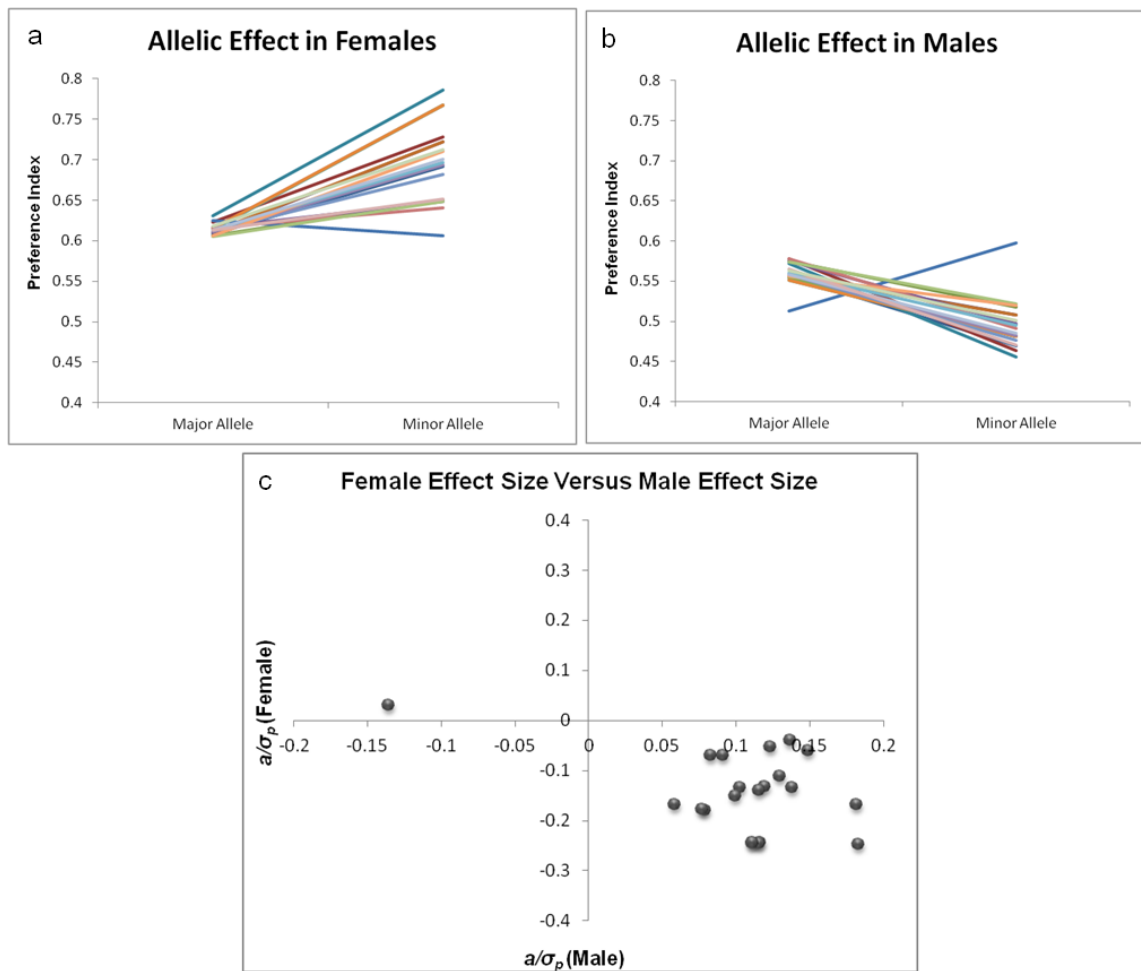


Figure 3.4: Alleles for SNPs significant under SNP by sex interaction term associated with olfactory behavioral response to ethanol. (a) and (b) show the effect of the major and minor alleles within females and males, respectively. (c) Shows

Figure 3.4: Continued

female effect size versus male effect size. (Effect size is calculated as one-half the difference in trait mean between the major and minor allele).

We found some overlap between males and females at the level of SNPs associated with olfactory preference for ethanol. There was overlap in six SNPs, two of which fell within genes (*CG32987* and *CG32082*) and the rest were intergenic.

Additionally, different SNPs in one gene (*CG6216*) were associated with olfactory response to ethanol.

SNPs associated with preference for ethanol consumption

We found 137 SNPs associated with phenotypic variation in behavioral response to ethanol consumption at $p < 10^{-5}$ (Figure 3.5), out of which 67 SNPs were present within genes. When we considered the sexes separately, 11 SNPs were significant for females and 32 SNPs were significant for males. Just as in the case for the significant SNPs associated with variation in olfactory response to ethanol, we did not observe any long range LD between the significant SNPs identified for this behavioral trait (Figure 3.5).

Similar to what we found for olfactory response to ethanol, a majority of the significant SNPs associated with variation in response to ethanol consumption are at intermediate frequency (Figure 3.6). The SNP effects for females, males, and sexes pooled taken together were more positive than negative (such that there was

reduced aversion to ethanol consumption [i.e. higher preference scores] in lines with the major allele relative to the lines with the minor allele). Among females, males, and sexes pooled, it was only in females that the number of SNPs showing negative effects (where lines with the minor allele exhibited reduced aversion to ethanol consumption [i.e. higher preference scores] compared to the lines with the major allele) was more than the SNPs showing positive effects. In the females, 5 SNPs showed positive effects and 6 SNPs showed negative effects (Figure 3.7(a)). In the males, 19 SNPs showed positive effects and 13 SNPs showed negative effects (Figure 3.7(b)). In sexes pooled, 32 SNPs showed positive effects and 5 SNPs showed negative effects (Figure 3.7(c)).

As observed from the quantitative genetic analyses in Chapter Two, there was significant genetic variation in sexual dimorphism for preference to ethanol consumption. From the GWA analysis, we identified 65 SNPs with significant SNP by sex interaction terms at $p < 10^{-5}$. The major allele conferred similar preference for ethanol consumption in both males and females (Figure 3.8(a) and (b)). Out of the 65 SNPs, the minor allele associated with 58 SNPs increased aversion to ethanol consumption (i.e. lower preference score) in the females compared to the males and the same alleles decreased aversion to ethanol consumption (i.e. high preference score) in males relative to females (Figure 3.8(c)). A similar effect was observed for the minor allele associated with the remaining 7 SNPs, but here the minor allele increased aversion to ethanol consumption (i.e. lower preference score) in the males compared to the females and the same alleles decreased aversion to ethanol

consumption (i.e. high preference score) in females relative to males (Figure 3.8(c)). Thus, the minor allele for all the SNPs significant for the SNP by sex interaction term exhibit sexually antagonistic effects. Additionally, while the major allele conferred similar sensitivity to ethanol in both sexes, a majority of the minor alleles conferred increased aversion to ethanol consumption in females (i.e. high preference score) relative to the major allele and a majority of the minor alleles conferred reduced aversion to ethanol consumption in males compared to the major allele (Figure 3.8(c)).

Preference for Ethanol Consumption

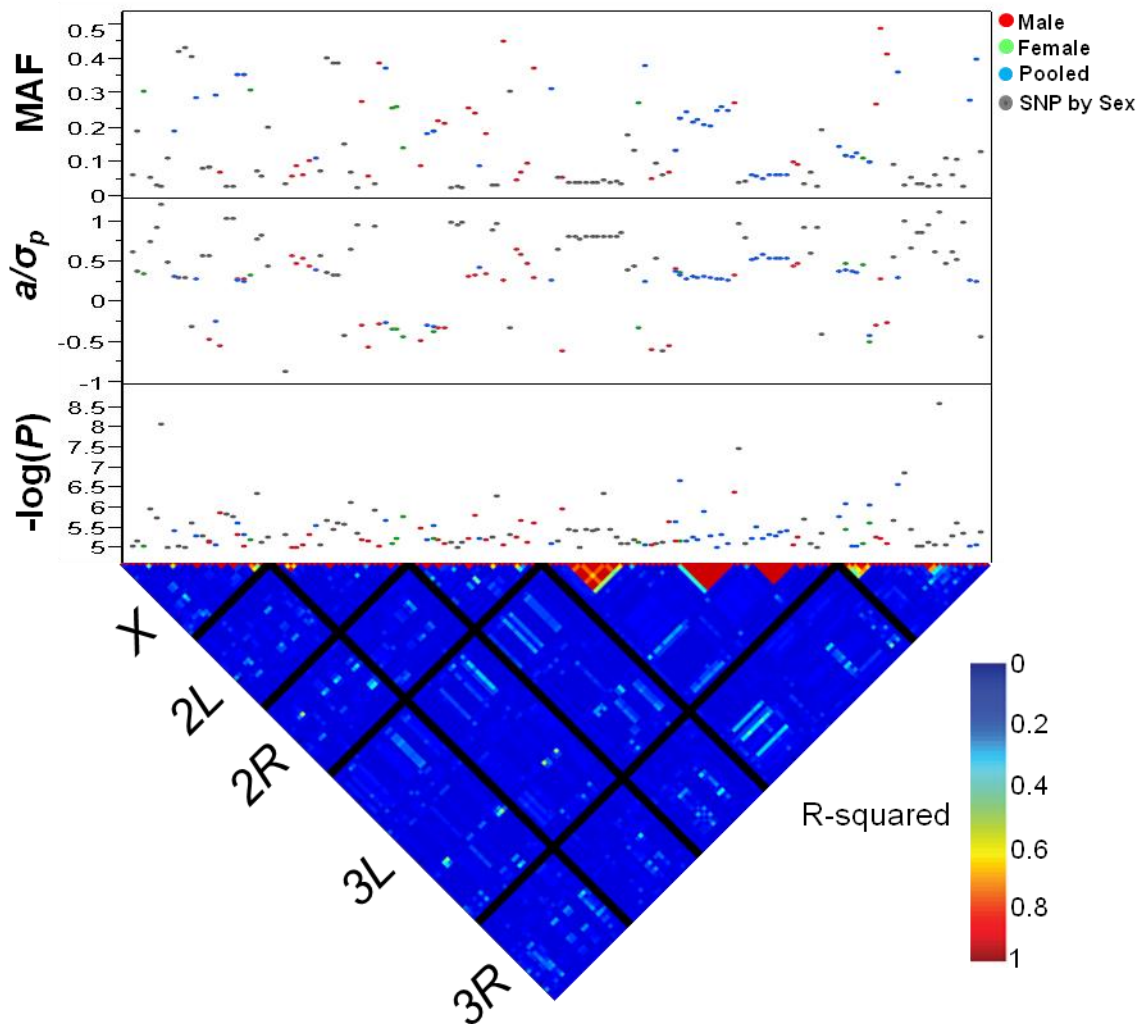


Figure 3.5: Genome wide association analyses for variation in behavioral response to ethanol consumption. All SNPs from single marker analysis with a nominal $p < 10^{-5}$ are shown. Significant associations for males are depicted by red dots; females by green dots; sexes pooled by blue dots; and SNP by sex interactions by grey dots. The lower triangle shows the degree of LD between the SNPs as measured by r^2 , with the five major chromosome arms demarcated by black lines. The heat map

Figure 3.5: Continued

indicates the magnitude of LD with blue signifying absence of LD and red signifying complete LD. The upper panel shows the significance threshold ($-\log_{10} p$ -value), the middle panel shows the effect size in phenotypic standard deviation units (a/σ_p), and the lower panel shows the minor allele frequency (MAF).

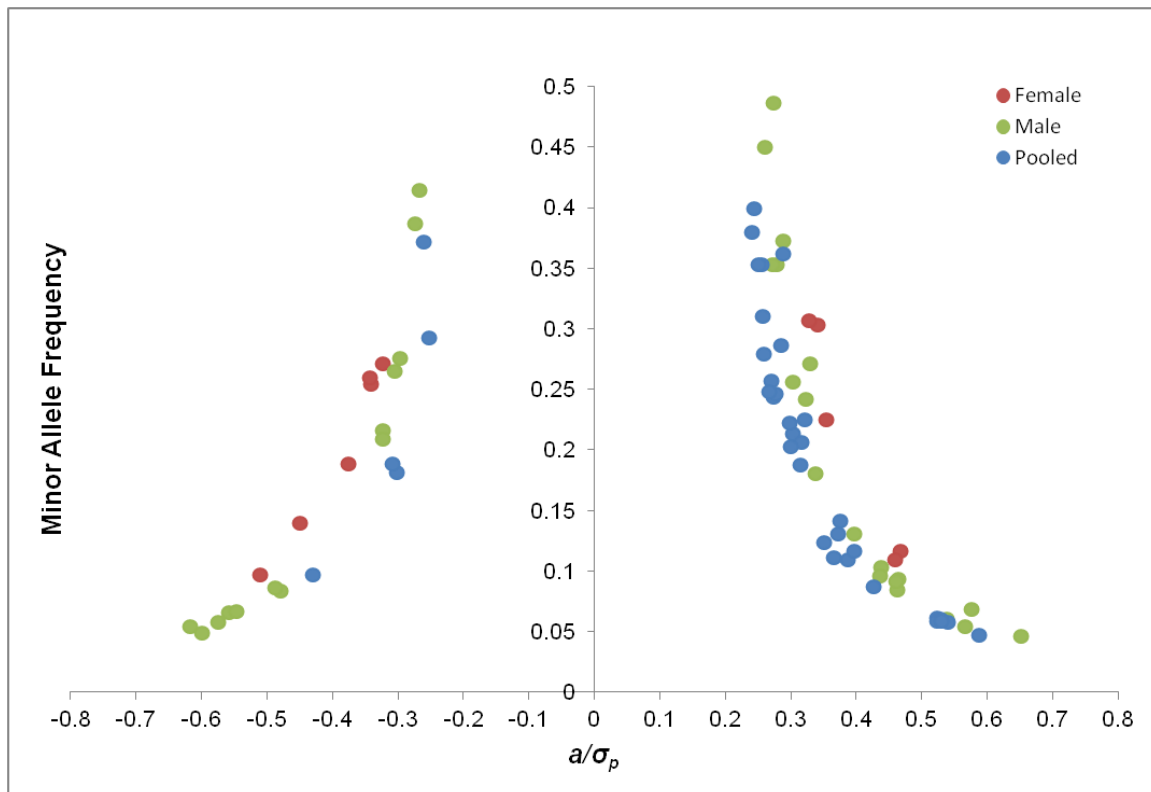


Figure 3.6: Minor allele frequency versus effect size. All main SNP effects of females, males, and sexes pooled for behavioral response to ethanol consumption are shown. (Effect size is calculated as one-half the difference in trait mean between the major and minor allele).

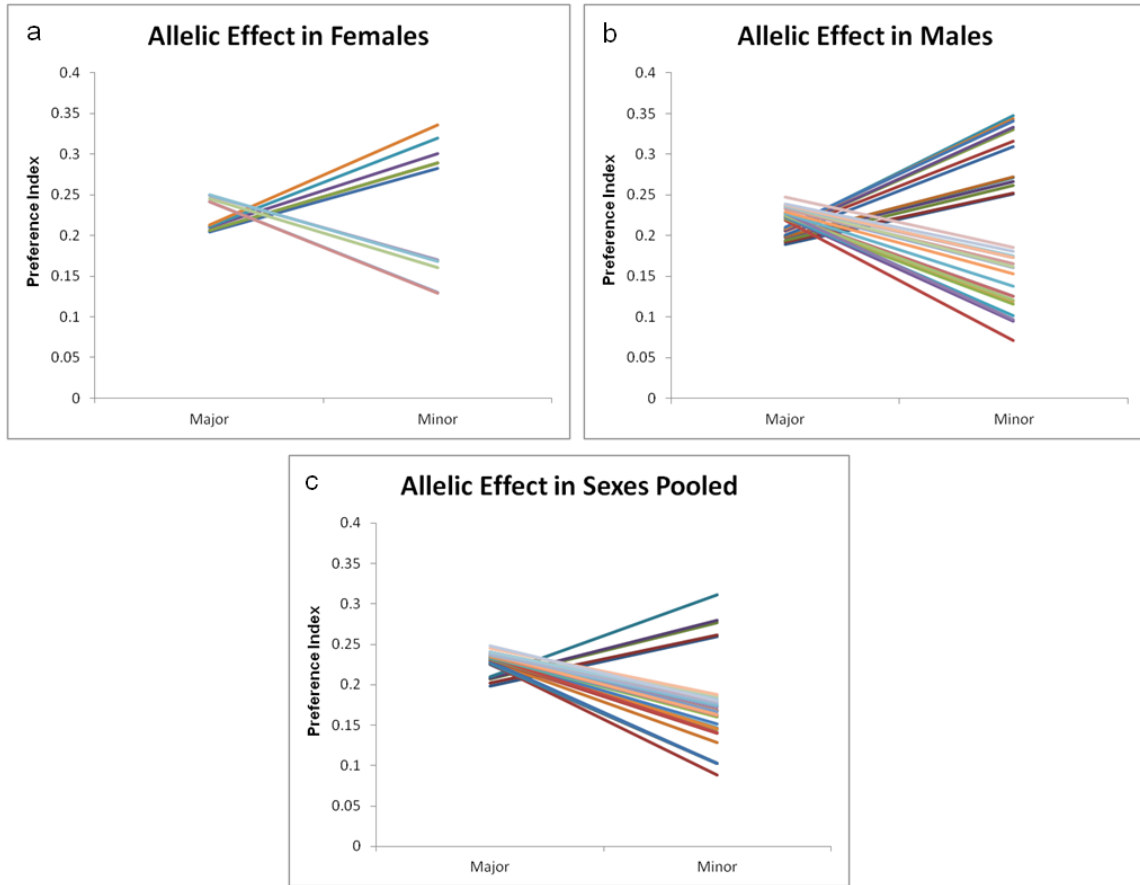


Figure 3.7: Effect of major and minor allele for significant SNPs (females, males, and averaged across the sexes) associated with preference for ethanol consumption. (a) Shows allelic effect in females. 5 major alleles show higher preference scores compared to their minor allele and 6 minor alleles show higher preference scores relative to their major allele. (b) Shows allelic effect in males. 19 major alleles show higher preference scores compared to their minor allele and 13 minor alleles show higher preference scores relative to their major allele. (c) Shows allelic effect in sexes pooled. 32 major alleles show higher preference scores

Figure 3.7: Continued

compared to their minor allele and 5 minor alleles show higher preference scores relative to their major allele.

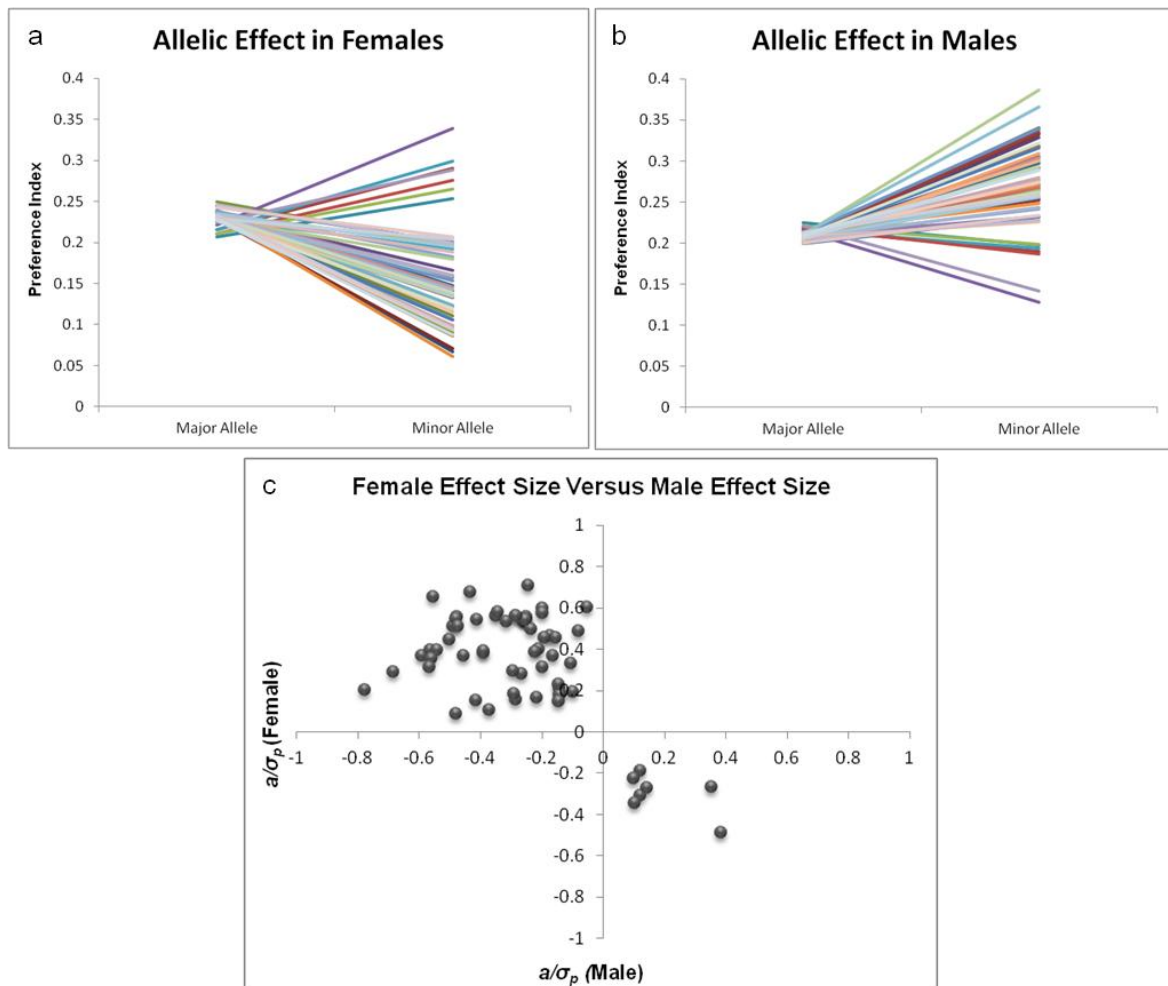


Figure 3.8: Alleles for SNPs significant under SNP by sex interaction term associated with behavioral response to ethanol consumption. (a) and (b) show the effect of the major and minor alleles within females and males, respectively.

Figure 3.8: Continued

(c) Shows female effect size versus male effect size. (Effect size is calculated as one-half the difference in trait mean between the major and minor allele).

We did not find overlap between males and females at the level of SNPs associated with preference for ethanol consumption. However, we did find different SNPs in one gene (*muscleblind*) and within 2kb of two genes (*CG1314*, *Ionotropic receptor 67b*) that overlapped between males and females.

