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Exploring the *Drosophila*-yeast mutualism in natural contexts

by

Allison S. Quan

A dissertation submitted in partial satisfaction of the

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of the

University of California, Berkeley

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Spring 2018

Exploring the *Drosophila*-yeast mutualism in natural contexts

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Allison S. Quan

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## Abstract

### Exploring the *Drosophila*-yeast mutualism in natural contexts

by

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Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Professor Michael B. Eisen, Chair

Mutualisms are among the most ubiquitous interactions in nature yet the general rules governing them have remained elusive. The natural mutualism between fruit flies and yeast, two common model organisms in molecular biology, is a particularly useful relationship for investigating these parameters. *Drosophila* feed on yeasts throughout their entire lifecycle and nonmotile yeasts depend on *Drosophila* to vector them to new, sugar-rich substrates. In the laboratory, *Drosophila melanogaster* can discriminate between and prefer different strains of *Saccharomyces cerevisiae*. However, in nature, *Drosophila* are associated with a variety of yeast species in addition to *S. cerevisiae*. The efforts detailed in this thesis are focused on characterizing the natural associations between wild *Drosophila* and yeast and testing the relevance of the fine-scale specificity between flies and yeast observed in the laboratory under more natural contexts.

First, I present a detailed dissection of the associations between *Drosophila* and natural yeasts over two harvest seasons in organic wineries. Using targeted, amplicon sequencing methods, I found that the fungal communities vectored by *Drosophila* in wineries are distinct between winery microhabitats. However, the structure in these fungal communities is not a direct result of *Drosophila* behavior. Instead, a diversity of yeast species, even those that are not commonly associated with flies, are adequate partners in the fly-yeast mutualism at a wide range of relevant temperatures. While many yeast species can stimulate oviposition in *Drosophila*, ovipositional responses vary depending on the volatile profile emitted by a particular yeast species. Using gas chromatography-mass spectrometry and synthetic compounds, I found that a minimal blend of isoamyl acetate, acetic acid, 2-phenylethyl ester, and ethanol is sufficient for stimulating oviposition. However, the strength of the oviposition response is dependent on the volatile composition of the overall blend.

Flies initially locate yeast through olfaction and a large portion of *D. melanogaster* odorant receptors are dedicated to detecting yeast metabolites. Kelly Schiabor, a former graduate student in the lab, demonstrated a correlation between two allelic variants of

the odorant receptor 22 (Or22) in *D. melanogaster* and sensitivity to the volatiles produced by yeast grown under different nitrogen conditions. In collaboration with Carolyn Elya, another graduate student in the lab, I tested the hypothesis that the chimeric allele of Or22 confers heightened sensitivity to yeast grown on sugar-rich but nitrogen-limited substrates (YVN), a nutrient composition much like a natural, fruity substrate. Through extensive genotyping of natural populations, bidirectional crosses between chimeric and non-chimeric lines, and functional allele replacement of a non-chimeric Or22 allele with a chimeric allele, we found that Or22 alone cannot account for behavioral sensitivity to YVN. Even so, the signs of selection at the Or22 locus across wild *D. melanogaster* populations suggest that this receptor confers some kind of adaptive function in wild flies.

Because wineries are not purely natural ecosystems, I next characterized the fly-yeast mutualism in Hawaiian *Drosophila*. The Hawaiian *Drosophila* are a diverse species group that has radiated across the Hawaiian Islands and exhibit very specific host plant adaptations. It has been hypothesized that the microbial communities on host plants, rather than host plants themselves, mediate host plant discrimination in Hawaiian *Drosophila*. I characterized the fungal communities associated with three closely related species of Hawaiian *Drosophila* and their respective host plants but found no clear association between the fungi associated with flies and host plants. However, the yeast species isolated from host plants and flies produce distinct volatile profiles, suggesting that different yeast species would affect the overall volatile bouquet of a plant substrate. Unfortunately, direct behavioral assays were not possible because the Hawaiian *Drosophila*-yeast system is limited by sample size and the ability to rear healthy fly populations in the laboratory. Nonetheless, the role of fungi in Hawaiian *Drosophila* host plant discrimination remains an ecologically significant question and an open area of investigation given the appropriate resources.

Initially, the range of suitable yeast species in the fly-yeast mutualism was a surprising observation of these studies. However, this flexibility is clearly beneficial to the fitness of both flies and yeast from an evolutionary standpoint, as both organisms exist in dynamic environments. Even though their mutualism is nonspecific, both flies and yeast have continually coevolved conserved mechanisms and fine-tuned behaviors for ensuring a close association with each other.

*For my grandfathers  
who came to this country,  
served it proudly,  
and helped make it great.*

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# CHAPTER 1: Introduction

## Animal-microbe interactions, evolution, and behavior

Our world is dominated by microbes so it is unsurprising that all animals interact with microbes at some point in their lifecycle whether it be endosymbiotically or externally [1]. We are just beginning to appreciate the ubiquity of animal-microbe interactions due to recent advances in genomic sequencing technologies, which have revealed microbes that are unculturable and were therefore undetectable through traditional methods [2]. However, much less is understood about the molecular mechanisms underlying animal-microbe interactions and how these relationships fit into evolutionary principles. In-depth case studies of animal-microbe interactions can provide insights into the rules governing microbial associations and their role in the evolution of both animal and microbe.

Close, long-term associations between microbes and animals can be very specific and in some cases, this specificity is played out through microbe-induced behavioral changes in the host. Some of the most well characterized examples of microbes influencing animal behavior are reminiscent of science fiction. For example, mice infected with *Toxoplasma gondii* lose their innate avoidance behavior of feline urine. Loss of this innate aversion results in mice being eaten by cats and subsequently, ensures the transmission of *T. gondii* back to its primary host, cats, where it can reproduce [3,4]. An even more bizarre example is the case of “zombie ants,” which are infected by fungal *Ophiocordyceps*. Infected ants exhibit summiting behaviors and eventually die clamped down to a substrate before the fungus sprouts out of the ants’ head and ejects infectious spores onto other ants, thus continuing the infection cycle [5,6]. More recently, a fungus with a similar lifecycle, *Entomophthora muscae*, has been observed eliciting almost identical behavioral phenotypes in fruit flies [7]. Although the lifecycles of these fungi are remarkably analogous, they are not closely related taxonomically, suggesting that the ability to co-opt host behavior has evolved independently multiple times and is an evolutionary advantageous and efficient means of propagation.

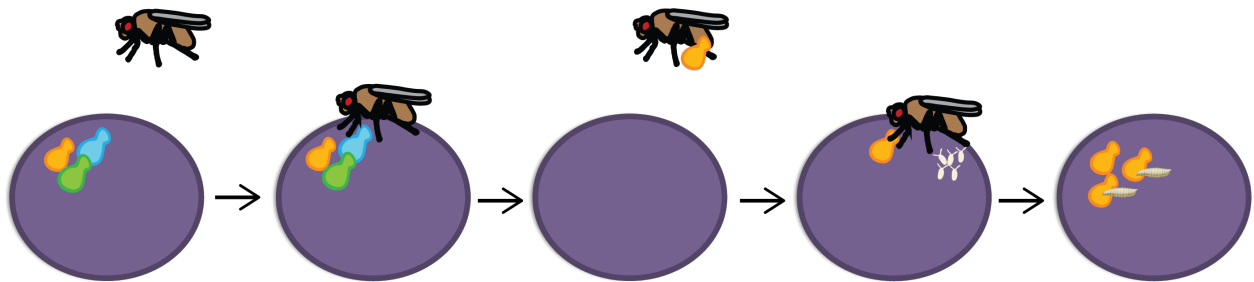
While these parasitic interactions dominate the animal-microbe literature, there are many mutualistic and symbiotic animal-microbe relationships that are equally compelling but less characterized. Mutualistic interactions are diverse and involve organisms from all five kingdoms of life [8]. You don’t have to look far for examples of microbes and hosts that have evolved to mutually benefit each other. The human body is home to a complex microbial community [9,10] that has coevolved with human hosts and play crucial roles in our physiology and metabolism [11-13]. While the composition of human gut microbiota can vary between individuals, some symbiotic animal-microbe interactions have evolved to be very specific and even obligate, as is the case with pea aphids and *Buchnera* bacteria. *Buchnera* provide pea aphids with essential amino acids they are unable to synthesize on their own and, in turn, reside in specialized structures within aphids that ensure transmission to aphid progeny [14,15]. The bobtail squid utilizes the light produced by the bioluminescent bacteria, *Vibrio fischeri*, to evade predators [16]. Like *Buchnera*, *Vibrio fischeri* are housed in a specialized light organ within the squid, however, the bacteria must recolonize their host each day [17]. In these symbiotic and



mutualistic relationships, the behavioral adaptations underlying the interactions are centered around locating and retaining beneficial partners. Clearly, these closely related organisms have evolved complex adaptations not only to coexist but to benefit mutually from each other. These examples indicate that animal-microbial interactions have evolutionary consequences and understanding the molecular and behavioral mechanisms underlying these relationships can provide insight into underappreciated evolutionary drivers.

## The *Drosophila*-yeast mutualism in the laboratory

One of the great challenges to studying animal-microbe interactions is recapitulating and investigating these relationships under controlled, laboratory conditions. Serendipitously, two quintessential model organisms in molecular biology, the fruit fly, *Drosophila melanogaster*, and baker's yeast, *Saccharomyces cerevisiae*, participate in a natural mutualism and offer a tractable, well-established study system. Both organisms are easily cultured in the laboratory and possess a suite of ready-made genetic tools that are ideal for dissecting the mechanisms underlying microbial interactions.



**Figure 1.1. Schematic of the *Drosophila*-yeast mutualism.**

The *Drosophila*-yeast interaction is classified as a guild mutualism in which each partner benefits from the interaction but several species can adequately serve in each role, contrary to the very specific associations of *Buchnera* and the pea aphid [18-20]. Nevertheless, this mutualism appears to be so strong that other organisms outside the fly-yeast partnership have evolved to mimic it. For example, the Solomon's lily tricks drosophilids for pollination by producing yeast fermentation volatiles [21].

While the fly-yeast mutualism is diffuse, it is still important to the viability of both partners. Both larvae and adult *Drosophila* feed on yeasts and the consumption of yeast is essential for the proper development of *Drosophila* larvae [22,23]. Anagnostou, Dorsch & Rohlf's [24] found that without the addition of dietary yeast, *Drosophila melanogaster* larvae are unable to pupate on sterile media. Conversely, the addition of yeast results in increased survival and decreased development time to adulthood. At the adult stage, consumption of the yeast species, *Issachenkia terricola*, can rescue lifespan in malnourished flies [25]. In addition to being nutritionally valuable, the ethanol produced by yeast during the process of fermentation inhibits the growth of microbes that may be harmful to larval development [26,27]. Contrary to their common name, adult fruit flies prefer yeast over sterile fruit or synthetic media. On its own, *Saccharomyces*

*cerevisiae*, can sufficiently induce *Drosophila melanogaster* attraction, oviposition, and larval development [28].

Yeast, the opposing partner in this mutualism, cannot be dispersed by wind and depend on insect vectors, such as *Drosophila*, to colonize new substrates [19,29-31]. Frequent migration to new habitats is essential for yeasts because it ensures transfer to new, nutrient-rich environments and the distribution of compatible mating types for sexual reproduction and outcrossing [19,32]. Like other animal-microbe symbioses and mutualisms, both flies and yeast have evolved specialized adaptations for attracting, sensing, and generally increasing the effectiveness of their association.

## Yeast volatile production and chemoecology: how yeast attract drosophilids

The chemical signals produced by yeast are imperative for attracting *Drosophila*, thus ensuring migration to novel substrates. Studies using a combination of neuronal recordings in *Drosophila* and gas chromatography – mass spectrometry (GC-MS) have characterized the yeast-produced compounds involved in attracting *Drosophila*. These attractants are volatile, fruity compounds such as ethyl acetate, isoamyl alcohol, isoamyl acetate, ethyl hexanoate, phenylethyl alcohol, and ethyl octanoate [33,34]. Most of these compounds are esters, which are produced by yeast as byproducts of several metabolic pathways, including the Ehrlich pathway [35]. Short-chain, acetate esters are produced during degradation of amino acids while medium-chain, fatty acid esters are formed during fatty acid synthesis or degradation [36,37]. While the metabolic pathways and the genes regulating ester synthesis have been well characterized, the biological function of esters in yeast remained unresolved.

At first glance, esters simply seemed to be metabolic byproducts without any obvious use to the cell. However, the energy required for ester synthesis and the tight regulation of these reactions within the cell suggested a more specific function. Saerens et al. [38] proposed ester production as a mechanism for attracting *Drosophila* for dispersal and hypothesized that yeast producing more fruity esters would achieve greater fitness. Several independent studies have provided evidence for this hypothesis. Buser et al. [30] found that strains of *Saccharomyces cerevisiae* producing more attractive volatile cues were more highly dispersed and stimulated more oviposition in *Drosophila simulans*. In the same year, Christiaens et al. [39] found that the deletion of ATF1, a gene coding for the alcohol acetyl transferases that catalyze acetate ester synthesis reactions, resulted in a decrease in *Drosophila melanogaster* attraction and reduced dispersal. Outcrossing rates of *Saccharomyces cerevisiae* can increase up to 10-fold when flies are present and fit patterns of genetic structure in natural yeast populations [32]. Conversely, when plants are moved outside their native range to a habitat where natural vectors are absent, the plants are not colonized by local yeasts [40]. *Saccharomyces cerevisiae* spores can survive the fly digestive tract, increasing their chances of successful transmittance to distant and novel habitats [32]. Together, these studies suggest that ester production in yeast plays an important role in the fly-yeast mutualism.

While most yeast are capable of producing esters, different yeast species and even different strains of *Saccharomyces cerevisiae* produce variable ester concentrations [41,42]. In nature, the evolution of yeast metabolism is shaped by the highly variable environments in which yeast reside and their interactions with microbial neighbors [27]. The concentration of ethyl esters

yeast produce is limited by fermentation conditions, such as nitrogen and amino acid availability [43-45]. For example, *S. cerevisiae* grown on media that is sugar limited, as is the case under laboratory conditions, compared to media that is nitrogen limited, as is the case in nature, produce different volatile profiles [34]. In turn, flies have evolved mechanisms to discriminate between and detect slight changes in the production of these compounds.

## The fruit fly is a yeast fly: how drosophilids sense yeast

At the most basic level of their interaction, *Drosophila* sense and locate yeast by olfaction. Odor perception, although a simple concept, involves the complex integration of several processes from the binding of odorant ligands to receptors, activation of olfactory neurons, processing of signals in the central nervous system, and finally, an output in the form of a behavioral response. Adult *Drosophila* have two olfactory organs: the maxillary palp and the third antennal segment. The surfaces of both of these structures are covered in sensory hairs called sensilla. Each sensillum is innervated by one to four odorant receptor neurons (ORNs), which project onto glomeruli in the antennal lobe, the *Drosophila* central olfactory processing center [46,47]. Each ORN expresses a single odorant receptor (OR) gene and Or83b, a coreceptor ubiquitously expressed in all ORNs and essential for odorant signal transduction [46,48,49]. All ORNs expressing a particular OR target a single glomerulus and the ORN projections onto these glomeruli have been completely mapped [50-52]. *Drosophila* odorant receptors distinguishing ORNs are part of the seven-transmembrane G-protein-coupled receptor (GPCR) super family but have an inverted membrane topology relative to the odorant receptors in other organisms [49,53]. The chain of events leading to olfactory sensing begins when an odorant receptor binds a specific odorant ligand.

Detailed analysis of the *Drosophila* olfactory signal transduction, particularly involving the ORs and the genes encoding them, has been and continues to be a rich and exciting field in biology. While the signal transduction pathway and organization involved in *Drosophila* olfaction has been known since 1969 [54], the genes encoding *Drosophila* odorant receptors remained elusive until the release of the *Drosophila* genome by the Berkeley Drosophila Genome Project in 1998 [55]. In two separate studies published less than a month apart, Clyne et al. [56] and Vosshall et al. [57] used a combination of computational and differential screening approaches to identify seventeen putative odorant receptor genes. The putative OR genes were very divergent from previously identified odorant receptor genes in *C. elegans* and vertebrates, explaining why traditional homology-based approaches has been previously unsuccessful.

The identification of the 60 genes encoding 62 odorant receptors opened new questions and tools for studying olfactory processing *Drosophila*. For example, Dobritsa et al. [58] developed a transgenic technique called the “empty neuron system,” which can be used to express ORs of interest and record the responses of a single sensillum to odorant stimuli in vivo. In a landmark paper, Hallem & Carlson [59] used this electrophysiology approach to extensively catalogue the *Drosophila* odorant receptor repertoire response to over 100 ecologically relevant odorants. This systematic characterization revealed that odorant receptor responses can be very specific or more broadly tuned depending on the stimulus. Many of the ligands activating ORs are yeast-produced volatiles. While the activation of ORs to some odor stimuli can be concentration dependent, yeast metabolites produced a neuronal response even at low concentrations.

In nature, flies must be able to sense yeast odors from far distances to locate food and suitable breeding sites so odorant receptors must be very sensitive to these volatiles. *Drosophila* can faithfully track even intermittent pulses of ethyl butyrate, a fatty acid ester produced by yeast during fermentation [60]. Odorant sensing is so sensitive that under laboratory conditions, *D. melanogaster* can discriminate between strains of *S. cerevisiae* based on volatile profile alone [34]. Other studies have shown that *Drosophila* also have the ability to discriminate between the volatile profiles of different yeast species as well as differentiate between more volatile bouquets involving more complex microbial communities [61-63]. Additionally, the neuronal response profiles of ester-binding odorant receptors are generally conserved across *Drosophila* species, suggesting that the ORs sensing yeast volatiles have important functions [64]. Together, these studies suggest that *Drosophila* have developed complex olfactory adaptations for localizing and discriminating between yeasts.

Sensitivity and attraction to yeast volatiles naturally varies between different *Drosophila melanogaster* lines [65]. Richgels and Rollmann [66] found that behavioral responses of *Drosophila melanogaster* to two yeast volatiles, methyl hexanoate and ethyl hexanoate, are associated with polymorphisms in *Or22a/b*, *Or35a*, and *Or47a*, demonstrating a genetic basis for differences in yeast volatile mediated behavior. Odorant receptors are evolutionary hotspots within *Drosophila melanogaster* genome and across the *Drosophila* phylogeny. Even the first OR genes to be identified in *Drosophila melanogaster* had relatively low amino acid sequence similarity to each other suggesting that they are rapidly evolving [46,56,57,67]. Comparative analysis of eleven *Drosophila* species genome assemblies revealed that *Drosophila* odorant receptor genes have undergone duplication, loss, pseudogenization, and relocation events [68]. While a core set of ORs seem to be functionally conserved between species, others seem to be less constrained and have evolved in response to different ecological contexts [69]. For example, host plant specializations in *Drosophila sechellia* and *Drosophila erecta* to the morinda fruit and the screw pine fruit, respectively, are both associated with changes to the olfactory receptor *Or22a* [70-72]. Similarly, the shift to herbivory in *Scaptomyza flava* is associated with gene loss and subsequent duplication of *Or67b* [73]. Transitions to host plant specialization in *Drosophila* seem to be linked to changes in odorant receptor specificity and sensitivity and/or relative proportions of ORN types so it is likely that similar changes underlie coadaptation in the fly-yeast mutualism.

## Specificity in the *Drosophila*-yeast mutualism and the potential evolutionary consequences

As described in the previous sections, both yeast and flies have evolved adaptations to increase their chances of association. In *Drosophila*, genetic changes in the olfactory system can have behavioral consequences. Disparities in *Drosophila* behavioral response towards yeast volatiles can lead to adaptation to local environments, subsequent reproductive isolation, and, in the most extreme cases, speciation. The potential role of the fly-yeast mutualism in *Drosophila* evolution and speciation is not a novel concept. In 1981, Starmer [74] argued that yeast must have a role in *Drosophila* diversification because *Drosophila* can utilize a diversity of habitats, such as fruits, stems, bark, wood, leaves, and flowers, and the single common factor of these habitats is yeast. Independently, Begon [22] hypothesized that *Drosophila* would behave differently towards different yeast species given that yeasts vary inherently in their nutritional constituents and their ability to metabolize different substrates. Therefore, the yeast present on and metabolizing the

substrates could be more important than the substrate themselves. Local adaptations to the yeast communities present on different host substrates can occur even within microhabitats, paving the way for partial reproductive isolation [75].

There is a surprisingly vast body of old literature driven by these hypotheses. In two studies from 1955 to 1956, Dobzhansky and colleagues [76,77] observed differential attraction to yeast in sympatric *Drosophila* species in Brazil and the Yosemite Valley. Later, *Drosophila* species from different habitats were shown to be associated with distinct microbial distributions, suggesting the partitioning of resources by different *Drosophila* species is due to differing interactions with the microbes on the host substrate [78]. Starmer [74] reasoned that resource partitioning by *Drosophila* and the associated yeast communities should track with *Drosophila* evolution. When comparing yeast communities present in different *Drosophila* habitats to *Drosophila* phylogenies, he found *Drosophila* evolution parallels the yeast communities and concluded that *Drosophila* habitats are constrained by the physiological properties of the yeast community metabolizing each substrate.

Each of these studies are supported by the ecology of cactophilic *Drosophila*, a species clade that depends on the microbial community on cacti to decay cactus tissue into food. These associations are so specific that each cactus species hosts a single *Drosophila* species [79] and in southern Arizona, catophilic *Drosophila* species are associated with distinct yeast communities [80]. Two sympatric species in Australia, *Drosophila buzzatii* and *Drosophila aldrichi*, are differentially attracted to yeast, suggesting that the niche separation between these species in nature is driven by yeast associations [81].

More recently, there have been new examples linking genetic olfactory changes to behavioral adaptations in microenvironments. For example, *Drosophila mojavensis* utilizes different cactus species across its natural range and each population exhibits variable olfactory receptor neuron specificity and sensitivity that track with the microhabitat from which each population was derived [82]. The best and most well-studied example of how olfactory sensing and behavioral responses can lead to reproductive isolation is the sympatric host shift in the apple maggot fly, *Rhagoletis pomonella*, from the native hawthorn to the domestic apple. The two *R. pomonella* populations are genetically distinct [83] and discrimination of host volatiles guides the behavioral shift from one host to another [84]. Interestingly, the populations do not differ in olfactory receptor neuron number, location, or excitatory activation response. Instead, changes in the sensitivity and temporal firing pattern of the ORNs mediate host preference between the two populations [85,86].

It is clear from these examples that changes in olfaction and behavior can lead to divergence between populations occupying different microclimates and eventual reproductive isolation. The fly-yeast mutualism can increase fly specificity for yeast and act as a driver for ecological separation [87]. Based on the examples reviewed in this section, changes in olfactory sensory cues can result in behavioral responses towards different yeast species and may preclude speciation events.

## Remaining questions and the contents of this dissertation

Recent studies between *Drosophila melanogaster* and *Saccharomyces cerevisiae* in the laboratory indicate that flies can discriminate between different yeast strains and species and exhibit natural preferences for some over others. However, whether flies actually show behavior this specific in nature is unknown. While a lot of progress has been made characterizing the fly-yeast mutualism in the laboratory, an important caveat to these studies is their relevance in a natural context. In his book chapter on the yeast and *Drosophila* association, written before most *Drosophila melanogaster* and *Saccharomyces cerevisiae* laboratory studies were done, Begon [22] warned, “There has been little consideration of the extent to which laboratory-reared species are cultured on yeasts and substrates unlike the ones to which they have been adapting for millions of generations. Therefore, it is hoped that, in the future, laboratory workers will recognize that their *Drosophila* do have an environment, that it is not the one to which they are adapted, and that it is not so simple that it can be ignored.”

Nonetheless, many recent studies on the fly-yeast mutualism use wild-type *D. melanogaster* lines that have been established in the laboratory for many years and laboratory *S. cerevisiae* strains to represent all yeast species in nature. While *S. cerevisiae* is undoubtedly the most important yeast species for humans, yeasts are actually a very diverse group defined as eukaryotic, single-celled fungi, which grow asexually through budding or fission or sexually without a fruiting body [88]. In nature, *Drosophila* carry a diversity of yeast species and it has been suggested that measuring fly interactions with *S. cerevisiae* is not actually indicative of natural associations [89,90]. In fact, Buser et al. [30] found that yeast isolated from flies caught in the field were more attractive and likely to be dispersed than laboratory strains. Characterizing the yeasts *Drosophila* are naturally associated with in nature and quantifying preference for these yeast species will add ecological context to laboratory studies of *D. melanogaster* and *S. cerevisiae*.

To understand relevance of the laboratory studies between *D. melanogaster* and *S. cerevisiae* in nature, we need to better understand the natural context in which the fly-yeast mutualism operates. This begins with considering the actual association of flies and yeast in nature. Are flies naturally associated with certain yeast species? If so, are there any patterns to this association? Wild *Drosophila* lines are more selective in the odorants they are attracted to [65] so do flies actively seek out particular yeast species?

The natural odors that the *Drosophila* olfactory system must decipher are complex mixtures of both compounds and microbes. In laboratory studies, *Drosophila* seem to respond more positively to complex volatile bouquets over single synthetic compounds [63,91]. Clearly, the *Drosophila* olfactory system has been tuned to sense the volatiles produced by yeast but which particular compounds are behaviorally relevant in driving the fly-yeast mutualism? How complicated is the yeast volatile code and how have flies adapted to recognize and distinguish ecologically relevant odorants or odorant combinations? What are the fly and yeast genetics underlying the adaptations that increase the beneficial mutualistic associations of both organisms?

Finally, we still do not have a full understanding of the rules governing animal-microbe interactions despite their ubiquity. A more comprehensive understanding of the fly-yeast mutualism, especially in a natural context, can provide a better framework for interpreting the evolution of other mutualistic or symbiotic relationships between animals and microbes.

In this dissertation, I strive to answer these questions by studying the fly-yeast mutualism in more natural contexts and combining hypotheses and approaches introduced in classic literature with new technologies and advances in molecular biology. I begin with an in-depth analysis of the association between *Drosophila* and natural yeast species in organic, Northern California wineries (Chapter 2 and 3). Then I will move onto a more natural study system – the Hawaiian *Drosophila*, which exhibit host plant preferences that are thought to be driven by the microbial communities on the plants rather than the plants themselves (Chapter 4). Finally, I will present previously published work on the *Drosophila melanogaster* odorant receptor, Or22a/b and its role in the discrimination of *S. cerevisiae* volatiles (Chapter 5). In conclusion, I offer my perspective on the fly-yeast mutualism and its implications on the field of animal-microbe interactions and suggest future directions (Chapter 6).

# CHAPTER 2: The ecology of the *Drosophila*-yeast mutualism in wineries

## Abstract

The fruit fly, *Drosophila melanogaster*, is preferentially found on fermenting fruits. The yeasts that dominate the microbial communities of these substrates are the primary food source for developing *D. melanogaster* larvae, and adult flies manifest a strong olfactory system-mediated attraction for the volatile compounds produced by these yeasts during fermentation. Although most work on this interaction has focused on the standard laboratory yeast *Saccharomyces cerevisiae*, a wide variety of other yeasts naturally ferment fallen fruit. Here we address the open question of whether *D. melanogaster* preferentially associates with distinct yeasts in different, closely-related environments. We characterized the spatial and temporal dynamics of *Drosophila*-associated fungi in Northern California wineries that use organic grapes and natural fermentation using high-throughput, short-amplicon sequencing. We found that there is nonrandom structure in the fungal communities that are vectored by flies both between and within vineyards. Within wineries, the fungal communities associated with flies in cellars, fermentation tanks, and pomace piles are distinguished by varying abundances of a small number of yeast species. To investigate the origins of this structure, we assayed *Drosophila* attraction to, oviposition on, larval development in, and longevity when consuming the yeasts that distinguish vineyard microhabitats from each other. We found that wild fly lines did not respond differentially to the yeast species that distinguish winery habitats in habitat specific manner. Instead, this subset of yeast shares traits that make them attractive to and ensure their close association with *Drosophila*.

## Introduction

All animals interact with microbes, and it is increasingly clear that the collection of microbes with which an animal interacts can have a dramatic impact on its physiology, behavior, and other phenotypes [1,12,13,92,93]. Some of the microbes associated with animals in the wild are highly specific and acquired through dedicated mechanisms that ensure the robust maintenance of their interaction [14-16]. Other associations, however, are more contingent, and involve microbes acquired as the animal navigates a microbe rich environment. While this latter class has received less attention, studying the contingent microbiome of wild animals can reveal important details of natural history, ecology, and behavior.

The microbiome of the fruit fly, *Drosophila melanogaster*, represents an interesting mix of obligate and contingent microbial associations [87]. In nature, *D. melanogaster* is found on or near fermenting substrates, on which they preferentially oviposit, as *D. melanogaster* larvae (and indeed those of most *Drosophila* species) feed on microbes, particularly yeasts [23,24]. Yeasts benefit from visits by adult flies, who vector them from site to site, enabling their dispersal and colonization of new substrates [29,32]. This association is mediated by a strong, olfactory-based



attraction of adult *D. melanogaster* to the volatile compounds produced during yeast fermentation [28,94].

A growing body of work has investigated the interaction between *D. melanogaster* and the brewer's yeast, *Saccharomyces cerevisiae*, in the laboratory. Adult fruit flies prefer substrates inoculated with yeast over any other sterile substrate [28], and under laboratory conditions, *D. melanogaster* can discriminate between and prefers some strains of *S. cerevisiae* over others based on volatile profile alone [34,39,61]. While the interaction between flies and yeasts is clear, the specificity of this interaction in nature has been poorly studied.

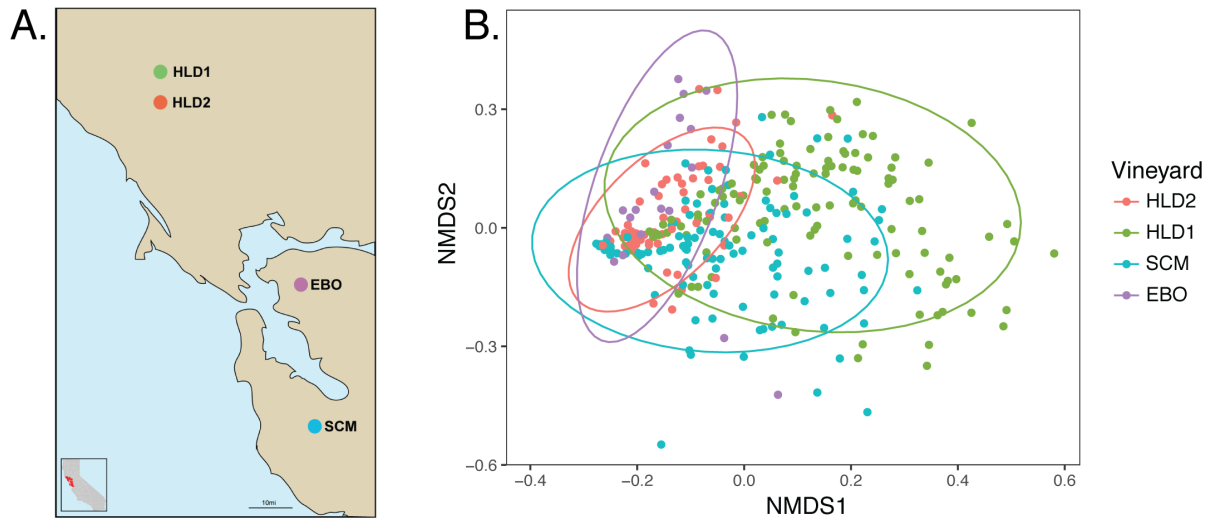
Both *D. melanogaster* and *S. cerevisiae* are found in abundance in wineries, a habitat more natural than a controlled laboratory, but more accessible than a completely wild ecosystem [95]. A variety of non-*Saccharomyces* yeasts are observed during spontaneous fermentation, a winemaking practice in which only the yeasts found on the grapes at the time of harvest, and those introduced naturally or incidentally after harvest, are used for fermentation [96,97]. However, little is understood about the movement of non-*Saccharomyces* yeasts in vineyards, although insects are acknowledged as potentially important vectors [31,89,98-100]. Given that drosophilids are closely associated with yeast throughout their entire lifecycle, flies are likely candidates for vineyard and winery yeast dispersal [32,76,79]. However, the yeasts associated with vineyard and winery *Drosophila* have yet to be thoroughly characterized.