The GWA analyses for both the traits did not reveal any overlap between SNPs or genes significantly associated with variation in olfactory behavioral response and the behavioral response for ethanol consumption.

Gene Ontology Analysis

Gene ontology (GO) analysis reveals both similar and diverse categories of genes that confer variation in olfactory and gustatory behavioral responses to ethanol. The GO categories prominently associated with variation in olfactory response to ethanol appear to be involved in fly development and transcript regulation (Figure 3.9). The GO categories prominently associated with variation in response to ethanol consumption include nervous system development and neurotransmitter activity (Figure 3.10).

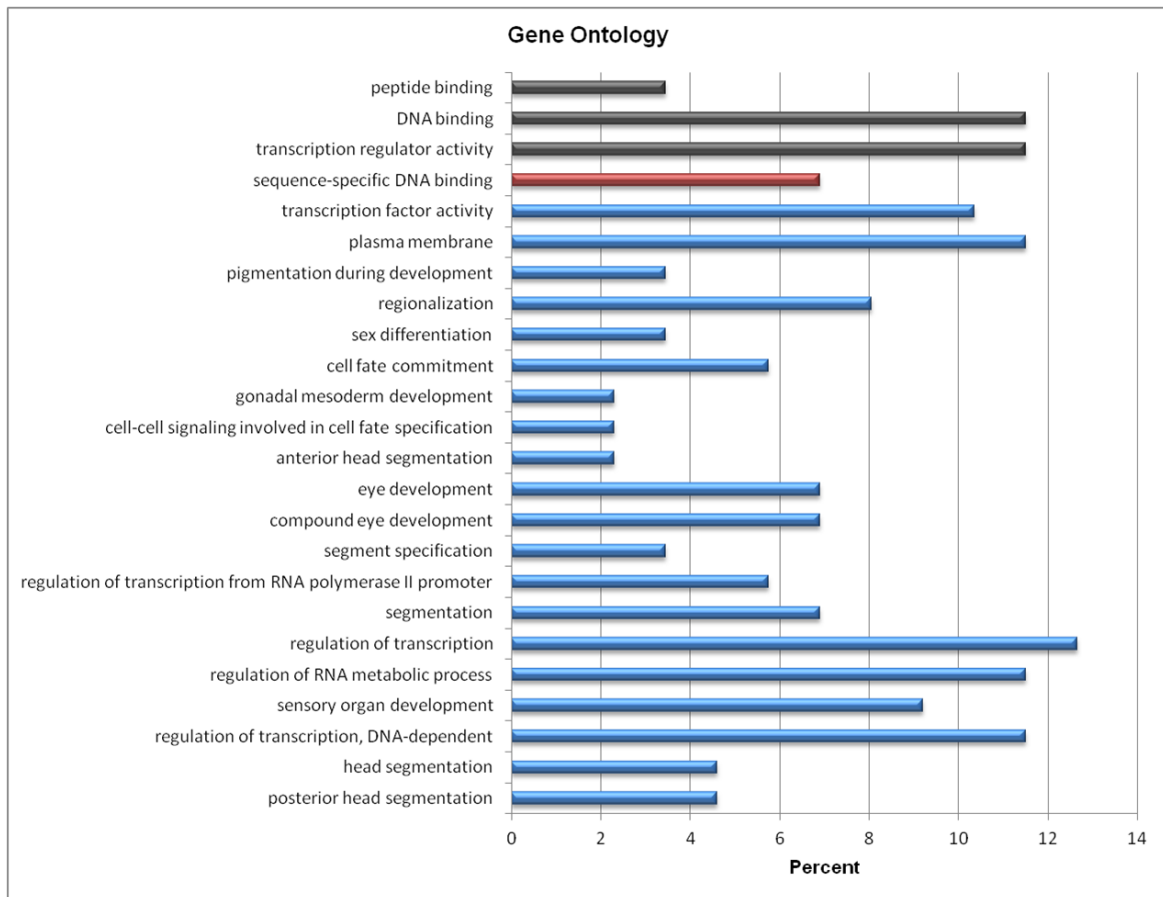


Figure 3.9: Gene ontology categories for candidate genes associated with variation in olfactory behavioral response to ethanol. The blue, red, and black bars indicate percentage of input genes that fall within each biological process, cellular component, and molecular function, respectively. The x-axis indicated the percent of genes in each category at $p < 0.05$.

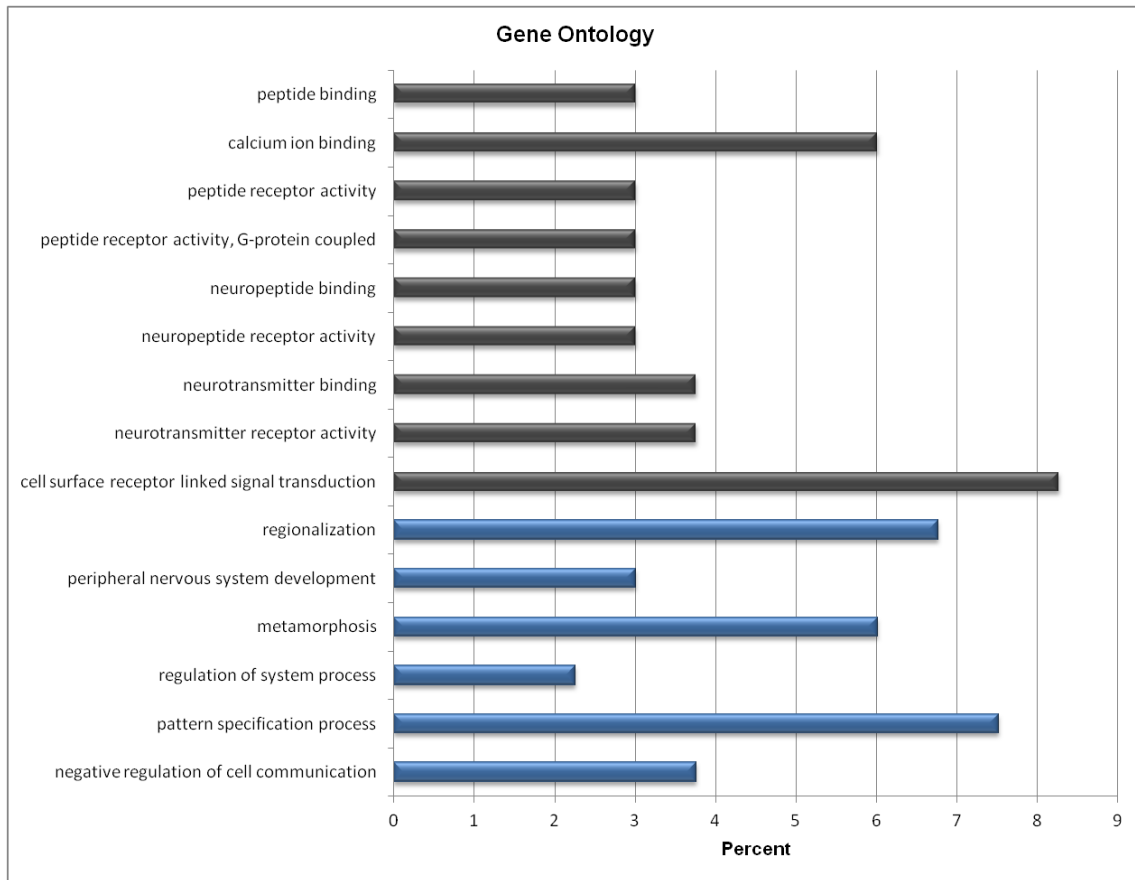


Figure 3.10: Gene ontology categories for candidate genes associated with variation in behavioral response to ethanol consumption. The blue and black bars indicate percentage of input genes that fall within each biological process and molecular function, respectively. The x-axis indicated the percent of genes in each category at $p < 0.05$.

Chemosensory Genes Associated with Variation in Olfactory and Gustatory Behavioral Responses to Ethanol.

As chemosensory genes (60 olfactory receptors genes [*Ors*], 61 ionotropic genes [*Irs*], 60 gustatory receptor genes [*Grs*], and 51 odorant binding protein genes [*Obp*]) are involved in the detection of olfactory and gustatory cues, we conducted a separate GWA analysis to identify SNPs significantly associated with olfactory and gustatory behavioral responses to ethanol at $p \leq 0.0005$. The p -value of 0.0005 is based on Bonferroni correction.

Using only chemosensory genes in the GWA analysis, we found eight chemosensory genes associated with the olfactory behavioral response (Table 3.1) and 23 chemosensory genes associated with behavioral response to ethanol (Table 3.2). We found no overlap between these genes.

Table 3.1: Significant SNPs associated with chemosensory genes for olfactory behavioral response to ethanol at $p \leq 0.0005$.

Chromosome	Position	Type	P-value	Source	Gene Symbol	FlyBase ID	SNP Location In Gene	SNP Location Intergenic	Site Class
X	15870003	OR	0.0004	Male	<i>Or13a</i>	FBgn0030715	<i>Or13a-in</i>		intronic
3L	2963721	OR	0.0005	Female	<i>Or63a</i>	FBgn0035382	<i>Or63a-u3</i>		UTR
2R	5447643	OR	0.0004	SNP by Sex	<i>Or45b</i>	FBgn0033422	<i>Or45b-in</i>		intronic
3L	12120757	IR	0.0002	Male	<i>Ir68b</i>	FBgn0036250		263	intergenic
2R	902292	IR	0.0002	Male	<i>Ir41a</i>	FBgn0040849	<i>Ir41a-in</i>		intronic
X	11202006	IR	0.0005	Pooled	<i>Ir10a</i>	FBgn0083979		-560	intronic
2R	16436577	OBP	0.0004	Male	<i>Obp57d</i>	FBgn0043536	<i>Obp57d-cds</i>		synonymous
2R	15755413	OBP	0.0002	Male	<i>Obp56i</i>	FBgn0043532	<i>Obp56i-in</i>		intronic

Source: Identifies the source of the significant p value: Male, female, pooled, or SNP by sex.

Table 3.2: Significant SNPs associated with chemosensory genes for behavioral response to ethanol consumption at $p \leq 0.0005$.

Chromosome	Position	Type	P-value	Source	Gene Symbol	FlyBase ID	SNP Location In Gene	SNP Location Intergenic	Site Class
2L	11113995	GR	0.0004	Female	<i>Gr32a</i>	FBgn0041246		-188	intergenic
2L	17178845	GR	0.0004	Pooled	<i>Gr36b</i>	FBgn0045486	<i>Gr36b-cds</i>	.	missense
2R	16575223	GR	0.0004	SNP by Sex	<i>Gr57a</i>	FBgn0041240		808	intergenic
3L	4029091	GR	0.0002	SNP by Sex	<i>Gr64b</i>	FBgn0045478	<i>Gr64b:2</i>	.	synonymous
3L	4036268	GR	0.0001	SNP by Sex	<i>Gr64f</i>	FBgn0052255	<i>Gr64f:3</i>	.	synonymous
3L	11744674	GR	0.0002	Male	<i>Gr68a</i>	FBgn0041231	<i>Gr68a-PA</i>	.	synonymous
3R	5823260	GR	0.0003	SNP by Sex	<i>Gr85a</i>	FBgn0045473		749	intergenic
3R	23313266	GR	<.0001	SNP by Sex	<i>Gr98a</i>	FBgn0039520		-383	intergenic
3L	10421942	IR	<.0001	Female	<i>Ir67b</i>	FBgn0036083		-847	intergenic
2R	10912162	OBP	0.0003	Pooled	<i>Obp51a</i>	FBgn0043530	<i>Obp51a-PA</i>	.	missense
2R	15591117	OBP	0.0002	Pooled	<i>Obp56d</i>	FBgn0034470	<i>Obp56d-PA</i>	.	missense
2R	16394248	OBP	0.0003	Pooled	<i>Obp57a</i>	FBgn0043535		983	intergenic
3R	1800191	OBP	0.0003	Female	<i>Pbprp3</i>	FBgn0011281	<i>Pbprp3-in</i>	.	intronic
2L	1520019	OR	0.0002	SNP by Sex	<i>Or22a</i>	FBgn0026398		-594	intergenic
2L	1523616	OR	<.0001	SNP by Sex	<i>Or22b</i>	FBgn0026397	<i>Or22b-in</i>	.	intronic
2R	19359602	OR	0.0002	Female	<i>Or59b</i>	FBgn0034865		369	intergenic
2R	19359602	OR	0.0002	Female	<i>Or59c</i>	FBgn0034866		-234	intergenic
3L	9525132	OR	<.0001	Pooled	<i>Or67a</i>	FBgn0036009		696	intergenic
3R	1223988	OR	<.0001	Pooled	<i>Or83a</i>	FBgn0037322	<i>Or83a:3</i>	.	nonsense
3R	1233105	OR	<.0001	Pooled	<i>Or83b</i>	FBgn0037324	<i>Or83b-u3</i>	.	UTR
3R	9936530	OR	<.0001	Pooled	<i>Or88a</i>	FBgn0038203	<i>Or88a:4</i>	.	synonymous
3R	18814359	OR	0.0001	Pooled	<i>Or94a</i>	FBgn0039033		134	intergenic
3R	18814359	OR	0.0001	Pooled	<i>Or94b</i>	FBgn0039034		-658	intergenic

Source: Identifies the source of the significant p value: Male, female, pooled, or SNP by sex.

Gene-Centered Prediction Models

Single marker association analysis can lead to biased estimates of allelic effects when multiple SNPs are associated with a trait and the SNPs are correlated (Mackay et al. 2012). Taking this into consideration, we computed gene-centered forward selection multiple regression models to assess what fraction of the total genetic variation was accounted for by the SNPs. We computed the multiple

regression models separately for females, males, and the average across sexes.

We allowed for a maximum of 12 SNPs in each model.

The proportion of phenotypic variation explained by each multiple regression model is given by r^2 . A relatively large portion of the phenotypic variation in olfactory behavioral response and behavioral response to ethanol consumption was explained by the model: between 52-60% (Table 3.3) and 48-64% (Table 3. 4), respectively.

We also estimated the fraction of total genetic variance explained by the models as the fraction of the total variance among the line means due to variance among haplotypes formed by the SNPs in the models. The genetic variance explained by each model was high between 66-78% for olfactory behavioral response to ethanol (Table 3.5, Figure 3.11) and 50-70% for behavioral response to ethanol consumption (Table 6, Figure 3.12).

For olfactory behavioral response to ethanol, three SNPs were present in both the female and male prediction models (*2L_8863843* (*CG32987*), *3L_11142680* (*CG32082*), and *3L_11427728*, present 129 base pairs upstream of *CG6149*). The overlap between three genes suggest that alleles with large effects are shared between the sexes which is reflective of the relatively strong phenotypic ($r_p = 0.76$) and cross-sex genetic correlation ($r_{MF} = 0.91$) we observed for this trait (described in Chapter Two). For behavioral response to ethanol consumption only one SNP was shared in both the males and female prediction model (*3L_10422175*, present 614 base pairs upstream of *Ir67b*) suggesting that alleles with relatively large effects are not common between the sexes. This was expected since the cross-sex phenotypic

($r_p = 0.43$) and genetic correlations ($r_{MF} = 0.47$) are not very strong (described in Chapter Two). We also found no overlap between the genes in the multiple regression models for both traits, suggesting that different genes are responsible for phenotypic variation in both behavioral traits as reflected by the low cross-trait phenotypic ($r_p = -0.21$) and genetic correlation ($r_G = -0.29$).

Table 3.3: Gene-centered predictive models for olfactory response to ethanol.

Trait	Analysis	Variable	SNP Location	Estimate	t	p-value
Olfactory Response to Ethanol	Males	Intercept		0.59274	107.34	<.0001
		<i>X_8052792</i>	<i>Gllspla2</i> (u3)	-0.05373	14.22	0.0002
		<i>2L_1157134</i>	<i>capt</i> (in)	0.03965	4.84	0.0293
		<i>2L_8863843</i>	<i>CG32987</i> (u5)	-0.03395	4.94	0.0277
		<i>2R_7717882</i>	<i>Sobp</i> (cds)#	0.05626	12.61	0.0005
		<i>2R_17655708</i>	<i>CG30395</i> (cds)#	-0.09468	23.13	<.0001
		<i>3L_11142680</i>	<i>CG32082</i> (in)	0.06117	10.87	0.0012
		<i>3L_11427728</i>	<i>CG6149</i> (-129)	-0.03995	7.28	0.0077
		<i>3R_9191831</i>	<i>poly</i> (in)	-0.08168	15.06	0.0002
	Females	Intercept		0.64671	1190.5	<.0001
		<i>X_10808734</i>	<i>CG11203</i> (in)	0.03804	8.22	0.0047
		<i>2L_8863843</i>	<i>CG32987</i> (u5)	-0.04281	9.91	0.002
		<i>2L_13309154</i>	<i>CG16970</i> (u5)	-0.05428	7.38	0.0074
		<i>2L_14309673</i>	<i>wb</i> (in)	0.05906	9.88	0.002
		<i>2R_4890688</i>	<i>CG34350</i> (in)	-0.04952	9.3	0.0027
		<i>2R_18093670</i>	<i>CG3290</i> (cds)	-0.04026	12.22	0.0006
		<i>3L_3936042</i>	<i>Eip63F-2</i> (50) / <i>CG10866</i> (-555)	-0.06173	8.43	0.0042
		<i>3L_11142680</i>	<i>CG32082</i> (in)	0.05964	13.26	0.0004
	<i>3L_11427728</i>	<i>CG6149</i> (-129)	-0.03923	8.33	0.0045	
	<i>3R_12439840</i>	<i>CG10340</i> (cds)	0.0283	6.45	0.0121	
	Pooled	Intercept		0.65806	2296.8	<.0001
<i>2L_8863843</i>		<i>CG32987</i> (u5)	-0.05718	21.08	<.0001	
<i>2L_13309154</i>		<i>CG16970</i> (u5)	-0.07066	14.71	0.0002	
<i>2L_14309673</i>		<i>wb</i> (in)	0.05876	11.79	0.0008	
<i>2R_4890688</i>		<i>CG34350</i> (in)	-0.04132	6.76	0.0102	
<i>2R_17655728</i>		<i>CG30395</i> (in)	-0.07706	23	<.0001	
<i>2R_19702860</i>		<i>CG4797</i> (in)	-0.09134	29.37	<.0001	
<i>3L_11142680</i>		<i>CG32082</i> (in)	0.05232	11.65	0.0008	
<i>3L_11427728</i>		<i>CG6149</i> (-129)	-0.04563	13.01	0.0004	

Markers are listed in the order in which they entered the model. Estimates of effects are for (Minor allele – Major allele). In: intronic; cds: coding sequence; +/-: base pairs upstream/downstream; #: Missense; u3/5: 3'/5' UTR.

Table 3.4: Gene-centered predictive models for response to ethanol consumption.

Trait	Analysis	Variable	SNP Location	Estimate	<i>t</i>	<i>p</i> -value
Gustatory Response to Ethanol	Males	Intercept		0.25611	839.77	<.0001
		<i>X</i> _18395970	<i>Wnt5</i> (cds)	0.02949	10.97	0.0012
		<i>2R</i> _10272012	<i>Dh44-R1</i> (in)	0.02717	10.82	0.0012
		<i>2R</i> _17235870	<i>CG17974</i> (in)	-0.01667	8.6	0.0039
		<i>2R</i> _18665337	<i>CG42260</i> (in)	-0.0127	8.38	0.0043
		<i>2R</i> _19611671	<i>Sesn</i> (in)	-0.02735	12.11	0.0007
		<i>2R</i> _19926921	<i>CG3173</i> (in)	-0.01246	7.35	0.0074
		<i>3L</i> _1098229	<i>bab1</i> (in)	0.03287	10.8	0.0013
		<i>3L</i> _10422175	<i>Ir67(b)</i> (-614)	-0.02429	24.86	<.0001
		<i>3L</i> _14107857	<i>Sox21b</i> (in)	-0.01934	6.77	0.0102
	<i>3R</i> _9612579	<i>Dip-B</i> (1178)	0.02013	16.52	<.0001	
	<i>3R</i> _10465601	<i>CG7886</i> (in)	-0.01712	14.9	0.0002	
	Females	Intercept		0.20822	429.51	<.0001
		<i>X</i> _20737533	<i>CG1314</i> (-1483)	-0.02903	18.96	<.0001
		<i>2L</i> _18486307	<i>CG10176</i> (in)	0.0172	5.6	0.0192
<i>2L</i> _19053996		<i>CG10492</i> (-85)	0.02289	6.25	0.0135	
<i>2R</i> _13164115		<i>mbl</i> (in)	0.03232	16.97	<.0001	
<i>3L</i> _2625204		<i>Pxn</i> (in)	0.02493	13.63	0.0003	
<i>3L</i> _10422175		<i>Ir67(b)</i> (-614)	-0.02081	9.03	0.0031	
<i>3R</i> _7977426	<i>dpr15</i> (in)	0.0334	0.0016	0.0016		
Pooled	Intercept		0.2276	881.26	<.0001	
	<i>X</i> _18287706	<i>CG32549</i> (in)	0.01679	10.64	0.0014	
	<i>2L</i> _7001589	<i>uif</i> (in)	-0.02527	11.33	0.001	
	<i>2R</i> _13164115	<i>mbl</i> (in)	0.02524	18.5	<.0001	
	<i>3L</i> _10422175	<i>Ir67(b)</i> (-614)	-0.01996	14.34	0.0002	
	<i>3L</i> _13420545	<i>CG14105</i> (772)	-0.02939	10.76	0.0013	
	<i>3R</i> _7977426	<i>dpr15</i> (in)	0.03004	14.1	0.0002	
<i>3R</i> _24820269	<i>CG1443</i> (in)	-0.0162	11.56	0.0009		

Markers are listed in the order in which they entered the model. Estimates of effects are for (Minor allele – Major allele). In: intronic; cds: coding sequence; -/+ : base pairs upstream/downstream; #: Missense; u3/5: 3'/5' UTR.

Table 3.5: Analyses of variance of haplotypes from gene-centered predictive models for olfactory response to ethanol.

Trait	Analysis	Source of Variation	d.f.	MS	F	<i>p</i> -value	σ^2
Olfactory Response to Ethanol	Sexes Pooled	Haplotype	37	0.216	7.34	<.0001	0.02587
		Line(Haplotype)	129	0.029	2.46	<.0001	0.0085925
		Error	167	1.992			0.01193
	Female	Haplotype	64	0.676	3.50	<.0001	0.02506
		Line(Haplotype)	102	0.194	2.91	<.0001	0.01303
		Error	1503	0.067			0.06661
	Males	Haplotype	38	1.236	6.70	<.0001	0.04242
		Line(Haplotype)	128	0.185	2.90	<.0001	0.01223
		Error	1503	0.064			0.06365

d.f.: degrees of freedom; MS: Type III Mean Squares; σ^2 : variance component.