In a broader context, investigating the degree of specificity of the fly-yeast mutualism in nature can help reveal both the constraints and plasticity of natural mutualisms. While several studies have characterized the yeasts vectored by *Drosophila* in vineyards and wineries using culture-based methods [95,101], we present here a comprehensive study of the relationship between flies and yeast in wineries, using high-throughput, amplicon sequencing of the fungi associated with flies, coupled with well-established *Drosophila* behavior assays using both the yeast isolates and fly lines isolated from the same wineries. We demonstrate that *Drosophila* vector a distinct set of yeasts in wineries and exhibit a generally positive behavioral response towards commonly vectored yeasts. This suggests that the fly-yeast mutualism is not as specific as laboratory experiments indicate, and that flies interact with a diversity of yeast species in different ecological contexts.

## Results

### *Drosophila* vector wine yeasts in wineries

To identify the fungi vectored by flies, we collected adult *Drosophila* in three areas – fermentation tanks, cellars, and pomace piles – in four different wineries over two harvest seasons in the San Francisco Bay Area, California, USA (Figure 2.1A). In our initial harvest season, we collected in two wineries, one in Healdsburg, CA (HLD1) and the other in the Santa Cruz Mountains (SCM). We collected adult *Drosophila* every two weeks from May 2015 to November 2015. To expand our study in 2016, we collected flies in a four wineries, HLD1, SCM, HLD2 (also in Healdsburg, CA), and EBO (Orinda, CA) at a single time point from each winery in late September 2016 - early October 2016.



**Figure 2.1. Fungal communities vectored by *Drosophila* are distinct between wineries.**

(A) Geographic locations of wineries sampled from 2015 and 2016 in the San Francisco Bay Area. (B) Bray-Curtis dissimilarity NMDS of fungal communities vectored by *Drosophila* in wineries (ADONIS:  $R^2 = 0.129$ ,  $p=0.001$ ). Each sample was rarefied to 1000 sequences and is represented by a single point, color-coded by winery. Note, HLD2 and EBO have fewer samples because these wineries were only sampled in 2016.

DNA from whole, adult flies was extracted and short-amplicon sequencing targeting the universal fungal internal transcribed spacer region (ITS) was performed to characterize fungal community composition [102,103]. After quality filtering and processing, we clustered a total of 7,609,820 fungal ITS reads into 399 operational taxonomic units (OTUs) (Table S2.1). When rarefied to 1000 sequences per sample, the overall mean OTU richness per fly associated fungal community for each winery ranged from 19.741  $\pm$  7.673 to 39.771  $\pm$  10.537 OTUs (Figure S2.1, Table S2.2). We found that *Drosophila* species that were not *D. melanogaster* or its sister species *D. simulans* carried subtly but significantly different fungal communities ( $R_{ANOSIM}=0.018$ ,  $p<0.001$ , Figure S2.2) so we omitted these samples from our subsequent analysis. Removal of these samples resulted in 308 OTUs.

As expected, yeast species dominate the fungal communities vectored by *Drosophila*. The phylum Ascomycota, which includes many yeast species, represented the bulk of the fly-associated fungal taxa (average relative abundance: 95.6%). At the species level, fungal communities were dominated by *Hanseniaspora uvarum* (30.2% average relative abundance across all samples), *Pichia manshurica* (11.5%), *Issatchenkia orientalis* (10%), and members of the genus *Pichia* we could not identify at the species level (4.4%). Although *S. cerevisiae* is the dominant yeast in late stage fermentations, it was unevenly represented in the fungal communities vectored by *Drosophila*. Only 8.8% of the samples collected contained *S. cerevisiae* reads and of these samples, the relative abundance of *S. cerevisiae* ranged from 0.1% to 81.9% with no correlation to any particular winery or winery microhabitat.

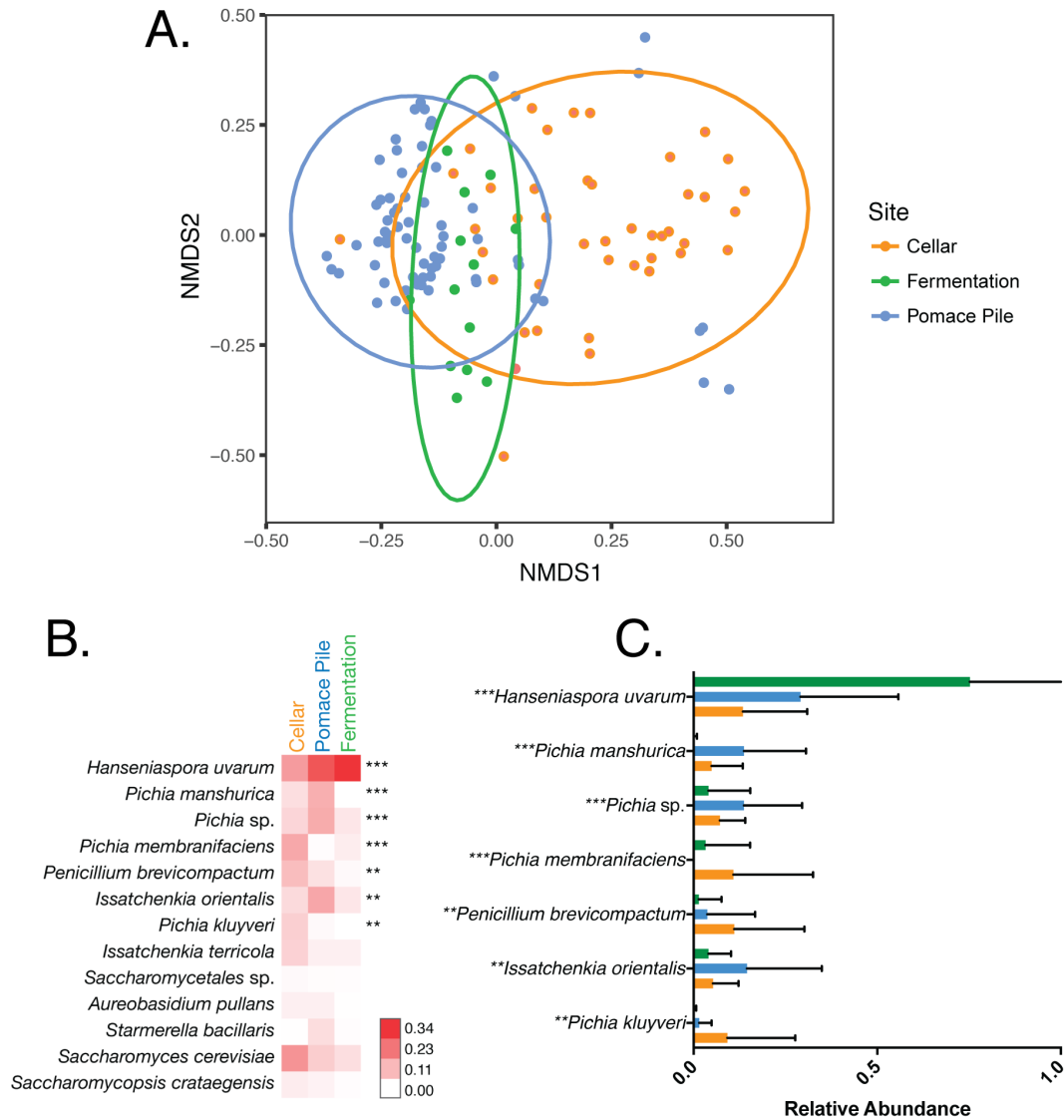
In the laboratory, flies exhibit a strong attraction to fermentation volatiles, so it was unsurprising to find that drosophilds were associated with a range of fermentative yeast species commonly found in winery environments. However, the weak association with *S. cerevisiae* we observed was noteworthy given that *S. cerevisiae* is almost exclusively used in behavior assays investigating the fly-yeast mutualism in the laboratory. As previously suggested by Hoang et al [90], it is possible that other yeast species may have a stronger association with *Drosophila* than *S. cerevisiae*. Since we did observe a strong association between flies and non-*Saccharomyces* yeasts, we next asked if these associations were homogeneous across all winery *Drosophila* or dynamic across space and time.

### Nonrandom structure in the fungal communities associated with *Drosophila* between and within wineries

To elucidate the spatial and temporal patterns of the fly-fungi relationship, we asked if fungal community patterns could be distinguished between winery *Drosophila* populations. We found that the fungal communities vectored by flies are not randomly distributed and are significantly different between wineries (Figure 2.1B, Bray-Curtis  $R_{ANOSIM}=0.129$ ,  $p<0.001$ ). These observations are consistent with what is known about the microbial communities present on wine grapes, which are predominantly defined by regional geography [104,105].

Unsurprisingly, fruit flies are found predominately in areas of active fermentation or containing products of fermentation. To reflect this, we focused our drosophilid collections in three main areas in each winery: fermentation tanks, where primary fermentation occurs; cellars, where wine is aged; and the pomace pile, where grape berry waste is discarded. Adult flies are abundant at all of these winery microhabitats during wine production.

Within the HLD1 winery, we observed fungal community structure in the fungi vectored by flies between these three winery areas (Figure 2.2A, Bray-Curtis  $R_{ANOSIM}=0.166$ ,  $p<0.001$ ). A single yeast species, *Hanseniaspora uvarum* (75.3%), dominated the fungal communities vectored by *Drosophila* collected from fermentation tanks. *Saccharomyces cerevisiae* (17.6%), *Hanseniaspora uvarum* (16.1%), *Pichia membranificiens* (14%), and *Penicillium brevicompactum* (10.7%) were overrepresented in the fungal communities carried by flies from the cellars while the pomace pile flies vectored primarily *Hanseniaspora uvarum* (28.2%), *Issachenkia orientalis* (14.6%), and *Pichia* species, such as *Pichia manshurica* (12.9%).



**Figure 2.2. Within wineries, the fungal communities vectored by *Drosophila* are distinct between winery habitat and distinguished by the relative abundances of a few yeast species.** (A) Bray-Curtis dissimilarity NMDS of fungal communities vectored by *Drosophila* in HLD1 in 2015 and 2016 (ADONIS:  $R^2 = 0.166$ ,  $p=0.001$ ). Each sample was rarefied to 1000 sequences and is represented by a single point, color-coded by winery area. (B) Heatmap comparing the average relative abundances of all fungal species representing >1% of the total fungal community in each winery area. Each row represents a single fungal species. Stars to the right denote fungal taxa that have significantly different relative abundances between winery areas (one-way ANOVA with Bonferroni error correction, ns: not significant, \*:  $p<0.05$ , \*\*:  $p<0.01$ , \*\*\*:  $p<0.001$ , \*\*\*\*:  $p<0.0001$ ). (C) Bar graphs of the relative abundances of fungal taxa that are significantly different between winery areas.

*Drosophila* in different winery areas carried many of the same fungal taxa but the relative abundances of these species distinguished fungal communities in one area from another (Figure 2.2B and 2C). Of these fungal species, the relative abundances of only six taxa were significantly

different between the fungal communities vectored by flies in these specific winery areas: *Hanseniaspora uvarum*, *Pichia manshurica*, *Pichia membranifaciens*, *Penicillium brevicompactum*, *Issatchenkia orientalis*, and *Pichia kluyveri* (Figure 2.2C). All of these yeast species are commonly found in vineyards and wineries [105]. Studies at other wineries have shown that winery equipment and processing surfaces harbor distinct microbial communities that change rapidly over time [104]. While we also observed distinct fly-associated fungal communities in different winery areas, the makeup of these communities do not fluctuate over time and do not completely mirror the previously identified fungal communities colonizing the fermentation tanks and cellar surfaces in other studies. Instead, *Drosophila* carry a subset of these taxa, suggesting that flies might play a role in shaping or maintaining the fungal community composition in these areas and only a subset of the yeasts from these fungal communities have a direct mutualistic relationship with flies.

#### *Drosophila melanogaster* do not prefer yeast species representative of the winery area from which they were collected

We next asked how the fungal community structure between different winery areas is established. If flies actively modulate their associated fungal communities, we expected flies to prefer the yeast species characteristic of the fungal communities in the winery area from which they were established. We expected those preferences to manifest themselves in fly behaviors that are closely associated with the presence of yeast, such as olfactory attraction, oviposition, larval development, and longevity.

To test these hypotheses, we quantified the behaviors of four isofemale *Drosophila melanogaster* lines that were established from the three winery areas towards yeast isolates that were cultured and isolated from flies in the winery (Table 2.1). Founders of the fly and yeast lines were collected from the SCM and HLD1 wineries. We selected a panel of six yeast species that most strongly distinguished each winery area from the others (Figure 2.2C). Each winery area was represented by a single yeast species except for the pomace pile, which was represented by two because *Pichia manshurica* (isolate P2) was unable to ferment in liquid grape juice. We also included two controls in the yeast panel. *Issatchenkia terricola* (yeast isolate CTLns) was included because it was vectored by all flies and did not distinguish one winery area from the others. Finally, *S. cerevisiae* (isolate CTLsc) was included as it is most often used in *Drosophila* behavior experiments in the laboratory and is generally attractive to *Drosophila melanogaster*, although we did not find that it was vectored frequently by wild flies in the winery.

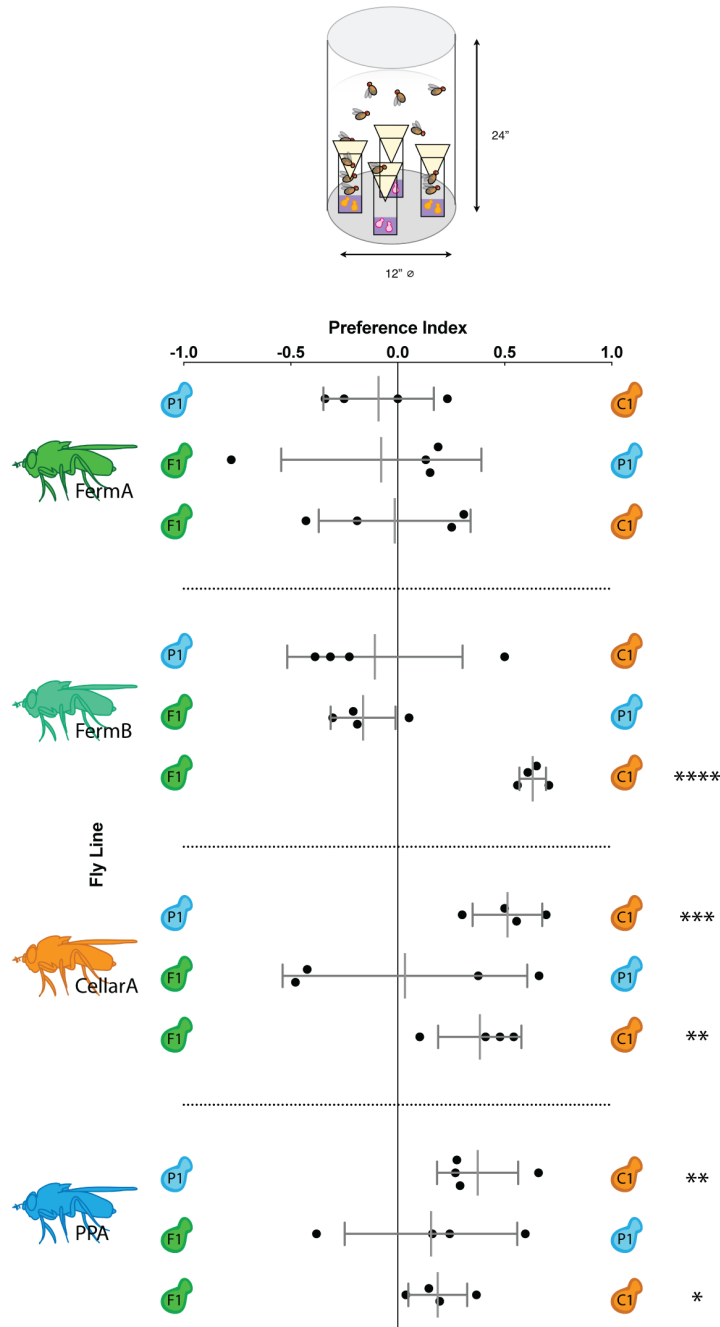
**Table 2.1. Fly lines and yeast isolates used in the behavior assays.**

<b>Isolate/ Fly Line</b>	<b>Type</b>	<b>Species</b>	<b>Vineyard</b>	<b>Winery Habitat</b>	<b>Year Collected</b>	<b>Month Collected</b>
C1	Yeast isolate	<i>Pichia kluyveri</i>	HLD1	Cellar	2015	August
F1	Yeast isolate	<i>Hanseniaspora uvarum</i>	SCM	Fermentation	2014	October
P1	Yeast isolate	<i>Issachenkia orientalis</i>	SCM	Pomace pile	2015	September
P2	Yeast isolate	<i>Pichia manshurica</i>	HLD1	Pomace pile	2015	August
CTLsc	Yeast isolate	<i>Saccharomyces cerevisiae</i>	SCM	Fermentation	2014	October
CTLns	Yeast isolate	<i>Issachenkia terricola</i>	SCM	Fermentation	2015	September
FermA	Isofemale fly line	<i>Drosophila melanogaster</i>	SCM	Fermentation	2014	October
FermB	Isofemale fly line	<i>Drosophila melanogaster</i>	SCM	Fermentation	2014	October
CellarA	Isofemale fly line	<i>Drosophila melanogaster</i>	HLD1	Cellar	2015	August
PPA	Isofemale fly line	<i>Drosophila melanogaster</i>	HLD1	Pomace pile	2015	August

### Olfactory preference

Because *Drosophila* initially rely on olfactory cues to locate yeasts [94,106], we first tested differential attraction for the yeast species that distinguish the fly-associated fungal communities from different winery areas. We used a simple, olfactory-based assay previously developed in our lab [34] to quantify fly preference, and tested pairwise comparisons of a smaller yeast panel, with a single yeast representing each winery area.

Although we did find significant preferences between yeast species, these preferences did not reflect winery area and were variable between fly lines (Figure 2.3). In all lines except for FermA, *Pichia kluyveri* (yeast isolate C1) was more attractive than both *Hanseniaspora uvarum* (yeast isolate F1) and *Issachenkia orientalis* (yeast isolate P1). Interestingly, FermA was the only fly line that was equally attracted to all three yeast species.



**Figure 2.3. Based on olfactory cues, *Drosophila* do not prefer the yeast associated with their winery area.**

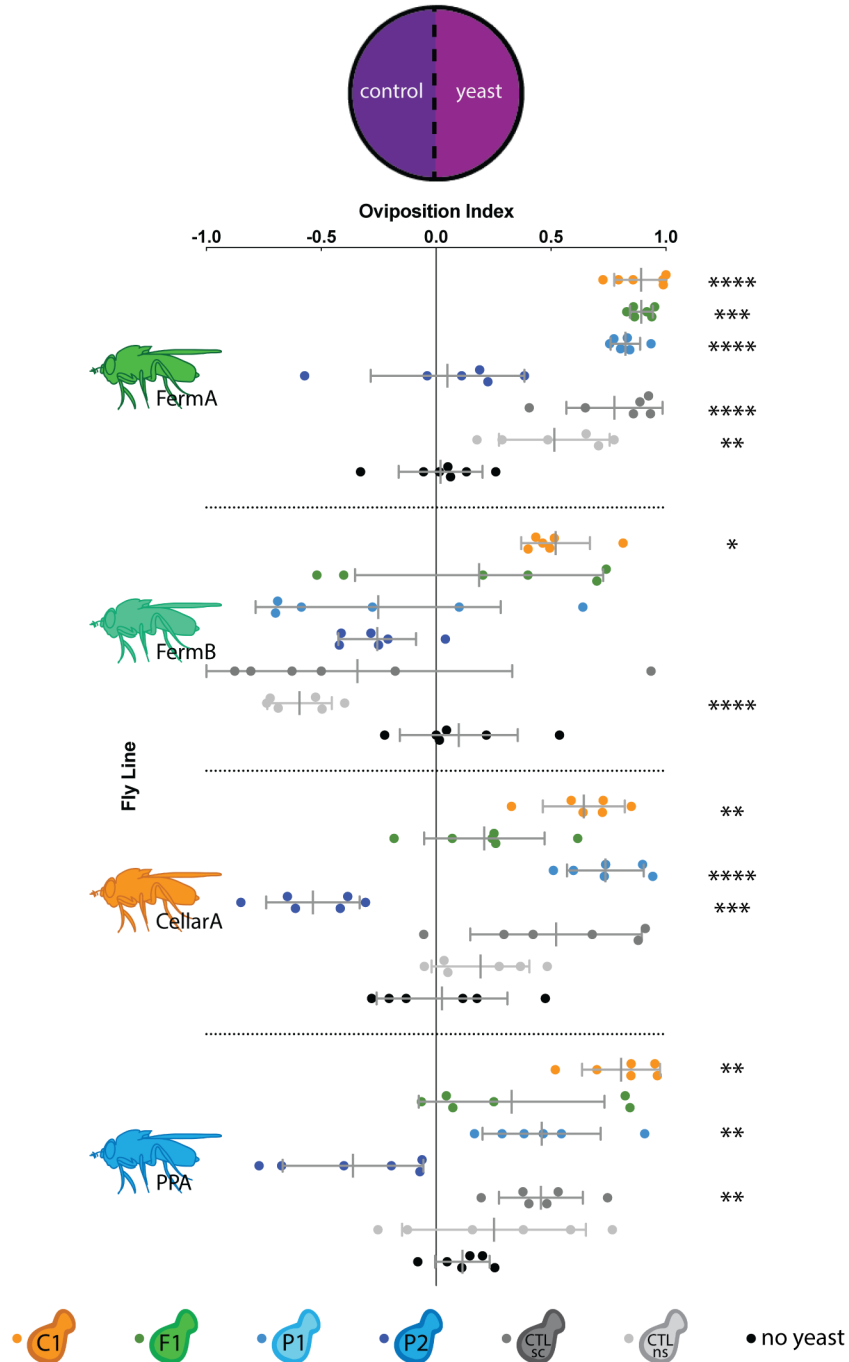
*Drosophila* lines tested are denoted by fly icons to the left and yeast species being compared are denoted by yeast symbols on the left and right axes. For a given comparison between yeast species A and B, a positive PI indicates a preference for yeast A, a negative PI indicates a preference for B, and a PI of 0 indicates no preference. Black dots indicate trial replicates. Short grey lines represent standard deviation and longer grey lines represent the mean of all trials. Stars to the left denote significantly different preferences between the two yeast species being tested (multiple t-tests with a Bonferroni correction, \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ , \*\*\*\*:  $p < 0.0001$ ).

Fly lines did not prefer the yeast species that we had designated as representative of the fungal communities vectored by flies in their winery area. Instead, fly lines were more attracted to some yeasts over others with variability between lines. While the attractiveness of these yeast species varies, these data suggest that the yeast species tested in this study produce metabolites that are generally attractive to *Drosophila*.

### Oviposition

Where female flies choose to lay eggs is strongly coupled with offspring fitness [107,108]. We hypothesized that if *Drosophila* actively modulate the fungal communities they vector, female flies would prefer to oviposit on substrate inoculated with yeast representative of their winery area. To determine if this specificity exists, we tested female oviposition preference for sterile grape substrate or grape substrate inoculated with a single yeast species from our yeast panel (Figure 2.4).





**Figure 2.4. Most fly-associated yeasts elicit a generally positive oviposition response with variability between fly lines.**

A positive OI indicates an ovipositional preference for the yeast side, a negative OI indicates an ovipositional preference for the control side, and an OI of 0 indicates no preference. *Drosophila* lines tested are denoted by fly icons to the left. Individual replicates are represented by dots and are color-coded by yeast species. Short grey lines represent standard deviation and longer grey lines represent the mean of all trials. Stars to the left denote significantly different oviposition preferences between the two sides (multiple t-tests with a Bonferroni correction, \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ , \*\*\*\*:  $p < 0.0001$ ).

*Pichia kluyveri* (yeast isolate C1) was the only yeast species on the panel that elicited a significant, positive oviposition response (Figure 2.4) for all fly lines tested, which is consistent with olfactory preference results. In contrast, *Pichia manshurica* (yeast isolate P2) was the only yeast that elicited no preference or a negative oviposition response in all fly lines. Analysis of the metabolites produced by *Pichia manshurica* using gas-chromatography, mass-spectroscopy (GC-MS) showed that this yeast species is unable to ferment and produce volatile metabolites in liquid grape media (Figure S2.3). The lack of volatile attractants may explain why *Pichia manshurica* elicits oviposition responses that mirror those of a sterile media control. Other yeasts in the panel, which fermented successfully, elicited intermediate ovipositional responses. These ovipositional preference patterns are not correlated with winery area. Instead, *Drosophila* seem to follow a general behavioral trend in response to the yeast panel, where some yeasts are more or less desirable oviposition substrates than others.

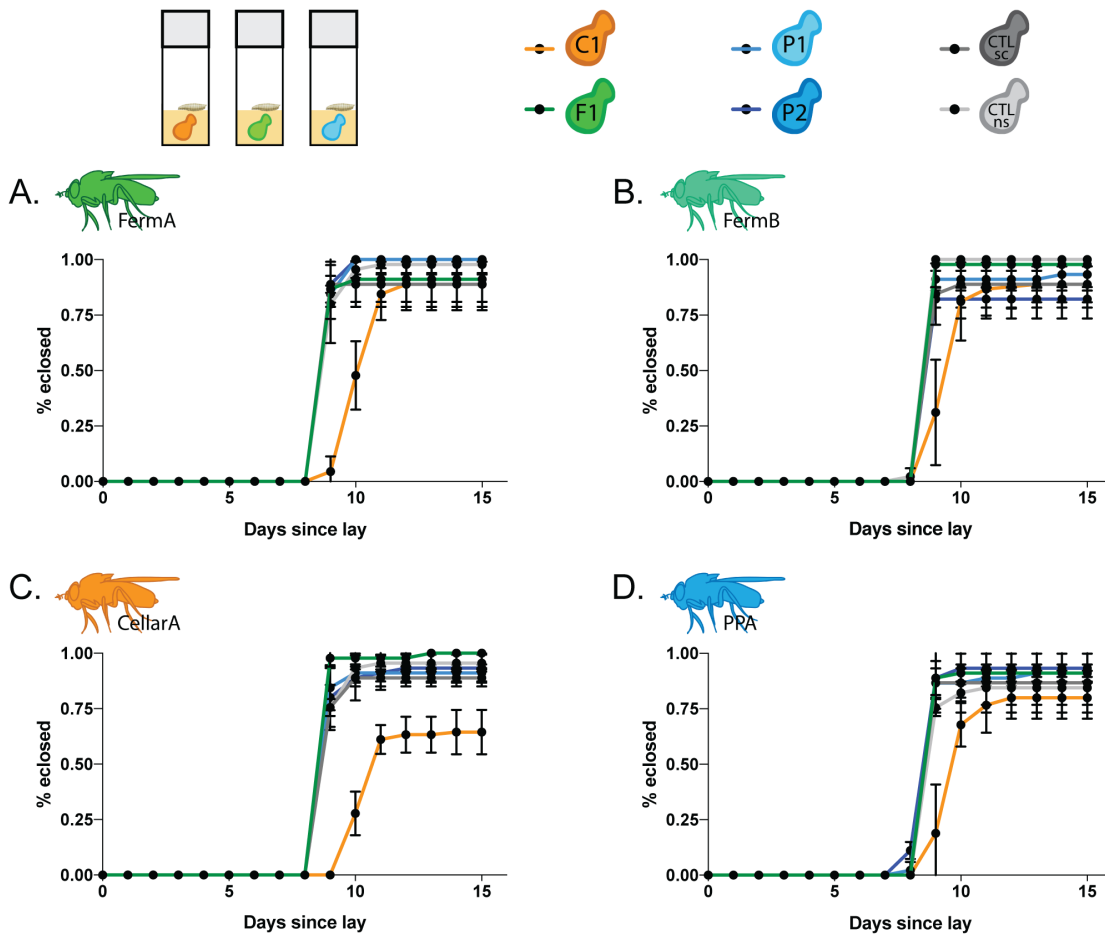
While all fly lines generally preferred to lay on yeast-inoculated media over sterile media (Figure 2.4), there was significant variation in oviposition response between fly lines (Table S2.3). For example, the two fly lines derived from the fermentation tanks, FermA and FermB, exhibited conflicting oviposition responses despite being collected at the same time. CellarA and PPA, which were collected in a different winery, winery area, and time, exhibited more similar, intermediate oviposition behavior. These data show that while there is natural heterogeneity in yeast volatile sensitivity between fly lines, the variation in ovipositional preference is not specific to winery area.

### Larval development

While oviposition substrate is important for larval fitness, larval development success and time is an indicator of nutritional health. *Drosophila* larva eclose both faster and more successfully when larval diet is supplemented with yeast [23,24]. To test if the yeasts associated with *Drosophila* in different areas of the winery have effects on larval development, we measured the development of winery fly lines when fed active monocultures of the yeast panel.

In corroboration with previous studies, we found that all fly lines develop more successfully on a diet supplemented with either live or dead yeast than on sterile media (Figure S2.4, Table S2.4). In fact, larvae grown on sterile media did not pupate at all, except for larvae from a single fly line, PPA, which only exhibited a 20% eclosion success on sterile media.

If a fly-yeast specificity existed in the winery, we hypothesized that larvae would develop faster and more successfully when supplemented with yeast species that are overrepresented in the fungal communities vectored by *Drosophila* in their winery area. For the subsequent statistical analyses, we omitted the no yeast and dead yeast controls from our dataset, as we were only interested in differential effects of the yeast species in our panel. We found that all yeast species on the yeast panel were equally suitable for *Drosophila melanogaster* development, with the exception of CellarA on *Pichia kluyveri* (yeast isolate C1, Figure 2.5A). Only 64.4% of CellarA larvae supplemented with *Pichia kluyveri* successfully eclosed compared to greater than 88% successful eclosion on all other yeast species in the panel (Table S2.4,  $p < 0.0001$  when compared to all other yeast species on panel). Interestingly, *Pichia kluyveri* did not have a significantly different effect on eclosion success in other fly lines tested.



**Figure 2.5. Some yeasts are more suitable for *Drosophila* development but do not follow a winery area specific pattern.**

Short, horizontal black lines represent standard deviation and black points represent the mean of all trials. Accompanying statistics for larval development time in Table S2.5. Larval development of fly lines (A) FermA (B) FermB. (C) CellarA. (D) PPA.

Similar to the eclosion success, we found no significant differences in median development time between larva from all fly lines raised on active monocultures of the yeast species on our yeast panel except for *Pichia kluyveri* (Figure 2.5, Table 2.2). While the median developmental time of all four fly lines on other yeast species was nine days, larva raised on *Pichia kluyveri* had a delayed development time of 10-11 days (Table S2.5).

**Table 2.2. *Drosophila* larval development time from lay to eclosion when diet is supplemented with a single yeast species.**

Fly Line	Yeast treatment	No. of larva	Median time to eclosion, days
FermA	C1	80	10
FermA	F1	41	9
FermA	P1	45	9
FermA	P2	45	9
FermA	CTLsc	40	9
FermA	CTLns	44	9
FermB	C1	80	10
FermB	F1	44	9
FermB	P1	42	9
FermB	P2	37	9
FermB	CTLsc	40	9
FermB	CTLns	45	9
CellarA	C1	58	11
CellarA	F1	45	9
CellarA	P1	41	9
CellarA	P2	42	9
CellarA	CTLsc	40	9
CellarA	CTLns	43	9
PPA	C1	72	10
PPA	F1	41	9
PPA	P1	41	9
PPA	P2	42	9
PPA	CTLsc	39	9
PPA	CTLns	38	9

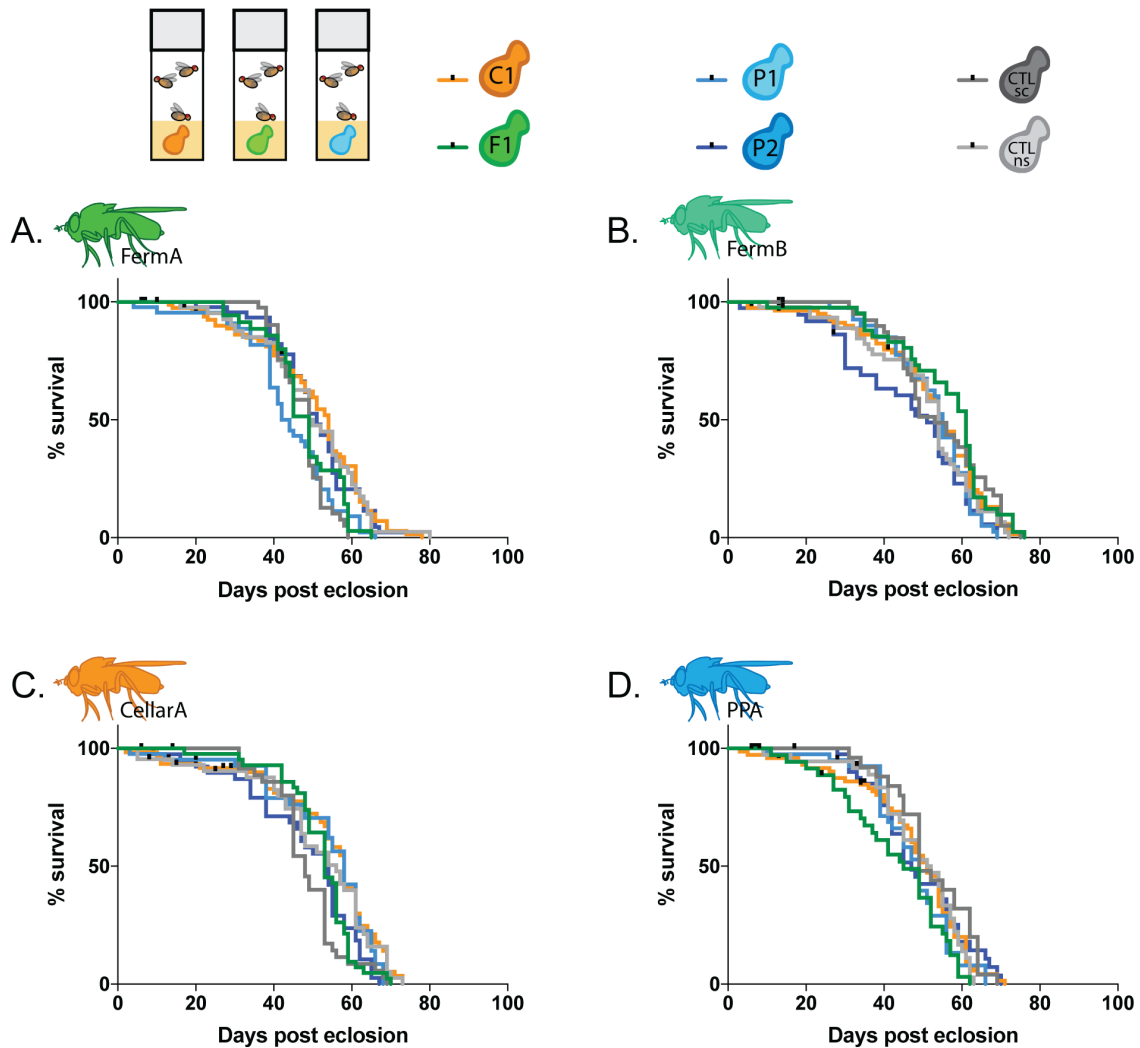
Accompanying statistics in Table S2.5.

In our the behavior assays we described above, we showed that *Pichia kluyveri* is both more attractive to adult flies and preferred as an oviposition substrate compared to other yeasts in the panel. So it is surprising that it has a negative effect on developmental timing. In addition, CellarA, a fly line established from a single female collected in a cellar, was the only line in which eclosion success was negatively affected by *Pichia kluyveri*, which is commonly vectored by *Drosophila* in cellars. Together, these data suggest effects of yeast on *Drosophila* larval development are nonspecific to winery area. Instead, a broad range of yeasts are suitable for *Drosophila* larval development and while most are equally beneficial, some are less favorable than others.

### Longevity

Finally, we tested if the yeast species in our panel had winery area specific effects on *Drosophila* lifespan. Supplementing diet with yeast can rescue undernutrition and extend lifespan in *D. melanogaster* [25]. To measure if winery area yeasts have differential effects on *Drosophila* longevity, we maintained the adult flies that eclosed from the previous larval development assay on sterile media inoculated with the same yeast species throughout the lifetime of the fly. While

these wild fly lines exhibit natural variation in lifespans (ANOVA,  $p < 0.0001$ , Table S2.6), no particular yeast species had a significant effect on lifespan for a given fly line (Figure 2.6, Table 2.3). These results mirror those of the previously described olfactory preference, oviposition, and larval development assays.



**Figure 2.6. Fly-associated yeasts have no differing effects on *Drosophila* longevity.**

Accompanying statistics for longevity assay in Table S2.6. Lifespan of fly lines (A) FermA (B) FermB. (C) CellarA. (D) PPA.

**Table 2.3. *Drosophila* lifespan when monoassociated with a single yeast species.**

Fly Line	Yeast treatment	No. of flies	Median lifespan, days
FermA	C1	78	54
FermA	F1	35	49
FermA	P1	44	43
FermA	P2	44	51
FermA	CTLsc	40	49
FermA	CTLns	40	50
FermB	C1	75	55
FermB	F1	41	61
FermB	P1	40	55
FermB	P2	35	51
FermB	CTLsc	39	53
FermB	CTLns	45	54
CellarA	C1	57	58
CellarA	F1	42	53
CellarA	P1	36	58
CellarA	P2	38	54
CellarA	CTLsc	35	48
CellarA	CTLns	38	56
PPA	C1	70	51
PPA	F1	33	45
PPA	P1	38	49
PPA	P2	32	47
PPA	CTLsc	25	49
PPA	CTLns	36	51.5

Overall, we found that while different *Drosophila* lines vary in their behaviors towards some yeasts over others, these behaviors are not specific to winery area. These results consistently indicate that the structure in fungal communities vectored by *Drosophila* in different winery areas is not a direct result of fly behavior.

## Discussion

The interaction between fruit flies and yeast provides an ideal system in which to explore the parameters governing a natural mutualism. In the laboratory, *Drosophila melanogaster* exhibit a remarkable ability to discriminate between different strains of *Saccharomyces cerevisiae* based solely on volatile profile [34,39] but whether these preferences are relevant in nature is less clear.

Our first goal in conducting this study was to determine if the fungal communities associated with *Drosophila* varied in a predictable way among the different fly-rich habitats in wineries. Our data clearly demonstrate that they do, with different abundances of a generally shared set of yeast species serving as a signature of fermentation tanks, cellars, and pomace piles.

This observation raises the more interesting question of how distinct fly-associated fungal communities are determined. There are two possible, not mutually exclusive, explanations: flies could be selectively sampling fungi from these environments or flies could be passively sampling from environments with markedly different fungal populations.

Using a set of well-defined *Drosophila* behavioral assays, we found that the yeast species on our panel were generally attractive based on volatile cues, equally suitable for larval development, and have no differential effects on lifespan. Flies did not selectively sample the yeast species distinguishing the fungal communities of their winery habitats, suggesting that the stratification fly-associated yeast communities with habitat is not a direct result of fly behavior.

The alternative explanation is that flies passively sample fungal communities that are predetermined by the growth conditions and activity in a given winery habitat. Environmental factors, such as substrate nutrient composition and temperature, likely have a pronounced effect on yeast community composition. Wine production and the movement of processing equipment also affects the rate and time at which particular fungal species flow in and out of these environments. We cannot reject the hypothesis that passive sampling of the fungi that grow optimally in particular winery niches accounts for the observed structure in fly-associated fungal communities.

Even if flies do not deliberately associate with particular yeast species within wineries, they likely influence the overall yeast species composition in these environments. Many observations, including this study, have shown that flies carry diverse fungal populations, and that they can transfer these populations to new substrates [31,32,89]. It is possible that the yeast species tested here are commonly found in vineyards and wineries because of flies' general attraction to them. Interestingly, we found that winery *Drosophila* were associated with many non-*Saccharomyces* wine yeasts that are known to contribute aroma complexity to wine fermentations [96,97,105], suggesting that flies may play a role in microbial terroir of wine flavor and aroma.

Our observations indicate that a broad range of yeast species are beneficial to *Drosophila* and can serve adequately as a mutualistic partner. However, lack of specificity does not imply that the fly-yeast mutualism is a weak interaction. The function and maintenance of a mutualism requires each partner to constantly evolve traits that allow for more efficient interactions with the other [8,109,110].

In a constantly fluctuating environment, where there are seasonal changes in food sources and temperature throughout the year, it is important that *Drosophila* be able to locate and subsist on many yeast species. Conversely, yeast must be able to produce volatile cues that ensure a close association with flies. Many of the yeast species tested in this study, despite being phylogenetically distant, share the ability to attract flies, confirming recent work demonstrating that volatile attractant production is a conserved trait across many yeast species [111]. However, the ability to attract flies does not always confer a benefit to fly fitness. In this study, we observed a yeast species, *Pichia kluyveri*, that was more attractive in the olfactory preference and oviposition assays than other yeasts but was the only yeast species that had a negative impact on larval fitness.

Wineries are habitats where both flies and yeast co-occur in large numbers and while this was advantageous for the goals of our study, we recognize that the yeast species tested in this study have already undergone selection for successful growth in a specific niche. The yeast used in this

study were all commonly vectored by *Drosophila* in wineries. Whether flies are associated with the same yeast species in habitats where resources are scarce or habitats with other fruit substrates, such as apple orchards, remains open to investigation. Characterizing fly behavior towards yeast that are not associated with *Drosophila* or associated with *Drosophila* from very different habitats, would reveal whether the fly-yeast interactions we observed in this study are only relevant for this particular niche or more broadly applicable to the fly-yeast mutualism across many environments. Additionally, a comparison of the volatile profiles of these yeast species with those tested in this study could also elucidate whether the responses we observed are context-dependent and be used to identify specific compounds that influence fly behavior. We hope that future studies will continue to study the fly-yeast mutualism in other natural ecosystems to yield more insight into the parameters constraining this mutualism.

## Materials and Methods

### Field collections of *Drosophila* and yeasts

All the wineries participating in this study practice organic farming and use spontaneous fermentations in winemaking. Adult *Drosophila* were collected in individual, sterile vials by direct aspiration or netting in cellars, fermentation tanks, and pomace piles. In 2015, flies were collected from two wineries in Healdsburg, California (HLD1) and the Santa Cruz Mountains, California (SMC) every two weeks from May 2015 – November 2015. During the 2016 harvest season, flies were collected from all four winery sites (Figure 2.1A) at a single time point from late September to early October. Geographical coordinates for collection sites were as follows: HLD1: 38°38'55.2"N 122°53'36.6"W, HLD2: 38°36'01.2"N 122°53'30.6"W, EBO: 37°53'27.7"N 122°10'55.0"W, and SCM: 37°18'18.2"N 122°07'44.2"W. Flies were immediately transported back to the laboratory and processed within four hours of collection. Upon arrival at the laboratory, we documented sex and grouped samples into either *D. melanogaster*/*D. simulans* or other *Drosophila* species by eye.

### Isolation and identification of yeasts for behavior assays

Roughly one third of the total flies collected were cooled in individual vials on ice for two minutes to reduce activity and placed onto 5% YPD agar plates (Table S2.7). Roughly equal numbers of males and females were sampled. Flies were allowed to walk on plates overnight at ambient room temperature to deposit yeasts on plates and were aspirated off of plates in the morning. Yeast deposited on the plates were allowed to grow at ambient room temperature for 3-4 days. Single colonies, representing every yeast morphology present, were picked by eye and streaked onto fresh 5% YPD agar plates. If plates were overgrown with mold or single colonies were unable to be picked, a subsequent isolation was performed on a fresh plate. Isolated colonies were allowed to grow at ambient room temperature and stored at 4°C until molecular identification. Original plates were kept for an additional three days after picking to ensure slower growing yeast were sampled.

Yeast colonies were identified by Sanger sequencing of the internal transcribed spacer region (ITS) [102]. Colony PCR reactions were performed in 25uL reaction volumes as follows: 12.5uL GoTaq Colorless Master Mix (Promega), 2uL of ITS1 and ITS4 primer at 10uM, 8.5uL nuclease-free water (Promega), and colony spike-in. Reaction conditions were as follows: 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 60 s, and a final



extension of 72 °C for 4 min (Jeremy Roop, personal communication). Amplification was verified on an agarose gel before being sent for Sanger sequencing (ELIM Biopharmaceuticals). Resulting sequences were trimmed for quality and then identified using BLAST (NCBI). Hits with an identity score greater than 98% were documented. After positive identification, yeast isolates were frozen as -80°C glycerol freezer stocks using standard protocol (Methods in Yeast Genetics, 2005) until use in behavior assays.

#### Establishment of isofemale fly lines for behavior assays

Female *Drosophila* used for the yeast collection described above were aspirated onto fresh fly media (standard cornmeal-molasses-yeast medium, Bloomington *Drosophila* Stock Center) and used to establish isofemale lines. Each line underwent three rounds of intensive inbreeding where one to three virgin females were mated to three male siblings. After intensive inbreeding, lines were inbred at least 25 generations (10-40 female and male siblings) before being used in behavior assays.

#### DNA extraction for amplicon study

The remaining flies collected (about 2/3 of flies) were immediately frozen and stored in individual, sterile 1.5mL microtubes for amplicon analysis. DNA was extracted following QIAGEN's QIAamp Micro Kit tissue protocol with the modifications briefly described below (Elya et al. 2016). After the overnight digestion with proteinase K, samples were bead beat (0.5 mm Zirconium beads, Ambion) in 200uL of WLB (Table S2.7). The samples were beat beat (MoBio) twice for one minute at 4°C with a 30 second break in between, spun five minutes at ~14,000xg, and the supernatant was transferred to a new tube. Beads were resuspended in 1ml of buffer WLB and beat again an additional minute, spun down, and supernatant was pooled. Finally, beads were washed once more with 1mL of buffer WLB, spun down, and supernatant was pooled. The pooled supernatant was spun down to pellet any beads and transferred to a clean microtube. One ug of carrier RNA (QIAGEN) dissolved in buffer AE was added to the supernatant before proceeding to ethanol precipitation and elution per the manufacturer's protocol. DNA samples were quantified (Qubit dsDNA HS assay kit, ThermoFisher Scientific) and stored at -20°C.

#### Amplification and library construction

Fungal communities were characterized by amplifying the universal fungal internal transcribed spacer region I (ITS1) using BITS and B58S3 primers designed by Bokulich & Mills [103]. Each forward BITS primer includes a unique 8bp barcode connected to the universal forward primer with a 2bp linker sequence (sequences generously provided by Bokulich & Mills).

The following pre-PCR steps were carried out in a biosafety cabinet. The biosafety cabinet and laboratory supplies used were cleaned at the start of each day as follows to minimize PCR contamination: 10% bleach for 20mins, rinsed with autoclaved MilliQ water, 3% hydrogen peroxide for 10mins, and UV lamp for at least 5mins. PCR reactions were carried out in triplicate following the protocol previously used in [104] and described below.

For a single PCR reaction, reagents were added in the following order: 12.5uL GoTaq Colorless Master Mix (Promega), 2uL of B58S3 primer at 10uM, 5.5uL nuclease-free water (Promega), 2uL of BITS primer at 10uM, and finally 5–100 ng DNA template. Reaction conditions were as follows: 94°C for 3 min, 35 cycles of 94°C for 45s, 50°C for 60s, and 72°C for 90s, and finally

extension of 72°C for 10 min [104]. PCR reactions were performed in 96 well plates with sample columns randomized between replicates to control for potential well biases. Both positive mock cultures (based on design described in [112]) and negative controls (from extraction and amplification steps) were randomized in the PCR plates among real samples.

PCR replicates were pooled and cleaned using AMPure XP magnetic beads according to manufacturer's protocol (Beckman Coulter). Pooled PCR products were quantified using the Qubit dsDNA HS assay kit and the samples on a single PCR plate were pooled at 30ng equimolar concentration. Any samples with concentrations of <1ng/uL were omitted. Negative control samples with concentrations of <1ng/uL were pooled to the largest volume of real samples. After pooling, each pool was cleaned and concentrated according to the manufacturer's protocol (Zymo Clean and Concentrator), eluted in 22uL of sterile water, and quantified with the Qubit dsDNA HS assay kit.

Illumina sequencing libraries were prepared for each PCR plate pool using TruSeq RNA v2 kit (Illumina) beginning at the A-Tailing step of the manufacturer's protocol using at least 200ng of starting material. A different Illumina index was used for each PCR plate pool. Libraries were verified and quantified using the Qubit dsDNA HS assay kit and the Agilent 2100 Bioanalyzer (Agilent Technologies). Up to four libraries were pooled and sequenced on a single 250bp paired-end Illumina MiSeq lane. Samples from 2015 were sequenced at the Vincent J. Coates Genomics Sequencing Laboratory (UC Berkeley) and the 2016 samples were sequenced at the UC Davis DNA Technologies Core. To control for the change in sequencing services, we resequenced a library from 2015 at UC Davis with 2016 samples and achieved very replicable results (Figure S2.6).

#### Data analysis of amplicon study

We followed many of the same processing steps outlined in [105,113] as we used the primers designed in these studies and expected the fungal communities of *Drosophila* in wineries to be similar to the fungal communities in wineries and breweries. Raw and quality filtered sequence counts from the following steps are summarized in Table S2.1. Raw read pairs were merged using BBMerge (<https://jgi.doe.gov/data-and-tools/bbtools/>), demultiplexed in QIIME v1.9.1 [114], and primer sequences were trimmed using cutadapt [115]. Resulting reads were quality filtered in QIIME as follows: any read less than 80bp was removed, any read with more than 3 consecutive bases with a quality score <19 was removed, and any chimeric sequences were filtered against the UCHIME chimera reference dataset v7.1 [116] using the union method.

Open reference OTU picking was performed in QIIME using the UCLUST method [117] against a modified UNITE database [118,119] with a threshold of 97% pairwise identity. Sequence alignment and treebuilding were suppressed and taxonomy was assigned using BLAST (NCBI). After OTU picking, positive control mock culture samples and OTUs with 'no blast hit' were filtered. Using R version 3.2.4 [120], negative controls were removed, max sequence counts of all negative control OTUs were calculated, and then subtracted from all real samples to account for spurious sequences produced from possible PCR, sequencing, or spillover contamination [112,121]. Finally, an OTU threshold of 0.001% was applied [105,113].

Alpha diversity measurements of observed OTU richness were calculated and visualized in QIIME to reveal that all samples had been sequenced to saturation (Figure S2.1). Community analyses were conducted using the vegan [122] and biom [123] packages in R. To determine

relationship between the fungal communities vectored by *Drosophila*, samples were evenly subsampled to 1000 reads per sample and Bray-Curtis dissimilarity was calculated and visualized with nonmetric multidimensional scaling (NMDS) using ggplot2 [124]. ADONIS was used to calculate the relative effects of factors that distinguished fly-associated communities from others. The fungal communities associated with *Drosophila simulans*/*Drosophila melanogaster* samples were significantly different than those of other *Drosophila* species (Figure S2.2) so other *Drosophila* samples were filtered out.

The relative abundances of fungal taxa in different winery areas were clustered using hierarchical clustering by taxa (Cluster 3.0) and visualized in Java TreeView and Prism 7 (GraphPad). To identify fungal taxa with significantly different relative abundances between winery areas, OTUs were collapsed by species name and the Kruskal-Wallis test was employed (with Bonferroni correction) in QIIME.

All raw reads are available from the NCBI Sequence Read Archive under accession SRP136413.

### Olfactory preference assay

A custom trap-based olfactory preference assay previously designed by Schiabor et al was used to measure *Drosophila* olfactory preference [34]. Yeast species were plated onto agar grape juice plates (Table S2.7) and grown at 30°C for 22 hours. The following day, plates were removed from the incubator, fitted with a custom printed lid, and secured with Parafilm. Lids were topped with a 50mL conical centrifuge tube (Falcon) with the end removed and covered in mesh. A funnel was fashioned from 150mm filter paper (Whatman, 150mm, Grade 1) with a 5mm hole snipped off the tip and secured to the top of the centrifuge tube with tape.

*Drosophila melanogaster* lines were raised at room temperature (21°C-23°C) on standard cornmeal-molasses-yeast media and aged at room temperature for at least four days under ambient lighting conditions (i.e. adjacent to a window) before being used in behavior assays. One hundred and twenty 4-10 day old mixtures of male and female flies were anesthetized with CO<sub>2</sub> and allowed to recover on cornmeal-molasses-yeast media for two hours before being used in behavior assays.

Pairwise comparisons of yeast were used to assay for olfactory preference. Two traps for each yeast species were placed into behavior arenas (*Drosophila* population cages, 24" x 12" clear acrylic cylinders, TAP plastics) and fitted with netting (Genesse Scientific) as shown in Figure 2.3 and Figure S2.5A. All four possible orientations of plates within the arena were tested to control for potentially confounding environmental variables such as a light (Figure S2.5B).

Flies were introduced into behavior arenas at 3pm and allowed survey traps. After 18 hours, traps were removed from the arena and the number of flies in each trap were counted, sexed, and recorded. Flies were only used in behavior assays once and were discarded after counting. A preference index was calculated from the number of flies in each trap as follows:

For **A** = total number of flies in traps baited with Yeast A

For **B** = total number of flies in traps baited with Yeast B

$$\text{Preference Index (PI)} = (\mathbf{A}-\mathbf{B})/(\mathbf{A}+\mathbf{B})$$

A positive PI indicates a preference for yeast A, a negative PI indicates a preference for B, and a PI of 0 indicates no preference. Multiple t-tests with Bonferroni correction were executed in Prism 7 and used to determine which yeast preferences were significant.

### Oviposition assay

The egg laying assay in this study was adapted from Joseph et al [125] and Fischer et al [63]. At 10am, 75mL of sterile liquid grape juice (Table S2.7) was inoculated with 1.5mL of yeast cells diluted to  $OD_{600}=1$  in sterile 1X PBS (Mediatech Inc). These cultures, and a negative control, were grown shaking at 30°C for 72 hours.

Oviposition assay cages were fashioned from polypropylene *Drosophila* bottles (6oz, square, Genesee Scientific) with the bottom cut out and covered with mesh. During acclimation, cages were capped with petri dishes (35x10mm, Falcon) filled with grape agar premix (Genesee Scientific) and topped with yeast paste (Red Star).

Similar to olfactory preference assays, *Drosophila melanogaster* lines were raised and aged for four to ten days at room temperature on standard cornmeal-molasses-yeast media. Twenty-four hours before the assay began, twenty non-virgin females were acclimated to oviposition cages at 25°C. Cages were kept on the top shelf of a 25°C incubator on a 12 hour light cycle, placed on the side, and positioned so the mesh bottom faced the back of the incubator and the plate faced the door of the incubator (S5C Fig).

At least one hour before the start of the behavior assay, acclimated cages were cleared by replacing the petri dish with a new grape agar premix plate without yeast paste and returned to 25°C. After 72 hours of fermentation, cultures were removed from 30°C, mixed 1:1 with a boiled water-agar solution (BD Bacto Dehydrated Agar) cooled to 65°C, to achieve a final agarose concentration of 1.6%. The lids of petri dishes (35x10mm, Falcon) were divided in half using laminated paper. Plates containing half uninoculated grape juice and half inoculated grape juice were created by pouring both sides simultaneously (S5C Fig).

Plates were cooled for 15 minutes at room temperature and the laminated paper was removed. Plates were immediately used in behavior assays by replacing the grape agar premix plate cage topper. *Drosophila* females were allowed to oviposit on plates for three hours before plates were removed for counting (usually from around 12noon to 3pm). Flies were only used once and discarded at the end of the assay.

An oviposition index (OI) was calculated from the number of embryos deposited on each side of the plate as follows:

For **Y** = total number of eggs oviposited on inoculated side  
For **N** = total number of eggs oviposited on uninoculated side

$$\text{Oviposition Index (OI)} = (\mathbf{Y-N})/(\mathbf{Y+N})$$

A positive OI indicates an oviposition preference for the yeast side, a negative OI indicates a preference for the control side, and a OI of 0 indicates no preference for either side. Multiple t-tests with Bonferroni correction were used to determine whether the yeast tested elicited a significantly different oviposition preference in relation to the control. One way ANOVA was

used to test for any differences in oviposition responses between fly lines for a given yeast species. If ANOVA results were statistically significant, Tukey's multiple comparisons test was used to identify the fly lines exhibiting oviposition responses that were significantly different than other lines. These statistical analyses and those described in the methods following were implemented in Prism 7 with a significance cutoff of  $p > 0.05$ .

#### Gas chromatography – mass spectrometry (GC-MS)

A subset of the oviposition plates used in the oviposition assays were also sampled by GC-MS in parallel with the behavior assays using a stirbar sorptive extraction (SBSE) and thermal desorption approach. Oviposition plates were placed in sterile 60 x 15mm petri dishes for headspace sampling. As previously described in [34], a conditioned, Twister stir bar (10 mm in length, 0.5mm film thickness, 24uL polydimethylsiloxane, Gerstel Inc) was suspended from the lid of the larger petri dish with rare earth magnets for 40 minutes at room temperature. The Twister bar was then dried using a Kimwipe, placed in a thermal desorption sample tube, topped with a transport adapter, and loaded onto sampling tray (Gerstel Inc).

Automated sampling and analysis was performed using the Gerstel MPS system and MAESTRO integrated into Chemstation software. Sample analysis was performed on an Agilent Technologies 7890A/5975C GC-MS equipped with a HP-5MS (30m x 0.25mm, i.d., 0.25micrometers film thickness, Agilent Technologies) column.

Samples were thermally desorbed using the Gerstel Thermal Desorption Unit (TDU) in splitless mode, ramping from 30°C to 250°C at a rate of 120°C/min, and held at the final temperature for 5 minutes. The Gerstel Cooled Injection System (CIS-4) was cooled to -100°C with liquid nitrogen before ramping to 250°C at a rate of 12°C/min and held for 3 mins for injection into the column. The injector inlet was operated in the Solvent Vent mode, with a vent pressure of 9.1473 psi, a vent flow of 30mL/min, and a purge flow of 6mL/min.

The GC oven temperature program was set to 40°C for 2 min, raised to 140°C at 4 °C/min, and finally raised to 195°C at 15°C/min and held for 10 min. A constant helium flowrate of 1.2 mL/min was used as carrier gas. The MSD transfer line temperature was set at 280°C. The MS was operated in EI mode with the electron voltage set at autotune values. The detector was set to scan from 30 to 300amu at a threshold of 150 at a scanning rate of 2.69 scans/second. The ion source and quadrupole temperatures were set at 230°C and 150°C, respectively.

GC-MS data files were visually inspected using Chemstation and peaks were identified using the NIST O8 database. Datafiles were transferred, parsed, and analyzed using custom written Matlab scripts in [34]. Every chromatogram trace represents, at minimum, the average of 6 replicates.

#### Larval development assay

In order to test the effects of each yeast species on larval development time and success, *Drosophila* larvae were raised on sterile, yeast-free media supplemented with a single yeast species. As in the previously described assays, *Drosophila melanogaster* lines were raised and aged for four to ten days at room temperature on standard cornmeal-molasses-yeast media. Twenty-four hours before embryo collection, at least 50 adults flies were acclimated to the oviposition assay cages capped with grape agar premix plates and yeast at 25°C as described above.

At 9am the following morning, plates in oviposition cages were replaced with new grape agar premix with yeast plates to clear any hoarded eggs. After 30 minutes, clearing plates were replaced with new grape agar premix with yeast plates and flies were allowed to lay for two hours at 25°C for embryo collection. After two hours, collection plate was removed and aged at 25°C for two hours. Aged embryos were washed off plates with MilliQ water into a embryo wash basket fashioned out of a 50mL conical (Falcon) with the end cut off and the top of the lid removed and covered with thin mesh. In the wash basket, embryos were dechorionated with 30% bleach for three minutes, consequently removing any previously associated yeast. Embryos were washed with sterile, autoclaved MilliQ and then with sterile PBS-t (1X PBS, 0.5% triton). Using a sterile paintbrush, embryos were moved onto sterile agar plates and allowed to hatch at 25°C overnight. For data analyses, this day was considered Day 0 of the assay.

On the same day at 9:30am, 5mL starter cultures of liquid 5% YPD (Table S2.7) were inoculated with the yeast species of interest and grown shaking at 30°C. At 3:30pm, cultures were removed and diluted to  $OD_{600}=0.5$ . *Drosophila* vials with sterile, yeast-free GB media (Table S2.7) were spotted with 50uL of diluted culture and grown at 30°C overnight. Three replicate vials for each yeast species and each fly line were set up (S5E Fig).

At 11am the next day, embryo plates were removed from the incubator. Using a sterile paintbrush (dipped in 50% bleach, 75% ethanol, autoclaved MilliQ, and sterile PBS-t between each vial), 15 larvae were moved into each vial and allowed to develop at 25°C. Due to the extensive setup and time constrains of fly development, we tested the larval development of all four fly lines for each yeast species on the panel in three groups over 2 months. In each group, a positive control on standard cornmeal-molasses-yeast media (dead yeast) and a negative control on sterile GB media with no yeast supplement was run in parallel with experimental conditions.

We opted to start our assays with larvae instead of embryos in an effort to control for any death after dechorionation. For data analyses, this day was considered Day 1 of the assay. Each day, vials were checked for emerged adults and randomized within the incubator until the assay was terminated on Day 16. Adults that eclosed successfully were moved into sterile GB media vials daily and subsequently used for the longevity assays described below.

Eclosion curves and statistics were plotted in Prism 7. To test whether larvae given any yeast eclosed more successfully than those given no yeast and whether some yeast species resulted in greater eclosion success than others, one way ANOVA followed by Tukey's multiple comparisons test was used to calculate significance values for each yeast species and controls within each fly line.

### Longevity assay

To study the effects of each yeast species on the lifespan of *Drosophila*, adults that eclosed successfully from the larval development assay were fed a diet supplemented with the same yeast species throughout their lifetime. At 9am on Day 8 of the larval development assay, 5mL starter cultures of liquid 5% YPD were inoculated with the yeast species of interest and grown shaking at 30C. At 3:30pm, cultures were removed and diluted to  $OD_{600}=0.5$ . *Drosophila* vials with sterile, yeast-free GB media (Table S2.7) were spotted with 50uL of diluted culture and grown at 30°C overnight (S5E Fig).

As flies hatched off of the larval development assay, they were moved onto the inoculated media and checked every day. Each day, vials were randomized within the incubator to control for positional effects. Flies were maintained at 25°C and pushed onto fresh media twice a week, once into sterile GB media and once into inoculated media prepared as described above.

Survival curves and statistics were plotted in Prism 7. One way ANOVA followed by Tukey's multiple comparisons test was used to test whether different fly lines had significantly different lifespans. The effect of single yeast species on the lifespan of a single fly line was tested using one way ANOVA but found no significant differences.

## Acknowledgements

We are grateful for field assistance from Patrick O'Grady, Kelly Schiabor, Kyle Barrett, Elizabeth Roeske, Carolyn Elya, Addie Norgaard, Ciera Martinez, and Jesse Rau. We are also indebted to Nick Bokulich, David Mills, Angus Chandler, Rachel Adams, Sydney Glassman, Shana McDevitt, and Dylan Smith for their advice in fungal amplicon experimental design and data analysis. David Hembry and Will Ludington provided valuable feedback on the drafts of this manuscript. None of this would have been possible without the generosity of Ridge Vineyards, DaVero Farms & Winery, Les Lunes Wine, and Populis Wine with special thanks to Shun Ishikubo, Eric Baugher, Mike Bairdsmith, and Will Thomas.