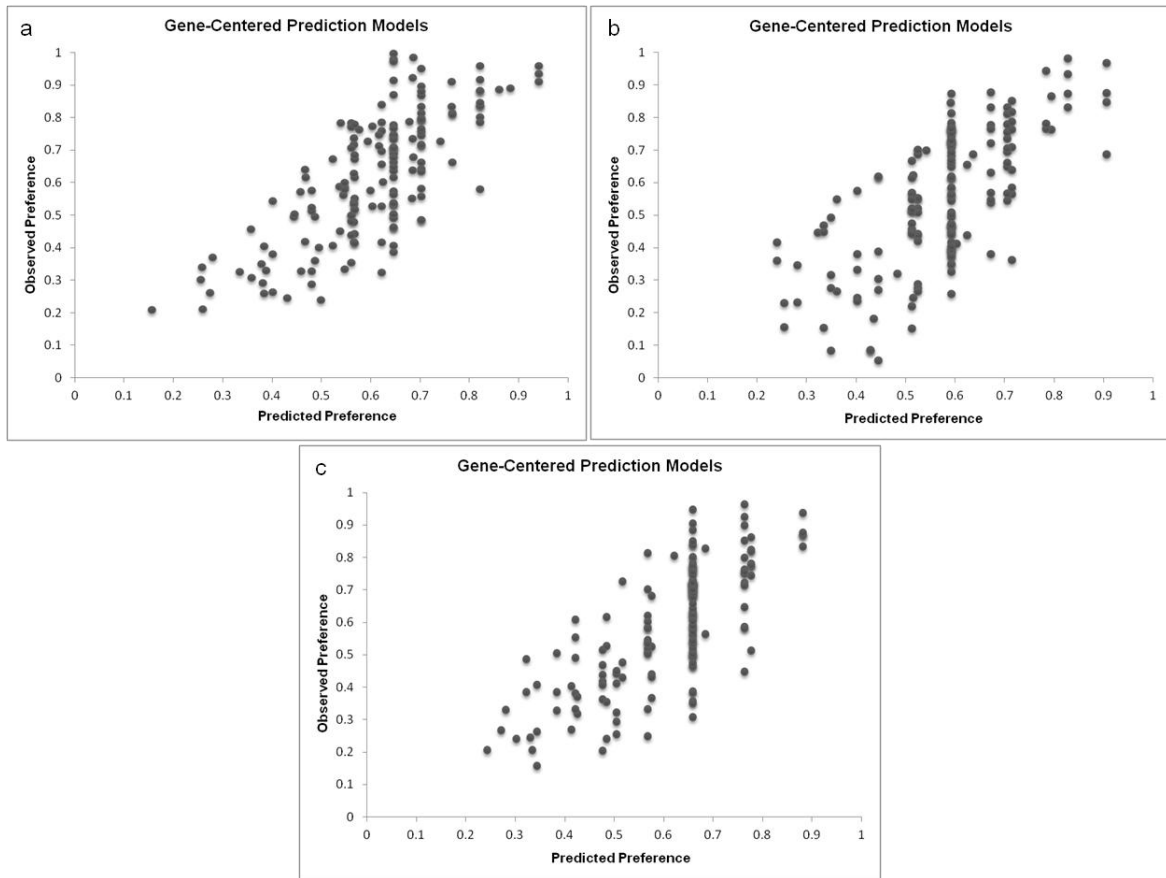


Figure 3.11: Gene-centered prediction models for olfactory response to ethanol. (a) Females ($r^2 = 0.582$). (b) Males ($r^2 = 0.517$). (c) Pooled ($r^2 = 0.60$) pooled.

Table 3.6: Analyses of variance of haplotypes from gene-centered predictive models for response to ethanol consumption.

Trait	Analysis	Source of Variation	d.f.	MS	F	p-value	σ^2
Gustatory Response to Ethanol	Sexes Pooled	Haplotype	38	0.033	4.97	<.0001	0.0049471
		Line(Haplotype)	128	0.007	7.08	<.0001	0.0029421
		Error	167	0.001			0.0009516
	Female	Haplotype	50	0.174	3.78	<.0001	0.0049828
		Line(Haplotype)	116	0.046	9.98	<.0001	0.0050508
		Error	1169	0.005			0.0046032
	Males	Haplotype	84	0.094	3.83	<.0001	0.0056905
		Line(Haplotype)	82	0.025	3.98	<.0001	0.002432
		Error	1169	0.006			0.0061674

d.f.: degrees of freedom; MS: Type III Mean Squares; σ^2 : variance component.

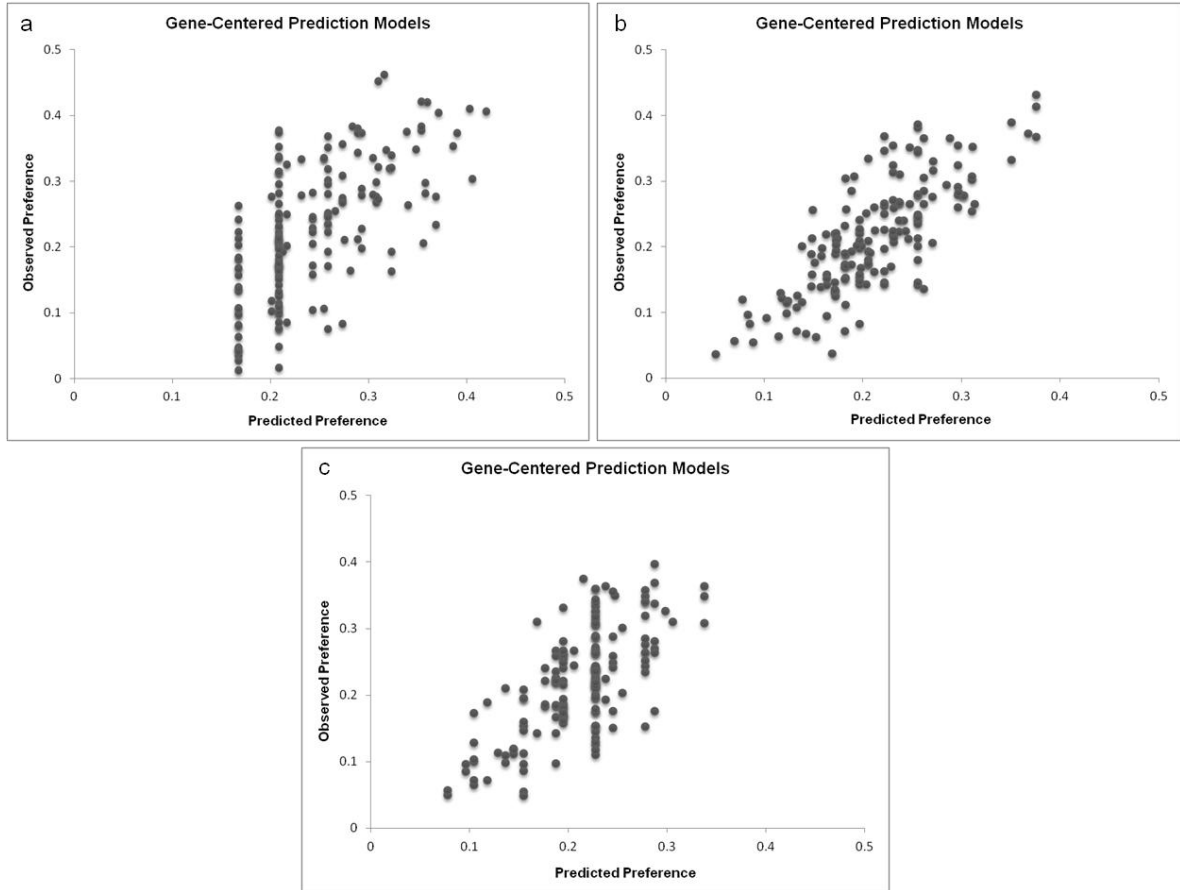


Figure 3.12: Gene-centered prediction models for response to ethanol consumption. (a) Females ($r^2 = 0.48$). (b) Males ($r^2 = 0.64$). (c) Pooled ($r^2 = 0.50$) pooled.

Validation of Candidate Genes

For functional validation of candidate genes, I selected 11 genes associated with variation in olfactory response to ethanol and 20 genes associated with variation in response to ethanol consumption. These genes were selected based on the significance level of their association with phenotypic variation, their known

functions, and the availability of co-isogenic *P*-element, *Minos*-element, or RNAi knockdown alleles.

The 11 genes associated, with variation in olfactory preference, selected for function tests were *Glutamate receptor I (Glu-R1)*, *katanin p60-like 1 (kat-60L1)*, *CG32082*, *CG3290*, *CG34381*, *Ionotropic receptor 10a (Ir10a)*, *Tachykinin-like receptor at 99D (Tacr99D)*, *wing blister (wb)*, *CG10340*, *Dicarboxylate carrier 1 (Dic1)*, and *Odorant receptor 63a (Or63a)*. All 11 genes and corresponding controls were tested for both sexes. All the genes, except for *CG34381*, showed significant reduction in olfactory preference for ethanol between RNAi knockdown, *P*-element, and *Minos*-element mutants and their respective controls for at least one sex (Figure 3.13). The absence of significant difference between the control and *CG34381 Minos*-element mutant does not necessarily mean that this gene does not have an effect on olfactory preference of ethanol. The mutant line has a different genetic background than the DGRP population and differences in genetic background can have profound effects on behavioral phenotypes (Mackay 2004).

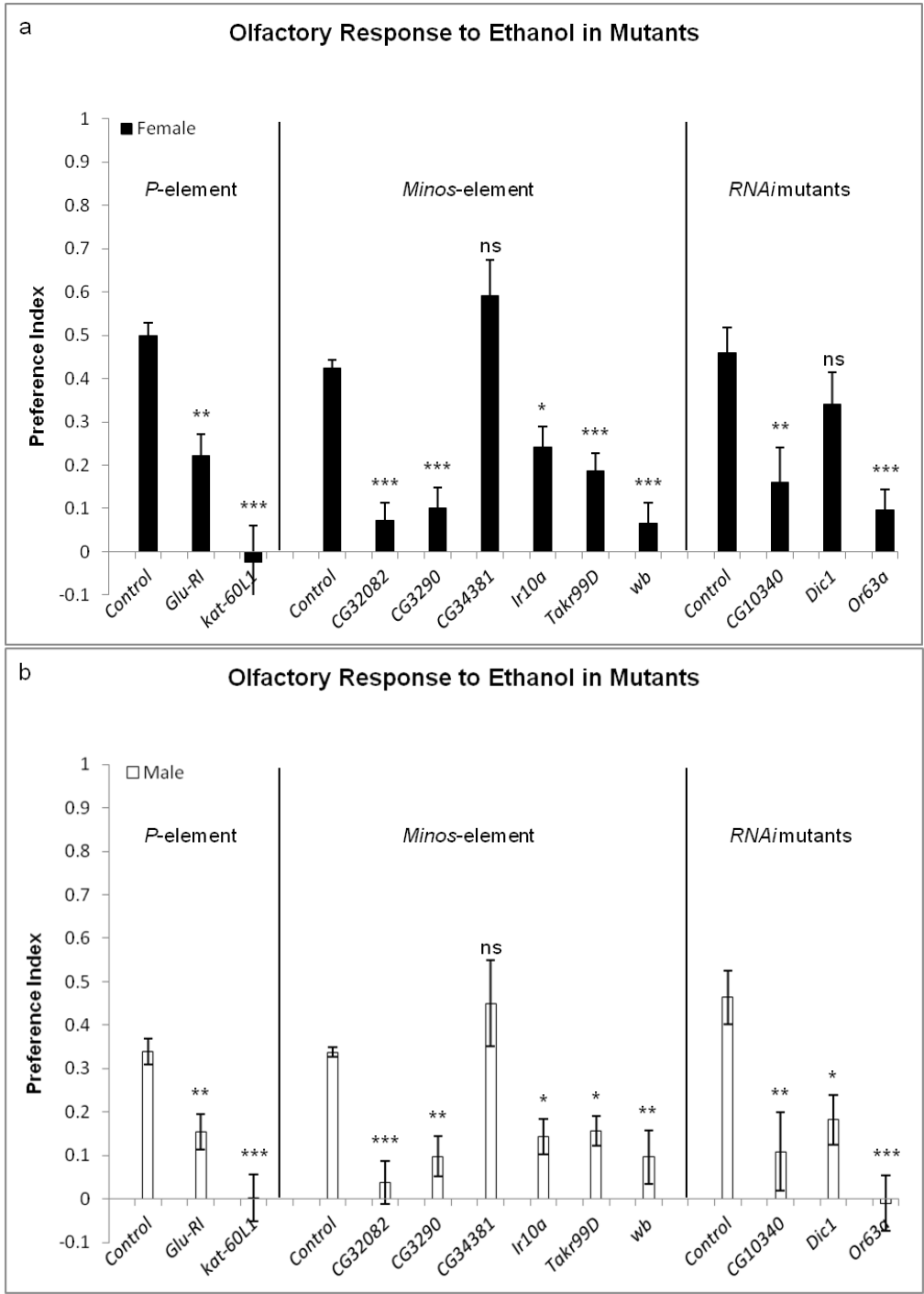


Figure 3.13: Validation of candidate genes associated with variation in olfactory behavioral response to ethanol. (a) Shows olfactory behavioral response of

Figure 3.13: Continued

females. (b) Shows olfactory behavioral response of. First, second, and third panel contain *P*-element, *Minos*-element, and RNAi knockdown lines, respectively. Each mutant allele was compared relative to its control. (Dunnet's test; *: $0.01 \leq p \leq 0.05$; **: $0.001 \leq P \leq 0.01$; ***: $0.0001 \leq p \leq 0.001$; ns: $p > 0.05$).

The 20 genes associated with variation in behavioral preference for ethanol consumption selected for function tests were *Ccn*, *CG42260*, *bric a brac 1 (bab1)*, *CG34411*, *dpr15*, *dpr9*, *Gustatory receptor 36b (Gr36b)*, *Ionotropic receptor 67b (Ir67b)*, *CG4168*, *CR31084*, *Odorant-binding protein 57a (Obp57a)*, *Odorant receptor 22b (Or22b)*, *Odorant receptor 59b (Or59b)*, *Odorant receptor 59c (Or59c)*, *Odorant receptor 83a (Or83a)*, *Odorant receptor 88a (Or88a)*, *Pheromone-binding protein-related protein 3 (Pbprp3)*, *muscleblind (mbl)*, *Sestrin (Sesn)*, and *Wnt oncogene analog 5 (Wnt5)*. All 20 genes and corresponding controls were tested for both sexes. All the genes showed significant reduction in avoidance of the food containing ethanol (i.e. significantly higher preference scores) between RNAi knockdown, *P*-element, and *Minos*-element mutants and their respective controls for at least one sex (Figure 3.14).

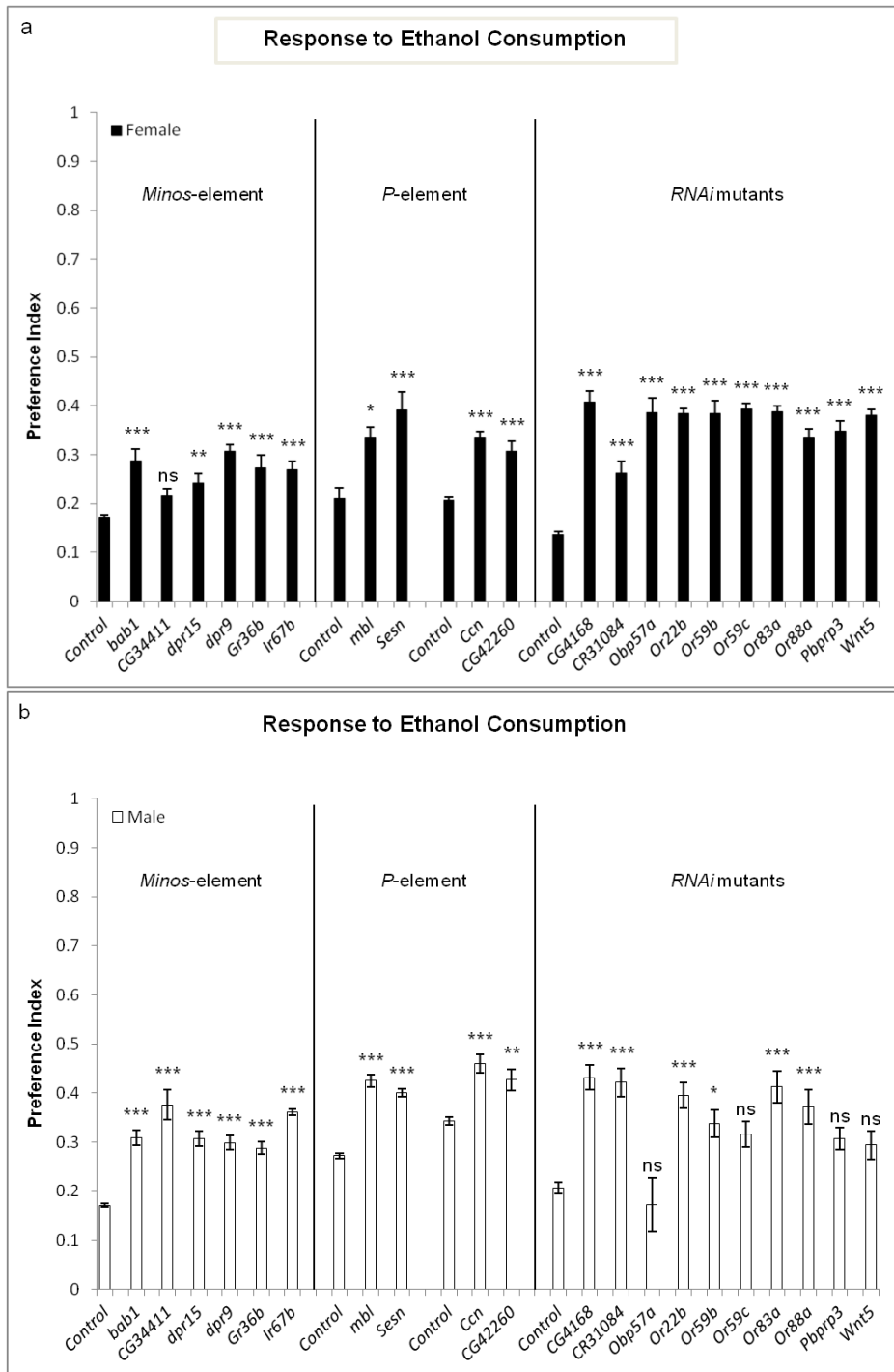


Figure 3.14: Validation of candidate genes associated with variation in behavioral response to ethanol consumption. (a) Shows behavioral response of females.

Figure 3.14: Continued

(b) Shows behavioral response of males. First, second, and third panel contain *Minos*-element, *P*-element, and RNAi knockdown lines, respectively. The second panel contains two different *P*-element collections and their corresponding controls. Each mutant allele was compared relative to its control. (Dunnet's test; *: $0.01 \leq p \leq 0.05$; **: $0.001 \leq P \leq 0.01$; ***: $0.0001 \leq p \leq 0.001$; ns: $p > 0.05$).

DISCUSSION

This study is one of the largest efforts conducted to date utilizing genome wide natural genetic variation in a model system to uncover the genetic architecture underlying variation in olfactory behavioral response and behavioral response to consumption of ethanol, an ecologically relevant compound encountered by flies in and around their food source. By conducting genome wide association analyses, we were able gain valuable insights into the genetic basis of chemosensory behavioral responses to ethanol and identify novel genes associated with variation in these traits.

GWA Analysis for Variation in Chemosensory Behavioral Responses to Ethanol

From the GWA analyses, we identified 80 and 137 SNPs significantly associated with natural variation in olfactory and gustatory behavioral responses to ethanol, respectively. For variation in olfactory behavioral response, 34 SNPs were significant for females and 24 SNPs were significant for males. SNP effects, calculated as one-half the mean difference in the trait between the major and minor allele class, were mostly positive for males (20 SNPs had positive effects compared to 4 SNPs with negative effects) indicating that most of the major alleles are associated with higher preference for ethanol when detected as an olfactory cue. In females, the number of SNPs with negative effects (19 SNPs) was slightly more than SNPs with positive effects (SNPs 15) indicating that both major and minor alleles are associated with increased preference for ethanol. For both sexes, the SNPs with positive effects had larger effect sizes than the SNPs with negative effects (Figure 3.2 and Figure 3.3).

For behavioral response to ethanol consumption, we identified 11 SNPs that were significant for females and 32 SNPs that were significant for males. Five SNPs had positive and six SNPs had negative effects on female behavioral response and 19 SNPs had positive and 13 SNPs had negative effects on male behavioral response to ethanol consumption (Figures 3.6 and 3.7). These results indicate that, for both sexes, the major and minor alleles are associated with reduced aversion (i.e. higher preference scores). Additionally, the range of effect sizes was similar for SNPs with

positive and negative effects (Figure 3.6). For both the chemosensory traits, a majority of the SNPs had moderate to large effects with an inverse relationship between minor allele frequency and effect size, such that the less common alleles had the largest effects (Figures 3.2 and 3.6).

For both males and females, majority of SNPs associated with variation in olfactory and ethanol consumption behavior were at intermediate frequency suggesting that they might be maintained by a balance of selective forces (Mitchell-Olds et al. 2007). We can test whether these alleles (SNPs) are maintained by balancing selection by going back to the same population, from which the DGRP was derived in 2002, ten years later and determining whether the same alleles are segregating in the population. If we find these alleles segregating in the population, then there will be support for the hypothesis that they are maintained by balancing selection. This can be done by collecting males and females from the same site and genotyping them for the causal SNPs identified in the present study to determine whether these SNPs are segregating in the population. If we find these alleles segregating in the population then the assumption would be that they are being maintained under balancing selection. However, it is important to keep in mind that epistasis is a common hallmark of the genetic architecture underlying complex traits (Sambandan et al. 2006; Swarup et al. 2012) and can mask the genotypic effects at one locus by genotypes of another locus (Phillips 2008). Since flies in a natural population have mixed genetic backgrounds, epistatic interactions might mask the effects of the alleles identified in the GWA analysis.

Phenotypic and Genetic Variation Explained by Gene-Centered Prediction

Models

The SNPs associated with variation in olfactory and ethanol consumption behavioral responses are not necessarily independent. As a result, single marker analysis can lead to biased estimates of allelic effects when multiple markers affecting a trait are correlated (Mackay et al. 2012). To deal with this, we computed gene-centered forward selection multiple regression models to estimate effect sizes when multiple SNPs are simultaneously evaluated in the model and the fraction of the total variation accounted for by the SNPs. We only include SNPs that were within 2 kb of an annotated gene. For cases where there were multiple SNPs within a gene, we included only one SNP and allowed for a maximum of 12 SNPs in each model. We computed the multiple regression models for females, males, and as averaged across sexes. These models explained between 52-60% (Table 3.3) and 48-64% of the phenotypic variation associated with olfactory and ethanol consumption behavioral responses, respectively. The models also explained between 66-78% of the genetic variance associated with olfactory behavioral response to ethanol (Table 3.5, Figure 3.11) and between 50-70% of the genetic variance associated with behavioral response to ethanol consumption (Table 3.6, Figure 3.12). Relatively few SNPs were significantly associated with variation in behavioral responses to ethanol and account for a large portion of the phenotypic and genetic variance in both traits, suggesting that the genetic architecture of segregating variation in chemosensory

behavioral responses to ethanol within the DGRP might be relatively simpler than expected (Beavis 1994; Flint and Mackay 2009).

Another way to confirm the effects of the significant SNPs on phenotypic variation in the chemosensory traits is to determine if these SNPs can be used to predict the chemosensory behavioral responses in flies for ethanol. The haplotypes formed by the SNPs used in the gene-centered prediction models explain a large portion of the phenotypic and genetic variance associated with variation in olfactory and gustatory responses to ethanol consumption and should predict the chemosensory behavioral responses among the remaining lines within the DGRP that were not phenotyped in this study (for the present study, I phenotyped 167 out of the 192 DGRP lines).

Similarly, we might also be able to use this information to predict the chemosensory behavior responses of flies collected from different populations. However, as mentioned above, different combination of alleles and their effects in different genetic backgrounds (flies collected from different locations) might prevent us from predicting the chemosensory behavioral responses of flies, especially, if these difference result in different and/or new epistatic interactions among alleles which affect the behavior.

Validation of Candidate Genes Identified in the by Gene-Centered Prediction Models

I have functionally validated four genes (*wb*, *CG3290*, *CG32082*, and *CG10340*) that were included in the gene-centered predictive models for olfactory response to

ethanol (Table 3.3) using RNAi knockdown and *Minos*-element lines. All the *Minos*-element mutant lines and the RNAi knockdown line showed reduced olfactory preference for ethanol confirming their role in olfactory behavioral response to ethanol (Figure 3.13). Experimental evidence suggests that *wb* is involved in digestive tract mesoderm development and gonad development (Weyers et al. 2011). It has also been implicated in conferring resistance in flies to ethanol exposure (Morozova et al. 2007; Morozova et al. 2011), suggesting that it might play a role in the behavioral response of flies to ethanol by increasing or decreasing their resistance to ethanol. Not much information is available on the other three genes. *CG3290* is computationally predicted to be involved in metabolic processes (Flybase). *CG32082* is predicted to be involved in signal transduction (Flybase). *CG10340* is predicted to be involved in protein complex assembly (Flybase). I also functionally validated seven genes (*Ir67b*, *bab1*, *dpr15*, *mb1*, *Sesn*, *Wnt5*, and *CG42260*) that were included in the gene-centered predictive models for behavioral response to ethanol consumption (Table 3.4) using RNAi knockdown, *Minos*-element, and *P*-element lines. The RNAi knockdown, *Minos*-element, and *P*-element lines showed reduced preference for ethanol consumption, in at least one sex, confirming their role in behavioral response for ethanol consumption (Figure 3.14). The genes and their possible role in response to ethanol consumption are briefly described below.

Ionotropic receptors belong to a family of chemosensory receptors that detect ammonia and different acids (Benton et al. 2009; Ai et al. 2010). Transcripts from

this gene family are expressed in the fly's antenna and proboscis (Benton et al. 2009). To date, no ionotropic receptor has been implicated in ethanol detection. *Ir67b* is the first reported gene from this family to be implicated in aversive behavioral response to ethanol consumption. Further experiments need to be conducted to determine whether *Ir67b* is expressed in the antenna, the proboscis, or both. This will shed light on whether ethanol is detected by IR67b as an olfactory or a gustatory cue. *bab1* is involved in female gonad development (Chatterjee et al. 2011), sex differentiation, regulation of pigmentation (Gibert et al. 2007), and leg and antenna pattern formation in *Drosophila* (Godt et al. 1993). *bab1* mutants have leg and antennae segmentation defects (Godt et al. 1993). A fly's chemosensory organs include the antennae and legs, and segmentation defects in such organs can affect its chemosensory responses to external stimuli such as ethanol. *dpr15* belongs to a family of genes that are involved in the sensory perception of chemical stimuli in flies (Nakamura et al. 2002). Genes within this family are expressed in gustatory sensilla on the labellum of flies and are involved in inducing an aversive behavior response to salt (Nakamura et al. 2002). Our results suggest that *dpr15* also plays a role in inducing an aversive behavioral response to ethanol consumption in flies. *mb1* is involved in DNA binding and is associated with the apoptotic process (Vicente-Crespo et al. 2008), muscle organ development, embryo development (Begemann et al. 1997), peripheral nervous system development (Prokopenko et al. 2000), and regulation of female receptivity (Juni and Yamamoto 2009). It has also been implicated in conferring resistance to ethanol exposure

(Morozova et al. 2007; Morozova et al. 2011) and affecting aggressive behaviors among flies (Zwarts et al. 2011). The peripheral nervous system is important in mediating behavioral responses. Ethanol induced behaviors are mediated by neurotransmitter systems, neuropeptides, synaptic proteins, channels and signaling processes (Scholz and Mustard 2011) and aggressive behaviors are modulated by neurotransmitters (Kravitz and Huber 2003), all of which form part of the peripheral nervous system. Similarly, chemosensory behaviors are also mediated via the peripheral nervous system. As *mbf* plays a role in peripheral nervous system development, mutations in this gene will probably affect an organism's normal behavioral response to external stimuli. Thus, it is not surprising to see this gene exhibiting pleiotropic effects and implicated in three different behavioral responses among flies. Additionally, pheromones are detected as olfactory and gustatory cues and play an important role in mediating aggressive behaviors (Guillot and Chapouthier 1996; Hasen and Gammie 2009; Liu et al. 2011). A mutation in a gene (such as *mbf*) that affects the chemosensory systems ability to detect and/or process such cues can affect aggressive behaviors. *Sesn* is involved in inter-male aggressive behavior (Edwards et al. 2009), regulation of reactive oxygen species, multicellular organismal aging, negative regulation of cell growth, and mitochondrion degradation (Lee et al. 2010). Improper regulation of reactive oxygen species can lead to DNA and protein damage and disrupt critical cellular signaling pathways (Finkel 2003) which can have an adverse impact on an organism's nervous system. As mentioned above, interference in the normal functioning of the nervous system

can affect both chemosensory and aggressive behaviors. *Wnt5* is involved with salivary gland morphogenesis (Harris and Beckendorf 2007), axon guidance, axon extension, and negative chemotaxis (Yoshikawa et al. 2003). Additionally, *wnt5* signaling controls neural circuit formation, including glomeruli patterning in the *Drosophila* olfactory system and loss of *wnt5* results in severe derangement of the glomerular pattern (Yao et al. 2007). Glomeruli play an important role in odor recognition (described in Chapter One). They are spherical structures present in the antennal lobes which are innervated by olfactory receptor neurons expressing the same odorant receptor (Vosshall et al. 2000). Activity in the glomeruli is transferred to higher brain centers via projection neurons (Smith 2007). Since the organization within the olfactory system is important for odor recognition, irregular patterning of the glomeruli can affect the processing of chemical cues and thereby affect chemosensory behavior responses to chemical stimuli. Our results show that variation in gustatory response to ethanol consumption is mediated by both gustatory and olfactory receptors (Table 3.2) (described in a later section) and genes (such as *wnt5*) that might affect the processing of olfactory information can cause variation in chemosensory behaviors. The last gene that I confirmed within this group was *CG42260*. This gene does not have any known function, though it is predicted to be involved transmembrane transport (Flybase).

Sexual Dimorphism in Chemosensory Behaviors

We observed significant phenotypic and genetic variation in sexual dimorphism for both chemosensory traits (Chapter 2, Tables 3.1 and 3.3; Chapter 3, Figures 3.4 and 3.8). There appear to be selective pressures on both males and females to utilize ethanol as an olfactory cue to locate transient food sources, which also act as places to find potential mates because it attracts other adult flies (Dudley 2002). However, there might be added selective pressures on females to utilize ethanol as a cue to locate oviposition sites (McKenzie and Parsons 1972). On the other hand, there appear to be selective pressures acting on both males and females to minimize ethanol consumption, since ethanol is a toxin and when consumed at high concentrations has fitness costs associated with it (Bokor and Pecsénye 2000). Thus, similar and different selective pressures might be acting on males and females and contributing to the variation in sexual dimorphic behaviors that we observe.

Sexual Dimorphism in Olfactory Behavioral response to Ethanol:

Under the SNP by sex interaction term, we identified 21 SNPs significantly associated with olfactory behavioral response to ethanol. The minor alleles were primarily associated with increase olfactory preference for ethanol in females relative to the major alleles (Figure 3.4). However, in males, we observed that the minor alleles were primarily associated with lower preference scores relative to the major alleles (Figure 3.4). Thus, these SNPs exhibit sex-biased or sex-specific effects, with the minor alleles exhibiting a change in magnitude across the sexes. A change

in the rank of alleles across environments (in this case across sex) might suggest their role in local adaptation if they are associated with fitness (Kawecki and Ebert 2004; Turelli and Barton 2004). It has been previously shown the female *D. melanogaster* flies exhibit a preference for laying eggs on ethanol-containing media (McKenzie and Parsons 1972) suggesting that ethanol might be used as oviposition cue. If ethanol is a cue utilized by females to locate oviposition sites and certain alleles, associated with fitness, increase the ability of females to detect such cues, then there might be selective pressures to maintain or increase the frequency of these alleles among females within the population.

To test the hypothesis, we first need to determine if ethanol acts as an oviposition cue in females within the DGRP lines. We can do this by conducting experiments where female flies from each DGRP line are given a choice between food sources without ethanol and different concentration of ethanol that are ecologically relevant (Gibson, May et al. 1981). Then, we can test whether there is a correlation between oviposition preference and olfactory preference for ethanol among the DGRP lines. A strong positive correlation between the two traits would suggest an overlap between genes and common biological processes that underlie these traits (Falconer and Mackay 1996). We can further determine whether these traits are correlated with fitness traits. Fitness can be measure in terms of number of viable progeny produced in the different DGRP lines or longevity of the lines (provided longevity is positively correlated with number of viable offspring produced). Again, a positive correlation will suggest that there is overlap between genes that underlie

these traits and further suggest that these genes have pleiotropic effects that affect both fitness and preference for ethanol.

Another way to test whether alleles associated with olfactory preference for ethanol among females are also associated with fitness, would be to go back to the same population from which the DGRP was derived in 2002 and determine whether these alleles are maintained at the same frequencies in the population or have increased in females over a period of ten years. An increase in the frequency of these alleles will suggest that they are associated with fitness.

The GWA analysis has identified candidate causal variants affecting the magnitude of sexual dimorphism for olfactory preference of ethanol. Knowledge of these variants enables us to assess the strength and nature of selection that might be acting on this trait in the laboratory setting. We can do this by creating a synthetic outbred population enriched for variants that exhibit sexual dimorphic effects and are associated with olfactory response to ethanol. We can then subject this population to laboratory evolution experiments. From the GWA analysis, we will be able to determine which lines harbor most of the alleles that exhibit sexual dimorphic effects and are associated with variation in the trait. We can use this data to select lines that harbor a majority of the molecular variants associated with variation in this trait and exhibit the highest attraction and the least attraction to ethanol. We can then construct an outbred population by crossing these lines in a half diallel design to create the base population. Such a design will ensure that all causal alleles are present at intermediate frequency in the base population. From the base population,

we will create 2 replicates by randomly mating a large number of individuals (500 males and 500 females) to minimize effects of genetic drift and subject them to different selection regiments. Each replicate will include 10 bottles of 50 males and 50 virgin females. We will rear the replicate populations on food with different concentrations of ethanol under conditions of strong larval crowding, adult crowding, and competition for females. We know that ethanol concentrations can vary in the wild anywhere from 0.02% to 6% (Gibson, May et al. 1981) depending on the type of fruit and its state of ripeness. To replicate the ethanol concentrations in nature and test whether specific alleles are involved in local adaptation to different ethanol concentration, we will rear each replicate on food containing 0%, 0.1%, 1%, 5%, and 10% ethanol. For the controls we will use un-crowded conditions and females will be allowed to lay eggs for one day on food containing the above mentioned ethanol regiments (i.e. 0% to 10%) to produce the next generation. Individuals emerging from these regiments will be used to produce next generation. For the larval crowding conditions, females will be allowed to lay eggs for 3 days. For the adult crowding condition, we subject flies to overcrowded conditions before selecting individuals to lay eggs on the different ethanol regiments. To create competition for females, bottles will be set up with a ratio of 4 males for every virgin female for an allotted time and then they will be randomly mixed and allowed to lay eggs on the different ethanol regiments. Every 10 generations, we will collect individuals from each replicate and ethanol regiment for genotyping and repeat this for 50 generations. Through genotyping every 10 generations, we can track changes in

the frequency of alleles identified through the GWA analyses under different ethanol regiments. We can expect that under normal food condition of 0% ethanol (i.e. no selective pressures), the alleles within the replicates will be maintained at the same intermediate frequencies at which they started. Under the different conditions and ethanol regiments, the alleles that afford a fitness advantage to the flies will increase in frequency over the period of the experiment. On the other hand if they are deleterious, they should decrease in frequency. By tracking the frequency of the alleles we should be able to determine the kind of selective pressure acting on them and if they offer any selective advantage or not. However, the above design ensures that all causal alleles are present at intermediate frequency in the base population and this prevents us from distinguishing between neutral evolution and balancing selection since, in both cases, the alleles will be maintained at intermediate frequencies throughout the experiment. One way to deal with this is to design the experiment in such a way that we have different starting frequencies of the candidate alleles in the base population. For example, we can cross three DGRP lines which have major alleles at different loci to generate progeny that are fixed for those alleles. Similarly, we can cross three lines to generate progeny that are fixed for the minor allele at those same loci. Then we can take 450 individuals having the major allele and mate them with 125 individuals having the minor allele to generate a base population. The base population should have the major allele frequency of ~ 0.75 and minor allele frequency of ~ 0.25 . As mentioned above, we will maintain large population sizes and test replicates to account for genetic drift.

We can then put the base population through the different conditions and ethanol regiments described above and track the allele frequencies over 50 generations. In the case of balancing selection we will expect the major and minor allele frequencies to move from 0.75 and 0.25 to more intermediate levels (i.e. ~0.5). If the alleles are advantageous under the selection regimes they should increase in frequency over time, if they are deleterious they should decrease in frequency, and if they are neutral then they should stay at the same frequency.

However, one caveat to keep in mind is that by creating a synthetic outbred population we have mixed different genotypes which might create new epistatic interactions that change/mask the effect of the alleles. One way to account for this is to repeat the above set-up using different set of lines and tracking the frequency of the candidate alleles under the above mentioned selection regiments and seeing if we can replicate the results from the first experiment. The results will provide information on whether specific alleles influence fitness, are involved in local adaptation, and what kind of selective pressures are acting on them.

Validation of Candidate Genes Associated with Variation in Sexual

Dimorphism for Olfactory Behavioral Response to Ethanol

I have functionally validated three genes (*kat-60L1*, *Glu-RI*, and *Takr99D*) for SNPs, identified under the SNP by Sex interaction term, that were significantly associated with variation in olfactory behavioral response to ethanol using a *Minos*-element and two *P*-element mutants (Figure 3.13). *kat-60L1* is involved in neuron remodeling.

Kat-60L1 is from the Katanin family of microtubule severing proteins and is required for proper dendrite severing. During metamorphosis, certain *Drosophila* sensory neurons undergo dendrite pruning to remove their larval branches before regeneration of adult dendrites (Lee et al. 2009). Dendrites are branched projections of a neuron and play an important role in integrating synaptic inputs from other neural cells and determining the extent to which action potentials are produced by the neuron. They play an important role in the normal functioning of the nervous system and defects in them can affect an organism's behavioral response to chemical stimuli. The second gene, *Glu-R1* is involved in glutamate receptor activity (Schuster et al. 1993), however the biological processes in which it is involved are not known. The third gene, *Takr99D* belongs to the G-protein-coupled receptor family and is involved in the tachykinin receptor signaling pathway (Li et al. 1991; Hewes and Taghert 2001). Tachykinins are members of a family of peptides, that act as neurohormones in mammals and insects, which are critical for motor and sensory functions and are related to central and peripheral neuronal activity (Torfs et al. 2000). Olfactory receptor neurons (ORNs) express tachykinins which are involved in synaptic transmission within the ORNs (Ignell et al. 2009). All the three genes mentioned above are involved in processes affecting the nervous system. Impairment in the normal development of the nervous system or of processes involved in the proper functioning of the nervous system can impact an organism's behavioral response to external cues, such as ethanol. The mutant lines for all the three genes exhibited reduced preference for ethanol, in both males and females,

confirming the role of these genes in olfactory behavioral response to ethanol (Figure 3.13).

Sexual Dimorphism in Behavioral response to Ethanol Consumption:

Under the SNP by sex interaction term, we identified 65 SNPs significantly associated with behavioral response to ethanol consumption. While the major alleles conferred similar sensitivity to ethanol in both sexes, the minor alleles conferred both reduced aversion (i.e. high preference scores) and increased aversion (i.e. low preference scores) to ethanol consumption in both males and females relative to the major alleles (Figure 3.8). Thus, the minor alleles exhibit sexually antagonistic effects. Additionally, the minor allele frequency (MAF) for most of the SNPs ranges between 0.02 and 0.1. These observations suggest that these alleles might be maintained under a mutation-selection balance model (Falconer and Mackay 1996) and might be involved in the local adaptation of flies to changing ethanol concentrations present in their food source. Ethanol is a toxin and has fitness costs associated with it when consumed at high concentrations (Bokor and Pecsénye 2000), thus selective pressures might be acting on both males and females to minimize ethanol consumption. Change in rank fitness of alleles can maintain polymorphisms at a locus on which selection can act to produce phenotypic diversity and adaptation to local environments (Turelli and Barton 2004).

As mentioned above, we can use information gained from the GWA analysis to create a synthetic outbred population enriched for variants that exhibit sexual

dimorphic effects, are associated with gustatory response to ethanol consumption, and subject it to laboratory evolution experiments. This experiment will be conducted exactly the same way and under the same conditions, as described above for olfactory behavioral response to ethanol. The only difference here will be in the lines selected to create the synthetic outbred population, since different DGRP lines harbor the alleles that exhibit sexual dimorphic effects and are associated with variation in gustatory response to ethanol consumption. The results from these experiment will valuable information on whether these alleles are associated with fitness, are involved in local adaptation, and the kind of selective pressures acting on them.

Additionally, we can look at correlations between gustatory behavioral response and other fitness traits, within the DGRP, to determine if the same genes and pathways underlie the different traits.

Validation of Candidate Genes Associated with Variation in Sexual Dimorphism for Behavioral Response to Ethanol Consumption

I have functionally tested three genes (*Or22b*, *Ccn*, and *CR31084*), using RNAi knockdown and a *P*-element line (Figure 3.14), identified through significant SNPs associated with variation in behavioral response to ethanol consumption under the SNP by sex interaction term. *OR22b* is an olfactory receptor that is coexpressed along with *OR22a* in the same neuron (Dobritsa et al. 2003). *OR22a*, which is activated by ethanol (Hallem and Carlson 2006), was also identified in the present

study (Table 3.2). Though previous studies have not identified any odorant that activates OR22b, its coexpression in the same neuron as OR22a and its significant association with variation in gustatory response to ethanol consumption suggests its role in ethanol detection. Furthermore, OR22a and OR22b are sexually dimorphic in their expression, with females having higher expression of the receptors compared to males (Dobritsa et al. 2003), and in the present study both OR22a and OR22b were identified through SNPs significant for the SNP by sex interaction term suggesting that differences in expression of receptors between the sexes might be one of the reason for the observed sexually dimorphic responses among males and females. From the above evidence, it appears that OR22a and OR22b produce sexually dimorphic responses to ethanol consumption between males and females. The reduced aversion to ethanol consumption in the *Or22b* RNAi knockdown flies strongly suggests that activation of OR22b by ethanol induces an aversive behavioral response in flies.

The RNAi knockdown and P-element mutant line for *CR31084* and *Ccn* showed reduced aversion to consumption of ethanol (Figure 3.14). Not is much known about these genes. *Ccn* has relatively high levels of expression in brain and head tissue (FlyAtlas) and is predicted to be involved growth factor activity. There is some experimental evidence to suggest its role in neurogenesis (Neumuller et al. 2011). *CR31084* is a non-coding RNA with no known or predicted function (Flybase), however, non-coding RNAs are involved in many functions including transcriptional

regulation, post-transcriptional regulation, and chromatin modification (Esteller 2011).

Chemosensory Genes Associated with Variation in Olfactory and Gustatory Behavioral Responses to Ethanol

From the initial GWA analyses using all the 2,481,491 SNPs (Mackay et al. 2012), two chemosensory genes (*Ir67b* and *Or22b*) were identified as significantly associated with variation in gustatory behavioral response to ethanol consumption at a p -value of $<10^{-5}$. Since chemosensory behavioral responses to ethanol involve the detection of ethanol, both as an olfactory and gustatory cue, and we did not identify any chemosensory gene associated with variation in olfactory behavioral response to ethanol, we decided to conduct a separate GWA analysis including only chemosensory genes. We used a stringent p -value of 0.0005, based on Bonferroni correction, as a cut-off to identify significant chemosensory genes associated with olfactory and gustatory behavioral responses to ethanol. From the analysis, we identified eight chemosensory genes significantly associated with variation in olfactory behavioral response (Table 3.1) and 23 chemosensory genes significantly associated with variation in behavioral response to ethanol consumption (Table 3.2). We found no overlap between the chemosensory genes identified for both traits.