Supporting Information

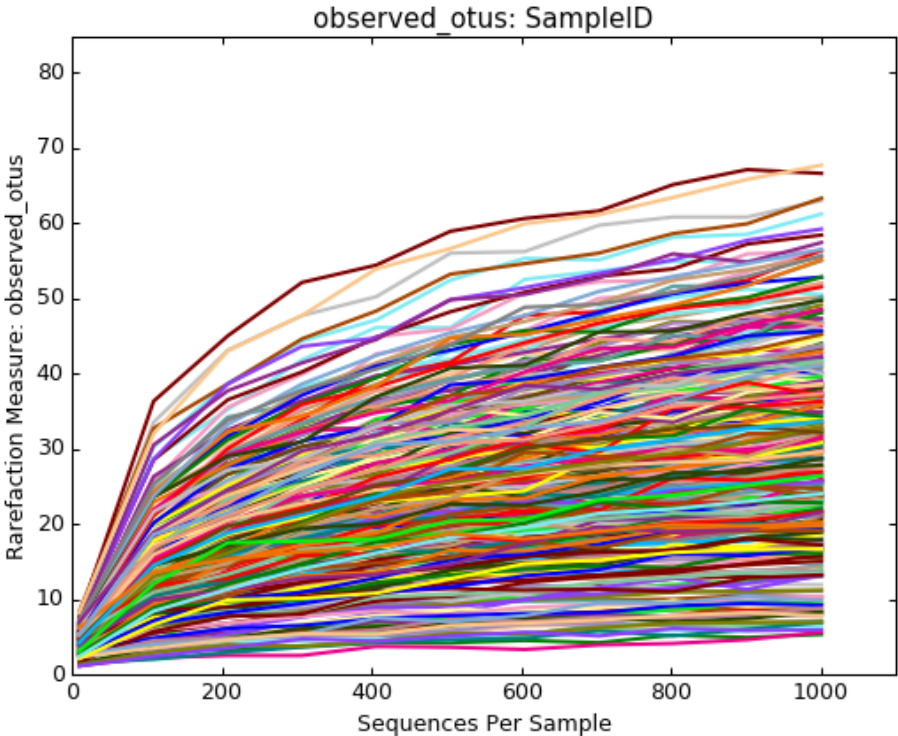
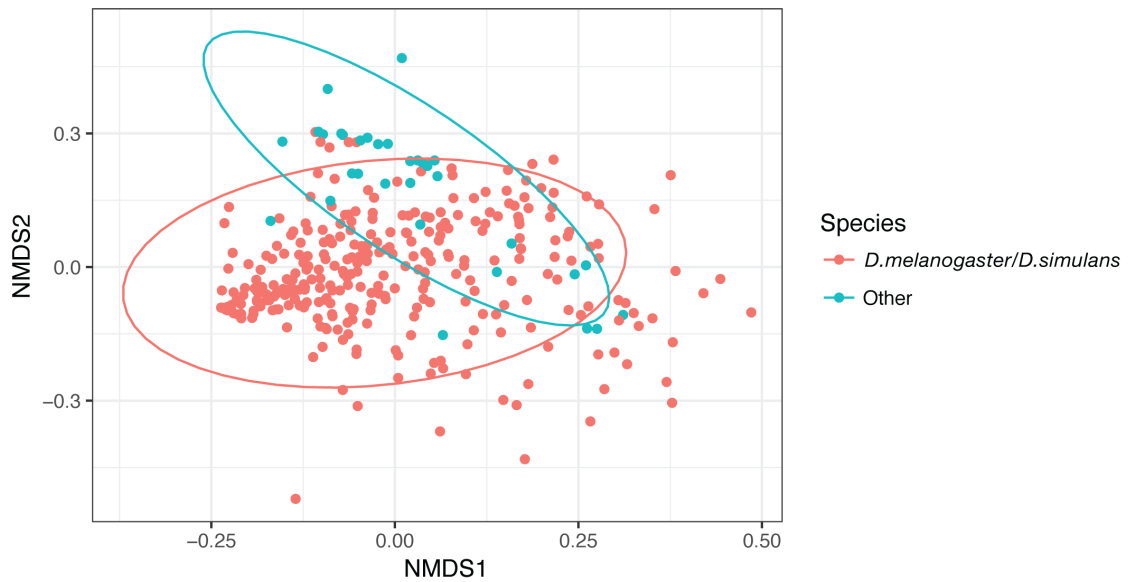


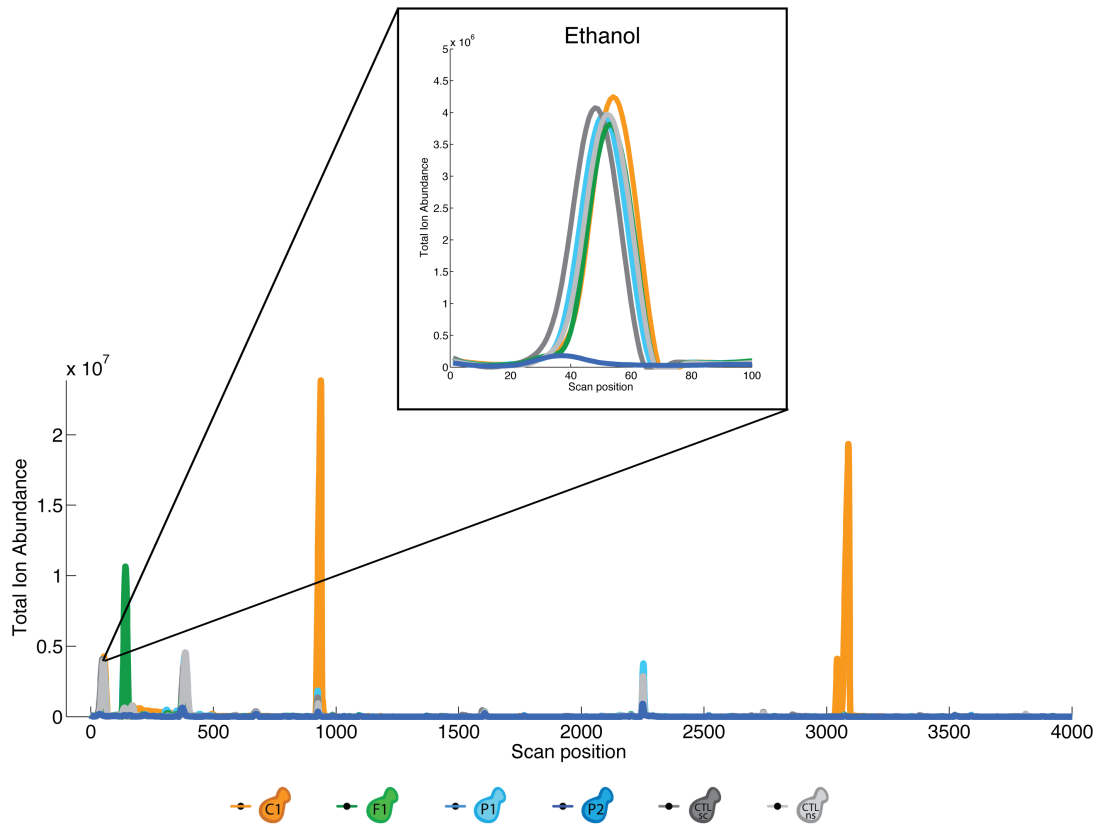
Figure S2.1. Alpha diversity richness QIIME rarefaction curves.





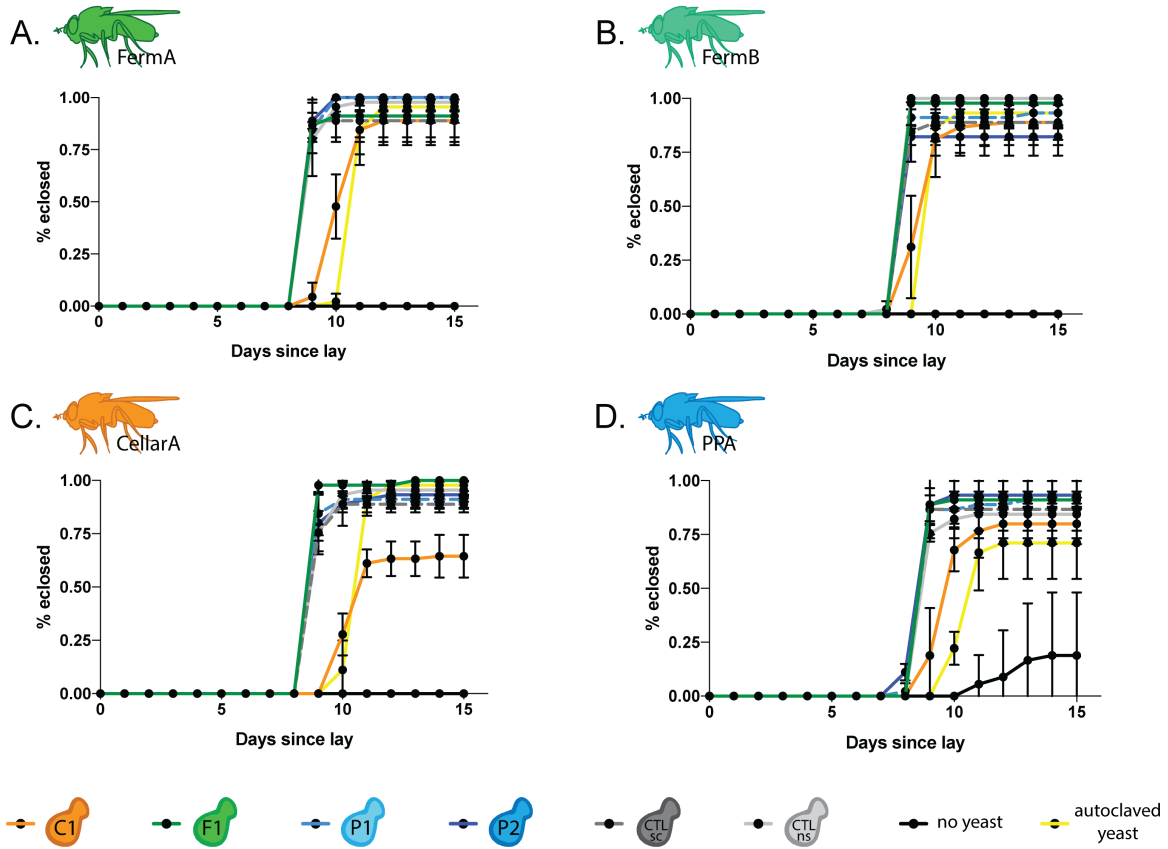
**Figure S2.2. The fungal communities vectored by *Drosophila melanogaster/Drosophila simulans* are different than other *Drosophila* species.**

Bray-Curtis dissimilarity NMDS of fungal communities vectored by *Drosophila* in all vineyards in 2015 and 2016. Each sample was rarefied to 1000 sequences and is represented by a single point, color-coded by species. ADONIS:  $R^2 = 0.018$ ,  $p=0.001$ .



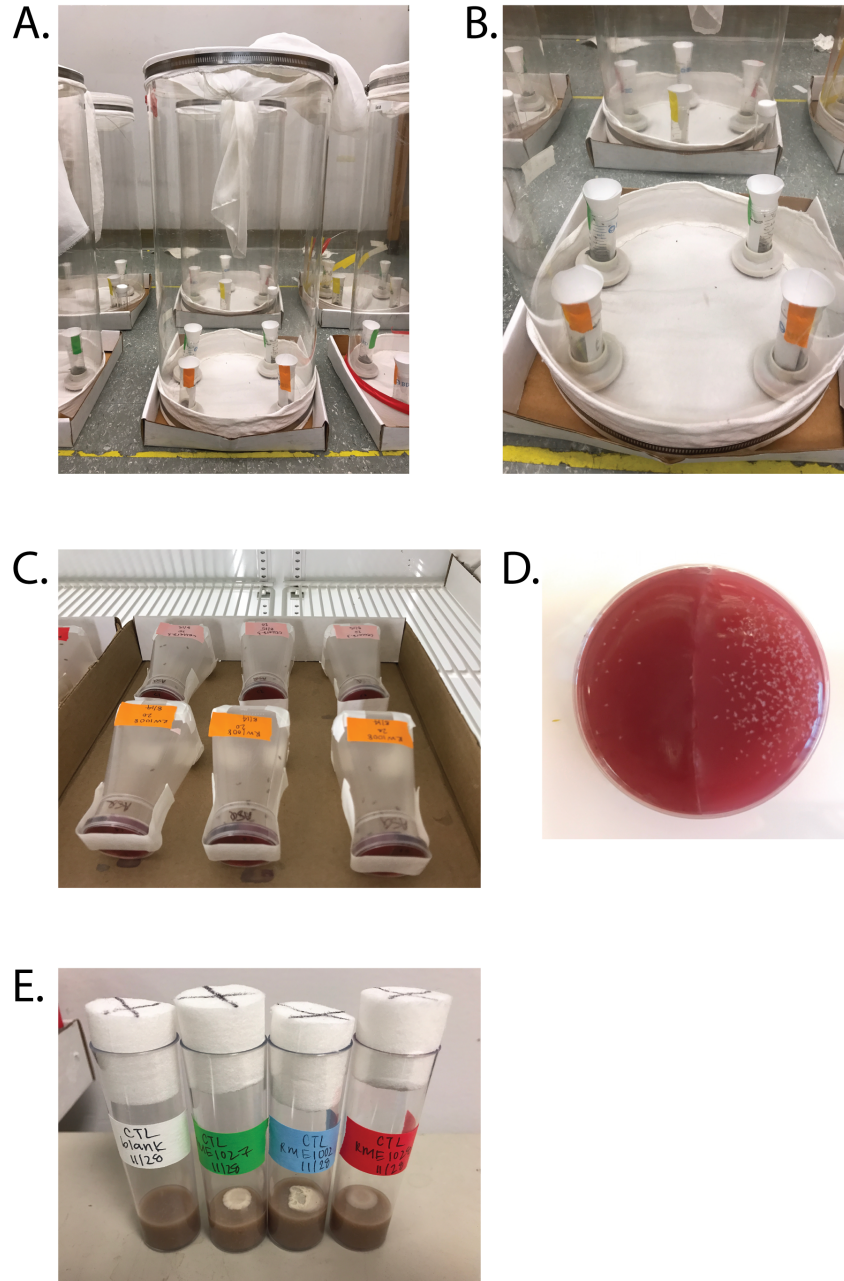
**Figure S2.3. *Pichia manshurica* (yeast isolate P2) does not ferment well in liquid grape juice.**

When measured by GC-MS, *Pichia manshurica* produces very low levels of ethanol (inset) and other volatile metabolites compared to other yeast species on the panel. Each line represents the average of eight GC-MS replicates for a given yeast species. Replicates were sampled for GC-MS in parallel with oviposition assays.



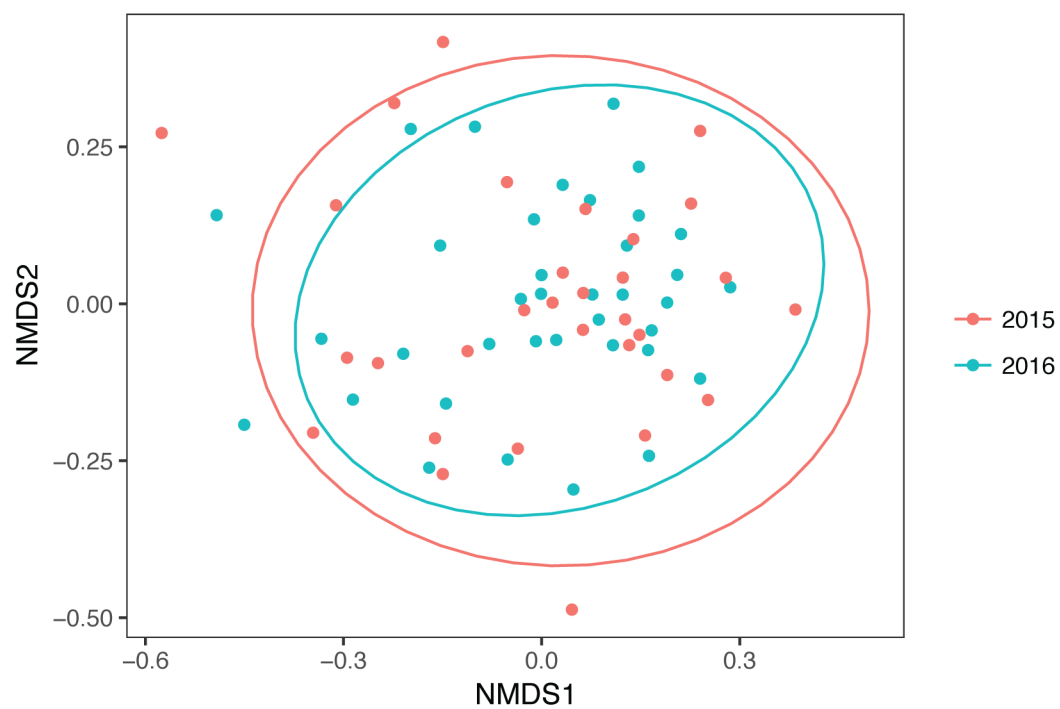
**Figure S2.4. *Drosophila* larvae supplemented with any species yeast species, dead or alive, develop more successfully than those fed no yeast at all.**

Note that data for larvae that were not given yeast only exist for PPA line because no larvae eclosed without the addition of yeast in any other lines.



**Figure S2.5. Photographs of behavior assays.**

(A) Setup of a single, trap-based olfactory assay. (B) Close up of trap-based olfactory assay. Traps can be arranged in four possible combinations, two of which are depicted here. (C) Setup of six oviposition assays. (D) Example agar plate after oviposition assay. Left side is uninoculated grape juice agar, right side is yeast inoculated grape juice agar. (E) Larval development and longevity assays were performed in wide vials shown here. Both larvae and adults were exposed to a live monoculture of yeast spotted onto sterile banana media. Depicted are Day 7, negative control vials that had no larvae or adult flies but grew alongside behavioral assays to monitor bacterial or mold contamination.



**Figure S2.6. Bray-Curtis dissimilarity NMDS of PCR plate sequenced with samples collected in 2015 at UC Berkeley and the same plate resequenced with samples collected in 2016 at UC Davis.**

Each sample was rarefied to 200 sequences and is represented by a single point, color-coded by year.

**Table S2.1. Library and quality filtering statistics.**

	<b>2015 total reads</b>	<b>2016 total reads</b>	<b>Total OTUs</b>	<b>% original reads retained</b>
<b>Raw sequences</b>	8724595	15570284	--	--
<b>After merging pairs</b>	8034126	14261774	--	91.77%
<b>After demultiplexing</b>	5389671	7311698	--	52.28%
	<u>Combined total reads</u>			
<b>Removal of resequenced control</b>	12037090		--	49.55%
<b>After quality filtering</b>	11302418		--	46.52%
<b>After chimera filtering</b>	11293777		6159	46.49%
<b>Post OTU picking quality filtering</b>	7609820		399	31.32%
<b>Final sequence count</b>	7609820		399	31.32%

**Table S2.2. Alpha richness of fungi vectored by *Drosophila* by vineyard and harvest season. Rarefied to 1000 sequences per sample.**

<b>Vineyard</b>	<b>Year Collected</b>	<b>PCR plate name</b>	<b>mean richness</b>	<b>SE</b>	<b>No. samples</b>
HLD1	2015	L15	31.502	9.516	84
SCM	2015	M15	33.253	17.22	74
HLD1	2016	L16	39.771	10.537	41
HLD1	2016	LF16	28.8	NA	1
SCM	2016	M16	33.953	7.672	47
SCM	2016	LF16	23.15	2.55	2
HLD2	2016	D16	19.741	7.673	54
HLD2	2016	LF16	25.9	5.193	10
EBO	2016	O16	22.027	7.209	44

**Table S2.3. Statistics accompanying the oviposition index responses of each fly line in Figure 2.4 for each yeast species.**

Each table represents a different yeast species. Within each yeast species, ANOVA was first used to test for any differences in oviposition responses between fly lines for a given yeast species and denoted by a \* next to each yeast isolate. If ANOVA results were statistically significant, Tukey's multiple comparisons test was used to identify the fly lines exhibiting oviposition responses that were significantly different than other lines and are depicted with \* within the table. ns: not significant, \*: p<0.05, \*\*: p<0.01, \*\*\*: p<0.001, \*\*\*\*: p<0.0001.

Yeast Isolate: C1**				
	FermA	FermB	CellarA	PPA
FermA	-	**	ns	ns
FermB	-	-	ns	*
CellarA	-	-	-	ns
PPA	-	-	-	-

Yeast Isolate: F1**				
	FermA	FermB	CellarA	PPA
FermA	-	*	*	ns
FermB	-	-	ns	ns
CellarA	-	-	-	ns
PPA	-	-	-	-

Yeast Isolate: P1****				
	FermA	FermB	CellarA	PPA
FermA	-	****	ns	ns
FermB	-	-	***	**
CellarA	-	-	-	ns
PPA	-	-	-	-

Yeast Isolate: P2**				
	FermA	FermB	CellarA	PPA
FermA	-	ns	**	ns
FermB	-	-	ns	ns
CellarA	-	-	-	ns
PPA	-	-	-	-

Yeast Isolate: CTLsc***				
	FermA	FermB	CellarA	PPA
FermA	-	***	ns	ns
FermB	-	-	**	*
CellarA	-	-	-	ns
PPA	-	-	-	-

Yeast Isolate: CTLns****				
	FermA	FermB	CellarA	PPA
FermA	-	****	ns	ns
FermB	-	-	***	***
CellarA	-	-	-	ns
PPA	-	-	-	-

**Table S2.4. Average percentage of *Drosophila* larvae that eclose successfully when developing on different yeast species.**

Control conditions are shaded grey. (ANOVA when compared to no yeast control followed by Tukey's multiple comparisons test was used to calculate significance values, \*\*\*\*\*:  $p < 0.0001$ ).

Fly Line	ANOVA	Yeast treatment						no yeast	Dead yeast, cornmeal media
		C1	F1	P1	P2	CTLsc	CTLns		
FermA	****	88.9%	100.0%	100.0%	100.0%	100.0%	97.8%	0.0%	94.4%
FermB	****	88.9%	100.0%	93.3%	82.2%	88.9%	100.0%	0.0%	93.3%
CellarA	****	64.4%	100.0%	91.1%	93.3%	88.9%	95.6%	0.0%	96.7%
PPA	****	80.0%	91.1%	91.1%	93.3%	86.7%	84.4%	20.0%	67.8%



**Table S2.5. Statistics to accompany Table 2.2 on effects of yeast species on larval development time for each fly line.**

One way ANOVA followed by Tukey's multiple comparisons test was used to calculate significance values, \*\*\*\*:  $p < 0.0001$ .

<b>FermA</b>	C1	F1	P1	P2	CTLsc	CTLns
C1	-	****	****	****	****	****
F1	-	-	ns	ns	ns	ns
P1	-	-	-	ns	ns	ns
P2	-	-	-	-	ns	ns
CTLsc	-	-	-	-	-	ns
CTLns	-	-	-	-	-	-

<b>FermB</b>	C1	F1	P1	P2	CTLsc	CTLns
C1	-	****	****	****	****	****
F1	-	-	ns	ns	ns	ns
P1	-	-	-	ns	ns	ns
P2	-	-	-	-	ns	ns
CTLsc	-	-	-	-	-	ns
CTLns	-	-	-	-	-	-

<b>CellarA</b>	C1	F1	P1	P2	CTLsc	CTLns
C1	-	****	****	****	****	****
F1	-	-	ns	ns	ns	ns
P1	-	-	-	ns	ns	ns
P2	-	-	-	-	ns	ns
CTLsc	-	-	-	-	-	ns
CTLns	-	-	-	-	-	-

<b>PPA</b>	C1	F1	P1	P2	CTLsc	CTLns
C1	-	****	****	****	****	****
F1	-	-	ns	ns	ns	ns
P1	-	-	-	ns	ns	ns
P2	-	-	-	-	ns	ns
CTLsc	-	-	-	-	-	ns
CTLns	-	-	-	-	-	-

**Table S2.6. Comparisons of fly line lifespans to each other.**

One way ANOVA followed by Tukey's multiple comparisons test was used to calculate significance values, ns: not significant, \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ , \*\*\*\*:  $p < 0.0001$ .

	FermA	FermB	CellarA	PPA
FermA	-	*	*	ns
FermB	-	-	ns	***
CellarA	-	-	-	**
PPA	-	-	-	-

**Table S2.7. Media and buffers used in this study.**

Media name	Purpose	Volume	Recipe
<b>5% YPD, agar</b>	Solid media for yeast isolation	1L	20g Peptone (BD Bacto Peptone), 10g Yeast Extract (Amresco Yeast Extract, Bacteriological, Ultra Pure Grade), 50g Dextrose (Fisher Scientific Dextrose Anhydrous), 20g Agar (BD Bacto Agar), MilliQ water to 1L. Autoclaved and poured into 100 x 60mm petri dishes (Falcon).
<b>WLB</b>	Buffer for DNA extraction	-	2M Guanidinium thiocyanate (Fisher Scientific), 0.5 M EDTA (Fisher Scientific), 1.8% Tris base (Promega), 8% NaCl (Sigma Aldrich), 150mL of MilliQ water, and adjust to pH 8.5. Autoclaved and filter sterilized (Nalgene 75mm filter unit, 0.2aPES). (Will Ludington, personal communication).
<b>Agar grape juice</b>	Olfactory preference assay	1L	1.7g Yeast nitrogen base without amino acids or ammonium sulfate (Difco, BD), 20g Agar (BD Bacto Agar), 355mL Organic Cascadian Farms Concord grape juice concentrate (1 can), and 645mL MilliQ water. Heated to a boil and poured into 60 x 15mm petri dishes (Falcon).
<b>Liquid grape juice</b>	Oviposition assay	1L	(Prepare as instructed on can) 1 can of Organic Cascadian Farms Concord grape juice concentrate, 3 parts MilliQ water. Heated to a boil.
<b>5% YPD, liquid</b>	Liquid media for yeast starter cultures	1L	20g Peptone (BD Bacto Peptone), 10g Yeast Extract (Amresco Yeast Extract, Bacteriological, Ultra Pure Grade), 50g Dextrose (Fisher Scientific Dextrose Anhydrous), and MilliQ water to 1L. Autoclaved and filter sterilized (Nalgene 75mm filter unit, 0.2aPES).
<b>GB media</b>	Larval development and longevity assay	see recipe	40% weight by volume (w/v) fresh, pureed organic banana, 60% w/v MilliQ water, and 1.25% agar (BD Bacto Agar). Autoclaved and poured into wide mouth <i>Drosophila</i> vials (wide mouth, K-resin, Genessee Scientific).

# CHAPTER 3: The parameters governing the fly-yeast mutualism

## Abstract

Under optimal laboratory conditions, flies can utilize a wide range of yeast species that are found to be associated with wild *Drosophila*. Here, I tested the boundaries of the winery fly-yeast mutualism by measuring *Drosophila melanogaster* larval fitness in response to changes temperature, testing behavioral responses towards non-*Drosophila* associated yeast species, and identifying the minimal volatile blend that stimulates oviposition. Even at the lowest temperature at which *Drosophila melanogaster* can be raised in the lab, the panel of *Drosophila*-associated yeasts tested in Chapter 2 was generally beneficial to *D. melanogaster* larval fitness. Yeast species that are not commonly associated with *Drosophila* can stimulate oviposition and serve as adequate larval nutrition sources, suggesting that that ability to attract flies is a conserved trait in yeasts. Finally, I found that physical yeast cells are not required to stimulate oviposition in *Drosophila melanogaster*. Instead, a minimal synthetic blend of isoamyl acetate, acetic acid, 2-phenylethyl ester, and ethanol is sufficient for stimulating oviposition. However, the strength of the oviposition response is context-dependent and changes based on volatile composition and the relative concentrations of compounds present in the overall blend. In corroboration with Chapter 2, the experiments in this chapter demonstrate the impressive flexibility of the yeast-fly mutualism.

## Introduction

The previous chapter focused on characterizing the *Drosophila*-yeast mutualism in wineries and testing for specificity between flies and yeast species present in different winery microhabitats. I demonstrated that fly-associated fungal communities are structured but a diverse range of yeast species can serve as adequate mutualistic partners to flies. In this chapter, I explore the factors constraining and mediating this partnership on both a macro and micro scale. I focused on gaining a more in-depth understanding of the effects of 1) temperature, 2) non-fly associated yeasts, and 3) chemoeology of the fly-yeast mutualism. Together, the experiments in this chapter aim to address the boundaries of the fly-yeast mutualism under natural conditions and contexts.

### The effects of temperature on the fly-yeast mutualism

*Drosophila* species are found in a diverse array of habitats and have adapted to meet the climatic fluctuations of the habitats in which they reside [126,127]. These habitats are also colonized by yeast communities [74], which are also affected by environmental changes. The temperature of a given habitat is a particularly important factor for yeast, as temperature can impact growth rate, metabolism, and competition between yeast species. These changes affect yeast volatile production and subsequently influence fly perception of potential oviposition or feeding sites. Because both the wild fly lines and yeast species used in the previous chapter are naturally found

in microhabitats that range widely in temperature, I tested for temperature dependent effects on the larval development assays performed in Chapter 2.

### Non-fly associated yeasts as mutualistic partners to *Drosophila*

In molecular biology, the term “yeast” commonly refers only to a single species, *Saccharomyces cerevisiae*. However, yeasts are actually a diverse, polyphyletic group defined by Kurtzman & Boekhout [128] as “fungi that asexually reproduce by budding or fission and have a sexual state not enclosed in a fruiting body” [20,129]. Not all yeast species are associated with drosophilids, suggesting that coevolution has occurred between *Drosophila* and particular yeast species [111,130]. All of the yeast species tested in Chapter 2 were directly vectored by wild *Drosophila*, so it is unsurprising that they all conferred a general, positive benefit to the *Drosophila* lifecycle.

What are the traits, if any, that fly-associated yeasts share but non-fly associated yeasts do not? Are these traits signatures of coevolution between particular yeast species and *Drosophila*? To address this, I tested *Drosophila* oviposition responses towards and larval development on two non-fly associated yeast species.

### The chemoeecology underlying the fly-yeast mutualism

Chemoeecology drives many host plant specializations in *Drosophila* and presumably underlies the *Drosophila*-yeast mutualism [21,62,63,111]. *Drosophila sechellia* has evolved hypersensitivity to methyl hexanoate, one of the primary volatiles emitted by its primary host plant, morinda fruits [72]. The shift to herbivory in *Scaptomyza flava* is associated with a loss of sensitivity to common yeast volatile compounds [73]. While the *Drosophila*-yeast mutualism is more diffuse than these examples, identifying the specific yeast-produced volatile compounds that guide *Drosophila* behaviors can elucidate the parameters of their relationship.

In this chapter, I focused on characterizing the yeast volatiles mediating ovipositional choice, as it has a direct impact on larval fitness - where females lay their eggs dictates where their larva feed. Previous studies on the compounds driving *Drosophila* egg laying behavior have focused on individual compounds [108,125] or fruit volatiles [131]. Here, I characterize the volatiles produced by yeast associated with wild *Drosophila*, grown on natural substrates, and at relevant concentrations. To identify the minimal optimal oviposition blend, I compared the volatile profiles of the yeast on the Chapter 2 yeast panel, generated synthetic blends of potentially important volatiles, and measured *Drosophila* ovipositional responses to these blends.

## Results

### Vineyard *Drosophila*-associated yeasts are generally suitable for *Drosophila* larval development at relevant temperatures

In Chapter 2, I found that a panel of fly-associated yeast species was suitable for *Drosophila* larval development when larva were raised at the same temperature. However, in the winery, flies reside in microhabitats that vary in temperature. Cellars are maintained at a constant temperature of 55°F (about 13°C). The buildings housing fermentation tanks are kept at ambient room temperature, although the inside of an active fermentation tank can reach 70°F - 85°F (about 20°C - 30°C). Pomace piles are outside and therefore exposed to the fluctuations of the environment, which ranged from 40°F to 90°F (about 4°C - 32°C) in the vineyards where I

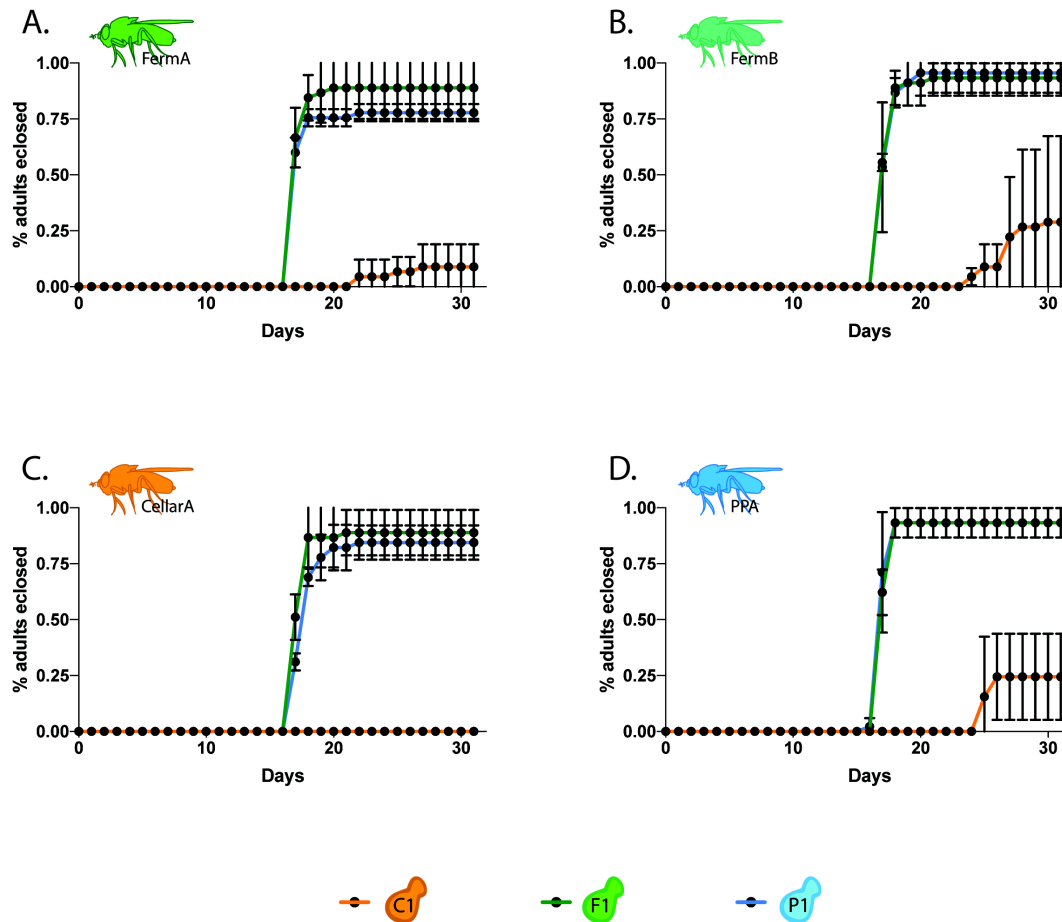
collected. The yeast species colonizing these winery microhabitats grow at different optimal temperatures (Table 3.1), so the temperature of a particular microenvironment can potentially influence the way *Drosophila* sense and interact with particular yeast species.

**Table 3.1. Documented growth temperatures of yeast used in Chapter 2 panel.**

Isolate/ Fly Line	Type	Species	Winery Habitat	Documented growth temperatures	Reference
C1	Yeast isolate	<i>Pichia kluyveri</i>	Cellar	14C - 30C	[132,133]
F1	Yeast isolate	<i>Hanseniaspora uvarum</i>	Fermentation	8C - 37C	[134]
P1	Yeast isolate	<i>Issachenkia orientalis</i>	Pomace pile	37C - 40C	[134]
P2	Yeast isolate	<i>Pichia manshurica</i>	Pomace pile	20C - 28C	[135,136]
CTLsc	Yeast isolate	<i>Saccharomyces cerevisiae</i>	Fermentation	12C - 42C	[134,137]
CTLns	Yeast isolate	<i>Issachenkia terricola</i>	Fermentation	24C - 40C	[134,138]

In the larval development assays described in Chapter 2, temperatures were held consistent across yeast species and fly lines in an effort control as many variables as possible. Briefly, yeast were initially grown at 30°C overnight, a general culturing condition, to ensure as equal growth as possible. In Chapter 2 experiments, fly larva were allowed to develop on those cultures at 25°C, the optimal temperature for fly development. To determine if the mutualistic benefits of the Chapter 2 yeast panel were temperature dependent, I measured *Drosophila* larval development at 18°C, the lowest temperature at which flies are raised in the laboratory. I tested larval development on three yeasts from the Chapter 2 panel: *Pichia kluyveri* (isolate C1), *Hanseniaspora uvarum* (isolate F1), and *Issachenkia orientalis* (isolate P1). If the fly-yeast relationship is temperature dependent, I expected the Cellar fly line (CellarA), which naturally resides at a cooler temperature, to be more successful at developing on *Pichia kluyveri* (isolate C1), the yeast species most commonly vectored by flies in cellars, than the fermentation tank fly lines (FermA and FermB) and pomace pile fly line (PPA), which naturally reside at warmer temperatures.

Instead, larval development at 18°C gave the same overall result as 25°C except with larger variation between replicates (Figure 3.1). Variation in both time and success of eclosion was expected because fly larva are generally less healthy at lower temperatures.



**Figure 3.1. *Drosophila* larval development at 18°C on the Chapter 2 yeast species panel.**

Fly lines are indicated by the fly icons in the top left corner of each graph. Short, horizontal black lines represent standard deviation and black points represent the mean of all trials.

Larva from all fly lines fed both *Hanseniaspora uvarum* (isolate F1) and *Issachenkia orientalis* (isolate P1) eclosed successfully in about 17 days, as expected at a low temperature. There was a more pronounced negative effect on fly lines raised on *Pichia kluyveri* (isolate C1) at 18°C. At 25°C, the Cellar fly line (CellarA) was the least successful at reaching eclosion when raised on monocultures of *Pichia kluyveri* (Figure 2.5). Lower temperatures exacerbated this effect, as CellarA larvae did not eclosed at all on *Pichia kluyveri* when raised at 18°C, clearly demonstrating that the fly-yeast mutualism is not habitat specific or temperature dependent. Instead, many yeast species can serve as mutualistic partners to *Drosophila* at a range of temperatures, although some are more beneficial than others.

Because larval development at 18°C did not indicate any temperature effects, I did not pursue longevity, oviposition, and olfactory preference assays at lower temperatures. Instead, I aimed to understand whether the panel of fly-associated yeasts share distinct traits that make them attractive and beneficial to flies, or if this is a general trait shared by all yeast species.

Yeast species that are not commonly associated with *Drosophila* are also suitable partners in the fly-yeast mutualism

In Chapter 2, I found that a panel of yeast species isolated from wild, vineyard *Drosophila* affected the *Drosophila melanogaster* lifecycle positively. However, the close, natural association of the yeasts on the panel with *Drosophila* already suggests that they confer a benefit to flies. Are yeast species that are not closely associated with flies missing traits that attract and benefit flies?

To test the interactions between flies and non-fly associated yeasts, I gathered a panel of yeast species isolated from a variety of non-*Drosophila melanogaster* sources around the world and were not captured in the Chapter 2 amplicon study (Table 3.2). I first screened the panel for growth and fermentation ability in the oviposition assay using gas chromatography – mass spectrometry (GC-MS). Then I selected two yeast species, *Saccharomyces paradoxus* (isolate EL76) and *Torulaspora delbrueckii* (isolate HI1514), which produced odor profiles that were distinct from the yeast species in the original Chapter 2 panel. Using the same four, wild *Drosophila melanogaster* lines used in Chapter 2, I tested the ability of these non-fly associated yeasts to stimulate oviposition and serve as adequate nutrition sources for developing larvae.

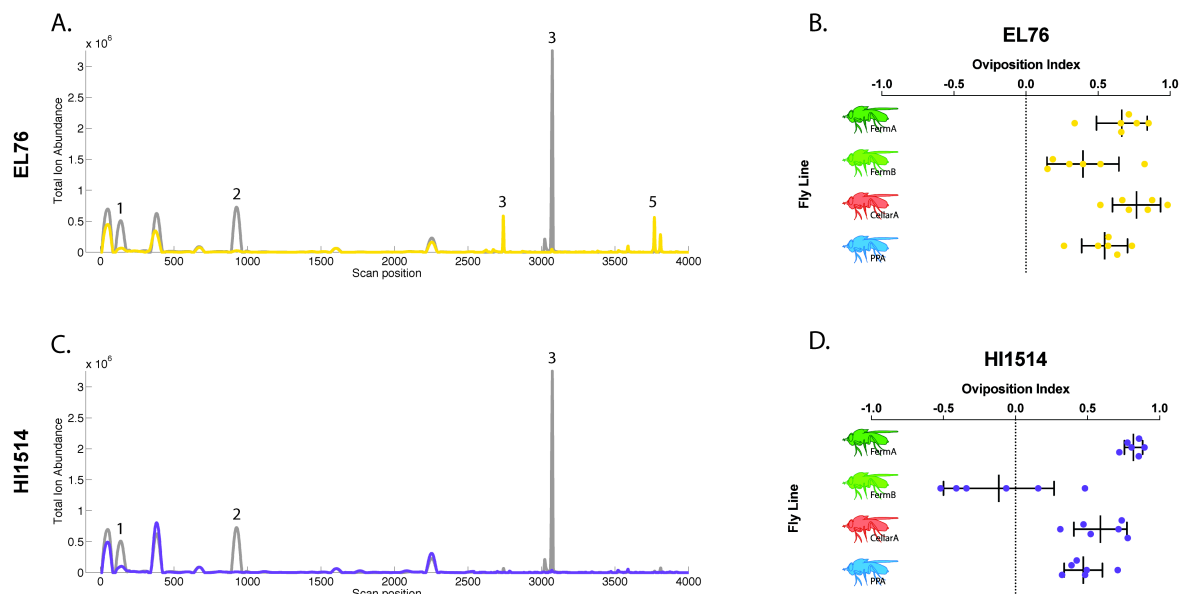
**Table 3.2. Panel of non-*Drosophila* associated yeasts used in this chapter.**

Yeast species in bold were used in behavior assays.

Isolate Name	Species	Collection Site	Collection Substrate	Fermentation activity (Y/N)	Reference
EL24	<i>S. bayanus</i>	Spain	Mesophylax	Y	[139]
<b>EL76</b>	<b><i>S. paradoxus</i></b>	<b>Russia</b>	<b>Flux of Querus</b>	<b>Y</b>	[139]
EL19	<i>S. mikatae</i>	Japan	leaf	Y	[139]
EL138	<i>S. castelli</i>	unknown	unknown	Y	[140]
EL351	<i>S. arboricolus</i>	unknown	unknown	Y	[140]
HI1501	<i>Trichosporon aquatile</i>	Hawaii, USA	<i>Drosophila soonae</i>	N	this study, Chapter 4
HI1504	<i>Yarrowia lipolytica</i>	Hawaii, USA	<i>Drosophila kambysellisi</i>	N	this study, Chapter 4
HI1507	<i>Zygowilliopsis californica</i>	Hawaii, USA	<i>Sapinus saponaria</i> fruit	Y	this study, Chapter 4
<b>HI1514</b>	<b><i>Torulaspora delbrueckii</i></b>	<b>Hawaii, USA</b>	<b><i>Sapinus saponaria</i> fruit</b>	<b>Y</b>	<b>this study</b>
PT_1	<i>Aureobasidium sp.</i>	Berkeley, CA, USA	Pine Tree	Y	this study
PT_2	<i>Hormonema carpetanum</i> (99%)	Berkeley, CA, USA	Pine Tree	N	this study

*Saccharomyces paradoxus* and *Torulaspora delbrueckii* shared some volatiles with fly-associated yeasts but completely lacked others (Figure3.2A and Figure3.2C). Both yeast species produced very little or none of ethyl acetate, isoamyl acetate, and acetic acid, 2-phenylethyl ester, compared the some of the yeasts in the original panel tested in Chapter 2 (see numbered peaks in Figure3.2A and Figure3.2C). *Saccharomyces paradoxus* (isolate EL76) produced two unique volatiles: octanoic acid, ethyl ester and decanoic acid, ethyl ester (Figure3.2A peaks 4 and 5 respectively).

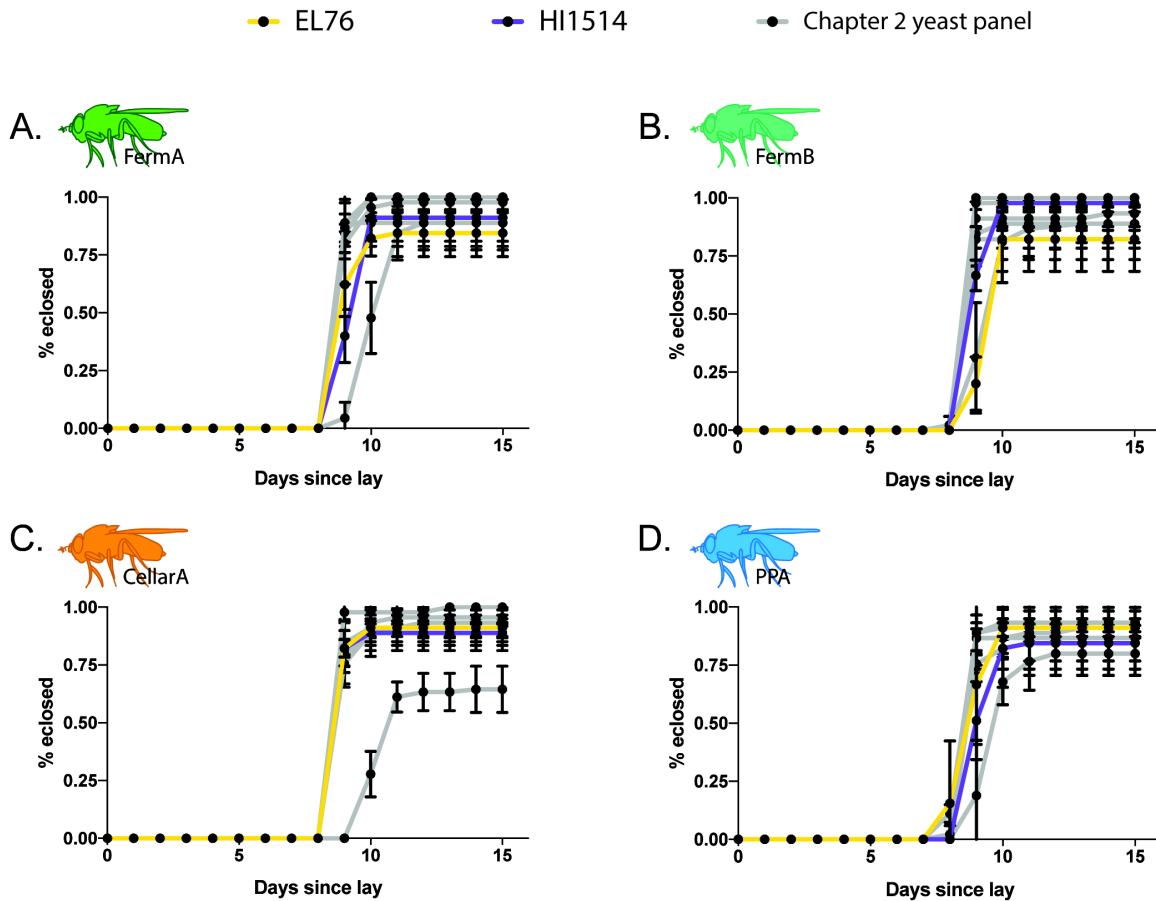
Despite these differences in odor profile, both non-fly associated yeast species elicited a generally positive oviposition response (Figure 3.2B and Figure 3.2D). In fact, *Saccharomyces paradoxus* (isolate EL76) induced an oviposition response equal to that of the most attractive yeast tested in Chapter 2, *Pichia kluyveri* (isolate C1). Similarly, larva fed monocultures of *Torulaspora delbrueckii* (isolate HI1514) and *Saccharomyces paradoxus* (isolate EL76) developed as successfully and at the same rate as larva that were fed yeasts in the original panel (Figure 3.3).



**Figure 3.2. Non-fly associated yeast species volatile profiles and *Drosophila* oviposition responses.**

*Saccharomyces paradoxus* (isolate EL76) GC-MS profile (A) and ovipositional response (B). *Torulaspora delbrueckii* (isolate HI1514) GC-MS profile (C) and ovipositional response (D). For (A) and (C), grey lines indicate the volatile profiles of yeast species tested in the original Chapter 2 panel. Numbers indicate specific volatiles of interest: 1) ethyl acetate, 2) isoamyl acetate, 3) acetic acid, 2-phenylethyl ester, 4) octanoic acid, ethyl ester, and 5) decanoic acid ethyl ester. For (B) and (D), a positive OI indicates an ovipositional preference for the yeast side, a negative OI indicates an ovipositional preference for the control side, and an OI of 0 indicates no preference. *Drosophila* lines tested are denoted by fly icons to the left. Individual replicates are represented by dots and are color-coded by yeast species. Short black lines represent standard deviation and longer black lines represent the mean of all trials.





**Figure 3.3. *Drosophila* larval development on non-fly associated yeasts.**

Grey lines indicate the volatile profiles of yeast species tested in the original Chapter 2 panel. Fly lines are indicated by the fly icons in the top left corner of each graph. Short, horizontal black lines represent standard deviation and black points represent the mean of all trials.

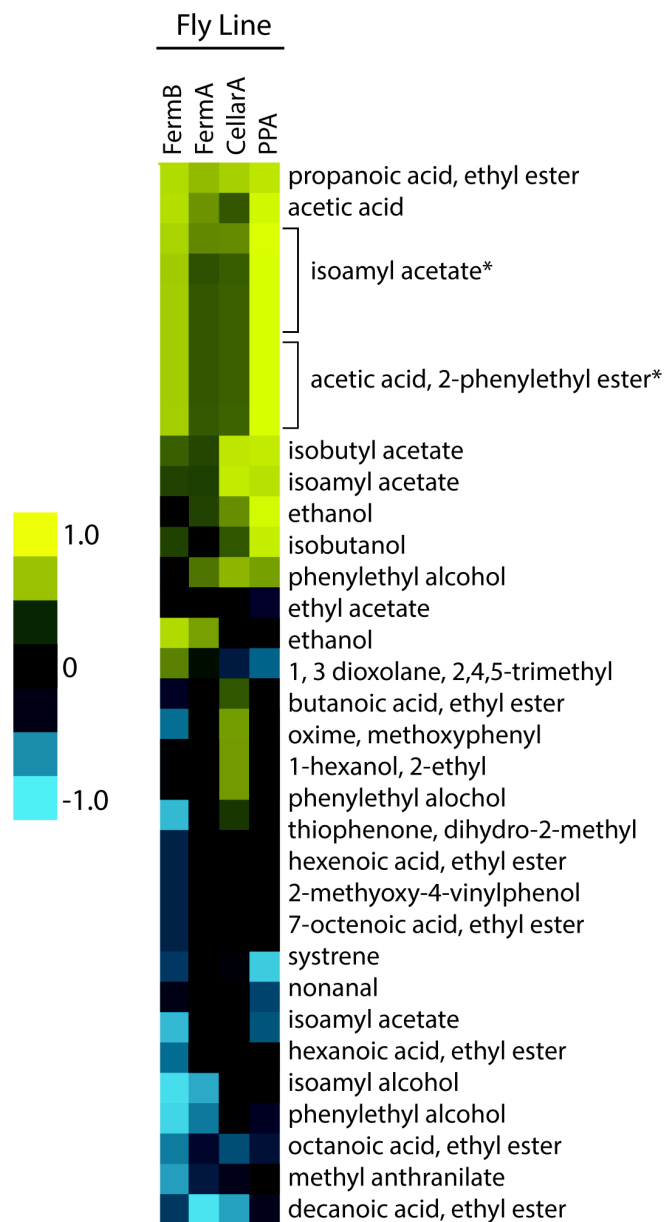
Overall, yeast species that are not known to be closely associated with *Drosophila* can still act as adequate partners in the fly-yeast mutualism, corroborating the results of the previous chapter and a recent study by Becher et al [111], which found that *D. melanogaster* is attracted to yeast species spanning a broad phylogenetic range. Together, these results suggest that the production of attractive compounds is a common and conserved trait in yeasts and that flies have coevolved chemosensory systems that can detect a diversity of these compounds in order to successfully locate yeasts. However, not all yeast volatile profiles are equal, as different yeast species elicit variable degrees of oviposition. I next sought to identify the specific volatiles that are most important for stimulating egg laying.

The yeast-produced volatile compounds, isoamyl acetate and acetic acid, 2-phenylethyl ester, are strongly correlated with a positive *Drosophila melanogaster* oviposition index

Female flies rely heavily on volatile cues from yeasts to make initial egg laying decisions and lay few eggs on substrates lacking these volatiles [107,108,125,130]. Yeast species that do not produce volatiles elicit the same ovipositional behavior as a negative, sterile media control (see

*Pichia manshurica* (isolate P2) Figure 2.4). While a wide range of yeast species stimulated female *Drosophila* oviposition in my assays, some yeast species elicited stronger responses than others. Each of these yeast species produced a distinct odor profile, consisting of a blend of volatile compounds at varying concentrations. However, which volatiles, or mixture of volatiles, mediate the variation in egg laying behavior is unclear.

To narrow down the volatiles associated with positive oviposition responses, I looked for correlations between the volatile profiles of each yeast species tested in Chapter 2 and the oviposition responses of each fly line (Figure 3.4). This analysis revealed two volatiles, isoamyl acetate and acetic acid, 2-phenylethyl ester, which were highly associated with positive oviposition indices (Figure 3.4 denoted with \*).

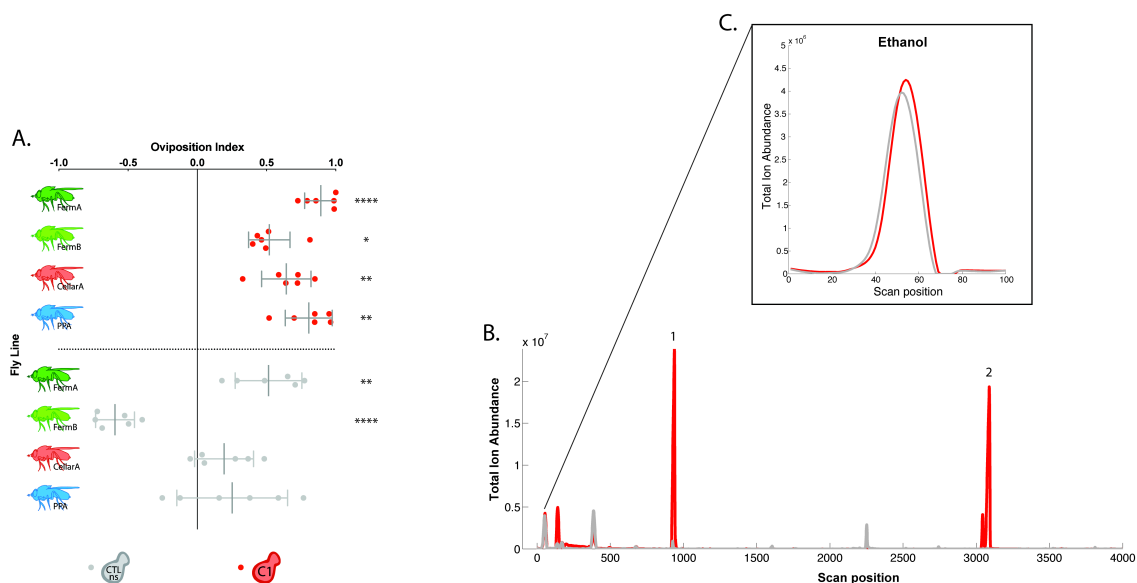


**Figure 3.4. Pearson's correlation coefficients of yeast-produced compounds and oviposition behavior by fly line.**

Positive correlations are represented by yellow blocks, negative correlations are represented by blue blocks, and no correlation is represented by black blocks, as indicated on the color scale. Compounds marked with \* were tested in synthetic blends. Note, some compounds have more than one correlation coefficient because they elute as multiple peaks on the GC-MS.

Interestingly, isoamyl acetate and acetic acid, 2-phenylethyl ester dramatically distinguish the volatile profiles of two yeasts, *Pichia kluyveri* (isolate C1) and *Issachenkia terricola* (isolate CTLns) (Figure 3.5B). These yeast species elicit very different egg laying behaviors (Figure 3.5A) despite fermenting at comparative rates (Figure 3.5C). *Pichia kluyveri* (isolate C1)

produced the greatest concentrations of both isoamyl acetate and acetic acid, 2-phenylethyl ester and was the only yeast species to elicit strong, positive oviposition responses in all fly lines tested. *Issachenkia terricola* (isolate CTLNs), comparatively, elicits a weak or negative ovipositional response. These observations suggested that isoamyl acetate and acetic acid, 2-phenylethyl ester are strong candidates for mediating egg-laying behaviors in *Drosophila melanogaster*.



**Figure 3.5. *Pichia kluyveri* (isolate C1) and *Issachenkia terricola* (isolate CTLNs) volatile profiles and *Drosophila* oviposition responses.**

(A) Oviposition behavior. A positive OI indicates an ovipositional preference for the yeast side, a negative OI indicates an ovipositional preference for the control side, and an OI of 0 indicates no preference. *Drosophila* lines tested are denoted by fly icons to the left. Individual replicates are represented by dots and are color-coded by yeast species. Short grey lines represent standard deviation and longer grey lines represent the mean of all trials. (B) GC-MS profiles. Numbered compounds are as follows: 1) isoamyl acetate and 2) acetic acid, 2-phenylethyl ester. (C) both species produce equal amounts of ethanol.

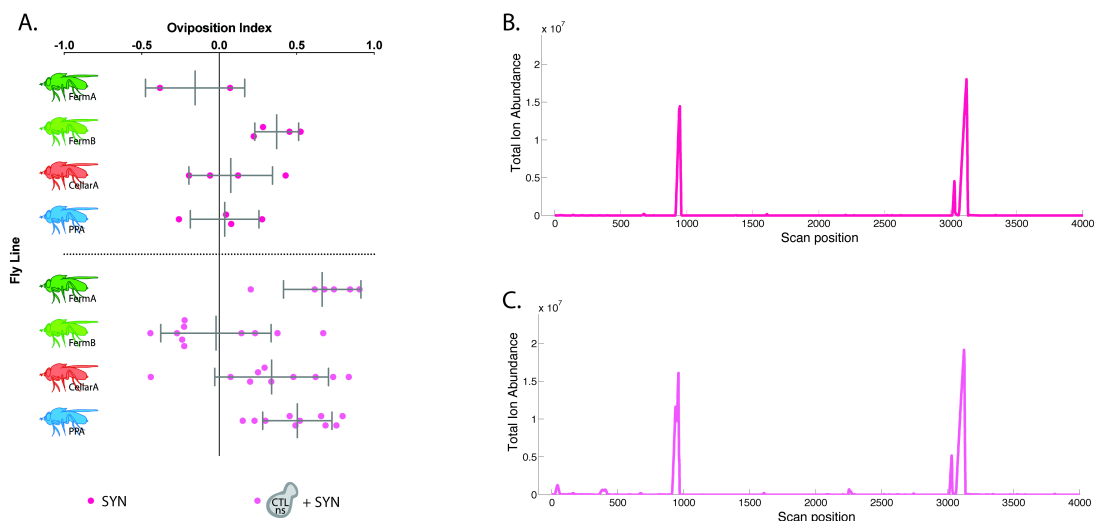
Synthetic blends of isoamyl acetate and acetic acid, 2-phenylethyl ester alone do not phenocopy behavioral responses to *Pichia kluyveri* (isolate C1)

To functionally test the correlation between oviposition behavior and isoamyl acetate and acetic acid, 2-phenylethyl ester, I performed a series of oviposition assays using synthetic blends of these two compounds to find the minimal optimal ovipositional blend.

In these assays, it was crucial that I use synthetic blends at concentrations that were relevant to what a fly might encounter in nature. Seemingly small changes in odor concentration can have a dramatic effect on *Drosophila* behaviors – a volatile compound can be attractive at one concentration and repulsive at another [33]. I used GC-MS to titrate the concentrations of synthetic isoamyl acetate and acetic acid, 2-phenylethyl ester to match the relative abundance of

these compounds produced naturally by *Pichia kluyveri* (isolate C1). In the following assays, “SYN” designates a synthetic blend of isoamyl acetate and acetic acid, 2-phenylethyl ester diluted to a concentration of  $1 \times 10^{-4}$  in organic grape juice (see Materials and Methods).

If isoamyl acetate and acetic acid, 2-phenylethyl ester are the only volatiles mediating female oviposition behavior, a synthetic blend of these volatiles should be sufficient to produce a positive egg laying response. I began by measuring ovipositional responses to two simple blends: a minimal blend of SYN and an additive blend of SYN.



**Figure 3.6. *Drosophila* oviposition responses to synthetic blends of isoamyl acetate and acetic acid, 2-phenylethyl ester.**

(A) Oviposition behavior. A positive OI indicates an ovipositional preference for the yeast side, a negative OI indicates an ovipositional preference for the control side, and an OI of 0 indicates no preference. *Drosophila* lines tested are denoted by fly icons to the left. Individual replicates are represented by dots and are color-coded by blend. Short grey lines represent standard deviation and longer grey lines represent the mean of all trials. (B and C) GC-MS profiles to accompany (A) and color-coded by blend.

Female *D. melanogaster* showed no ovipositional preference between SYN alone and a negative control (Figure 3.6A and B). Therefore, isoamyl acetate and acetic acid, 2-phenylethyl ester alone are not sufficient to produce a positive ovipositional response.

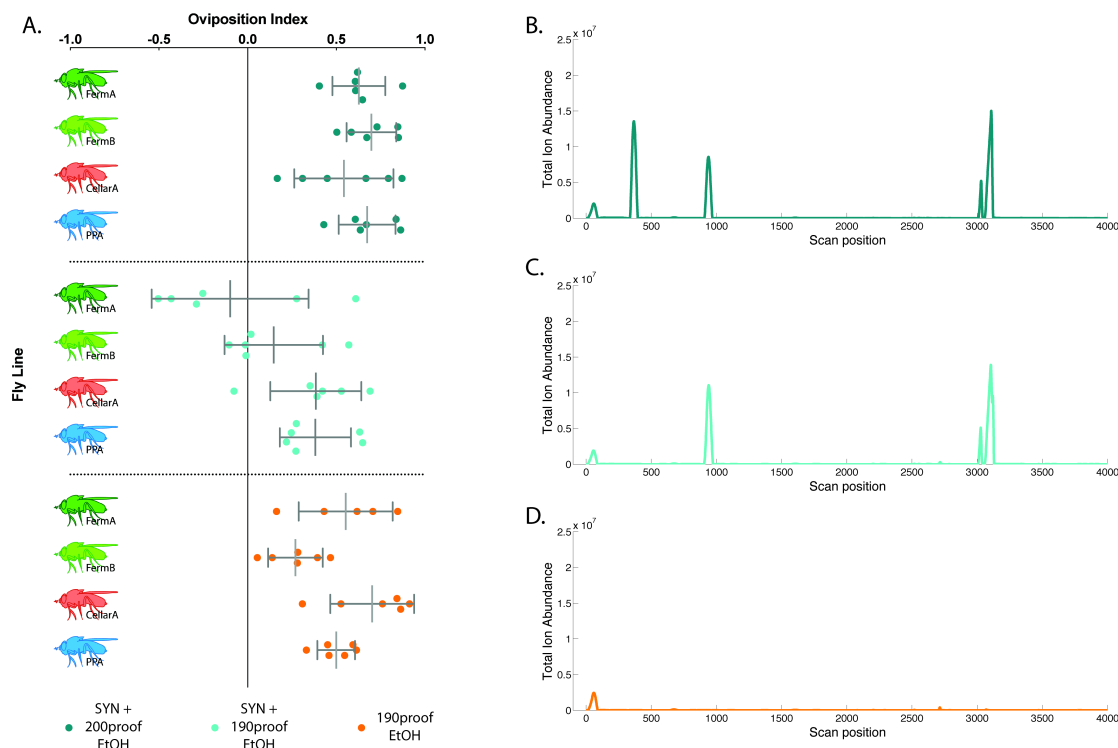
Alone, a culture of *Issachenkia terricola* (isolate CTLNs) elicits a weak or negative ovipositional response (Figure 3.5A). To test if isoamyl acetate and acetic acid, 2-phenylethyl ester could rescue the oviposition response to *Issachenkia terricola*, I spiked SYN into to a culture of *Issachenkia terricola* (hereby referred to as “CTLNs + SYN”). CTLNs + SYN shifted ovipositional responses more positively but did not completely phenocopy the ovipositional response to *Pichia kluyveri* (isolate C1, Figure 3.6A and C).