Validation of Candidate Genes Associated with Variation in Chemosensory Behavioral Responses to Ethanol

I functionally validated two chemosensory genes (*Or63a* and *Ir10a*) that were significantly associated with olfactory behavioral response to ethanol using a RNAi knockdown and a *P*-element line. Both lines showed significantly lower preference for ethanol relative to the controls (Figure 3.13). These results support the role of OR63a and IR10a in detection of ethanol as an olfactory cue and in inducing attractive behavioral responses in flies. This is the first time that either receptor has been implicated in ethanol induced chemosensory behavioral responses.

For the gustatory behavioral response to ethanol consumption, I functionally tested one gustatory receptor gene (*Gr36b*), one ionotropic receptor gene (*Ir67b*), two odorant binding protein genes (*Obp57a* and *Pbprp3*), and five olfactory receptors genes (*Or22b*, *Or59b*, *Or59c*, *Or83a*, and *Or88a*) using RNAi knockdown, *Minos*-element, and *P*-element lines. All RNAi knockdown, *Minos*-element, and *P*-element lines showed significant reduction in aversion to ethanol consumption (i.e. higher preference scores) relative to their controls in at least one sex (Figure 3.14). None of these chemosensory genes have been previously implicated in ethanol detection or ethanol induced chemosensory behavioral responses.

This is the first instance where a gustatory receptor (*Gr36b*) has been implicated in ethanol induced behavioral response. Further evidence for the role of *Gr36b* in gustatory behavioral response to ethanol comes from experiments showing its expression in neurons limited to the small (S) taste sensilla (describe in Chapter

One) that are activated through gustatory receptors which detect noxious compounds and produce aversive behavioral responses in flies (Weiss et al. 2011). We identified two odorant binding protein genes (*Obp57a* and *Pbprp3*) involved in gustatory behavioral response to ethanol consumption. Odorant binding proteins (OBPs) play an important role in mediating chemosensory behavioral responses through their interaction with odorants and chemosensory receptors (described in Chapter One). I functionally tested *Obp57a* and *Pheromone-binding protein-related protein 3* (*Pbprp3*). RNAi knockdown of both these genes showed reduced aversion to ethanol consumption in only females relative to their controls (Figure 3.14). This is not surprising as previous studies have shown that *Obps* show sexual dimorphic differences in expression levels (Zhou et al. 2009) and are implicated in sexually dimorphic behaviors responses to chemical stimuli (Swarup et al. 2011). In addition to ethanol detection, *Obp57a* has been implicated in detection of 1-hexanol, 2-heptanone, benzaldehyde, isoamylacetate, and phenyl-ethanol (Swarup et al. 2011). Based on sequence similarity, *Pbprp3* is predicted to be involved in pheromone binding and odorant binding (FlyBase) and our results suggest that it is also involved in mediating behavioral responses to ethanol.

As described in Chapter Two, the gustatory choice assay measures the gustatory behavioral response of ethanol consumption in flies when they detect it in their immediate proximity, both as a gustatory and an olfactory cue. Therefore, while this assay appears to primarily quantify gustatory preference of ethanol consumption in flies, we cannot rule out the role of the olfactory system in modulating the gustatory

behavioral response of the flies. Thus, it was not very surprising that our results identified olfactory receptors associated with variation in gustatory preference for ethanol consumption (Table 3.2). I functionally tested five *Ors* (*Or22b*, *Or59b*, *Or59c*, *Or83a*, and *Or88a*) using RNAi knockdown lines and observed significant reduction in aversion to ethanol consumption (i.e. higher preference scores) in all the lines relative to their controls for at least one sex (Figure 3.14). Since flies exhibit an aversive gustatory behavioral response to ethanol consumption and RNAi knock down of the *Ors* resulted in reduced aversion to ethanol consumption, it appears that activation of these ORs, by ethanol, induces an aversive behavioral response in the flies. These results also suggest that these ORs are modulating the gustatory behavioral response on the flies.

Our results suggest that OR63a, OR22b, OR59b, OR59c, OR83a, and OR88a are all activated by ethanol but induce different behavioral responses. Activation of OR63a induces an attractive behavioral response to ethanol and activation of OR22b, OR59b, OR59c, OR83a, and OR88a induces an aversive behavioral response to ethanol in flies. These ORs might be activated at similar or different concentrations of ethanol (Hallem et al. 2006), however we could not assess that since we do not know how many molecules of the odorant were detected by the ORs in our behavioral assays.

Additionally, from our results, we would expect that if OR22b, OR59b, OR59c, OR83a, and OR88a induce aversive behavioral response to ethanol vapors, then we should observe an aversive olfactory response to ethanol in the *OR63a* RNAi

knockdown flies. However, the *Or63a* RNAi knockdown flies exhibited no preference in females and a slight preference for ethanol in males (Figures 3.13). Possible explanations of why we observed these result include: (1) other chemosensory genes, such as *Ir10a* identified in this study (Figure 3.13), might induce an attractive olfactory response to ethanol that override the effects of OR22b, OR59b, OR59c, OR83a, and OR88a, (2) the concentration of ethanol used was not sufficient enough to activate OR22b, OR59b, OR59c, OR83a, and OR88a, especially since the RNAi knockdown flies are in a different genetic background than the DGRP lines, (3) the olfactory trap assay is not suitable for measuring aversive behavioral response to ethanol, or (4) a combination of all the above factors.

These questions can be answered by measuring the olfactory behavioral response of the above mentioned chemosensory genes using an assay which is more appropriate to measure aversive olfactory behavioral response, such as the 'dipstick assay' (Anholt et al. 1996), and establishing their dose response curves for ethanol. We can also use, if available, or generate *Or63a/Ir10a* double knockout mutants and test them to ensure that, at different concentrations of ethanol, *Ir10a* is not countering the otherwise aversive olfactory behavioral response induced by OR22b, OR59b, OR59c, OR83a, and OR88a.

Results from the above experiments should provide insights on whether: (a) activation of OR22b, OR59b, OR59C, OR83a, and OR88a induces an aversive behavioral response to ethanol vapors, (b) activation of OR63a, and the resulting attractive behavioral response in flies, at certain ethanol concentration, overrides the

aversive behavioral response produced by activation of OR22b, OR59b, OR59c, OR83a, and OR88a, or (c) a combination of the above is occurring.

Some further insights can be gained by testing the behavioral response of *Or63a* and *Ir10a* using the gustatory choice assay. For example, RNAi knockdown of OR22b, OR59b, OR59c, OR83a, and OR88a reduced aversion to ethanol consumption in flies suggesting that these ORs modulate the gustatory behavioral response in flies. Similarly, it will be interesting to test whether *Or63a* and *Ir10a* are modulating the gustatory response of the flies to ethanol consumption. We can test that by asking whether: (a) RNAi knockdown of *Or63a* increases the gustatory aversion to ethanol consumption in flies, and (b) if we observe a similar increase in gustatory aversion to ethanol consumption in *Ir10a* mutants. Answers to these questions will provide insights into the dynamic interplay that exists between inputs from different chemosensory organs and genes in producing appropriate and sexually dimorphic behavioral responses to chemical stimuli.

Conclusions

This study has helped us identify genetic variants that explain a relatively large portion of the phenotypic and genetic variance associated with olfactory and gustatory behavioral responses to ethanol. This is in marked contrast with results from human association studies, where significant SNPs explain only a small fraction of the total phenotypic variance (Manolio et al. 2009) and illustrates the power of conducting marker-trait association using the *Drosophila* model system.

We have gained insights into the genetic architecture underlying olfactory and gustatory chemosensory behavioral responses to the same ecologically relevant chemical by revealing the effects of alleles on the traits, its sexually dimorphic characteristics, identification of genes with pleiotropic effects, and possible selective pressure that affect allele frequency within the population.

This study also reveals the power of exploiting naturally occurring allelic variation to identify genes affecting variation in chemosensory traits. Using this approach, we were able to identify novel genes, including chemosensory genes, involved in chemosensory behavioral responses to ethanol. Our results provide insights into the dynamic interplay that exists between inputs from different chemosensory organs and genes in producing appropriate behavioral responses to chemical stimuli.

Finally, knowledge of the causal variant affecting olfactory and gustatory behavioral responses to ethanol afford us the opportunity to derive random mating population from the DGRP lines to conduct laboratory evolution experiment to assess the type of selective pressures that act on these traits (Burke et al. 2010).

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CHAPTER FOUR

QUANTITATIVE TRAIT TRANSCRIPTS ASSOCIATED WITH CHEMOSENSORY BEHAVIORAL RESPONSES TO ETHANOL

Chemosensory behaviors are quantitative traits which are influenced by numerous genes, the environment, and gene by environmental interactions. Natural variation in such behavioral responses is attributable to segregating variants at multiple interacting loci, the effects of which are sensitive to the environment to which the individuals are exposed (Falconer and Mackay 1996). Association of DNA sequence variation, such as single nucleotide polymorphisms (SNPs), with variation in organismal phenotypes using a Genome Wide Association (GWA) mapping approach can help identify candidate causal polymorphisms and provide information about the genetic architecture underlying phenotypic variation (Mackay 2010). However, associating DNA sequence variation with variation in organismal phenotype does not take into account the intermediate steps that are involved in producing such variation (Mackay et al. 2009). For example, polymorphisms associated with naturally occurring variation in quantitative traits do not affect the trait directly, but do so by impacting the transcription of genes that interact to produce the trait.

Systems Genetics Analysis of Chemosensory Behavior

Quantitative trait nucleotides (QTNs) are causal molecular variants that affect variation in a quantitative trait, and can be identified using a GWA approach (as detailed in Chapter Three). However, identifying a list of all genes and QTNs associated with a quantitative trait does not place them in a biologically relevant context. QTNs do not affect the traits directly but do so through interrelated

networks of transcriptional, protein, metabolic, and other molecular endophenotypes (Mackay et al. 2009). A systems genetic approach will enable us to integrate DNA sequence variation, variation in transcript abundance, and other molecular phenotypes to variation in chemosensory behavioral responses to ethanol and allow us to interpret genetic variation in terms of biologically meaningful causal networks of correlated transcripts (Sieberts and Schadt 2007).

As described in Chapters Two and Three, the *Drosophila melanogaster* Genetic Reference Panel (DGRP) has been fully sequenced (Mackay et al. 2012) and phenotyped for olfactory and gustatory behavioral responses to ethanol.

Additionally, whole-genome transcript abundance for each line within the DGRP has been obtained (Carbone et al. 2012, in preparation). Transcripts do not function in isolation and the level of expression of many genetically variable transcripts co-vary between individuals in a mapping population (Rockman and Kruglyak 2006; Sieberts and Schadt 2007; Ayroles et al. 2009). Genetically correlated transcripts that fall within a regulatory network might be coexpressed, and identifying such networks can provide insights into the underlying biology of quantitative traits by providing information about relevant pathways, gene ontology categories, tissue-specific expression patterns, and transcription factor binding sites. Statistical methods can be used to group genetically correlated transcripts into modules such that each module contains a group of transcripts with higher correlations to each other than the rest of the transcriptome (Stone and Ayroles 2009).

Quantitative trait transcripts (QTTs) are transcripts for which variation in expression is correlated with variation in a quantitative trait (Mackay et al. 2009) and we can identify candidate QTTs, by associating variation in transcript abundance to variation in the trait. QTTs provide information about the different transcripts affecting a trait and can be used to construct modules of correlated QTTs to identify pathways and processes associated with organismal quantitative traits (Ayroles et al. 2009; Harbison et al. 2009). Additionally, DNA sequence data and transcriptional profiling facilitate the identification of regions within the genome, known as expression quantitative trait loci (eQTLs), containing one or more genes that affect variation in gene expression through linkage to polymorphic marker loci (Gilad et al. 2008; Hansen et al. 2008). Information about the location of the molecular variant (QTN) can help determine whether an eQTL is *cis* or *trans* acting. *cis* acting eQTLs are molecular variants located within a gene region that regulated the expression of that gene, whereas *trans* acting eQTLs are polymorphisms associated with variation in a transcript of another gene (Rockman and Kruglyak 2006). Information about *cis* and *trans* acting eQTLs can be used to determine the flow of information within networks of correlated transcripts and infer which transcripts are directly affected by genetic perturbations and which are coregulated by genetic perturbations (Mackay et al. 2009). Thus, in the case where variation in organismal phenotype is influenced by variation in gene expression, a systems genetic approach can be used to identify the causal molecular variant (a QTN) that is associated with variation of an organismal phenotype (a QTL) and associated with variation in gene expression (an eQTL).

This approach can also be used to identify variation in gene expression that is correlated with variation in the organismal phenotype (a QTT) (Rockman 2008; Mackay et al. 2009).

As mentioned above, I have measured the olfactory and gustatory behavioral responses to ethanol and identified SNPs associated with variation in these traits. Whole-genome transcript abundance data for each line within the DGRP have been obtained (Carbone et al. 2012, in preparation) and will facilitate the identification of eQTLs, coexpression networks of genetically correlated transcripts, and QTTs. In this Chapter, I report the QTTs that are associated with variation in olfactory and gustatory behavioral responses to ethanol.

MATERIALS AND METHODS

***Drosophila* Rearing and Collection**

All lines were reared on standard cornmeal molasses agar medium at 25°C, 60-75% relative humidity, 12 hour light/dark cycle, and controlled larval densities. For each line, we collected two replicates per sex for analysis of gene expression, consisting of 25 female flies or 40 male flies per replicate (~25 mg each), for a total of 768 samples. As all replicates from all the lines could not be collected simultaneously, we used a strict randomized experimental design for sample collection. Three to five day old mated flies were collected between 1-3 pm and stored at -80°C until they were ready to be processed.

RNA Extraction

The flies were homogenized with 1 ml of QIAzol lysis reagent (Qiagen). Total RNA was extracted using the miRNeasy 96 kit (Qiagen). The RNA was eluted with 45 μ l of RNase-free water. The eluted samples contain total RNA including miRNAs and other small RNAs (\geq 18 nucleotides). Total RNA was quantified using a NanoDrop 8000 spectrophotometer (Thermo Scientific) and the concentrations of the RNA samples adjusted to 1 μ g/ μ l for preparation of biotin-labeled double-stranded cDNA.

Preparation of Whole Transcript Double-Stranded cDNA

First strand cDNA was prepared from 7 μ g of total RNA (1 μ g/ μ l) with 1 μ l of random primers (3 μ g/ μ l) (Invitrogen) and incubating at 70°C (5 minutes) followed by 25°C (5 minutes) and 4°C (10 minutes). We added 5x first-strand buffer (4 μ l; Invitrogen), 0.1 M dithiothreitol (2 μ l; Invitrogen), 10 mM dNTP+dUTP (1 μ l; Promega), RNase Inhibitor (1 μ l; Invitrogen) and SuperScript II (4 μ l; Invitrogen) and incubated the reactions in a thermal cycler. Second-strand cDNA was synthesized by adding 17.5 mM MgCl₂ (8 μ l; Sigma), 10 mM dNTP+dUTP (1 μ l; Promega), DNA Polymerase I (1.2 μ l; Promega), RNase H (0.5 μ l; Promega) and RNase-free water (9.3 μ l; Ambion) to the first-strand cDNA reactions. The reactions were incubated in a thermal cycler at 16°C for 2 hours (without heated lid) followed by 75°C for 10 minutes (with heated lid) and 4°C for 10 minutes. Double-stranded cDNA was purified using the QIAquick 96 PCR kit (Qiagen) by following the manufacturer's protocol except that buffer PN was used instead of buffer PM. The cDNA was eluted

with 45 μ l of RNase-free water and quantified using a NanoDrop 8000 spectrophotometer (Thermo Scientific).

Fragmentation and Biotin-Labeling of Double-Stranded cDNA

The double-stranded cDNA (7.5 μ g) was fragmented with 4.8 μ l 10 X fragmentation buffer (Affymetrix), 1.5 μ l UDG (10 U/ μ l; Affymetrix), 2.25 μ l APE 1 (100 U/ μ l; Affymetrix) and RNase-free water (up to 48 μ l; Affymetrix) using a thermal cycler.

The fragmented double-stranded DNA (45 μ l) was biotin-labeled by incubation with 12 μ l of 5X TdT buffer (Affymetrix), 2 μ l of 30 U/ μ l TdT (Affymetrix) and 1 μ l of 5 mM DNA labeling reagent (Affymetrix) in a thermal cycler using the following protocol: 37°C (1 hour), 70°C (10 minutes) and 4°C (10 minutes). Hybridization cocktail (164 μ l) was added to 7 μ g of fragmented and labeled double-stranded cDNA for hybridization to *Drosophila* 2.0R Tiling Arrays (Affymetrix). We randomized RNA extraction, labeling and hybridization across all samples.

Quality Control and Normalization

We visualized the spatial distribution of probe intensities using the R package STARR (Zacher et al. 2010) to identify technical artifacts on the arrays (e.g., salt rings from reagents). Arrays were considered to be outliers if the mean expression of probes on the array was \pm two standard deviations of the sample mean from all arrays in the study; or if the variance of probe expression was \pm two standard deviations from the sample mean variance of arrays used in the study. Outlier

arrays and arrays with visible spatial artifacts were not considered for the analysis and samples from these arrays were re-hybridized to new arrays. We normalized raw \log_2 expression values for each array by mean centering.

Analysis to Identify QTTs

We used regression models in the form of $Y = \mu + S + T + S \times T + \epsilon$, where S denotes sex and T chemosensory traits to identify QTTs significantly ($P < 0.01$) associated with variation in olfactory and gustatory behavioral responses to ethanol. Reduced analyses of the form $Y = \mu + T + \epsilon$ were also performed for males and females separately.

Bioinformatics

Statistical analyses were performed using SAS software (SAS, Cary, NC, USA). Functional gene annotations are based on FlyBase (Drysdale and Crosby 2005; Wilson et al. 2008; Tweedie et al. 2009). Gene ontology enrichment analysis used the DAVID program (Huang da et al. 2009). Tissue specific gene expression data were obtained from FlyAtlas (Chintapalli et al. 2007).

RESULTS

QTTs Associated with Variation in Chemosensory Behavioral Responses to Ethanol

We used regression models and tested 14,639 transcripts to identify QTTs significantly associated with variation in olfactory and gustatory preference for ethanol consumption in 167 DGRP lines at $p < 0.01$. We performed the analysis for males and females separately and also pooled across sexes.

QTTs associated with olfactory preference for ethanol

We found 108 QTTs associated with phenotypic variation in olfactory behavioral response to ethanol. 26 transcripts were significant for females (Table 4.1), 34 transcripts were significant for males (Table 4.2), 52 were significant pooled across sexes (Table 4.3), and 17 were significant for the interaction term (Table 4.4).

There was no overlap between the QTTs in males and females. From the GWA analyses that identified candidate SNPs associated with variation in olfactory behavioral response to ethanol (described in Chapter Three), we found an overlap between the gene *CG6149*, identified through SNP *3L_11427728* which is 129 base pairs upstream of the gene, and the QTT identifying the transcript for the same gene. Since this SNP is immediately upstream of the *CG6149*, it might be regulating the expression of this gene and acting as a *cis* eQTL.

Three candidate chemosensory genes (*Or24a*, *Obp51a*, and *Obp99a*) were identified as QTTs associated with variation in olfactory response to ethanol. Out of these, *Obp51a* was identified as a candidate chemoreceptor associated with variation in the gustatory response to ethanol (Chapter Three), suggesting its role in mediating both olfactory and gustatory chemosensory behavioral responses to ethanol. Interestingly, *Obp99a* exhibits sexual dimorphic expression (Anholt et al. 2003) and was identified as a candidate QTT under the trait by sex interaction term (Table 4.4), suggesting that differences in expression among the sexes might be a reason for the observed sexually dimorphic responses among males and females. *Or24a* was also identified as a candidate QTT under the trait by sex interaction term (Table 4.4). Further experiments to determine *Or24a* expression pattern among males and females might reveal the mechanisms through which it causes sexual dimorphic chemosensory behavioral responses. However, the role of *Or24a* in olfactory behavioral response to ethanol has to be validated since it was identified through statistical associations with the trait.

Table 4.1: Female specific QTTs significantly associated with olfactory behavioral response to ethanol.

No.	SYMBOL	NAME	QTT <i>P</i> -value (Female)
1	<i>CG9463</i>	-	0.0002
2	<i>Spn43Aa</i>	<i>Serine protease inhibitor 43Aa</i>	0.0016
3	<i>Obp51a</i>	<i>Odorant-binding protein 51a</i>	0.0017
4	<i>CG12009</i>	-	0.0021
5	<i>CG6508</i>	-	0.0022
6	<i>CG3984</i>	-	0.0025
7	<i>NT1</i>	<i>Neurotrophin 1</i>	0.0026
8	<i>CG32277</i>	-	0.0026
9	<i>Adhr</i>	<i>Adh-related</i>	0.0028
10	<i>snoRNA:Me28S-C2645a</i>	-	0.0028
11	<i>Uhg3</i>	<i>Uhg3</i>	0.0028
12	<i>CG12929</i>	-	0.0029
13	<i>bgn</i>	<i>benign gonial cell neoplasm</i>	0.0029
14	<i>snoRNA:Psi18S-996</i>	<i>snoRNA:Psi18S-996</i>	0.0036
15	<i>CG42763</i>	-	0.0036
16	<i>CG6912</i>	-	0.0042
17	<i>CR42756</i>	-	0.0045
18	<i>tRNA:CR30449</i>	<i>transfer RNA:CR30449</i>	0.0052
19	<i>neo</i>	<i>neyo</i>	0.0052
20	<i>Pros28.1A</i>	<i>Proteasome 28kD subunit 1A</i>	0.0054
21	<i>CG4942</i>	-	0.0070
22	<i>CG15550</i>	-	0.0073
23	<i>CG43273</i>	-	0.0074
24	<i>tRNA:S7:23Eb</i>	<i>transfer RNA:ser7:23Eb</i>	0.0075
25	<i>CG9323</i>	-	0.0079
26	<i>CG11529</i>	-	0.0089

Table 4.2: Male specific QTTs significantly associated with olfactory behavioral response to ethanol.