Together, the synthetic minimal and rescue assays suggested two hypotheses. First, the physical presence of yeast is an important factor in egg laying decisions. Or second, another volatile, in addition to isoamyl acetate and acetic acid, 2-phenylethyl ester, has an important role in stimulating oviposition. The main difference between SYN and CTLNs + SYN was the presence of ethanol due to *Issachenkia terricola* fermentation (Figure 3.6B and C). Female flies exhibit ovipositional preferences for media containing ethanol [108,141]. Together, these results suggested that ethanol was the additional volatile compound necessary to recapitulate the ovipositional response to *Pichia kluyveri* (isolate C1).

### Synthetic compounds of isoamyl acetate, acetic acid, 2-phenylethyl ester, and ethanol shift oviposition responses positively in all fly lines

Both hypothesis can be tested by measuring *Drosophila* ovipositional responses to blends of SYN and ethanol. Two pure grades of ethanol were used (200 proof and 190 proof) and concentrations were titrated to match the relative abundance of ethanol produced by *Pichia kluyveri* (isolate C1) using GC-MS. In the following assays, ethanol was diluted 1:10 into final synthetic blends and/or grape juice.

The addition of 200 proof ethanol into the synthetic blend (hereby referred to as “SYN + 200 proof EtOH”) produced a strong, positive ovipositional response in all fly lines, completely phenocopying the ovipositional response to *Pichia kluyveri* (Figure 3.7A and B). These results were very exciting and indicated that physical yeast are not necessary for stimulating oviposition. Instead, isoamyl acetate, acetic acid, 2-phenylethyl ester, and ethanol are the minimum compounds sufficient for optimal *Drosophila* oviposition responses.



**Figure 3.7. *Drosophila* oviposition responses to synthetic blends of isoamyl acetate, acetic acid, 2-phenylethyl ester, and ethanol.**

(A) Oviposition behavior. A positive OI indicates an ovipositional preference for the yeast side, a negative OI indicates an ovipositional preference for the control side, and an OI of 0 indicates no preference. *Drosophila* lines tested are denoted by fly icons to the left. Individual replicates are represented by dots and are color-coded by blend. Short grey lines represent standard deviation and longer grey lines represent the mean of all trials. (B - D) GC-MS profiles to accompany (A) and color-coded by blend.

Through GC-MS characterization, I discovered that 200 proof purified ethanol also contained 1,1-diethoxyethane, a volatile byproduct of production (Figure 3.7B, Figure S3.1 peak #2). Because 1,1-diethoxyethane was not produced by any of the yeast in the Chapter 2 panel, I did not expect there to be a large behavioral difference between the two grades of ethanol blended with SYN. However, this compound was still a potential contaminate, so I repeated the assay using 190 proof ethanol, which has a single ethyl alcohol peak and no 1,1-diethoxyethane peak (hereby referred to as “SYN + 190 proof ethanol”, Figure 3.7C, Figure S3.1 peak #1).

Surprisingly, the ovipositional response to SYN + 190 proof ethanol was distinctly weaker than SYN + 200 proof ethanol (Figure 3.7A), suggesting that 1,1-diethoxyethane may play a role in ovipositional behavior. While 1,1-diethoxyethane is not commonly produced by the yeast species I have characterized, *Saccharomyces cerevisiae* is able to produce it during wine fermentation [142].

Slight changes in ethanol purity had unexpectedly significant effects on fly behavior. To really understand the effects of ethanol on *Drosophila* oviposition, I took a step back and directly tested oviposition in response to 190 proof ethanol alone, the most pure ethanol in terms of volatile composition. Surprisingly, 190 proof ethanol alone, without SYN, produced an ovipositional response equal to that of *Pichia kluyveri* (Figure 3.7A and D), suggesting that only ethanol is important for stimulating ovipositional behavior.

These results seemingly contradict each other but convey two general conclusions. First, all of these compounds - isoamyl acetate, acetic acid, 2-phenylethyl ester, and ethanol - are important cues that female *Drosophila* assess when deciding where to lay eggs. Second, the strength of these responses is contextually dependent on other volatiles present.

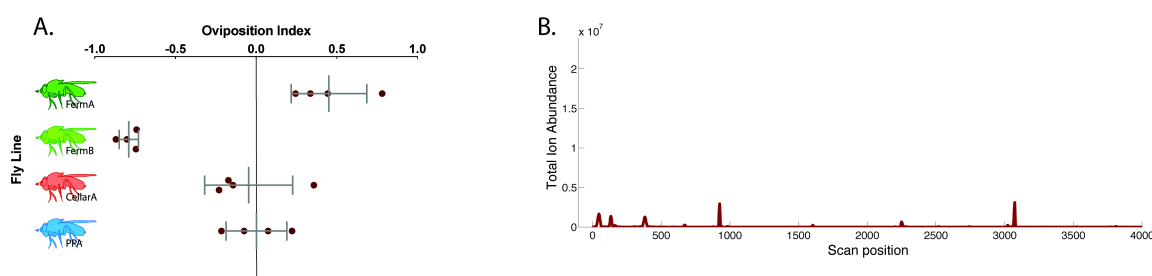
The amplicon study in Chapter 2 clearly indicates that the yeast species tested do not exist as single, isolated species but rather as variable mixtures of many species. Therefore, the volatile bouquet sensed by a fly in the winery would be a combination of the odor profiles of all the yeast species present. Testing ovipositional responses to a more complex mixture may be more ecologically relevant and elucidate more about volatile concentration and composition effects on egg laying behavior.

Oviposition responses to specific volatiles are concentration and context dependent.

In nature, the volatile bouquet of a potential oviposition substrate is a complex mixture of compound produced by a community of microbial residents [63]. To imitate this effect, I performed oviposition assays on a mixture of the five yeast species fermented independently and mixed at equal volume. These yeast species were selected from the Chapter 2 panel because they

successfully fermented in the oviposition assay media: *Pichia kluyveri* (isolate C1), *Hanseniaspora uvarum* (isolate F1), *Issachenkia orientalis* (isolate P1), *Issachenkia terricola* (isolate CTLns), and *Saccharomyces cerevisiae* (isolate CTLsc).

Despite the presence of isoamyl acetate, acetic acid, 2-phenylethyl ester, and ethanol in the mixed yeast blend, fly oviposition responses towards the mixture were very weak (Figure 3.8). FermA was the only fly line to respond positively to the mixture while FermB exhibited a very strong negative response to the mixture. Because each culture was mixed at equal volumes after fermentation, the relative abundances of the compounds produced by each yeast species were comparatively smaller than when each yeast species was tested alone. The discrepancy in oviposition behavior in response to lower concentrations of important volatiles demonstrates that the cues isoamyl acetate, acetic acid, 2-phenylethyl ester, and ethanol produce are concentration dependent and possibly diminished when the compounds produced by other yeasts are present.



**Figure 3.8. *Drosophila* oviposition responses to a mixed blend of *Pichia kluyveri* (isolate C1), *Hanseniaspora uvarum* (isolate F1), *Issachenkia orientalis* (isolate P1), *Issachenkia terricola* (isolate CTLns), and *Saccharomyces cerevisiae* (isolate CTLsc) cultures.**

(A) Oviposition behavior. A positive OI indicates an ovipositional preference for the yeast side, a negative OI indicates an ovipositional preference for the control side, and an OI of 0 indicates no preference. *Drosophila* lines tested are denoted by fly icons to the left. Individual replicates are represented by dots. Short grey lines represent standard deviation and longer grey lines represent the mean of all trials. (B) GC-MS profiles to accompany (A).

## Discussion

*Drosophila* can use a diverse set of yeast species as food sources at a wide range of temperatures, consistent with the conclusions of the previous chapter. Both non-fly associated yeast species tested in this chapter, *Torulaspora delbrueckii* (HI1514) and *Saccharomyces paradoxus* (EL76), are suitable nutrition for developing larvae and stimulate oviposition. In fact, *Saccharomyces paradoxus* more strongly stimulates oviposition than some of the *Drosophila*-associated yeasts tested in Chapter 2. Despite producing unique compounds or lacking others, these two non-fly associated yeast species still produce volatiles that are known yeast attractants [34,61],



suggesting that the ability to attract flies is a conserved trait in many yeast species. Of course, the positive responses in the laboratory to both non-*Drosophila* associated yeasts are artificial because it's unlikely that these interactions would actually occur in nature. However, flies' ability to utilize a diverse range yeast species expressing attractive traits, is an attestation to the importance and strength of this mutualism, rather than its weakness.

Generally, flies will oviposit in response to any yeast species that ferments and produces esters. Presumably because these volatiles signal to female flies that larvae will be provided an adequate food source. The only time I observed very little oviposition was when the yeast species was unable to grow and produce fermentation volatiles in the liquid grape juice used in the oviposition behavior assays (see *Pichia manshurica* (isolate P2) in Chapter 2).

Physical yeast cells are not necessary to stimulate oviposition responses in *Drosophila melanogaster*. I found that synthetic blends of isoamyl acetate, acetic acid, 2-phenylethyl ester, and ethanol are sufficient for stimulating oviposition. Availability of nitrogen sources, in the form of amino acids or ammonia, have strong effects on yeast volatile compound production, as most fly attractants are synthesized from the catabolism of sugars and nitrogen [44]. Fruity substrates encountered by flies and yeast in nature are sugar-rich but nitrogen limited. Nitrogen starvation results in the delay of fermentation – a sign of an unhealthy yeast culture. However, the addition of amino acids to fermenting grape must promotes the production of both isoamyl acetate and acetic acid, 2-phenylethyl ester [44,45]. Perhaps flies have adapted sensitivity to these two volatiles as a way to predict the overall health of a yeast community and nutrient quality of potential oviposition substrates.

The strength of the oviposition response is dependent on the compounds present and the relative concentrations of those compounds in the overall volatile bouquet. Oviposition behavior is complicated [108,125,143]. For example, subsets of neurons and sensory modalities compete to modulate oviposition responses to ethanol and acetic acid, respectively [108,125]. Innate avoidance behavior of CO<sub>2</sub> in *Drosophila melanogaster* is inhibited by 1-hexanol and 2,3-butanedione, two compounds produced when yeast and bacteria ferment fruit substrates [143]. These oviposition studies, in addition to those described in this chapter and the previous one, demonstrate that female flies are clearly sensitive to volatile composition and concentration of potential oviposition substrates. Whether flies collected from different habitats respond to distinct volatile blends remains open to investigation. In the future, characterizing these responses would require testing combinations of synthetic blends or controlled blends of the yeast species representative of each habitat.

## Materials and Methods

### *Drosophila melanogaster* lines used in this chapter

The same wild, isofemale *Drosophila melanogaster* lines used in Chapter 2 were also used in this chapter (see Chapter 2 Materials and Methods).

### Larval development assays at 18°C

All larval development assays were performed as described in Chapter 2 with the following modifications. Three replicates for each fly line were grown at 18°C on *Pichia kluyveri*

(isolate C1), *Hanseniaspora uvarum* (isolate F1), and *Issachenkia orientalis* (isolate P1). As in Chapter 2, yeast species were grown at 30°C overnight on sterile, yeast-free GB media and 15 larvae were moved into each vial. These larvae were allowed to develop at 18°C (not 25°C) and monitored daily for eclosion. The most extreme natural temperatures in winery habitats were not tested because other factors begin to affect larval fitness at these temperatures [127].

#### Collection of non-*Drosophila* associated yeast species

An initial panel of 11 non-*Drosophila* associated yeasts was gathered by myself or from other sources (see Table 3.2 for references). Five *Saccharomyces* species were included from Ed Louis' collection. Four yeast species I collected from endemic Hawaiian *Drosophila* species or soapberry fruits on Big Island of Hawaii were also included (see Chapter 4 Materials and Methods). Finally, I included two yeast species I swabbed and isolated from a redwood tree in Berkeley, CA.

#### Non-*Drosophila* associated yeast panel screening approach

The two non-*Drosophila* associated yeast species initially used in behavioral assays were selected from the non-*Drosophila* associated yeast panel. Because yeast species that don't grow simply do not produce volatiles (see *Pichia manshurica* (isolate P2) in Chapter 2), the panel was first screened for growth on the organic grape juice used in oviposition assays. Briefly, 75mL of organic liquid grape juice (Table S2.7) was inoculated with 1.5mL of yeast cells diluted to OD<sub>600</sub>=1 in sterile 1X PBS (Mediatech Inc). These cultures and a negative control were grown shaking at 30°C for 72 hours. After 72 hours of fermentation, cultures were mixed 1:1 with a boiled water-agar solution (BD Bacto Dehydrated Agar) that was cooled to 65°C, to achieve a final concentration of 1.6% agarose. Cultures were poured into the lids of petri dishes (35x10mm, Falcon), cooled for 15 minutes, and sampled by GC-MS following the sampling and GC-MS protocols detailed in the Chapter 2 Materials and Methods.

From the GC-MS results, I excluded yeast species that were unable to ferment in the organic grape juice media (Table 3.2). Of the yeast species remaining, I excluded yeast species that were recovered from my amplicon study in Chapter 2. Finally, I selected yeast species that produced volatile profiles that were most unlike the volatile profiles of the original yeast species from Chapter 2 (Figure 3.2A and C). This screening process resulted in two non-*Drosophila* associated yeast isolates: EL76 (*Saccharomyces paradoxus*) and HI1514 (*Torulasporea delbrueckii*).

#### Oviposition assays with non-*Drosophila* associated yeasts

*Drosophila melanogaster* oviposition responses to *Saccharomyces paradoxus* (EL76) and *Torulasporea delbrueckii* (HI1514) were tested following the protocol detailed in the Chapter 2 Materials and Methods. Six replicates were performed for each yeast species tested. Three replicates (technical) per fly line were tested on two different days (biological) to control for day-to-day variation. In parallel with each oviposition assay, technical replicates of the same plates were characterized by GC-MS following the GC-MS protocol detailed in the Chapter 2 Materials and Methods.

### Larval development assays with non-*Drosophila* associated yeasts

*Drosophila melanogaster* larval development responses to *Saccharomyces paradoxus* (EL76) and *Torulaspora delbrueckii* (HI1514) were tested following the protocol detailed in the Chapter 2 Materials and Methods. In addition to dead yeast and negative controls, these assays were run with a yeast species that was previously tested in Chapter 2, *Hanseniaspora uvarum* (isolate F1), as a positive control. Larval development on this control mirrored the results of Chapter 2 (data not shown).

### Correlations between oviposition index and yeast-produced compounds

Pearson correlation coefficients were calculated in R [R Development Core Team] between the median ovipositional indexes for each fly line and the volatiles produced by yeast species tested in Chapter 2. The volatile profile of *Pichia manshurica* (isolate P2) was excluded from the analysis because it produced little or no volatile compounds. Correlation coefficients for siloxane peaks were manually removed from the dataset because these compounds originate from the GC-MS sampling process rather than yeasts. Correlation coefficients were clustered using hierarchical clustering by compound (Cluster 3.0) and visualized in Java TreeView.

### Titration of synthetic volatiles

Synthetic compounds of isoamyl acetate, acetic acid, 2-phenylethyl ester, 200 proof ethanol, and 190 proof ethanol were titrated to match the relative abundance of these compounds naturally produced by *Pichia kluyveri* (isolate C1, Figure 3.5B in red).

All synthetic compounds were diluted in organic liquid grape juice (Cascadian Farms organic grape juice concentrate prepared as per the manufacture's protocol). Other studies use paraffin oil to dilute synthetic volatiles (eg. [34]) but I found that this altered the texture of the egg laying substrate and fly behavior. Single compounds were titrated at 1:10, 1:100, 1:1,000, 1:10,000, and 1: 100,000 dilutions and measured by GC-MS. Synthetic blends were mixed 1:1 with a boiled water-agar solution that was cooled to 65°C, to achieve a final concentration of 1.6% agarose. Cultures were poured into the lids of petri dishes (35x10mm, Falcon), cooled for 15 minutes, and sampled by GC-MS following the sampling and GC-MS protocols detailed in Chapter 2 Materials and Methods.

**Table 3.3. Table of synthetic compounds used in this chapter.**

Synthetic compound	CAS #	Supplier	Purity/Grade	Final working concentration
Isoamyl acetate	123-92-2	Sigma-Aldrich	analytical standard, ≥99.7%	1:10000
Acetic acid, 2-phenylethyl ester	103-45-7	Sigma-Aldrich	analytical standard, ≥97.0%	1:10000
Ethanol	64-17-5	Koptec	200 proof	1:10
Ethanol	64-17-5	Koptec	190 proof	1:10

### Oviposition assays with synthetic blends

Synthetic blends were diluted and mixed deliberately, in the following order, to minimize loss of volatile synthetic compounds. A breakdown of the exact blend constituents used in this chapter is summarized in Table 3.4. Oviposition assays were carried out as described in the Chapter 2

Materials and Methods with the following adjustments made to prepare synthetic egg laying substrate.

**Table 3.4. Table of synthetic blend combinations tested in this chapter.**

Blend	Isoamyl acetate	Acetic acid, 2-phenylethyl ester	<i>Issachenkia terricola</i> (isolate CTLns)	200 proof ethanol	190 proof ethanol
SYN	✓	✓			
CTLns + SYN	✓	✓	✓		
SYN + 200 proof EtOH	✓	✓		✓	
SYN + 190 proof EtOH	✓	✓			✓
190 proof EtOH alone					✓

To be consistent with oviposition assays using yeast cultures, two replicates of 75mL of sterile, organic liquid grape juice were incubated, shaking at 30°C for 72 hours. One replicate was used to dilute all synthetic compounds and the other replicate was used as a negative control. After a 72 hour incubation, 75mL of a boiled water-agar solution that was cooled to 65°C, was added to the negative control flask to achieve a final concentration of 1.6% agarose.

For synthetic blends containing SYN, an initial 1:1,000 dilution was performed by diluting 10uL of isoamyl acetate and 10uL of acetic acid, 2-phenylethyl ester into 10mL of sterile, organic grape juice in a 20mL amber glass vial (Sigma-Aldrich). The full 10mL 1:1,000 dilution was diluted to a final concentration of 1:10,000 in a total volume of 100mL.

For blends containing ethanol, 10mL of ethanol was spiked into a total volume of 100mL for final 1:10 concentration. If ethanol was added to the blend, it was always spiked in AFTER the addition of agarose (exactly prior to when plates are poured) to minimize loss by vaporization.

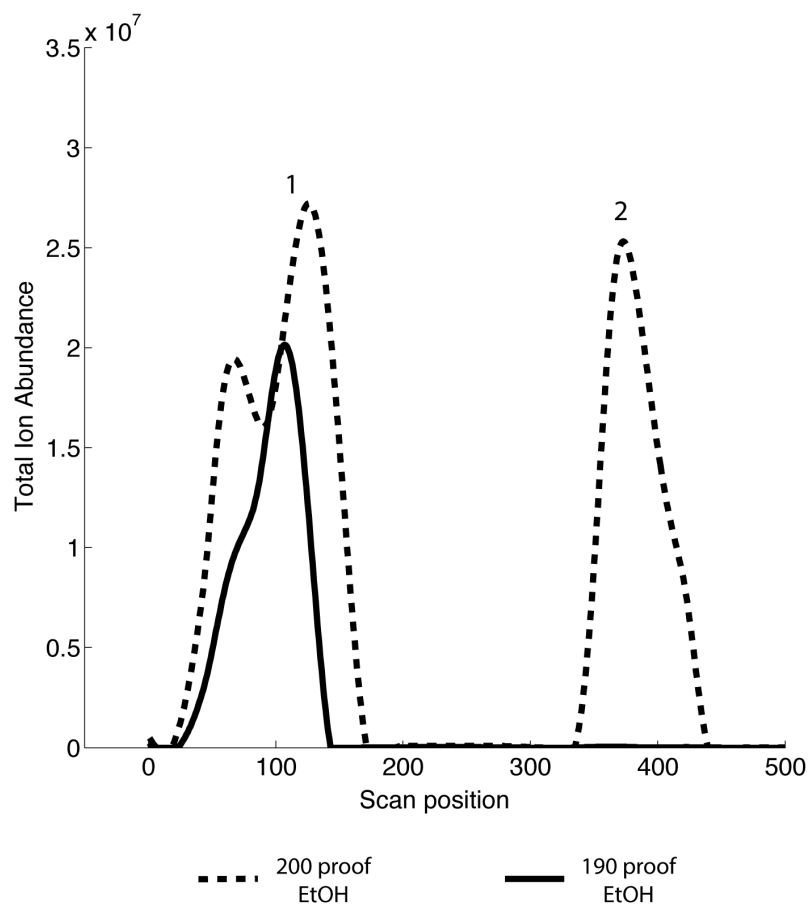
Synthetic blends were always made to a final volume of 100mL using a mixture of sterile grape juice mixed 1:1 with boiled water-agar solution that was cooled to 65°C, to achieve a final concentration of 1.6% agarose. Plates were poured, cooled, and used in oviposition behavior assays as described in the Chapter 2 Materials and Methods. Six replicates were performed for each yeast species tested. Three replicates (technical) per fly line were tested on two different days (biological) to control for day-to-day variation. In parallel with each oviposition assay, technical replicates of the same plates were characterized by GC-MS following the GC-MS protocol detailed in the Chapter 2 Materials and Methods.

#### Oviposition assays with mixed cultures

Individual cultures of *Pichia kluyveri* (isolate C1), *Hanseniaspora uvarum* (isolate F1), *Issachenkia orientalis* (isolate P1), *Issachenkia terricola* (isolate CTLns), and *Saccharomyces cerevisiae* (isolate CTLsc) were grown in individual 75mL cultures shaking, overnight at 30°C as described in the Chapter 2 Materials and Methods. After 72 hours of incubation, 15mL of each yeast species was combined into a new flask for a total of 75mL of mixed yeast culture. 75mL of a boiled water-agar solution (BD), cooled to 65°C, was added to the mixed culture to achieve a final concentration of 1.6% agarose. Plates were poured, cooled, and used in oviposition

behavior assays as described in the Chapter 2 Materials and Methods. Six replicates were performed for each yeast species tested. Three replicates (technical) per fly line were tested on two different days (biological) to control for day-to-day variation. In parallel with each oviposition assay, technical replicates of the same plates were characterized by GC-MS following the GC-MS protocol detailed in the Chapter 2 Materials and Methods.

## Supporting Information



**Figure S3.1. GC-MS peaks present in 200 proof ethanol and 190 proof ethanol.** Peaks are numbered as follows: 1) ethanol and 2) 1,1-diethoxyethane.

# CHAPTER 4: The role of fungi in the host plant specialization of Hawaiian *Drosophila*

## Abstract

The Hawaiian *Drosophila* are a diverse clade consisting of an impressive number of endemic species that have radiated across the Hawaiian Islands. Many Hawaiian *Drosophila* species exhibit very specific host plant adaptations, in some cases only utilizing a particular part of a plant. Microbial communities have long been hypothesized to mediate host plant discrimination in Hawaiian *Drosophila* through volatile cues, although direct evidence is lacking and mechanisms are unknown. In this chapter, I characterized the fungal communities associated with three Hawaiian *Drosophila* species, *Drosophila mimica*, *Drosophila kambysellisi*, and *Drosophila soonae*, and their respective host plants using both traditional isolation and culturing methods and high-throughput, short-amplicon sequencing of the universal fungal internal transcribed spacer (ITS) region. Targeted, DNA amplicon sequencing revealed a more diverse fungal community than culturing surveys, however, based on the amplicon study, there was no clear association between the fungi associated with Hawaiian *Drosophila* and their respective host plants. *Saccharomyces cerevisiae* was commonly isolated from *D. mimica* and *D. kambysellisi* and their respective host plants. I found that a *S. cerevisiae* strain isolated from *D. mimica* was genetically distinct from previously characterized sake, wine, and oak *S. cerevisiae* strains. Finally, the fungi vectored by Hawaiian *Drosophila* produce distinct volatile profiles and most likely contribute to the overall volatile bouquet of host plants. While the Hawaiian *Drosophila*-yeast system faces many of the challenges that limit the investigation of other endemic ecological systems, the role of fungi in Hawaiian *Drosophila* host plant discrimination remains an ecologically significant question and should continue to be an open area of investigation given the appropriate resources.

## Introduction

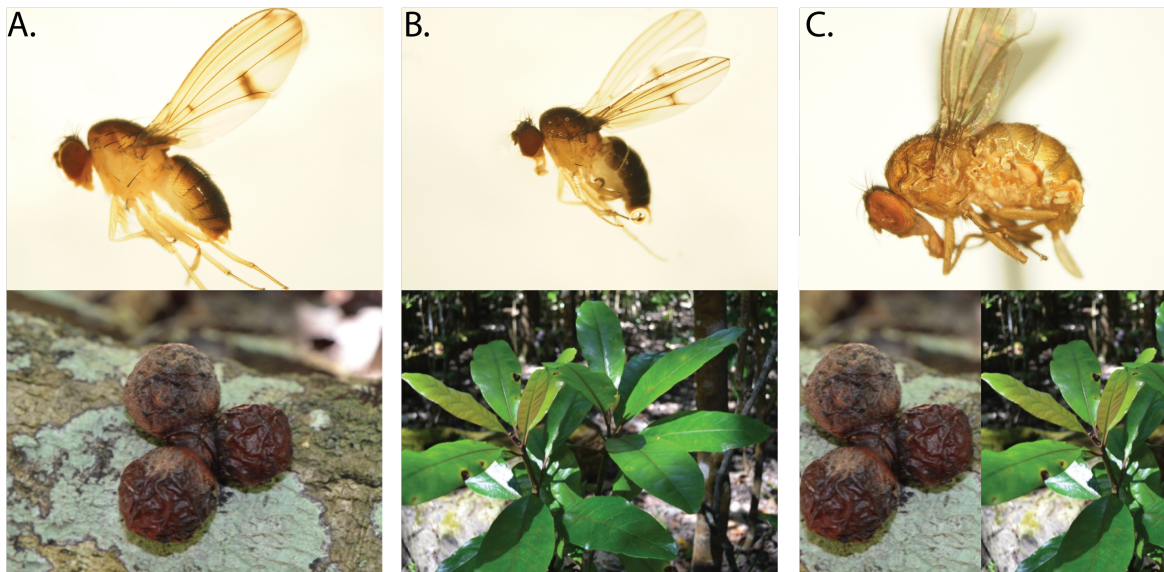
With an estimated 1000 species, the Hawaiian *Drosophila* clade has emerged as one of the most well known examples of extensive adaptive radiation [144-146]. The species group is thought to have arisen from a single colonization event that occurred about 25 million years ago [147,148]. Recent molecular phylogenetic reconstruction coupled with the well-characterized biogeography of the Hawaiian Islands has made the Hawaiian *Drosophila* a strong model for speciation theory [145,149,150].

In addition to a remarkable diversity of morphological and behavioral characteristics, many Hawaiian *Drosophila* exhibit very specific host plant specializations, exploiting almost 40% of native Hawaiian plant families [151,152]. Host plant specialization occurs on a continuum throughout the islands and is restricted by a variety of factors depending on the fly species, ranging from the ovipositional behavior of females to the nutritional requirements of larvae [153]. In the most dramatic cases, a fly species will only feed, mate, and oviposit on the leaves, stems, or bark of a particular plant species. The ability to occupy these narrow niches is thought

to reduce interspecies competition and maximize resource utilization [154,155]. Consequently, this habitat selection maintains reproductive isolation and can result in speciation, facilitating rapid adaptive radiation [156,157].

Like other *Drosophila* species, many Hawaiian *Drosophila* depend on fungi, mainly yeast, as nutrition for developing larvae, so it has been long hypothesized that volatiles produced by the microbial communities on host plants – rather than the host plant volatiles themselves – mediate host plant discrimination in Hawaiian *Drosophila* [74,151,153,156,158,159]. While this hypothesis has not been directly tested, past studies investigated the yeast communities associated with Hawaiian *Drosophila* host plants. In 1981, Starmer [74] found that the diversity of Hawaiian *Drosophila* habitats parallels the diversity in physiology of the yeast communities associated with those habitats. More recently, Ort et al [158] used cloning methods to survey the fungal diversity associated with common Hawaiian *Drosophila* host plants and found little overlap in the fungal communities present. Because these host substrates differ in both nutrient composition and fungal species composition, it's likely that interactions between the plant and microbes colonizing it produce a distinct volatile signature that can be detected by flies. Beyond this, little work has been done to directly investigate the role of microbes in host plant specification of Hawaiian *Drosophila*.

To further explore the hypothesis that Hawaiian *Drosophila* host plant discrimination is mediated by microbial volatiles, I performed a case study on the fly-fungal relationship of three closely related species in the *modified mouthpart* clade (Figure 4.1). In nature, these species are sympatric but exhibit assortative mating and are specialized to specific host plants [156,160,161]. Two of these species are considered specialists: *Drosophila mimica* is associated with soapberry fruits (*Sapindus saponaria*) and *Drosophila kambysellisi* is associated with fermenting *Pisonia brunonianum* leaves [151,156,160]. *Drosophila soonae*, by comparison, is considered a generalist and has been reared off of both soapberry fruits and *Pisonia* leaves (P. M. O'Grady, personal communication). I compared the fungal communities associated with Hawaiian *Drosophila* and their host plants using both culture-based and amplicon sequencing methods and characterized the volatile profiles of the yeast species vectored by Hawaiian *Drosophila* using gas chromatography – mass spectrometry (GC-MS) to better elucidate the mechanisms underlying Hawaiian *Drosophila* host plant discrimination.



**Figure 4.1. The *modified mouthpart* species and host plants characterized in this chapter.**

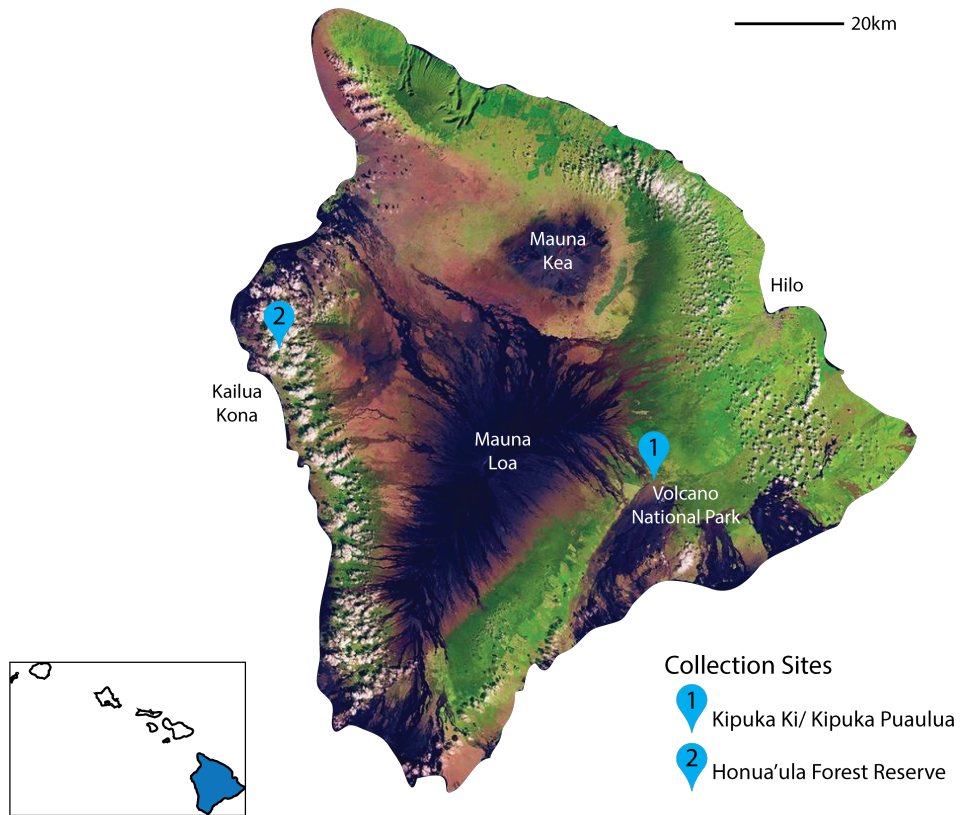
A) *Drosophila mimica* is associated with soapberry fruits (*Sapindus saponaria*). B) *Drosophila kambysellisi* is associated with fermenting *Pisonia brunonianum* leaves. C) *Drosophila soonae* has been reared off of both soapberry fruits and *Pisonia* leaves.

## Results

### Hawaiian *Drosophila* and associated fungi field collection and rearing Hawaiian *Drosophila* in the laboratory

In July of 2013 and January of 2015, I collected *Drosophila mimica*, *Drosophila kambysellisi*, and *Drosophila soonae* from two sites on the Big Island of Hawaii (Figure 4.2, Table 4.1). I also collected *S. saponaria* fruits and *Pisonia* leaves from a single site, Kipuka Puaulua. During both collections, my goals were two fold: 1) to collect the fungi associated with Hawaiian *Drosophila* and their host plants using both culturing and DNA extraction methods and 2) to establish isofemale lines of all three fly species in the laboratory to use in behavioral assays.





**Figure 4.2. Map of collection sites on the Big Island of Hawaii.**  
Original image by NOAA Coastal Services Center and NASA’s Earth Observatory.

**Table 4.1. Collection summary for July 2013 and January 2015.**

Collection Date	Site	Site Coordinates	Elev.	<i>D. mimica</i>	<i>D. kamb.</i>	<i>D. soonae</i>	Soap-berry	<i>Pisonia</i>
July 2013	Kipuka Puaulua	19° 26.251' N -155° 18.194' W	4058	20	12	0	3	0
July 2013	Honua'ula Forest Reserve	19° 43.084' N -155° 56.922' W	3284	0	0	5	0	0
January 2015	Kipuka Puaulua	19° 26.251' N -155° 18.194' W	4058	35	32	0	12	20
January 2015	Honua'ula Forest Reserve	19° 43.084' N -155° 56.922' W	3284	0	0	13	0	0
January 2015	Kipuka Ki	19° 26.576' N -155° 19.041' W	4334	1	0	0	0	0

I was successful in culturing fungi from the field, preserving samples for targeted, DNA amplicon sequencing, and transporting live *Drosophila mimica*, *Drosophila kambysellisi*, and *Drosophila soonae* back to our lab in Berkeley, CA. However, establishing isofemale lines of these three fly species under laboratory conditions proved to be one of the most challenging aspects of this project.

Hawaiian *Drosophila* species are known to be difficult to rear in the laboratory and require strict temperature, nutritional, and humidity conditions [145]. Adults are maintained at low temperatures and a high relative humidity, to recapitulate the cool, wet forests of their natural habitat. Females oviposit in Wheeler-Clayton media [126], which I custom made in our lab, and larva will “jump” from media vials into sand prior to pupation (Figure 4.3). The generation time of *D. mimica* is significantly longer than *D. melanogaster*. At 18°C, *D. melanogaster* develops from embryo to adult in about 19 days but it takes *D. mimica* about 35 days to complete the same lifecycle stages (Figure 4.4).

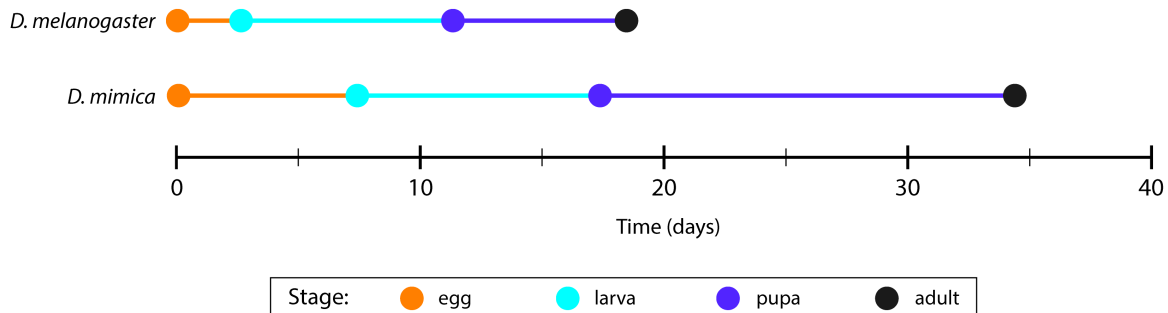


**Figure 4.3. *Drosophila mimica* development.**

*Drosophila mimica* females oviposit on Wheeler-Clayton media. Following the third instar, larvae will “jump” out of vials into sand for pupation.

Of the *D. mimica*, *D. kambysellisi*, and *D. soonae* females I transported back to Berkeley, CA, I was successful in inbreeding a single, *D. mimica* isofemale line for eight generations. At any given time, the population size of this *D. mimica* line in our lab was only 30-60 adult flies. Small population sizes are not likely to be a result of inbreeding depression but rather a matter of optimizing conditions or providing host plant extracts ([126] and K.Y. Kaneshiro, personal communication). With advice from experts in the field, I attempted a variety of conditions to

stimulate oviposition including providing host plant material, *Clermontia* leaf extracts, yeast, kimwipes, higher humidity, as well as testing different media refreshing schedules.



**Figure 4.4. *Drosophila mimica* lifecycle and development time.**

Despite my efforts, the *D. mimica* line in our lab eventually succumbed to bacteria contamination due low population sizes and high humidity. Behavioral experiments are necessary to directly test the responses of Hawaiian *Drosophila* to fungi on host plants so not having a viable fly line was a major limitation in understanding the mechanisms driving host plant discrimination. Overall, I did not have the adequate equipment to truly optimize the conditions necessary to maintain a stock of *D. mimica* that was healthy enough to be used in behavioral assays. The *Drosophila* Species Stock Center and labs that specialize in Hawaiian *Drosophila* have been successful in maintaining stocks of Hawaiian *Drosophila* species but have the appropriate facilities, such as dedicated incubators, and the ability to replenish stocks when lab populations decline. However, given the resources available, we ultimately decided that the behavioral aims of this project would be unobtainable.

#### Culturing survey revealed little diversity in Hawaiian *Drosophila*-associated fungi

While behavior assays were impractical given the challenges of rearing Hawaiian *Drosophila* in the laboratory, surveying the fungal communities associated with *D. mimica*, *D. kambysellisi*, and *D. soonae* and their respective host plants was successful. An initial culture-based fungal study was conducted by isolating single colonies of fungi from flies that had walked on solid, synthetic media, or from flies washed in liquid, synthetic media and then plated onto solid media. Combining the culturing results from 2013 and 2015, I found a relatively simple fungal community associated with both Hawaiian *Drosophila* and host plant substrate (Figure 4.5). A total of ten fungal species were isolated from both *D. mimica* and *D. kambysellisi* and fifteen fungal species were isolated from *D. soonae*. Host plant substrate harbored a less diverse fungal consortium, with only three fungal species isolated from rotting soapberry fruits and eight fungal species isolated from *Pisonia* leaves.

The fungal diversity revealed in the culturing study, or lack thereof, is similar to the diversity of fungal species cultured from Hawaiian *Drosophila* in other studies. *Scaptomyza calliginosa* and *Drosophila floricola* collected from morning glory flowers at Kipuka Puaulua were associated with four major fungal species and a few minor species [162]. The fungal

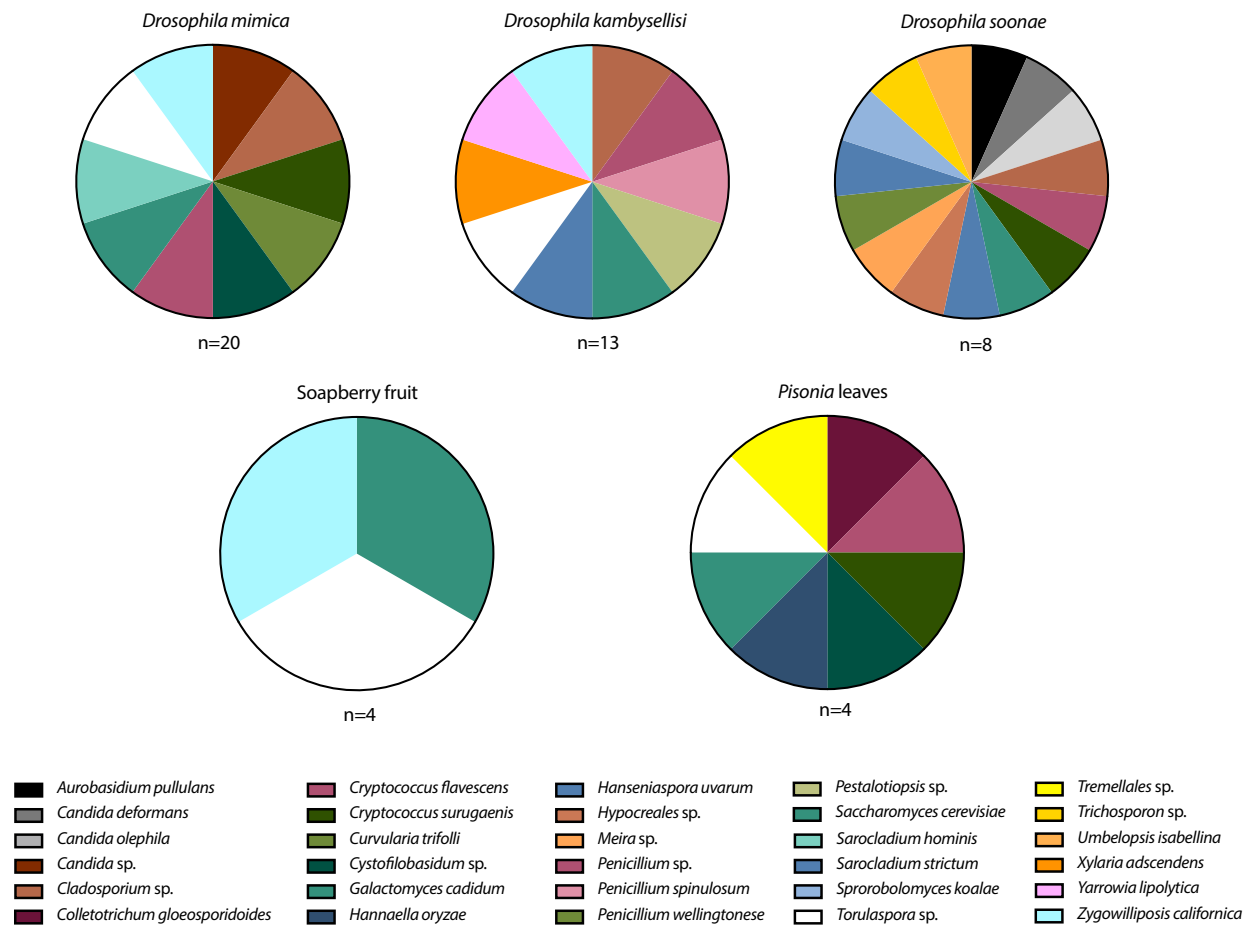
communities of *Drosophila imparisetae* and *Drosophila neutralis* were found to span only seven to eight fungal classes although the number of species was not specified [159].

If fungi played an important role in host plant specification, I expected that the fungi associated with a particular Hawaiian *Drosophila* species would be more similar to that of its respective host plant than to other Hawaiian *Drosophila* species. Comparison of the fungal communities of the three Hawaiian *Drosophila* species in this study revealed very little overlap. Only a single fungal genera, *Cladosporium*, which could not be identified down to the species level in our identification pipeline, was isolated from all three fly species. The genus *Cladosporium* are filamentous fungi that can disperse in the air and are ubiquitously found [163]. Therefore, *Cladosporium* species are unlikely to play a role in host plant discrimination in Hawaiian *Drosophila*. The lack of overlap between the fungal communities associated with *D. mimica*, *D. kambysellisi*, and *D. soonae* indicates that each fly species is associated with a distinct fungal community.

The fungal communities of Hawaiian *Drosophila* species and their respective host plants shared notably more fungal species. *Drosophila mimica* vectored all three of the fungal species isolated from its host plant, the soapberry fruit; *Saccharomyces cerevisiae*, *Zygowilliopsis californica*, and species of the genus *Torulaspora*. The fungal communities of *Drosophila kambysellisi* and its host plant, *Pisonia* leaves, also shared three fungal species: *Saccharomyces cerevisiae*, *Cryptococcus flavescens*, and species of the genus *Torulaspora*.

Surprisingly, the fungal communities of *D. soonae* and its presumed host plants, *Pisonia* leaves and soapberry fruits, had no overlapping fungal species. *Drosophila soonae*, unlike *D. mimica* and *D. kambysellisi*, is considered a generalist species and has been reared off of both soapberry fruits and *Pisonia* leaves (Patrick O'Grady, personal communication). Beyond these rearing observations, the host substrate range of *D. soonae* is relatively unknown [160]. The fungal diversity isolated from *D. soonae* was more broad than any other fly or plant species sampled in this study, consistent with generalist behavior and likely reflecting the utilization of a variety of substrates.

Conversely, *D. mimica* and *D. kambysellisi* are considered host plant specialists and clearly share fungal species with their respective host plants. However, both fly species carry other fungi in addition to those shared with their host plants. These fly-specific fungi may not colonize host plants because they are unable to metabolize plant substrates or are environmental community members, which become randomly associated with flies as they explore other substrates within the habitat. In either case, it's unlikely that these fungi are important for host plant discrimination since they were not isolated from host plant material. Instead, the shared fungal species are more likely to play active roles in distinguishing host plants with other substrates for Hawaiian *Drosophila* with very specific host plant specializations. If the fungal associations between *D. mimica* and *D. kambysellisi* and their respective host plants were important for host plant discrimination, each of these fungal species should produce a unique volatile signature.



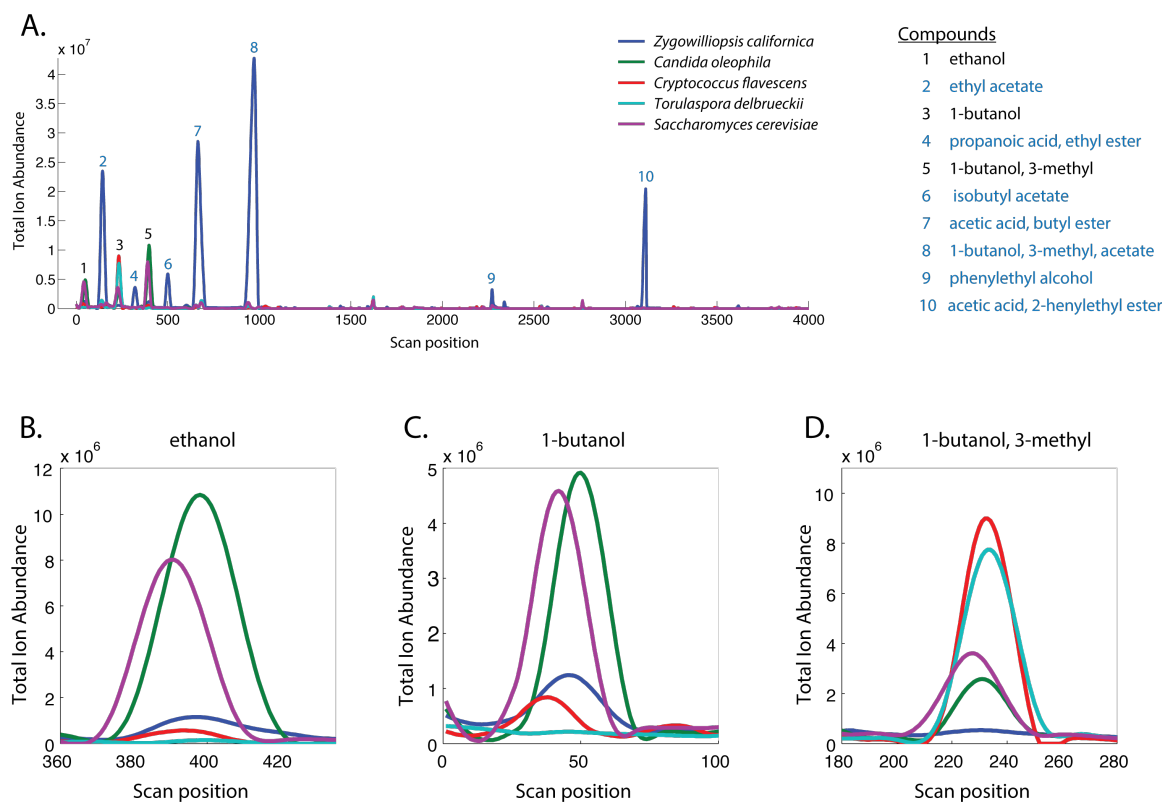
**Figure 4.5. Fungi cultured from Hawaiian *Drosophila* and host plants.** Fungi collected from flies and substrates collected in July 2013 and January 2015.

The fungi associated with Hawaiian *Drosophila* produce distinct volatile profiles

I used gas chromatography – mass spectrometry (GC-MS) to characterize the volatile compounds produced by five yeast species vectored by Hawaiian *Drosophila*: *Zygowilliposis californica*, *Candida oleophila*, *Cryptococcus flavescens*, *Torulaspora delbrueckii*, and *Saccharomyces cerevisiae*. All of these yeast species are shared between *D. mimica* and *D. kambysellisi* and their respective host plants, with the exception of *Candida oleophila*, which was unique to the generalist species, *D. soonae*. If fungi mediate host plant discrimination in Hawaiian *Drosophila*, these fungi should produce distinct volatile profile that can be sensed by *Drosophila*.

GC-MS analysis revealed that all five of these fungal species produce unique volatile profiles (Figure 4.6). Most of the yeast species produced the same volatile compounds but at variable concentrations (Figure 4.6B-D). *Zygowilliposis californica*, which was shared between *D. mimica* and its host plant, soapberry fruits, produced the most unique volatile profile. It produced very little of the volatile compounds shared by the other yeast species and instead, produced a unique set of volatiles at much higher concentrations (Figure 4.6A, in blue).

Because these yeast species produce distinct volatile profiles under ideal laboratory growth conditions, it is plausible that they would also produce different volatiles when metabolizing host plant substrate in nature. Many of volatile compounds produced by the yeast profiled here are known to be *Drosophila melanogaster* attractants [28,34,61]. Because olfactory neuron responses to these kinds of volatile ligands seem to be highly conserved across the *Drosophila* genus [59,64], it's likely that Hawaiian *Drosophila* would be able to detect them.



**Figure 4.6. GC-MS traces of five fungi commonly vectored by Hawaiian *Drosophila*.**

Each line represents the average of three GC-MS replicates for a given yeast species grown at room temperature for 22hrs on YPD media. (A) Full volatile profiles of all yeast species on the panel. Compounds designated in blue in the legend are unique to *Zygowillipsis californica*. (B) ethanol. (C) 1-butanol. (D) 1-butanol, 3-methyl.

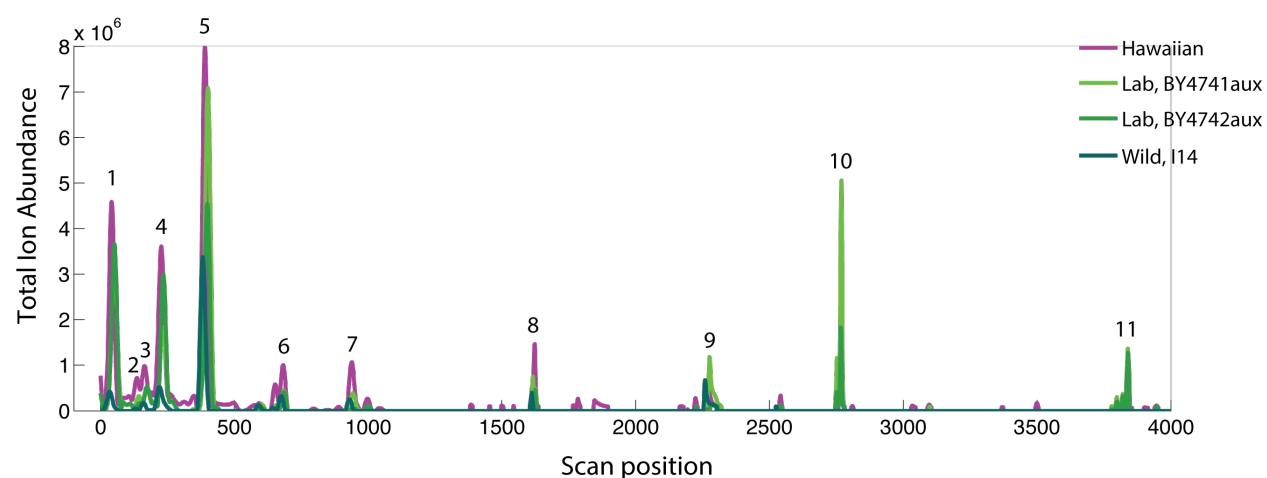
A *Saccharomyces cerevisiae* strain associated with Hawaiian *Drosophila* is distinct from both wild and laboratory strains

*Saccharomyces cerevisiae* was particularly interesting because it was shared between both *D. mimica* and *D. kambysellisi* and their respective host plants. Saccharomycetes, the fungal class in which *S. cerevisiae* belongs, is the most commonly vectored fungal class by two other Hawaiian *Drosophila* species, *Drosophila imparisetae* and *Drosophila neutralis* [159]. Conversely, an independent study of host plant fungal communities found that Saccharomycetes species are much less abundant on host plants [158]. Together, these studies suggest that flies vector a select

group of yeasts and, that Saccharomycetes species, such as *S. cerevisiae*, exhibit traits that ensure a close association to Hawaiian *Drosophila*.

To begin identify these attractive characteristics, I used GC-MS to compare the volatile profile of a *S. cerevisiae* strain isolated from a single *D. mimica* female collected in Kipuka Puaulua, herein referred to as “Hawaiian *S. cerevisiae* strain,” to wild and laboratory *S. cerevisiae* strains. These strains included two common laboratory isolates, BY4741aux and BY4742aux, which were previously used in our lab to demonstrate the importance of mitochondria in *Drosophila* attractant production [34,164], and a wild *S. cerevisiae* strain isolated from an Italian vineyard [165].

When grown under optimal conditions, all four strains produce distinct volatile profiles, distinguished by differences in relative abundance of compounds (Figure 4.7). The Hawaiian *S. cerevisiae* strain produces more short chain esters (Figure S4.1A-F) while the two laboratory strains produced more long volatile compounds (Figure S4.1G-I). The wild vineyard isolate produced relatively little or none of compounds compared to the Hawaiian and laboratory strains. Most of the volatiles produced are known *Drosophila* attractants, small differences in concentrations of which can be detected by *Drosophila melanogaster* [34,42].



#### Legend

1 ethanol	5 1-butanol, 3-methyl	9 phenylethyl alcohol
2 ethyl acetate	6 acetic acid, butyl ester	10 octanoic acid, ethyl ester
3 1-propanol, 2-methyl	7 1-butanol, 3-methyl, acetate	11 decanoic acid, ethyl ester
4 1-butanol	8 hexanoic acid, ethyl ester	

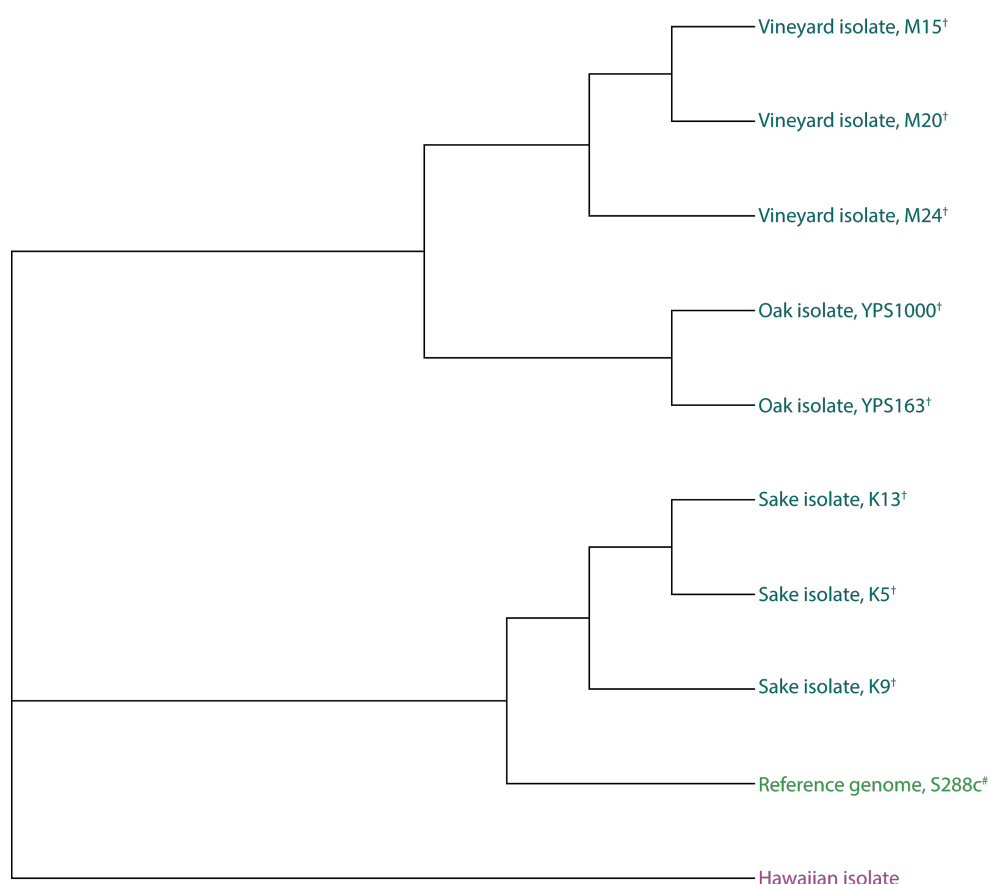
**Figure 4.7. GC-MS traces of Hawaiian *Saccharomyces cerevisiae* compared to laboratory and wild *S. cerevisiae* strains.**

Each line represents the average of three to four GC-MS replicates for a given *S. cerevisiae* strain grown at 30°C for 22hrs on YPD media.

The production of volatile *Drosophila* attractants in *S. cerevisiae* is genetically encoded [34,39] so I next sought to determine whether the Hawaiian *S. cerevisiae* strain was genetically distinct from the other *S. cerevisiae* strains. *S. cerevisiae* is predominately found to be closely associated

with human activity. However, wild *S. cerevisiae* populations are genetically distinct from domesticated strains, with wine, sake, and oak strains falling into three distinct lineages [165,166].

By sequencing and assembling the same five, unlinked loci Fay & Benavides [165] used to initially show population structure within *S. cerevisiae* strains, I phylogenetically placed the Hawaiian *S. cerevisiae* strain among vineyard, sake, oak, and laboratory strains. I found that the Hawaiian *S. cerevisiae* strain was genetically distinct from all other strains (Figure 4.8), indicating that the *S. cerevisiae* strain associated with Hawaiian *Drosophila* is diverged from other wild populations. Genetic divergence combined with a unique volatile profile suggests that the Hawaiian *S. cerevisiae* strain may have developed traits, such as the ability to produce more attractive volatile compounds that ensure a close association with Hawaiian *Drosophila* in nature.



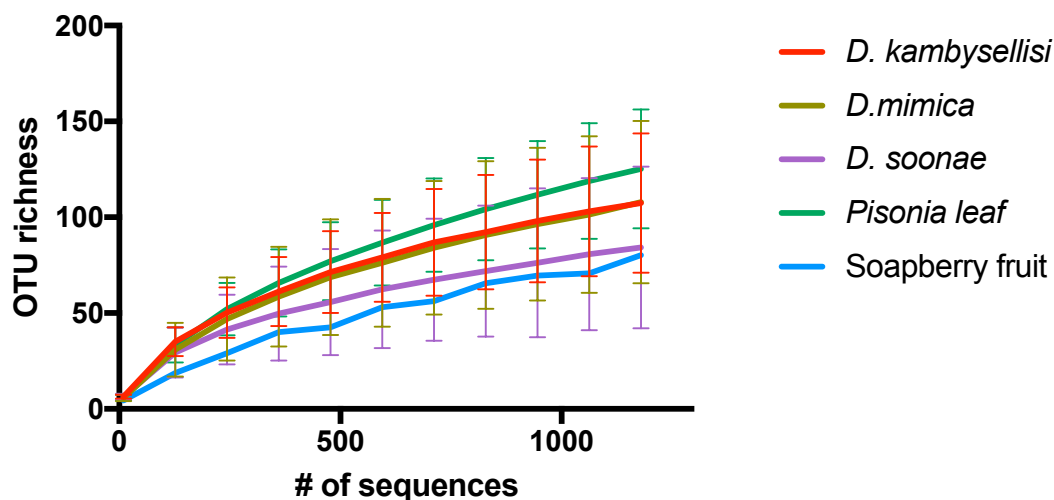
**Figure 4.8. Neighbor-joining tree of Hawaiian, laboratory, and wild *S. cerevisiae* strains.** Constructed from polymorphic sites at five unlinked loci. Isolates in dark green text and denoted with † were constructed with Genbank sequences from Fay & Benavides [165]. Isolates in light green text and denoted with # were constructed from the S288c reference *Saccharomyces cerevisiae* genome assembly (April 2011 release) [167,168]. Isolates in pink text are from this study.



## Amplicon survey reveals a more complicated picture of Hawaiian *Drosophila* associated fungal community structure

Although all of the fungal species captured in my culturing study have been isolated from both soapberries and *Pisonia* leaves in other studies (P.M. O' Grady, personal communication), it is likely that my culturing methods are biased towards fungi that are able to metabolize synthetic laboratory media more effectively and efficiently than other fungal species. Because this is a common confounding variable in culturing studies, I conducted targeted, high-throughput DNA sequencing to characterize the fungal species vectored by *D. mimica*, *D. kambysellisi*, *D. soonae*, and their respective host plants.

All amplicon collections were made over a single week in January 2015, frozen, and transported back to Berkeley, CA for DNA extraction. The fungal ITS region was targeted for short-amplicon sequencing [102,103]. In downstream read processing, a significant fraction of reads were lost when reads without a positive identification and reads present in the negative controls were filtered out (Table S4.1). After quality filtering and processing, I clustered a total of 578,329 fungal ITS reads into 1049 operational taxonomic units (OTUs). When rarefied to 1182 sequences per sample, the mean fungal OTU richness per sample ranged from 29 - 204 OTUs. Generally, I found that the average species diversity of the fungal communities associated with flies and *Pisonia* leaves were comparable (Figure 4.9). The results for soapberry fruits were inconclusive because the fungal community of only one soapberry sample was successfully sequenced. It is unlikely that soapberries are not colonized by yeast since yeast were successfully isolated from soapberries in the culturing study. Instead, it is possible that the foamy residue on the fruits may have inhibited DNA extraction or fungal ITS amplification.