No.	SYMBOL	NAME	QTT P-value (Male)
1	CG34326	-	0.0002
2	CG14673	-	0.0003
3	GstE2	Glutathione S transferase E2	0.0009
4	CG33276	-	0.0022
5	CG8800	-	0.0027
6	CG11951	-	0.0031
7	Cpr67Fa2	Cuticular protein 67Fa2	0.0033
8	fusl	fuseless	0.0033
9	CG33509	-	0.0036
10	Oseg6	Oseg6	0.0037
11	snoRNA:Me28S-G3255a	snoRNA:Me28S-G3255a	0.0039
12	snoRNA:Or-CD11	-	0.0044
13	O-fut1	O-fucosyltransferase 1	0.0045
14	CG34428	-	0.0046
15	CG30105	-	0.0048
16	CG7593	-	0.0049
17	CG17440	-	0.0049
18	CG42692	-	0.0053
19	NijA	Ninjurin A	0.0054
20	CG6225	-	0.0057
21	CG5114	-	0.0057
22	pad	poils au dos	0.0061
23	CG43343	-	0.0062
24	CG42372	-	0.0062
25	Lcch3	Ligand-gated chloride channel homolog 3	0.0067
26	CG31638	-	0.0072
27	CG7889	-	0.0075
28	PCID2	PCI domain-containing protein 2	0.0078
29	CG7407	-	0.0080
30	Mdr65	Multiple drug resistance 65	0.0085
31	snoRNA:U31:54Ed	snoRNA:U31:54Ed	0.0088
32	pie	pineapple eye	0.0095
33	tRNA:G3:35Bc	transfer RNA:gly3:35Bc	0.0095
34	CG7579	-	0.0098

Table 4.3: QTTs averaged across sexes significantly associated with olfactory behavioral response to ethanol.

No.	SYMBOL	NAME	QTT P-value (Pooled)
1	CG14673	-	0.0001
2	GstE2	Glutathione S transferase E2	0.0005
3	Adhr	Adh-related	0.0006
4	CG11951	-	0.0008
5	CG6508	-	0.0009
6	tRNA:CR30449	transfer RNA:CR30449	0.0011
7	CG3984	-	0.0012
8	CG6912	-	0.0014
9	Adh	Alcohol dehydrogenase	0.0015
10	gcl	germ cell-less	0.0017
11	neo	neyo	0.0018
12	Stlk	Ste20-like kinase	0.0019
13	CG31928	-	0.0019
14	CG32277	-	0.0019
15	CG6225	-	0.0021
16	CG13196	-	0.0023
17	CG13005	-	0.0024
18	CG7829	-	0.0025
19	cin	cinnamon	0.0026
20	CG12929	-	0.0030
21	se	sepia	0.0032
22	mago	mago nashi	0.0036
23	CG6843	-	0.0036
24	Cht7	Cht7	0.0037
25	CG17440	-	0.0039
26	CG16885	-	0.0042
27	tRNA:R2:84Fc	transfer RNA:arg2:84Fc	0.0046
28	CG33288	-	0.0047
29	CG14568	-	0.0051
30	CG6149	-	0.0051
31	CG9068	-	0.0052
32	BoYb	Brother of Yb	0.0053
33	CG32822	-	0.0057
34	CG42259	-	0.0057
35	CG18301	-	0.0059
36	CG33276	-	0.0061
37	Teh4	tipE homolog 4	0.0062
38	CG42692	-	0.0064
39	LysP	Lysozyme P	0.0067
40	crok	crooked	0.0067
41	CG9236	-	0.0069
42	CG6425	-	0.0072
43	CG31636	-	0.0081
44	CG34219	-	0.0082
45	wac	wee Augmin	0.0083
46	CG14323	-	0.0085
47	Pig1	Pre-intermoult gene 1	0.0088

Table 4.3: Continued

No.	SYMBOL	NAME	QTT P-value (Pooled)
48	CG32350	-	0.0089
49	CG43124	-	0.0090
50	<i>Lcch3</i>	<i>Ligand-gated chloride channel homolog 3</i>	0.0097
51	<i>Ripalpha</i>	<i>RPA-interacting protein alpha</i>	0.0098

Table 4.4: QTTs for interaction term (Trait by Sex) significant associated with olfactory behavioral response to ethanol.

No.	SYMBOL	NAME	QTT P-value (QTTbySex)
1	CG9463	-	0.0013
2	<i>Or98P</i>	<i>Odorant receptor 98a pseudogene</i>	0.0017
3	<i>capa</i>	<i>capability</i>	0.0023
4	CG14826	-	0.0033
5	<i>snRNA:U7</i>	<i>small nuclear RNA U7</i>	0.0039
6	CG11617	-	0.0040
7	CR42756	-	0.0046
8	<i>Or24a</i>	<i>Odorant receptor 24a</i>	0.0062
9	CG13022	-	0.0067
10	CG34428	-	0.0068
11	<i>Rbp6</i>	<i>RNA-binding protein 6</i>	0.0074
12	<i>snoRNA:Psi18S-640d</i>	<i>snoRNA:Psi18S-640d</i>	0.0085
13	CG34134	-	0.0086
14	<i>tRNA:S7:64D</i>	<i>transfer RNA:ser7:64D</i>	0.0087
15	CG43273	-	0.0087
16	<i>Obp99a</i>	<i>Odorant-binding protein 99a</i>	0.0092
17	<i>gcm2</i>	<i>gcm2</i>	0.0096

QTTs associated with preference for ethanol consumption

We found 216 QTTs associated with phenotypic variation in behavioral response to ethanol consumption. 108 transcripts were significant for females (Table 4.5), 46 transcripts were significant for males (Table 4.6), 61 were significant pooled across sexes (Table 4.7), and 48 were significant for the interaction term (Table 4.8).

As in the case of QTTs associated with olfactory response to ethanol, we found no overlap between the QTTs associated with behavioral response to ethanol consumption in males and females. From the GWA analyses that identified candidate SNPs associated with variation in behavioral response to ethanol consumption (Chapter Three), we found an overlap between the gene *Sex peptide receptor (SPR)*, identified through the SNP X_5367870 present in an intron within the gene, and the QTT identifying the transcript for the same gene. We also found overlap between *Or88a*, which was identified both as a candidate chemoreceptor associated with variation in the response to ethanol consumption and a QTT associated with the same trait. The role of *Or88a* in inducing an aversive behavioral response to ethanol consumption was functionally validated using RNAi knockdown (Chapter Three).

We found no overlap between the QTTs associated with variation in olfactory behavior and the behavioral response to ethanol consumption. While we found a few genes that were identified in both the GWA analyses and as a QTT for the same chemosensory trait (described above), we also found overlap between genes that were identified by the GWA analyses for one trait and as a QTT for the other chemosensory trait. These include the genes *CG34381 (2L_5855743)*, *Glu-RI (3L_6621548)*, and *wb (2L_14309673)* identified in the olfactory GWA analyses and as QTTs associated with variation in behavioral response to ethanol consumption. The association of these genes to both traits suggests that they are pleiotropic in nature. Similarly, we found overlap between the chemosensory genes *Ir68b*, *Or13a*,

and *Ir10a* which were identified in the olfactory GWA analyses and as QTTs associated with behavioral response to ethanol consumption.

As described in Chapters Two and Three, the gustatory choice assay measures the gustatory behavioral response of ethanol consumption in flies while they detect it in their immediate proximity, both as a gustatory and an olfactory cue. Thus, it is not surprising that through this assay we identified genes associated with both olfactory and gustatory behavioral responses to ethanol.

The role of *Ir10a*, identified in the olfactory GWA analyses and as a QTT associated with behavioral response to ethanol consumption, in olfactory behavioral response to ethanol was functionally validated using a *Minos*-element mutant. *Ir10a* mutants exhibited reduced olfactory preference for ethanol, suggesting that activation of IR10a by ethanol, as an olfactory cue, induces an attractive behavioral response to ethanol. The association of *Ir10a* with variation in behavioral response to ethanol consumption suggests that it interacts with other chemosensory genes to modulate the aversive behavioral response of flies to ethanol consumption. However, the role of *Ir10a* in behavioral response to ethanol consumption has to be validated to support the above assumption.

Table 4.5: Female specific QTTs significantly associated with behavioral response to ethanol consumption.

No.	SYMBOL	NAME	QTT P-value (Female)
1	<i>tRNA:L2:44EF</i>	<i>transfer RNA:leu2:44EF</i>	0.0000
2	<i>CG4462</i>	-	0.0001
3	<i>snoRNA:Psi18S-525i</i>	<i>snoRNA:Psi18S-525i</i>	0.0002
4	<i>CG34446</i>	-	0.0003
5	<i>Est-Q</i>	<i>Esterase Q</i>	0.0007
6	<i>Cyp6a13</i>	<i>Cyp6a13</i>	0.0007
7	<i>lft</i>	<i>lowfat</i>	0.0008
8	<i>CG18186</i>	-	0.0008
9	<i>CG33128</i>	-	0.0008
10	<i>kon</i>	<i>kon-tiki</i>	0.0009
11	<i>CG10550</i>	-	0.0009
12	<i>Rcd-1r</i>	<i>Rcd-1 related</i>	0.0009
13	<i>Dms</i>	<i>Dromyosuppressin</i>	0.0010
14	<i>CG12849</i>	-	0.0011
15	<i>CG14572</i>	-	0.0012
16	<i>PIP82</i>	<i>PIP82</i>	0.0013
17	<i>Cht4</i>	<i>Chitinase 4</i>	0.0017
18	<i>Lcp2</i>	<i>Larval cuticle protein 2</i>	0.0019
19	<i>Osi16</i>	<i>Osiris 16</i>	0.0019
20	<i>Notum</i>	<i>Notum</i>	0.0020
21	<i>CG34166</i>	-	0.0021
22	<i>CG8708</i>	-	0.0021
23	<i>chp</i>	<i>chaoptic</i>	0.0022
24	<i>CG10097</i>	-	0.0022
25	<i>Cyp9f3Psi</i>	<i>Cyp9f3Psi</i>	0.0023
26	<i>Lectin-galC1</i>	<i>Galactose-specific C-type lectin</i>	0.0023
27	<i>CG11253</i>	-	0.0023
28	<i>CG6865</i>	-	0.0024
29	<i>tap</i>	<i>target of Poxn</i>	0.0025
30	<i>CG18031</i>	-	0.0025
31	<i>Pif1B</i>	<i>PFTAIRE-interacting factor 1B</i>	0.0026
32	<i>Glu-RI</i>	<i>Glutamate receptor I</i>	0.0027
33	<i>CG5707</i>	-	0.0027
34	<i>CG13618</i>	-	0.0028
35	<i>CG34289</i>	-	0.0028
36	<i>Obp57c</i>	<i>Odorant-binding protein 57c</i>	0.0031
37	<i>Or13a</i>	<i>Odorant receptor 13a</i>	0.0033
38	<i>CG32483</i>	-	0.0033
39	<i>Fer2</i>	<i>48 related 2</i>	0.0034
40	<i>CG12643</i>	-	0.0035
41	<i>CheA87a</i>	<i>Chemosensory protein A 87a</i>	0.0036
42	<i>CG1461</i>	-	0.0036
43	<i>CG32626</i>	-	0.0036
44	<i>CG32201</i>	-	0.0036
45	<i>net</i>	<i>net</i>	0.0038
46	<i>CG14367</i>	-	0.0039
47	<i>Tsp42E1</i>	<i>Tetraspanin 42E1</i>	0.0040
48	<i>CG31710</i>	-	0.0040

Table 4.5 continued

No.	SYMBOL	NAME	QTT P-value (Female)
49	<i>tRNA:S4:12Ee</i>	<i>transfer RNA:ser4:12Ee</i>	0.0041
50	<i>Lcp1</i>	<i>Larval cuticle protein 1</i>	0.0041
51	<i>CG9316</i>	-	0.0041
52	<i>CG31021</i>	-	0.0042
53	<i>Muc26B</i>	<i>Mucin 26B</i>	0.0043
54	<i>Ir10a</i>	<i>Ionotropic receptor 10a</i>	0.0044
55	<i>CG6067</i>	-	0.0044
56	<i>CG9109</i>	-	0.0044
57	<i>snoRNA:Me18S-A934</i>	-	0.0045
58	<i>CG31404</i>	-	0.0048
59	<i>CG7607</i>	-	0.0049
60	<i>Gal</i>	<i>beta galactosidase</i>	0.0050
61	<i>Or88a</i>	<i>Odorant receptor 88a</i>	0.0050
62	<i>CG12691</i>	-	0.0051
63	<i>wb</i>	<i>wing blister</i>	0.0055
64	<i>CG10041</i>	-	0.0055
65	<i>CG14400</i>	-	0.0055
66	<i>ninaC</i>	<i>neither inactivation nor afterpotential C</i>	0.0056
67	<i>Tret1-1</i>	<i>Trehalose transporter 1-1</i>	0.0057
68	<i>tRNA:CR32456</i>	<i>transfer RNA:CR32456</i>	0.0057
69	<i>Gr23a</i>	<i>Gustatory receptor 23a</i>	0.0057
70	<i>CG2082</i>	-	0.0059
71	<i>CG43292</i>	-	0.0060
72	<i>CG42508</i>	-	0.0060
73	<i>CG15269</i>	-	0.0060
74	<i>CG43153</i>	-	0.0061
75	<i>CG10623</i>	-	0.0061
76	<i>Gyc-89Da</i>	<i>Guanylyl cyclase at 89Da</i>	0.0062
77	<i>CG34167</i>	-	0.0063
78	<i>CG43370</i>	-	0.0067
79	<i>Mal-B2</i>	<i>Maltase B2</i>	0.0068
80	<i>CG3259</i>	-	0.0069
81	<i>CG13239</i>	-	0.0070
82	<i>CG14054</i>	-	0.0071
83	<i>RabX4</i>	<i>RabX4</i>	0.0072
84	<i>Pjf1A</i>	<i>PFTAIRE-interacting factor 1A</i>	0.0073
85	<i>Lcp1Psi</i>	<i>Larval cuticle protein 1 pseudogene</i>	0.0076
86	<i>Gr97a</i>	<i>Gustatory receptor 97a</i>	0.0076
87	<i>CG17234</i>	-	0.0078
88	<i>snoRNA:Or-CD11a</i>	-	0.0078
89	<i>CG17324</i>	-	0.0078
90	<i>axo</i>	<i>axotactin</i>	0.0079
91	<i>CG9101</i>	-	0.0080
92	<i>CG8386</i>	-	0.0080
93	<i>CG30197</i>	-	0.0080
94	<i>CG17107</i>	-	0.0081
95	<i>CG7787</i>	-	0.0082
96	<i>CG18754</i>	-	0.0083

Table 4.5 continued

No.	SYMBOL	NAME	QTT P-value (Female)
97	<i>Ret</i>	<i>Ret oncogene</i>	0.0084
98	<i>CG15278</i>	-	0.0086
99	<i>CG14598</i>	-	0.0086
100	<i>CG43252</i>	-	0.0087
101	<i>CG2010</i>	-	0.0088
102	<i>CG43155</i>	-	0.0091
103	<i>Ir54a</i>	<i>Ionotropic receptor 54a</i>	0.0092
104	<i>CG12558</i>	-	0.0092
105	<i>Gr93d</i>	<i>Gustatory receptor 93d</i>	0.0093
106	<i>CG34234</i>	-	0.0095
107	<i>CG12116</i>	-	0.0098
108	<i>CG5160</i>	-	0.0100

Table 4.6: Male specific QTTs significantly associated with behavioral response to ethanol consumption.

No.	SYMBOL	NAME	QTT P-value (Male)
1	CG15741	-	0.0002
2	CG31279	-	0.0010
3	CG3999	-	0.0015
4	CG11458	-	0.0018
5	CG43689	-	0.0022
6	snoRNA:Me28S-A771	-	0.0025
7	tRNA:CR30240	transfer RNA:CR30240	0.0027
8	CG12768	-	0.0039
9	Jon25Biii	Jonah 25Biii	0.0039
10	CG32857	-	0.0042
11	CG33502	-	0.0043
12	CG32500	-	0.0043
13	rept	reptin	0.0047
14	CG34381	-	0.0048
15	lectin-46Cb	lectin-46Cb	0.0050
16	ast	asteroid	0.0050
17	Dip3	Dorsal interacting protein 3	0.0051
18	CG2694	-	0.0051
19	CG34216	-	0.0051
20	CG9286	-	0.0054
21	Manf	Mesencephalic astrocyte-derived neurotrophic factor	0.0056
22	sas	stranded at second	0.0056
23	CG11340	-	0.0058
24	CG13992	-	0.0059
25	pncr015:3L	putative noncoding RNA 015:3L	0.0061
26	Papss	PAPS synthetase	0.0061
27	CG10559	-	0.0065
28	CG31337	-	0.0067
29	CG9641	-	0.0067
30	mRpL39	mitochondrial ribosomal protein L39	0.0069
31	CG14500	-	0.0071
32	Pros26.4	Proteasome 26S subunit subunit 4 ATPase	0.0073
33	CG34144	-	0.0074
34	mms4	-	0.0074
35	CG33494	-	0.0075
36	ple	pale	0.0077
37	CR15280	-	0.0080
38	Chi	Chip	0.0082
39	CG42649	-	0.0086
40	CG1109	-	0.0090
41	CG2256	-	0.0093
42	CG30127	-	0.0095
43	CG11221	-	0.0096
44	CCHa2r	CCHamide-2 receptor	0.0097
45	CG11350	-	0.0099
46	CG17801	-	0.0099

Table 4.7: QTTs averaged across sexes significantly associated with behavioral response to ethanol consumption.

No.	SYMBOL	NAME	QTT P-value (Pooled)
1	CG17324	-	0.0003
2	CG15376	-	0.0005
3	<i>Lectin-galC1</i>	<i>Galactose-specific C-type lectin</i>	0.0005
4	CG14400	-	0.0007
5	<i>lectin-46Cb</i>	<i>lectin-46Cb</i>	0.0010
6	<i>ms(3)K81</i>	<i>male sterile (3) K81</i>	0.0010
7	<i>snoRNA:Psi18S-525i</i>	<i>snoRNA:Psi18S-525i</i>	0.0012
8	<i>PIP82</i>	<i>PIP82</i>	0.0013
9	<i>Cyp9f3Psi</i>	<i>Cyp9f3Psi</i>	0.0014
10	<i>Cpr65Av</i>	<i>Cuticular protein 65Av</i>	0.0014
11	CG18186	-	0.0016
12	CG8664	-	0.0017
13	CG42649	-	0.0017
14	CG43292	-	0.0022
15	CG12643	-	0.0023
16	<i>CheA7a</i>	<i>Chemosensory protein A 7a</i>	0.0025
17	CG42512	-	0.0028
18	<i>Osi19</i>	<i>Osiris 19</i>	0.0030
19	<i>tRNA:L2:44EF</i>	<i>transfer RNA:leu2:44EF</i>	0.0032
20	CG33128	-	0.0035
21	CG31782	-	0.0035
22	<i>tRNA:M2:48Bb</i>	<i>transfer RNA:met2:48Bb</i>	0.0036
23	CG14367	-	0.0036
24	<i>Osi1</i>	<i>Osiris 1</i>	0.0039
25	CG18754	-	0.0041
26	<i>Rcd-1r</i>	<i>Rcd-1 related</i>	0.0043
27	CG43707	-	0.0044
28	CR32835	-	0.0044
29	CG6763	-	0.0049
30	CG11694	-	0.0053
31	CG31728	-	0.0056
32	CG33494	-	0.0057
33	CG8854	-	0.0057
34	CG13239	-	0.0057
35	CG9642	-	0.0057
36	<i>B-H2</i>	<i>BarH2</i>	0.0059
37	<i>Cpr47Ea</i>	<i>Cuticular protein 47Ea</i>	0.0061
38	CG2256	-	0.0065
39	CG15741	-	0.0067
40	CG32246	-	0.0067
41	CG13088	-	0.0071
42	CG17639	-	0.0071
43	CR42874	-	0.0071
44	CG2975	-	0.0073
45	<i>snoRNA:Or-CD11a</i>	-	0.0073
46	CG12539	-	0.0074
47	CG4462	-	0.0079
48	<i>lig3</i>	<i>DNA ligase III</i>	0.0079

Table 4.7 continued

No.	SYMBOL	NAME	QTT P-value (Pooled)
49	<i>CG30033</i>	-	0.0079
50	<i>CG31279</i>	-	0.0080
51	<i>Prosbeta2R2</i>	<i>Proteasome beta2R2 subunit</i>	0.0085
52	<i>CG15186</i>	-	0.0086
53	<i>Lip4</i>	<i>Lipase 4</i>	0.0086
54	<i>net</i>	<i>net</i>	0.0089
55	<i>CG13386</i>	-	0.0089
56	<i>CG17801</i>	-	0.0090
57	<i>mRpL28</i>	<i>mitochondrial ribosomal protein L28</i>	0.0090
58	<i>CG13053</i>	-	0.0091
59	<i>Dip3</i>	<i>Dorsal interacting protein 3</i>	0.0093
60	<i>Cyp6t3</i>	<i>Cyp6t3</i>	0.0094
61	<i>CG30127</i>	-	0.0096

Table 4.8: QTTs for interaction term (Trait by Sex) significant associated with behavioral response to ethanol consumption.

No.	SYMBOL	NAME	QTT <i>P</i> -value (QTTbySex)
1	<i>Sclp</i>	<i>Sclp</i>	0.0011
2	CG31337	-	0.0013
3	CG3999	-	0.0016
4	CG11458	-	0.0016
5	CG11340	-	0.0018
6	<i>lft</i>	<i>lowfat</i>	0.0026
7	<i>Chi</i>	<i>Chip</i>	0.0027
8	CG34446	-	0.0031
9	CG15741	-	0.0033
10	CG31279	-	0.0033
11	<i>tRNA:CR30232</i>	<i>transfer RNA:CR30232</i>	0.0036
12	<i>kon</i>	<i>kon-tiki</i>	0.0036
13	CG11221	-	0.0040
14	<i>jeb</i>	<i>jelly belly</i>	0.0040
15	<i>tap</i>	<i>target of Poxn</i>	0.0041
16	CG10185	-	0.0044
17	CG15529	-	0.0049
18	<i>Est-Q</i>	<i>Esterase Q</i>	0.0050
19	<i>arg</i>	<i>arginase</i>	0.0051
20	CG32500	-	0.0051
21	CG17549	-	0.0051
22	CR43257	-	0.0054
23	CG10559	-	0.0057
24	CG13972	-	0.0057
25	CG8916	-	0.0058
26	<i>tra</i>	<i>transformer</i>	0.0058
27	CG32857	-	0.0062
28	CG33502	-	0.0062
29	<i>Gsc</i>	<i>Goosecoid</i>	0.0062
30	CG12849	-	0.0066
31	CG3940	-	0.0066
32	<i>Cad86C</i>	<i>Cad86C</i>	0.0067
33	CG31710	-	0.0069
34	CG42823	-	0.0070
35	<i>Ir68b</i>	<i>Ionotropic receptor 68b</i>	0.0074
36	<i>Cht5</i>	<i>Cht5</i>	0.0077
37	<i>yellow-e</i>	<i>yellow-e</i>	0.0079
38	CG43092	-	0.0079
39	<i>Pif1B</i>	<i>PFTAIRE-interacting factor 1B</i>	0.0083
40	CG4629	-	0.0083
41	<i>ade3</i>	<i>adenosine 3</i>	0.0084
42	CG5180	-	0.0089
43	CG34227	-	0.0091
44	<i>Dms</i>	<i>Dromyosuppressin</i>	0.0093
45	CG5162	-	0.0094
46	CG33226	-	0.0098
47	<i>SPR</i>	<i>Sex peptide receptor</i>	0.0098
48	<i>Neurochondrin</i>	<i>Neurochondrin</i>	0.0099

Gene Ontology Analysis

Gene ontology (GO) analysis reveals both similar and diverse categories of genes that confer variation in olfactory behavioral response and behavioral response to ethanol consumption. The GO categories prominently associated with variation in olfactory response to ethanol, for candidate QTTs, appear to be involved in metabolic processes and peptidase activity (Figure 4.1). GO analysis of candidate QTTs and the genes associated with variation in olfactory behavioral response to ethanol identified through the GWA analysis (Chapter Three) are shown in Figure 4.2. The genes are enriched for biological categories normally associated with chemosensory behaviors, such as sensory organ development, neuron development, and head segmentation.

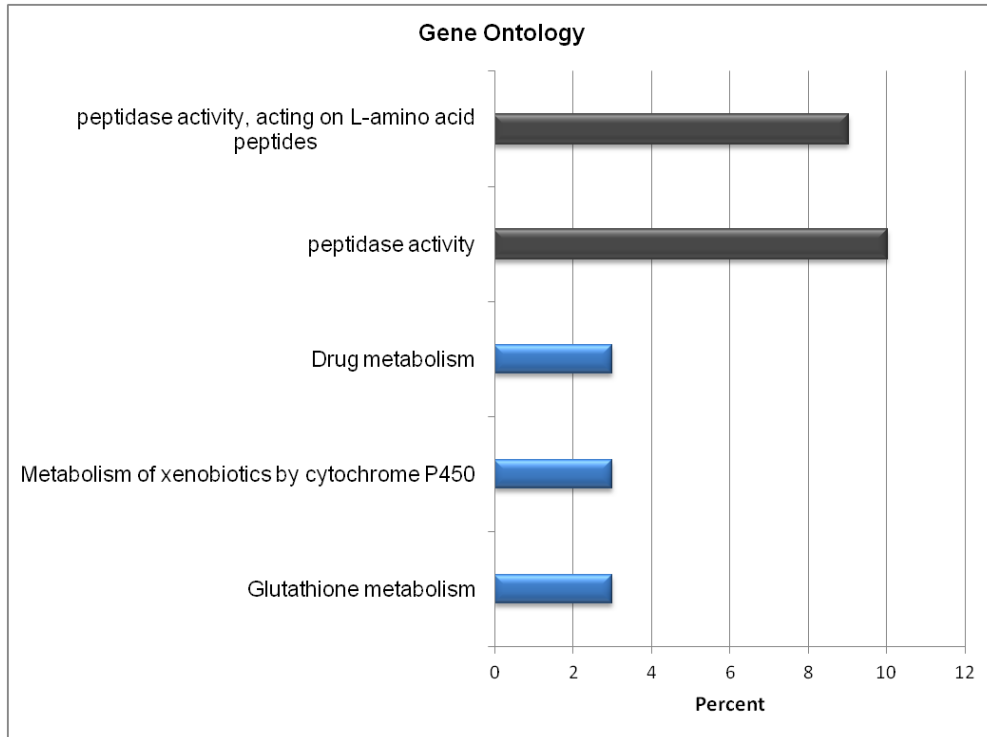


Figure 4.1: Gene ontology categories for candidate transcripts associated with variation in olfactory behavioral response to ethanol. The blue and black bars indicate percentage of input genes that fall within each molecular function and KEGG pathway, respectively. The x-axis indicated the percent of genes in each category at $p < 0.05$.

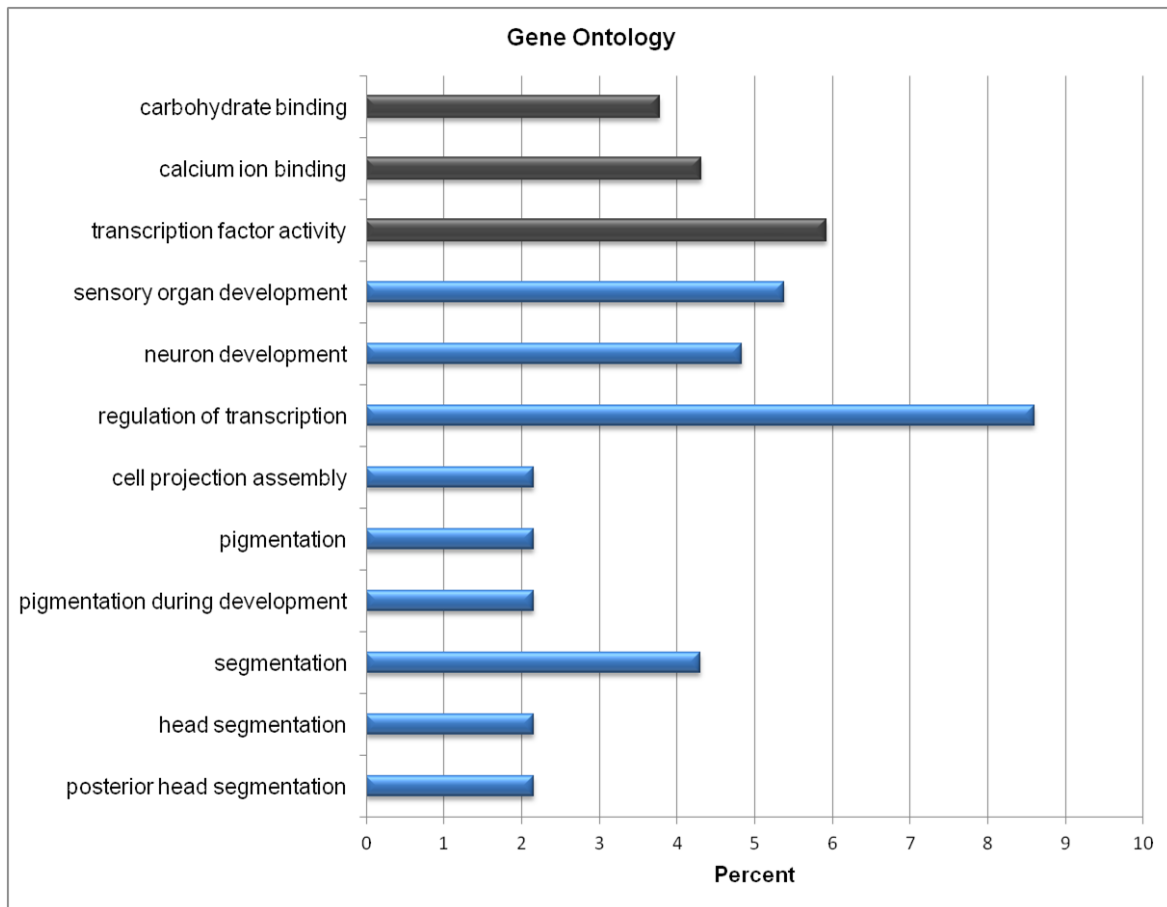


Figure 4.2: Gene ontology categories for candidate genes associated with variation in olfactory behavioral response to ethanol identified through GWA analysis and as QTTs. The blue and black bars indicate percentage of input genes that fall within each biological process, and molecular function, respectively. The x-axis indicated the percent of genes in each category at $p < 0.05$.

The GO categories prominently associated with variation in response to ethanol consumption include neurotransmitter binding, neurotransmitter receptor activity, proteolysis, sensory perception, and signal transduction (Figure 4.3).

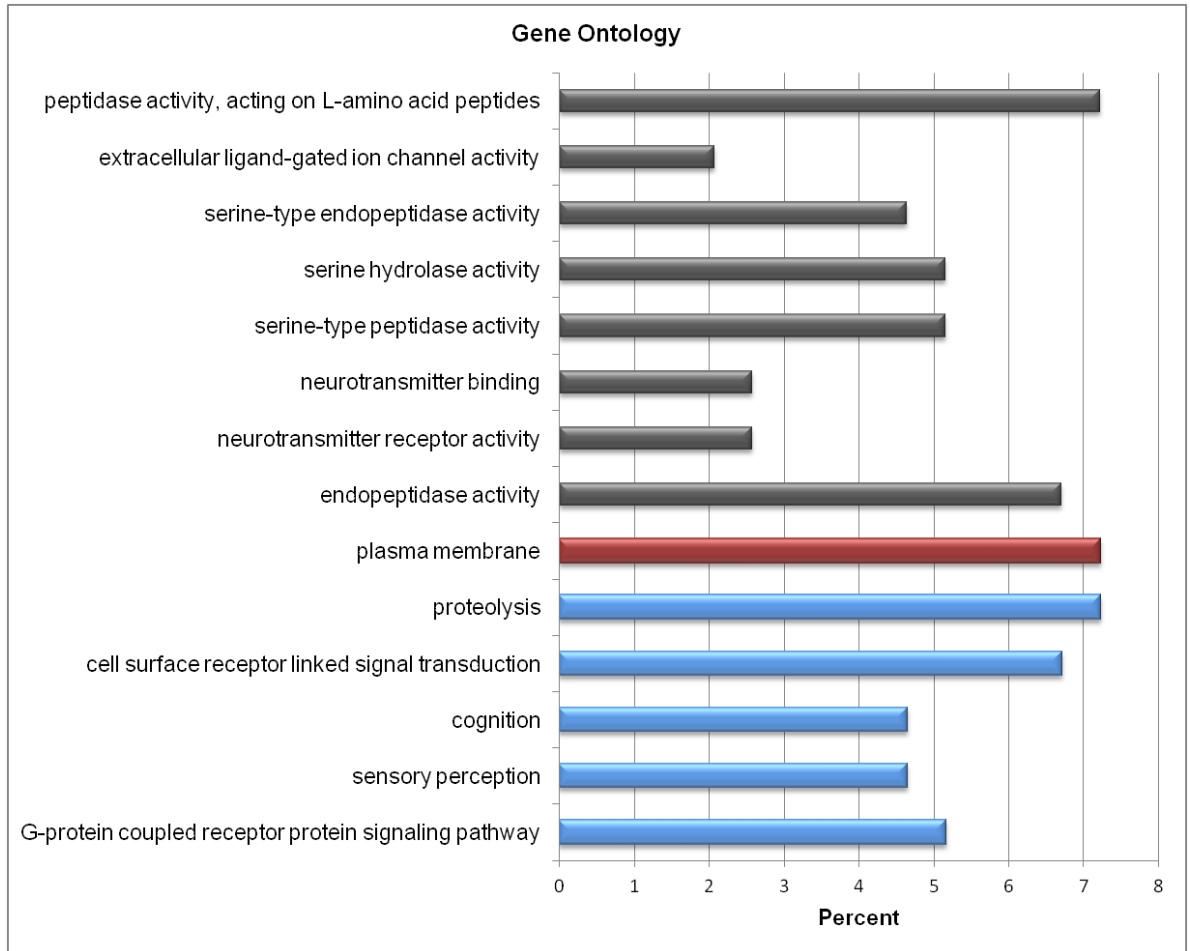


Figure 4.3: Gene ontology categories for candidate genes associated with variation in behavioral response to ethanol consumption. The blue, red, and black bars indicate percentage of input genes that fall within each biological process, cellular component, and molecular function, respectively. The x-axis indicated the percent of genes in each category at $p < 0.05$.

GO analysis of candidate QTTs and the genes associated with variation in behavioral response to ethanol consumption identified through the GWA analysis (Chapter Three) are shown in Figure 4.4. The genes are enriched for biological categories including G-protein coupled receptor activity, sensory perception, neuropeptide receptor activity, and signal transduction, all of which are essential in producing appropriate behavioral responses to chemical stimuli.

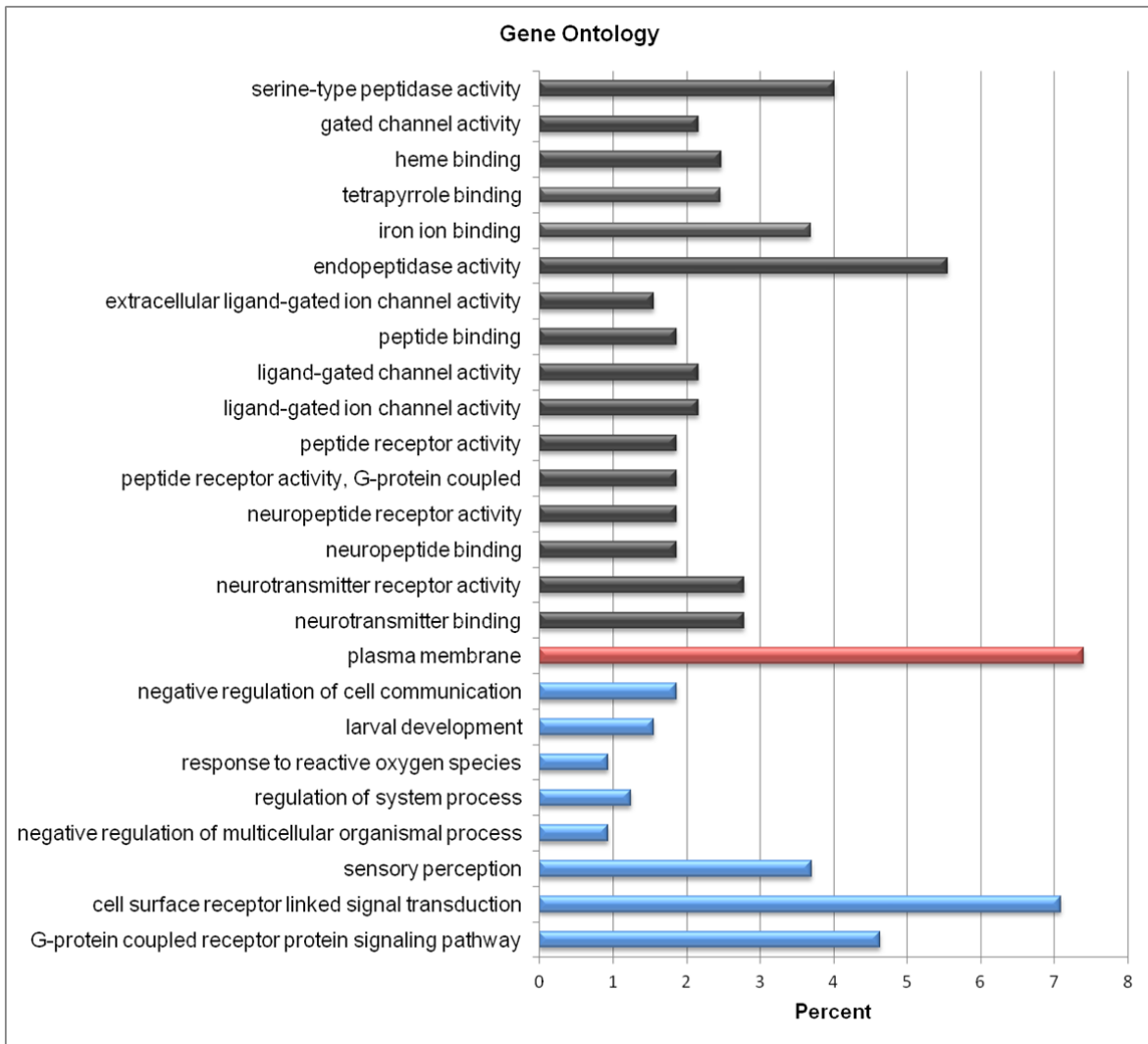


Figure 4.4: Gene ontology categories for candidate genes associated with variation in behavioral response to ethanol consumption identified through GWA analysis and as QTTs. The blue, red, and black bars indicate percentage of input genes that fall within each biological process, cellular component, and molecular function, respectively. The x-axis indicated the percent of genes in each category at $p < 0.05$.

Chemosensory behavioral responses involve processing information from the environment through sensory organs, and conveying it to the central nervous system via the peripheral nervous system. Ethanol-induced behaviors are mediated through neurotransmitter systems, neuropeptides, synaptic proteins, channels and signaling process (Scholz and Mustard 2011). Since both behavioral assays measured chemosensory behavioral responses to ethanol (Chapter Two), I included all the genes, identified through the GWA analyses and as QTTs for both chemosensory traits, and conducted a GO analysis to identify underlying biological processes that are shared in both olfactory and gustatory behavioral responses to ethanol. The analysis revealed that the genes are enriched for biological processes including posterior head segmentation, cell projection organization, G-protein coupled receptor activity, sensory perception, neuropeptide receptor activity, neuropeptide binding, neurotransmitter receptor activity, neurotransmitter binding, signal transduction, and GABA-A receptor activity (Figure 4.5). Interestingly, Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in mammalian brain and is associated with alcohol dependence (Agrawal et al. 2006). Antagonists of GABA B receptor reduce motion-impairing effect of ethanol in *Drosophila* (Dzitoyeva et al. 2003), suggesting the GABA signaling plays a role in regulation of direct ethanol action in the central nervous system of invertebrates (Scholz and Mustard 2011) and might also influence olfactory and gustatory behavioral responses to ethanol.

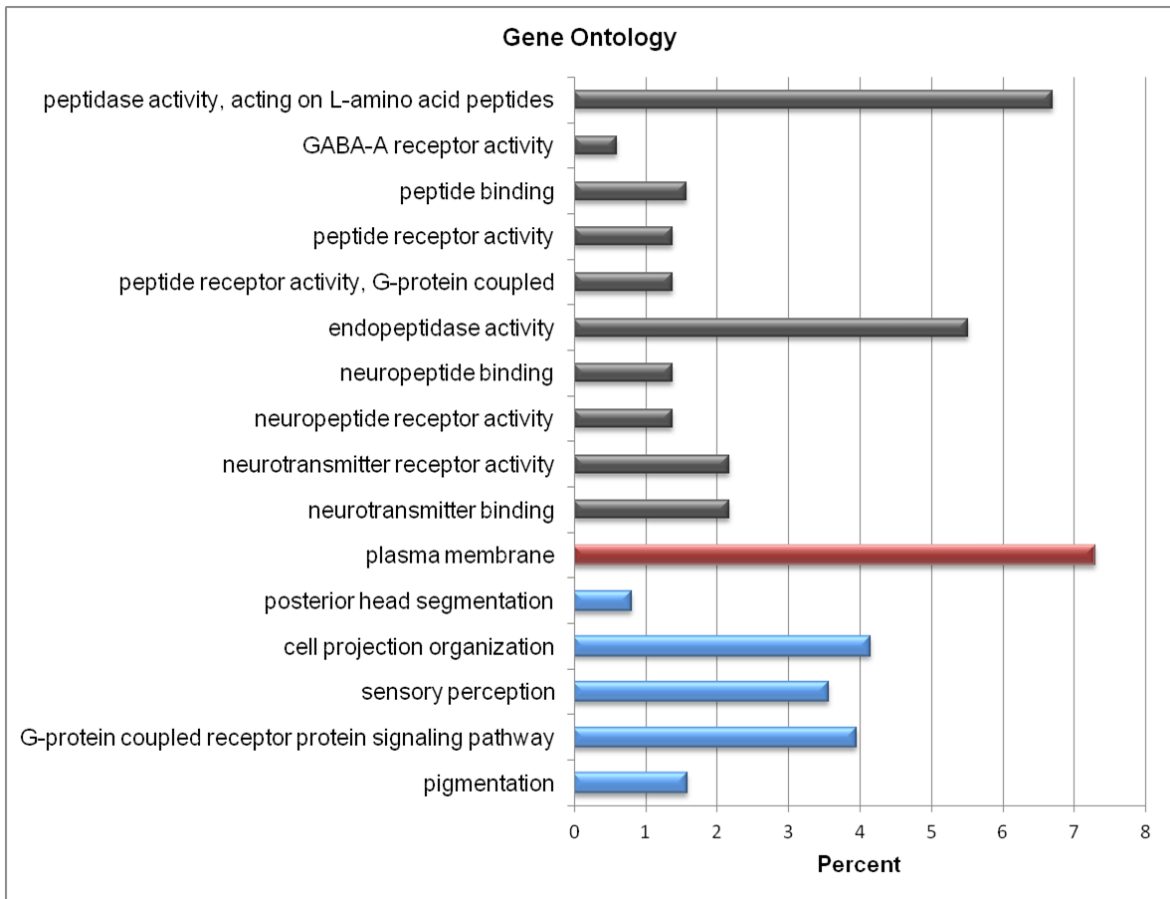


Figure 4.5: Gene ontology categories for candidate genes associated with variation in chemosensory behavioral responses to ethanol. The blue, red, and black bars indicate percentage of input genes that fall within each biological process, cellular component, and molecular function, respectively. The x-axis indicated the percent of genes in each category at $p < 0.01$.

DISCUSSION

This study utilizes whole-genome transcript data obtained from a large wild-derived inbred population of *D. melanogaster* (DGRP) (Carbone et al. 2012, in preparation) to identify quantitative trait transcripts that are genetically correlated with variation in olfactory behavioral response and behavioral response to ethanol consumption. This information along with information from the GWA analyses (Chapter Three) provides insights about relevant pathways and processes associated with chemosensory behaviors.

QTTs Associated with Variation in Chemosensory Preference for Ethanol

We identified 108 QTTs (Tables 4.1 to 4.4) associated with variation in olfactory behavioral response to ethanol and 216 QTTs (Tables 4.5 to 4.8) associated with variation in gustatory behavioral response to ethanol consumption.

We identified candidate genes that have been previously implicated in alcohol, glutathione, and xenobiotic metabolism. Flies encounter ethanol in their natural environment, in and around their food sources. As ethanol is toxic at high concentrations and has fitness cost associated with its consumption, flies need to minimize ethanol consumption and appropriately metabolize what they consume, (Bokor and Pecsénye 2000). *Alcohol dehydrogenase (Adh)* and *Adh-related (Adhr)* are involved in the main pathway of ethanol metabolism and are necessary for ethanol utilization as a nutrient and for ethanol tolerance throughout development

(Geer et al. 1989; Geer et al. 1993; Hernandez-Tobias et al. 2011). Experiments show that impaired Adh function is correlated with loss of ethanol preference at low concentrations and reduced aversion at high concentrations (Ogueta et al. 2010). *Glutathione S transferase E2 (GstE2)* is a detoxification enzyme that has been implicated in insecticide resistance (Hayes and Pulford 1995; Ranson et al. 2001) and believed to have evolved in response to toxins found in the food sources of the *Drosophila* species (Hayes and Pulford 1995; Low et al. 2007). We also identified three Cytochrome P450 genes (*Cyp6a13*, *Cyp6t3*, *Cyp9f3Ψ*). Since Cytochrome P450 enzymes are involved in xenobiotic metabolism (Coon et al. 1996; Tijet et al. 2001; Ranson et al. 2002), they might also play a role in ethanol metabolism. Tissue specific gene expression data (Chintapalli et al. 2007) reveals that *Adh*, *Adhr*, *GstE2*, *Cyp6a13*, and *Cyp9f3Ψ* are all expressed in the crop, mid-gut, and hind-gut which are tissues where ethanol metabolism would occur, suggesting their role in the metabolism and detoxification of ethanol consumed by the flies while feeding on rotting fruits.

Three candidate genes (*Or88a*, *CG6149*, and *Sex peptide receptor*) were associated with variation in the same chemosensory behavioral trait in the GWA analyses (described in Chapter Three) and as QTTs. Among these, I functionally validate *Or88a* using RNAi knockdown. The results suggest that activation of OR88a induces an aversive behavioral response to ethanol consumption (Chapter Three). The SNP (3R_9936530) within *Or88a*, associated with variation in behavioral response to ethanol consumption, is a synonymous substitution within the coding

region. Synonymous SNPs within coding regions can affect mRNA structure, stability, processing and/or translation efficiency (Duan et al. 2003; Wang et al. 2007) and thereby impact the functioning of a gene. Generally, SNPs in non-coding regulatory regions affect variation in expression of a transcript (Anholt 2011) and enable the identification of the transcript as a QTT associated with variation in an organismal phenotype. In the case of *Or88a*, we are not certain whether the synonymous substitution within the coding region of the gene is responsible for variation in its expression. Future analyses to identify eQTLs might help identify SNPs that affect the expression of *Or88a*. *CG6149* was identified as a candidate gene associated with variation in olfactory behavioral response to ethanol both as a QTT and through a SNP (*3L_11427728*) which is 129 base pairs upstream of the gene. This might be an example of a *cis* acting eQTL, where a SNP present within the regulatory region of a gene is the causal molecular variant and affects expression of that gene. The gene *Sex peptide receptor (SPR)*, which exhibits neuropeptide receptor activity and is involved in regulation of post-mating oviposition (Yapici et al. 2008), was identified as a candidate gene associated with variation in behavioral response to ethanol consumption both as a QTT and through a SNP (*X_5367870*) present in an intron within the gene. Further analysis needs to be conducted to determine whether this SNP is in or near a splice site, especially since SNPs within splice sites can potentially affect RNA splicing resulting in one or more mRNA which can be non-functional, have reduced stability, and/or translation efficiency.

The QTTs associated with variation in chemosensory behavioral responses to ethanol have identified candidate genes, but since these are statistical associations further studies using RNAi knockdown and/or *P*-element mutants will need to be conducted to validate their effect on the chemosensory traits.

Chemosensory Genes Associated with Variation in Olfactory and Gustatory Behavioral responses to Ethanol

We identified novel candidate chemosensory genes, that have not been previously implicated in ethanol induced behavioral responses, through QTTs associated with variation in olfactory (*Or24a*, *Obp51a*, and *Obp99a*) and gustatory (*Gr23a*, *Gr93d*, *Gr97a*, *Ir10a*, *Ir54a*, *Ir68b*, *Or13a*, *Or88a*, and *Obp57c*) behavioral responses to ethanol.

Out of the three chemosensory genes identified as QTTs associated with variation in olfactory response to ethanol, *Obp51a* was also identified as a candidate chemoreceptor associated with variation in behavioral response to ethanol consumption (Chapter Three), suggesting its role in mediating both olfactory and gustatory chemosensory behavioral responses to ethanol. Similarly, we found overlap between three chemosensory genes, *Ir68b*, *Or13a*, and *Ir10a*, which were identified in the olfactory GWA analyses and as QTTs associated with behavioral response to ethanol consumption. As discussed earlier, in the gustatory choice assay flies detect ethanol in their immediate proximity, both as a gustatory and olfactory cue. Therefore, it is not surprising that chemosensory genes activated by

both gustatory and olfactory cues are associated with variation in behavioral responses to ethanol consumption. *Ir10a* *Minos*-element mutants exhibited reduced olfactory preference for ethanol vapors (Chapter Three), suggesting that activation of *IR10a* as an olfactory cue induces an attractive behavioral response to ethanol. If *Ir10a* *Minos*-element mutants exhibit an enhanced aversive response to ethanol consumption compared to their controls, then this might suggest that it interacts with other chemosensory genes to modulate (in this case decrease) the aversive gustatory behavioral response of flies to ethanol consumption. However, the role of *Ir10a* in behavioral response to ethanol consumption has to be functionally tested to support the above hypothesis. Furthermore, association of a chemosensory gene with variation in olfactory or gustatory behavioral response to ethanol does not indicate whether its activation, by ethanol, will induce an attractive or aversive behavioral response in flies. We need to functionally test all of them to determine whether they induce attractive or aversive behavioral responses in flies.

We observed significant phenotypic and genetic variation in sexual dimorphic responses in olfactory and gustatory behavioral responses to ethanol (Chapter Two and Three). The sexually dimorphic expression of chemosensory genes might contribute to sexual dimorphic chemosensory behavioral responses to chemical stimuli (Bray and Amrein 2003; Schymura et al. 2010; Swarup et al. 2011). As described in Chapter Three, the GWA analyses identified *Or22b*, which is sexually dimorphic in its expression (Dobritsa et al. 2003), through SNPs significant for the SNP by sex interaction term. Similarly, *Or24a* and *Obp99a* were identified as

candidate QTTs associated with variation in olfactory behavioral response to ethanol and *Ir68b* was identified as a candidate QTT associated with variation in gustatory behavioral response to ethanol consumption, under the trait by sex interaction term. Interestingly, *Obp99a* also exhibits sexual dimorphic expression (Anholt et al. 2003), suggesting that differences in expression among the sexes might be a reason for the observed sexually dimorphic responses among males and females. Further experiments to determine *Or24a* and *Ir68b* expression pattern among males and females will provide information on the mechanisms through which they induce sexual dimorphic chemosensory behavioral responses. However, as mentioned above, these QTTs have to be functionally tested to confirm their role olfactory and gustatory behavioral responses to ethanol.

Gene Ontology Analysis

The gene ontology analyses to identify the biological processes associated with olfactory (Figures 4.1 and 4.2) and gustatory (Figures 4.3 and 4.4) behavioral responses to ethanol consumption reveal biological categories normally associated with chemosensory behaviors. When we combined all the candidate genes associated with variation in olfactory and gustatory behavioral responses to ethanol to identified GO categories shared between the traits, we identified biological categories including: posterior head segmentation, cell projection organization, G-protein coupled receptor activity, sensory perception, neuropeptide receptor activity, neuropeptide binding, neurotransmitter receptor activity, neurotransmitter binding,

signal transduction, and GABA-A receptor activity (Figure 4.5), many of which are associated with ethanol-induced behaviors (Scholz and Mustard 2011).

Interestingly, the GO analysis revealed that the genes associated with variation in olfactory and gustatory behavioral responses to ethanol include genes that exhibit GABA receptor activity, which is associated with alcohol dependence in humans (Agrawal et al. 2006). Also, antagonists of GABA B receptor reduce motion-impairing effect of ethanol in *Drosophila* (Dzitoyeva et al. 2003) suggesting that the GABA signaling plays a role in regulation of direct ethanol action in the central nervous system of invertebrates (Scholz and Mustard 2011). Taken together, this suggests GABA receptor activity might influence olfactory and gustatory behavioral responses to ethanol.

Future Analysis

Further analysis will be conducted utilizing whole-genome transcript data from the DGRP to construct genetically correlated transcriptional modules with higher correlations to each other than the rest of the transcriptome using statistical models (Stone and Ayroles 2009). This will enable us to group the QTTs identified in this study into modules consisting of transcripts with higher correlations to each other relative to the rest of the transcriptome. These coexpression networks should provide information about relevant pathways, gene ontology categories, tissue-specific expression patterns, and transcription factor binding sites (Ayroles et al. 2009; Harbison et al. 2009) and also help in predicting the function of

computationally predicted genes based on ‘guilt by association’ with well-annotated genes in the network (Ayroles et al. 2009; Harbison et al. 2009; Mackay et al. 2009). Additionally, incorporating information about *cis* and *trans* eQTLs, based on information from DNA polymorphisms, will enable us to determine the direction of flow of information within a network and infer which relationships are directly caused by genetic perturbations and which are coregulated by genetic perturbations (Mackay et al. 2009).

From our present analyses and that conducted in Chapter Three, we do not find much overlap between candidate genes identified as QTTs and from SNPs (except for *Or88a*, *SPR*, and *CG6149*) that were significantly associated with variation in olfactory and gustatory responses to ethanol. This is expected since variation in transcript expression is believed to be generally caused by SNPs in non-coding regulatory regions rather than within the coding regions of a gene (Anholt 2011). Analyses to identify eQTLs will help us determine which molecular variants affect variation in gene expression. From this information we will be able to dissect a QTN into its constituent eQTLs and QTTs (Mackay et al. 2009). While the eQTL analyses will help identify molecular variants that cause variation in a transcript, the transcript itself may not cause variation in the organismal phenotype but affect variation in other transcripts that are causal and lead to variation in the organismal phenotype (i.e. QTT). Using information about the SNPs significantly associated with the variation in both the chemosensory traits and information about eQTLs present in the DGRP, we will be able to identify transcripts for which variation in expression is

associated with this subset of significant SNPs. For example, take the case where a molecular variant affects the expression of a transcript which functions as a transcriptional factor but is not associated with variation in the chemosensory traits. Variation in this transcriptional factor might affect expression of its target genes. By identifying the transcriptional binding sites of this transcriptional factor, we will be able to identify transcripts containing those transcriptional binding sites and these transcripts might include the QTTs that are associated with variation in the organismal trait. Thus, we will be able to identify a causal variant associated with variation in the trait (a QTL), that is associated with gene expression (an eQTL), and variation in gene expression that is correlated with variation in olfactory or gustatory behavioral responses to ethanol (a QTT).

Another caveat to keep in mind is that as we obtained transcript data from whole adult flies that were between 3 to 5 days old, we will miss transcripts that are expressed at low levels in particular tissues and during different developmental stages. However, using gene expression levels in whole flies is an unbiased approach and has given us a wealth of information that we are starting to piece together and is providing invaluable insights into the genetic architecture underlying chemosensory behavioral responses to ethanol. With future decrease in costs of whole genome transcript profiling, subsequent studies on chemosensory behavioral phenotypes can be performed to examine variation in transcript expression at different developmental time points and in different tissues.

Conclusions

Ethanol is an ecologically relevant chemical encountered by flies in and around rotten fruits. Flies are attracted to ethanol as an olfactory cue and possibly use it to locate food sources. However, ethanol when consumed at high concentrations acts as a toxin and has fitness costs associated with it (Bokor and Pecsénye 2000). This creates an interesting situation where the same compound acts both as an attractant and a repellent depending on sensory modality through which and the context in which it is detected. This is reflected in the behavioral response of flies when they encounter ethanol. When ethanol is detected as an olfactory cue, flies are attracted to it. However, when it is detected in their food source as an olfactory and gustatory cue, flies try to minimize its consumption. These results suggest that these behavioral responses might have evolved as a mechanism to balance the need of finding food sources using olfactory cues while at the same time minimizing fitness costs associated with consuming a toxic compound producing such cues. Additional selective pressures might also be acting on females, to maintain preference for ethanol as an olfactory cue, especially if ethanol vapors are utilized to locate oviposition sites. This might be one of the reasons why we observed sexually dimorphic behavioral responses to ethanol. Future studies on use of ethanol as an oviposition site among females in the DGRP lines would provide insights into its role as an oviposition cue and whether it correlated with olfactory or gustatory behavioral responses to ethanol.

The present analyses have helped us identify candidate QTTs that were not identified in the GWA analyses. The GO ontology categories suggest that these genes are involved biological processes that are essential in producing appropriate behavioral responses to chemical stimuli. These genes enable the flies to detect ethanol, produce the appropriate behavioral responses, and metabolize and/or detoxify ethanol. It has also helped us identify candidate chemosensory genes that might provide insights into the dynamic interplay that exists between inputs from different chemosensory organs and genes in producing appropriate and sexually dimorphic behavioral responses to chemical stimuli.

Future analyses to identify eQTLs and coexpression networks will help us place causal variants in a biologically relevant context and allow us to predict the functions of computationally predicted genes based on 'guilt by association' with well-annotated genes within the coexpression networks (Ayroles et al. 2009; Mackay et al. 2009).

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CHAPTER FIVE

CONCLUSIONS AND FUTURE PERSPECTIVES

Behaviors are complex traits that are produced by the nervous system in response to internal and external environmental cues and enable animals to interact with and adapt to their environments. An animal's ability to interact and adapt to its environment is one of the most important factors in determining its fitness.

Behavioral traits provide the mechanisms through which evolutionary forces, such as natural selection, can act. Behavioral responses are influenced by many factors which include: the developmental history of the animal, its genetic makeup, its nervous system, its physiological state, and its physical and social environment. Animals encounter a wide array of odors in their natural environments and have evolved sophisticated chemosensory systems to detect and interpret such information. The ability to recognize and respond to chemical stimuli is critical to the fitness of most animals not only in terms of allowing an animal to locate food sources and identify mates, but also in detecting and avoiding predators and toxic compounds. Chemosensory behavioral responses involve perception of information from the environment, assessing its appropriate biological significance by integrating and processing that information in the brain, and directing motor outputs that translate into behavioral responses. Understanding the genetic architecture that underlies such traits involves identifying the genes (quantitative trait loci [QTLs]) regulating these traits, the subset of QTLs affecting naturally occurring variation in the trait, the homozygous, heterozygous, epistatic, and pleiotropic effects of QTL alleles, the molecular basis of allelic variation, and the evolutionary forces

responsible for maintaining genetic variation for quantitative traits in nature (Mackay 2004).

Chemosensory behaviors can be studied using a single gene mutational approach and/or an approach which exploits naturally occurring allelic variation to identify genes affecting variation in such behaviors (Anholt and Mackay 2004). Single gene mutation studies have been invaluable in functionally characterizing chemosensory genes (Sengupta et al. 1996; Dunipace et al. 2001; Stortkuhl and Kettler 2001; Hallem et al. 2004; Thorne et al. 2004; Wang et al. 2004; Xu et al. 2005; Hallem and Carlson 2006; Matsuo et al. 2007; Abuin et al. 2011; Weiss et al. 2011). However, since behaviors are complex traits influenced by numerous genes, the environment, and gene by environmental interactions (Falconer and Mackay 1996), single gene mutations studies are not appropriate to understand the genetic architecture underlying such behaviors.

The Genetic Architecture Underlying Chemosensory Behavioral Responses to Ethanol

In this study, I used a Genome Wide Association (GWA) mapping approach to study the genetic architecture underlying chemosensory behavioral responses to ethanol in *Drosophila melanogaster*. *Drosophila* encounters and is attracted to odors produced by rotten fruits, its primary food source, including ethanol which is produced by fermentation of yeast present in such fruits. Ethanol is probably used by *Drosophila* as a chemosensory cue to detect and locate transient food sources,

oviposition sites, and potential mates (McKenzie and Parsons 1972; Dudley 2002).

Ethanol is present in fermenting fruits at concentrations that can range anywhere from 0.02% to 6% (Gibson, May et al. 1981) depending on the type of fruit and its state of ripeness. However, ethanol is also a toxin and when consumed at high concentrations has fitness costs associated with it (Bokor and Pecsénye 2000). This creates an interesting scenario where on the one hand an ecologically relevant compound could be used to detect and locate food sources but on the other hand consuming such a compound can reduce fitness.

In order to use a GWA approach to identify natural occurring polymorphisms that are associated with phenotypic variation for a chemosensory trait, we need a population of flies which exhibited natural phenotypic variation in that trait. In addition, we need markers at the genome level to identify polymorphisms that are associated with variation in the trait. This is where tremendous resources of the *Drosophila melanogaster* Genetic Reference Panel (DGRP) (Mackay et al. 2012) were invaluable. It enabled me to precisely quantify naturally occurring phenotypic variation in olfactory and gustatory behavioral responses to ethanol and enabled the use of GWA analyses to gain insights into the genetic architecture underlying these traits.

My results revealed that when ethanol is detected as an olfactory cue, flies are attracted to it. However, when it is detected in their food source as an olfactory and gustatory cue, the flies try to minimize its consumption. Thus, ethanol is an example of ecologically relevant compound that acts both as an attractant and a repellent

depending on sensory modality through which and the context in which it is detected. The results further suggested that these behavioral responses might have evolved as a mechanism to balance the need of finding food sources, oviposition site, and potential mates using olfactory cues, while at the same time minimizing fitness costs associated with consuming a toxic compound producing such cues. Through this study, I identified genetic variants that explain a relatively large portion of the phenotypic and genetic variance associated with olfactory and gustatory behavioral responses to ethanol. This is in marked contrast with results from human association studies, where significant SNPs explain only a small fraction of the total phenotypic variance (Manolio et al. 2009) and illustrates the power of conducting marker-trait association using the *Drosophila* model system. The results revealed that genetic architecture of segregating variation in olfactory and gustatory behavioral responses within the DGRP is relatively simpler than expected since relatively few SNPs significantly associated with variation in olfactory and gustatory behavioral responses to ethanol accounted for a large portion of the phenotypic and genetic variance in both traits.

I did not observe any significant phenotypic and genetic correlation between olfactory and gustatory behavioral responses to ethanol, which was reflected in the limited overlap of genes between the two traits. However, the underlying biological processes associated with chemosensory behavioral responses to ethanol, which include: neurotransmitter systems, neuropeptides, synaptic proteins, channels and

signaling process all of which are known to mediate ethanol-induced behaviors (Scholz and Mustard 2011), were similar for both traits.

Chemosensory Genes Associated with Variation in Olfactory and Gustatory Response to Ethanol

This study also revealed the power of exploiting naturally occurring allelic variation to identify chemosensory genes associated with variation in chemosensory traits.

Using this approach, I was able to identify novel chemosensory genes that have not been previously implicated in chemosensory behavioral responses to ethanol. I functionally tested and confirmed the role of several chemosensory genes in olfactory and gustatory behavioral responses to ethanol. These results also provided insights into the dynamic interplay that exists between inputs from different chemosensory organs and genes in modulating and producing appropriate behavioral responses to chemical stimuli. Additionally, some of the chemosensory genes identified in this study have known sexual dimorphic expression, suggesting their role in the observed sexually dimorphic chemosensory behavioral responses to ethanol.

Sexually Dimorphic Chemosensory Behavioral Responses to Ethanol

We observed significant phenotypic and genetic variation in sexual dimorphism for both chemosensory traits. I observed sex-biased effects for the olfactory behavioral

response to ethanol and sexually antagonistic effects for the gustatory behavioral response to ethanol consumption.

Males and females flies appear to utilize ethanol as an olfactory cue to locate transient food sources and potential mates (Dudley 2002), suggesting there might be selective pressures to maintain alleles that enable this behavior. However, there might be added selective pressures on females to utilize ethanol as a cue to locate oviposition sites (McKenzie and Parsons 1972). On the other hand, there appear to be selective pressures acting on both males and females to minimize ethanol consumption, since ethanol is a toxin and when consumed at high concentrations has fitness costs associated with it (Bokor and Pecsénye 2000). Thus, similar and different selective pressures might be acting on males and females and contributing to the variation in the observed sexual dimorphic behaviors. Knowledge of the causal variants affecting these chemosensory behavioral responses will enable, for the first time, to conduct experiments to assess the strength and nature of selection acting on both these traits in a laboratory evolution setting.

Future Directions

The haplotypes formed by the SNPs used in the gene-centered prediction models explain a large portion of the phenotypic and genetic variance associated with variation in olfactory and gustatory responses to ethanol consumption. Can this information be used to predict chemosensory behavioral responses to ethanol of flies collected from different populations? If it can, this can be a strong tool to predict

specific phenotypes based on haplotype combinations. However, failure to do so might be attributed to non-additive gene action. If effects of alleles in different genetic backgrounds are not the same, then this has profound implications for the genetic basis underlying complex traits.

Identifying a list of all genes and causal molecular variants associated with a quantitative trait does not place them in a biologically relevant context. Molecular variants do not affect the traits directly but do so through interrelated networks of transcriptional, protein, metabolic, and other molecular endophenotypes (Mackay et al. 2009). A systems genetic approach enables us to integrate DNA sequence variation, variation in transcript abundance, and other molecular phenotypes to variation in chemosensory behavioral responses to ethanol and allow us to interpret genetic variation in terms of biologically meaningful causal networks of correlated transcripts (Sieberts and Schadt 2007). Future analyses utilizing whole-genome transcript data from the DGRP to construct genetically correlated transcriptional modules with higher correlations to each other than the rest of the transcriptome (Stone and Ayroles 2009) will enable us to group the quantitative transcripts identified in this study into modules consisting of transcripts with higher correlations to each other relative to the rest of the transcriptome. These coexpression networks should provide information about relevant pathways, gene ontology categories, tissue-specific expression patterns, and transcription factor binding sites (Ayroles et al. 2009; Harbison et al. 2009) and also help in predicting the function of computationally predicted genes based on 'guilt by association' with well-annotated

genes in the network (Ayroles et al. 2009; Harbison et al. 2009; Mackay et al. 2009). Additionally, incorporating information about *cis* and *trans* eQTLs, based on information from DNA polymorphisms, will enable us to determine the direction of flow of information within a network and infer which relationships are directly caused by genetic perturbations and which are coregulated by genetic perturbations (Mackay et al. 2009).

Finally, knowledge of causal variants affecting variation in chemosensory behavioral responses to ethanol enables us to assess the strength and nature of selection that might be acting on this trait in the laboratory setting. We can do this by creating a synthetic outbred population enriched for variants associated with chemosensory behavioral responses to ethanol. We can subject such a population to laboratory evolution experiments under different conditions. By tracking allele frequencies over several generations, we will be able to assess the strength and nature of natural selection acting on a trait.

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