**Figure 4.9. Mean OTU richness for flies and host plant substrate sampled.**

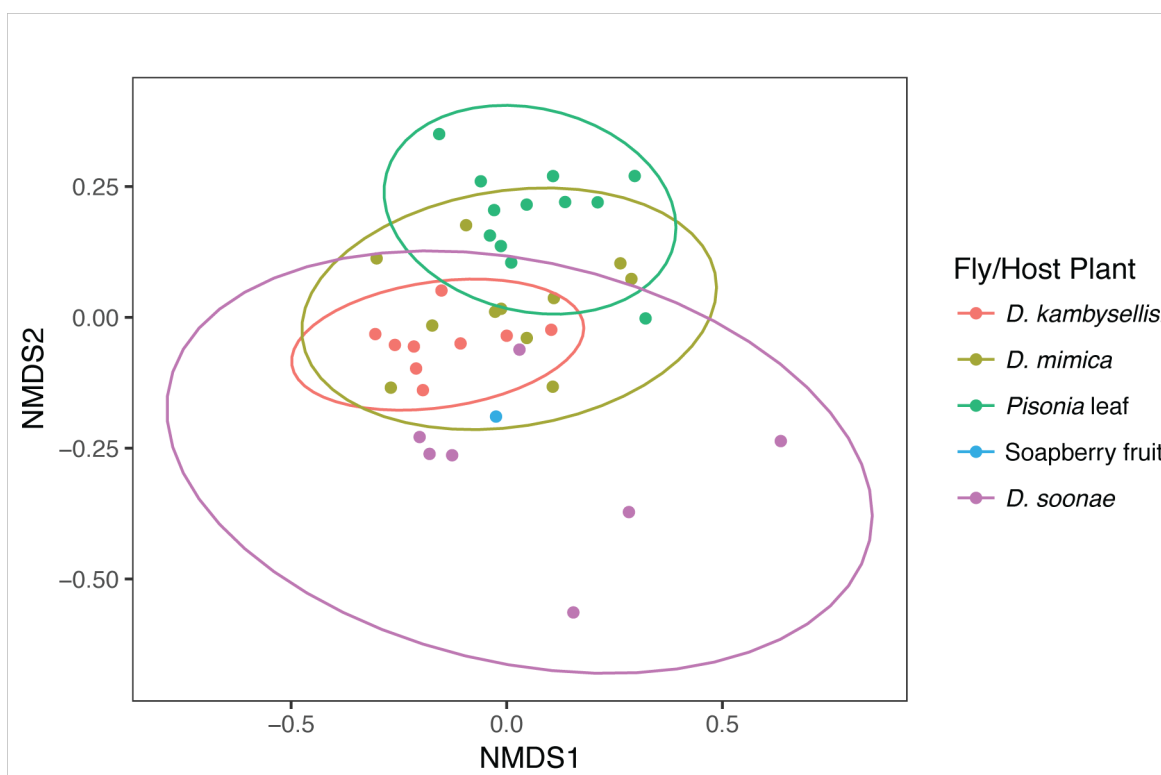
Note that the soapberry curve represents a single sample.

Although the amplicon survey revealed a great diversity of fungal species, only 56.7% of the fungal species identified from the culturing survey were recovered in the amplicon survey. The discrepancy could be explained by biases in both the culturing and amplicon methods or sampling season, although it is difficult to pinpoint a single explanation since the amplicon study was only performed for the January 2015 collection. Generally, the fungal communities of each

fly species or host plant clustered together (Figure 4.10) with fly or plant species accounting for the most variation in fungal community of all categorical factors (Bray-Curtis  $R_{ANOSIM}=0.218$ ,  $p<0.001$ , Table 4.2, Figure S4.2).

**Table 4.2. MANOVA (ADONIS) of Bray-Curtis diversity patterns for fungal communities associated with Hawaiian *Drosophila* and their host plants for known categorical factors.**  $R^2$  value represents the percentage of variation explained by each factor.

Factor	R <sup>2</sup>	p-value
Fly species/Host plant	0.218	0.001
Sex	0.148	0.001
Sample substrate	0.085	0.001
Collection site	0.079	0.006
Collection media	0.048	0.627

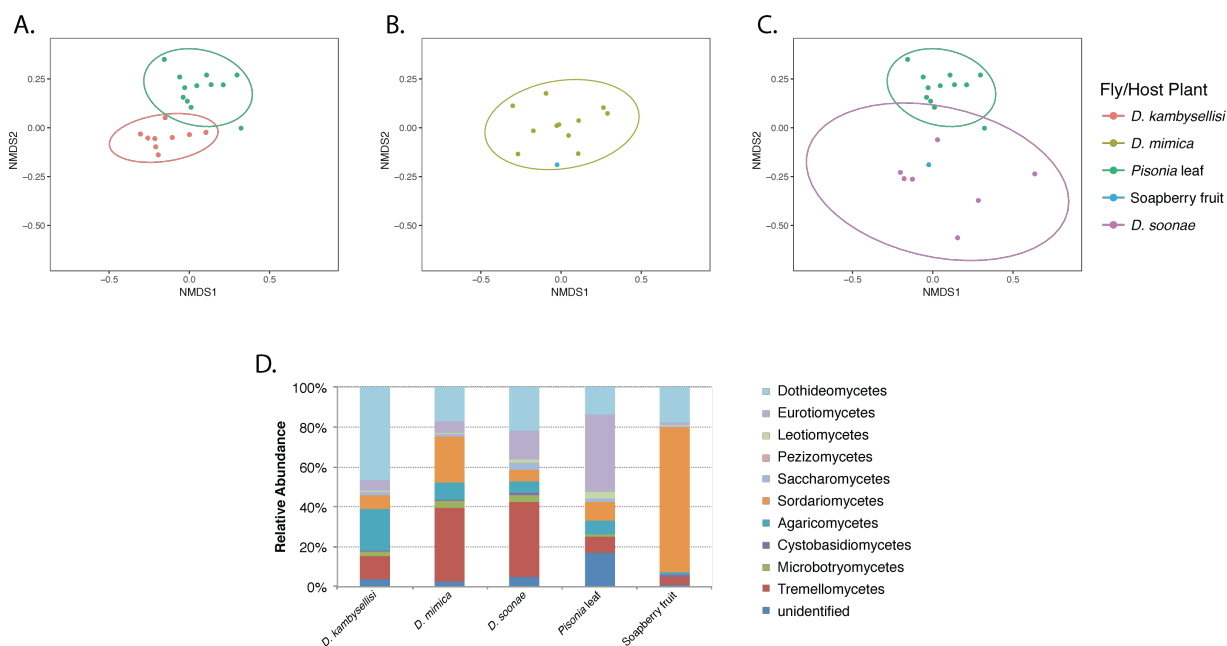


**Figure 4.10. Fungal Bray-Curtis dissimilarity NMDS of fungal communities associated with Hawaiian *Drosophila* or host plants.**

Each sample was rarefied to 1082 sequences and is represented by a single point, color-coded by fly species or host plant.

Contrary to the culturing study, the fungal communities of Hawaiian *Drosophila* species and their respective host plants did not cluster together (Figure 4.11 A-C). In fact, taxonomic breakdown of these fungal communities by class revealed that *D. mimica*, *D. kambysellisi*, and *D. soonae* harbor fungal communities that are more similar to each other than their host plants

(Figure 4.11D). These communities share many of the same fungal classes but are distinguished by relative abundances.

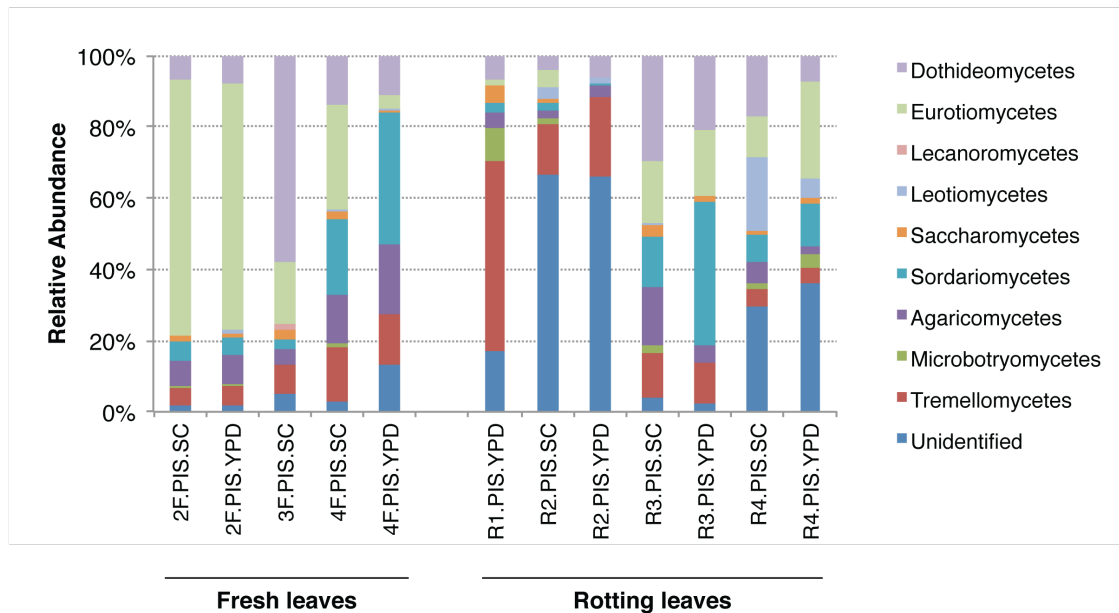


**Figure 4.11. Fungal communities associated with Hawaiian *Drosophila* species and respective host plants.**

(A) for *D. mimica* and its host plant, soapberry fruits. (B) for *D. kambysellisi* and its host plant, *Pisonia* leaves. (C) for *D. soonae* and its presumed host plants, *Pisonia* leaves and soapberry fruits. (D) Average, relative abundance of fungal classes associated with a fly species or host plant substrate and present in at least 1% of each sample type.

These results are similar to those reported by Yakym [169], who found no clear relationships between the fungal communities associated with two Hawaiian picture-wing *Drosophila*, *Drosophila sproati* and *Drosophila ochracea*, and their respective host plants, *Cheiodendron trigynum* and *Freycinetia arborea*. The lack of obvious similarity of between the fungal communities associated with Hawaiian *Drosophila* and their host plants suggest that Hawaiian *Drosophila* do not share fungal species with their respective host plants and that fungi may not play a role in host plant discrimination.

However, uncontrolled variables are important caveat to this study. There was large variation in the fungal communities associated with individual samples (Figure S4.3). While species and sex of individual fly samples could be verified, it is impossible to identify the age or mating history of a particular fly sample. These are just two confounding variables that could explain the inconsistency between individual flies. Even for host plant substrate, where rotting stage can be easily documented, a great deal of variability between plants still exists. Fresh and rotting *Pisonia* leaves clearly harbor a different fungal consortium, however, there is still a lot of variability from plant to plant (Figure 4.12).



**Figure 4.12. Average relative abundance of fungal classes associated with fresh or rotting *Pisonia* leaves.** Abundances shown for fungal classes present in at least 1% of all *Pisonia* samples.

## Discussion

One of the most striking characteristics of the Hawaiian *Drosophila* clade are the host plant specializations exhibited by many species throughout the radiation. Hawaiian *Drosophila* likely discriminate between host plants through volatile cues so the microbial communities colonizing host plants, particularly yeast, are hypothesized to mediate Hawaiian *Drosophila* host plant discrimination [74,151,153,156,158,159]. While the fungal communities present on common Hawaiian *Drosophila* host plants are distinct [74,158], whether these fungal communities overlap meaningfully with the fungal communities associated with Hawaiian *Drosophila* had not been previously investigated. In this chapter, I examined the fungal communities associated with three Hawaiian *Drosophila* species, *D. mimica*, *D. kambysellisi*, and *D. soonae*, and their respective host plants using both culturing and amplicon sequencing methods.

While the initial culturing survey showed little diversity in the fungal species associated with Hawaiian *Drosophila* and their host plants, DNA amplicon sequencing revealed much more extensive fungal communities. Based on the amplicon study, there was no clear association between fungi vectored by Hawaiian *Drosophila* and the fungi colonizing host plant substrate. Instead, the fungal communities of Hawaiian *Drosophila* species were more similar to each other than to their respective host plants. While these results suggest that fungi drive host plant discrimination in Hawaiian *Drosophila*, I cannot completely reject this hypothesis due to several confounding variables.

### Potential biases in fungal community survey methods

First, both culturing and amplicon fungal survey methods have inherent biases. Even the most nutrient-rich culturing media selects for fungi that can metabolize synthetic lab media most efficiently. While a diverse range of yeast have been isolated from the media used in this study, I only isolated a small portion of the fungi captured in the amplicon sequencing survey.

However, amplicon sequencing also has known caveats, such as biases in sequence length, error, and depth [121,170,171]. Additionally, post processing of raw reads also results in loss of potentially relevant information. A significant fraction of reads removed from the dataset were OTUs that were marked as unidentified fungal species. Without taxonomic information and a culturable isolate, it would be difficult to characterize the relationship of these fungi and Hawaiian *Drosophila* based on DNA sequence alone. In depth analysis of the *Saccharomyces cerevisiae* strain isolated from *D. mimica* suggested that the Hawaiian *S. cerevisiae* strain was genetically distinct from previously characterized sake, wine, and oak *S. cerevisiae* strains. So it is conceivable that Hawaiian *Drosophila* would be associated with fungal species that have high ITS sequence divergence from known fungal species and have yet to be characterized. It is possible that these fungi are shared between Hawaiian *Drosophila* and their host plants and could play a role in host plant discrimination.

### Identifying volatile compounds relevant to Hawaiian *Drosophila* host plant discrimination

The fungi vectored by Hawaiian *Drosophila* clearly produce different volatile profiles that would contribute to the overall volatile signature of a host plant. In this chapter, I characterized the volatile profiles of five yeast species grown individually on synthetic laboratory media. However, the fungal communities associated with host plants are undoubtedly more dynamic and complex. Interactions with other microbial community members and the nutrient composition of host plant substrate would have significant effects on the overall volatile bouquet detected by Hawaiian *Drosophila* in nature.

I attempted to run GC-MS on host plant material in the field but I was unable to adequately preserve volatile collections for successful sampling. Further, identifying the specific volatile compounds that are most significant for host plant discrimination would require controlled behavioral studies with Hawaiian *Drosophila*. Behavioral studies are constrained by the ability to raise a large population of Hawaiian *Drosophila* isofemale lines in the laboratory. While I was able to inbreed a single isofemale *D. mimica* line in our lab over eight generations, maintaining a long-term, healthy population was unsuccessful. Raising Hawaiian *Drosophila* under laboratory conditions is notoriously difficult so field experiments with combinations of yeast on host plants, such as the classic studies in the Yosemite Valley by Dobzhansky [76] and the more recent study by Batista [172] in Brazil, would likely be the most successful approach for directly testing these relationships further.

### The genetics underlying Hawaiian *Drosophila* host plant specialization

In the early stages of this project, I attempted to sequence and assemble the *Drosophila mimica* genome. However, the assembly was unsuccessful due to low coverage, bacterial contamination in unhealthy fly lines, and a high degree of heterozygosity in the isofemale line, as it was only inbred for six generations.

In future studies, I believe it would be more informative to focus on the assembly and analysis of OR genes across the Hawaiian *Drosophila* lineage. Because Hawaiian *Drosophila* presumably use volatile cues to locate host plants, analyzing the evolution of olfactory receptors (ORs) in Hawaiian *Drosophila* species would be an important next step to elucidating the genetic changes that occur with host plant specialization. Changes in ORs have been associated with host plant specialization in *Drosophila sechellia*, *Drosophila erecta*, and *Scaptomyza flava* [70,72,73,173]. When surveying the evolution of OR genes across *Drosophila* species, Guo & Kim [68] found that the Hawaiian *Drosophila* species, *Drosophila grimshawi* underwent the most dramatic OR gene duplication and loss events. A similar pattern of OR gene evolution has occurred in other *Drosophila* species that have evolved very specific host plant adaptations and suggests that changes in ORs may have occurred with host plant specialization in Hawaiian *Drosophila* species.

### The challenges of studying endemic species in fragile ecosystems

I was initially drawn to the fungal-Hawaiian *Drosophila* interaction because it offered a relatively unperturbed and ecologically significant study system in which to study the importance of the fly-yeast mutualism. However, like many study systems involving endemic species existing in very specific ecosystems, the ability to perform controlled laboratory experiments is very limited. Achieving adequate sampling depth is the most common challenge because collections and the transportation of collections are often restricted by policy (and rightfully so). Balancing sampling with self-regulation and respect for the ecosystems of interest was also a challenge faced by Yakym's study of the fungal communities associated with the picture-wing species, *Drosophila sproati* and *Drosophila ochracea* [169]. In this chapter, low sample number was an obstacle in almost every experiment. Seasonal variability in sampling and variation between individual samples were also factors that remained unresolved. Despite these challenges, I believe there is still more to understand about the interaction between Hawaiian *Drosophila*, fungi, and their host plants, provided the right resources and applications.

## Materials and Methods

### Field collection Hawaiian *Drosophila* and host plant substrate

All necessary permits were obtained from the Hawaii Volcanoes National Park, the State of Hawaii, and United States Department of Agriculture Animal and Plant Health Inspection Service. Adult Hawaiian *Drosophila* were collected in individual, sterile vials by direct aspiration or netting in July 2013 and January 2015. *Drosophila mimica* and *Drosophila kambysellisi* were collected in Kipuka Puaulua and Kipuka Ki. *Drosophila soonae* were collected from Honua'ula Forest Reserve. Rotting soapberry fruits were collected from Kipuka Puaulua. Both fresh and rotting leaves of *Pisonia* were collected from Kipuka Puaulua in January 2015. All sampling information is summarized in Table 4.1. Collection were either frozen, applied to culturing media, or maintained as isofemale lines within four hours of collection, as described below.

### Rearing Hawaiian *Drosophila*

Hawaiian *Drosophila* females were transported back to Berkeley, CA on sterile Wheeler-Clayton media (Table S4.2, original recipe from *Drosophila* Species Stock Center) in individual vials. In

the laboratory, adults were maintained in an incubator at 18°C and 70% relative humidity. Wheeler-Clayton media was made fresh every two weeks.

Females were allowed to lay in vials with a moist kimwipe and pushed every 3-4 days to stimulate oviposition as specified in [126]. Vials with embryos were kept at an angle in glass beakers fitted with a fabric topper with a base of fine sand (CaribSea Caribbean live sand) covered with an inch of coarse sand (CaribSea Aragonite Aquarium sand). Sand was watered with MilliQ water every day. Larva were allowed to “jump” from vials into sand for pupation. Eclosed adults were removed by aspirating and transferred to fresh Wheeler-Clayton media vials with less than eight adult flies per vial.

#### Culturing, isolation, and identification of the fungi associated with Hawaiian *Drosophila*

After collection, fungi were collected from Hawaiian *Drosophila* using two isolation methods. First, adult flies were allowed to walk on solid 5% YPD agar plates (Table S4.2) overnight at ambient room temperature and aspirated off of plates in the morning. Or, adult flies were dipped in microtubes with 1ml of sterile liquid 5% YPD or liquid 5% SC media (Table S4.2) and removed after five minutes. Liquid media was allowed to incubate overnight at ambient room temperature and placed at 4°C in the morning. Roughly equal numbers of males and females were sampled.

In the laboratory, plates were grown at ambient room temperature for 3-7 days. Two microliters of each liquid media sample were plated in triplicate on solid 5% YPD agar plates and allowed to grow at ambient room temperature for 3-7 days before single colonies were isolated.

Single colonies, representing every yeast morphology present on each plate, were picked by eye and streaked onto fresh 5% YPD agar plates. If plates were overgrown with mold or single colonies were unable to be picked, a subsequent isolation was performed on a fresh plate. Isolated colonies were allowed to grow at ambient room temperature and stored at 4°C until molecular identification. Original plates were kept for an additional three days after picking and monitored daily to ensure isolation of slower growing yeast.

Yeast colonies were identified by Sanger sequencing of the internal transcribed spacer region (ITS) using ITS1 and ITS4 primers [174,175]. Colony PCR reactions were performed in 25uL reaction volumes as follows: 12.5uL GoTaq Colorless Master Mix (Promega), 2uL of ITS1 and ITS4 primer at 10uM, 8.5uL nuclease-free water (Promega), and colony spike-in. Reaction conditions were as follows: 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 60 s, and a final extension of 72 °C for 4 min (Jeremy Roop, personal communication). Amplification was verified on an agarose gel before being sent for Sanger sequencing (ELIM Biopharmaceuticals). Resulting sequences were trimmed for quality and then identified using BLAST (NCBI). Hits with an identity score greater than 98% were documented. After positive identification, yeast isolates were frozen as -80°C glycerol freezer stocks using standard protocol (Methods in Yeast Genetics, 2005) until use in behavior assays.

#### Gas chromatography – mass spectrometry (GC-MS)

For Hawaiian fungal species volatile profile characterization, each species was plated on a 5% YPD agar plate and grown at ambient room temperature for 22 hours before GC-MS sampling. For *Saccharomyces cerevisiae* volatile profile characterization, each strain was plated on a 5%

YPD agar plate and grown at 30°C for 22 hours before GC-MS sampling. GC-MS sampling methods for both assays are described below.

Yeast volatiles were sampled using the stirbar sorptive extraction (SBSE) and thermal desorption method. As previously described in [34], a conditioned, Twister stir bar (10 mm in length, 0.5mm film thickness, 24uL polydimethylsiloxane, Gerstel Inc) was suspended from the lid of the larger petri dish with rare earth magnets for 40 minutes at room temperature. The Twister bar was then dried using a Kimwipe, placed in a thermal desorption sample tube, topped with a transport adapter, and loaded onto sampling tray (Gerstel Inc).

Automated sampling and analysis was performed using the Gerstel MPS system and MAESTRO integrated into Chemstation software. Sample analysis was performed on an Agilent Technologies 7890A/5975C GC-MS equipped with a HP-5MS (30m × 0.25mm, i.d., 0.25micrometers film thickness, Agilent Technologies) column.

Samples were thermally desorbed using the Gerstel Thermal Desorption Unit (TDU) in splitless mode, ramping from 30C to 250 °C at a rate of 120 °C/min, and held at the final temperature for 5 minutes. The Gerstel Cooled Injection System (CIS-4) was cooled to -100C with liquid nitrogen before ramping to 250 °C at a rate of 12 °C/min and held for 3 mins for injection into the column. The injector inlet was operated in the Solvent Vent mode, with a vent pressure of 9.1473 psi, a vent flow of 30mL/min, and a purge flow of 6mL/min.

The GC oven temperature program was set to 40 °C for 2 min, raised to 140 °C at 4 °C/min, and finally raised to 195 °C at 15 °C/min and held for 10 min. A constant helium flowrate of 1.2 mL/min was used as carrier gas. The MSD transfer line temperature was set at 280C. The MS was operated in EI mode with the electron voltage set at autotune values. The detector was set to scan from 30 to 300amu at a threshold of 150 at a scanning rate of 2.69 scans/second. The ion source and quadrupole temperatures were set at 230C and 150C, respectively.

GC-MS data files were visually inspected using Chemstation and peaks were identified using the NIST O8 database. Datafiles were transferred, parsed, and analyzed using custom written Matlab scripts in [34].

#### Hawaiian *Saccharomyces cerevisiae* phylogenetic strain characterization

Whole genome sequencing of the same Hawaiian *S. cerevisiae* isolate characterized by GC-MS above was used to place the strain within the known *S. cerevisiae* ecotypes. DNA was extracted using the Qiagen Genra Purgene Tissue DNA extraction Kit following manufacturer's protocol and sheared using the Diagenode Bioruptor Standard. Sheared DNA was quantified with Qubit (dsDNA HS Assay) and size and quality was verified with an Agilent 2100 BioAnalyzer (High Sensitivity DNA kit). Libraries were prepared with an Illumina TruSeq DNA PCR-Free Sample Preparation Kit (350 base pair insert size) and sequenced on an Illumina HiSeq 2000 (100 PE) at the Vincent J. Coates Genomics Sequencing Laboratory, UC Berkeley.

The relationship of the Hawaiian *S. cerevisiae* isolate to other *S. cerevisiae* strains was elucidated using five unlinked loci previously used to demonstrate population structure between wine, sake, and oak *S. cerevisiae* strains: CTY1, ZDS2, PDR10, MLS1, CCA1 [165]. Reference sequences for wine, sake, and oak strains were obtained as follows. Full sequences of each gene from Fay



& Benavides [165] were downloaded from Genbank for *S. cerevisiae* strains representing each ecotype:

- Sake *S. cerevisiae* strains: K5, K13, K9
- Wine *S. cerevisiae* strains: M24, M15, M20
- Oak *S. cerevisiae* strains: YPS163, YPS1000

S288c reference sequences for each gene were blat from the UCSC Genome Browser using the YPS163 sequences from Fay & Benavides [165].

Raw, sequenced reads of the Hawaiian *S. cerevisiae* isolate were extracted with blat using the S288c sequences for each gene as a reference [167]. Aligned reads were assembled in velvet (k-mer length of 27bp and default parameters) [176].

Gene sequences for each strain were trimmed to match the length of the YPS163 gene sequences. After trimming, all five genes for each strain were concatenated in this order: CTY1, ZDS2, PDR10, MLS1, CCA1. All concatenated sequences were aligned and a neighbor-joining tree was built in Geneious (version 5.1.7).

#### DNA extraction for amplicon study

Samples collected for amplicon study were collected in January 2015 and immediately frozen individually in sterile cryotubes (1mL, Corning) either alone, in 5% YPD liquid media, or in 5% SC liquid media. Sampling media did not affect amplicon results (Table 4.2, Figure S4.2D). Frozen samples were taken back to the laboratory and DNA was extracted from both whole fly and host plant substrate samples following QIAGEN's QIAamp Micro Kit tissue protocol with the modifications described below [177]. After the overnight digestion with proteinase K, samples were bead beat (0.5 mm Zirconium beads, Ambion) in 200uL of WLB (Table S4.2). Bead beating protocol: bead beat (MoBio) twice for one minute at 4C with a 30 second break in between, spun five minutes at ~14,000xg, and the supernatant was transferred to a new tube. Beads were resuspended in 1ml of buffer WLB and beat again an additional minute, spun down, and supernatant was pooled. Finally, beads were washed once more with 1mL of buffer WLB, spun down, and supernatant was pooled. The pooled supernatant was spun down to pellet any beads and transferred to a clean microtube. One ug of carrier RNA (QIAGEN) dissolved in buffer AE was added to the supernatant before proceeding to ethanol precipitation and elution per the manufacturer's protocol. DNA samples were quantified (Qubit dsDNA HS assay kit, ThermoFisher Scientific) and stored at -20C until PCR amplification.

#### Amplification and library preparation for amplicon study

Fungal communities were characterized by amplifying the universal fungal internal transcribed spacer region I (ITS1) using BITS and B58S3 primers designed by Bokulich and Mills [103]. Each forward BITS primer includes a unique 8bp barcode connected to the universal forward primer with a 2bp linker sequence (sequences generously provided by Bokulich and Mills).

The following pre-PCR steps were carried out in a biosafety cabinet. The biosafety cabinet and laboratory supplies used were cleaned at the start of each day as follows to minimize PCR contamination: 10% bleach for 20mins, rinsed with autoclaved MilliQ water, 3% hydrogen peroxide for 10mins, and UV lamp for at least 5mins. PCR reactions were carried out in triplicate following the protocol previously used in [104] and described below.

For a single PCR reaction, reagents were added in the following order: 12.5uL GoTaq Colorless Master Mix (Promega), 2uL of B58S3 primer at 10uM, 5.5uL nuclease-free water (Promega), 2uL of BITS primer at 10uM, and finally 5–100 ng DNA template. Reaction conditions were as follows: 94 °C for 3 min, 35 cycles of 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s, and finally extension of 72 °C for 10 min [104]. PCR reactions were performed in snapstrip PCR tubes (GeneMate) in triplicate. A single negative control PCR replicate was run in parallel with each primer pair used.

PCR products were quantified using the Qubit dsDNA HS assay kit and all samples with concentrations of >1ng/uL were pooled at 10ng equimolar concentration. After pooling, the pooled sample was cleaned and concentrated according to the manufacturer's protocol (Zymo Clean and Concentrator), eluted in 22uL of sterile water, and quantified with the Qubit dsDNA HS assay kit.

Illumina sequencing libraries were prepared using TruSeq RNA v2 kit (Illumina) beginning at the A-Tailing step of the manufacturer's protocol using 260ng of starting material. Libraries were verified and quantified using the Qubit dsDNA HS assay kit and the Agilent 2100 Bioanalyzer (Agilent Technologies). Libraries were sequenced single 150bp paired-end Illumina HiSeq2000 lane at the Vincent J. Coates Genomics Sequencing Laboratory (UC Berkeley).

#### Data analysis for amplicon study

Data analysis was based on processing steps detailed in [105,113] and followed that in the Materials and Methods section of Chapter 2. Raw and quality filtered sequence counts from the following steps are summarized in Table S4.1. Raw read pairs were merged using BBMerge (<https://jgi.doe.gov/data-and-tools/bbtools/>), demultiplexed in QIIME v1.9.1 [114] and primer sequences were trimmed using cutadapt [115]. Resulting reads were quality filtered in QIIME as follows: any read less than 80bp was removed, any read with more than 3 consecutive bases with a quality score <19 was removed, and any chimeric sequences were filtered against the UCHIME chimera reference dataset v7.1 [116] using the union method.

Open reference OTU picking was performed in QIIME using the UCLUST method [117] against a modified UNITE database [118,119] with a threshold of 97% pairwise identity. Sequence alignment and treebuilding were suppressed and taxonomy was assigned using BLAST (NCBI). After OTU picking, positive control mock culture samples and OTUs with 'no blast hit' were filtered. Using R version 3.2.4 [120], negative controls were removed, max sequence counts of all negative control OTUs were calculated, and then subtracted from all real samples to account for spurious sequences produced from possible PCR, sequencing, or spillover contamination [121,178]. Finally, an OTU threshold of 0.001% was applied [105,113].

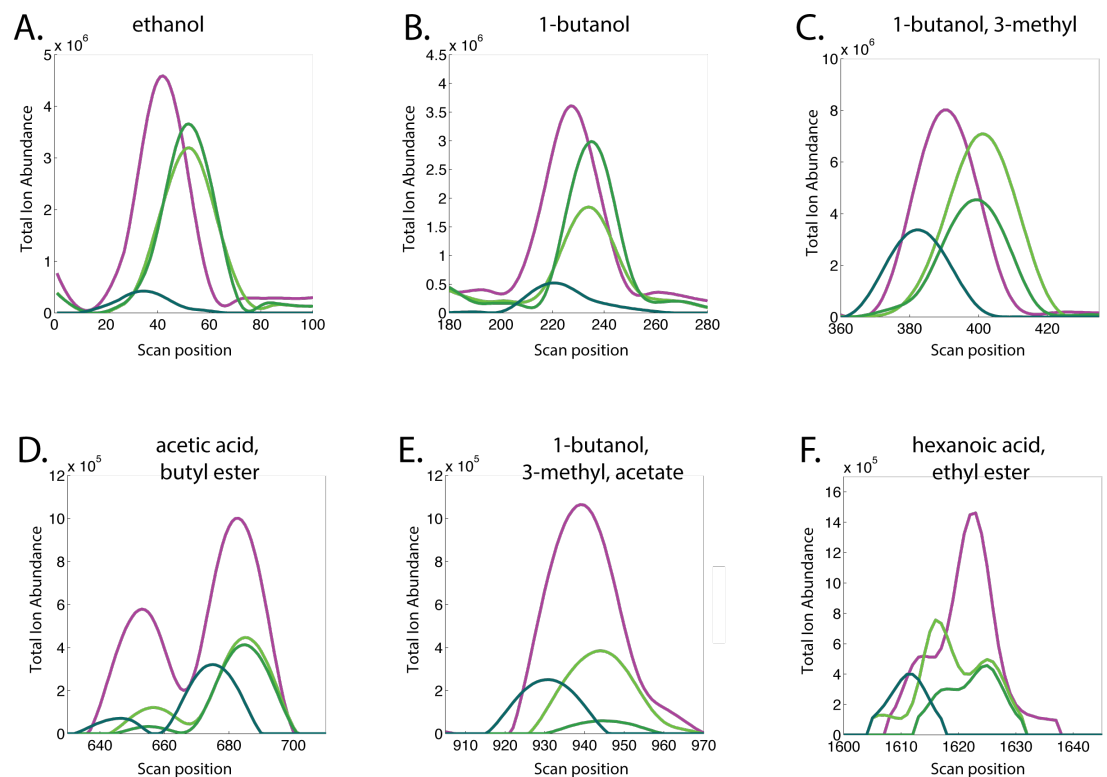
Alpha diversity measurements of observed OTU richness were calculated and visualized in QIIME to reveal that all samples had been sequenced to saturation, except for soapberry samples, which were inconclusive due to low sample number (Figure 4.9). Community analyses were conducted using the vegan [122] and biom [123] packages in R. To determine relationship between the fungal communities vectored by Hawaiian *Drosophila* and host plants, samples were evenly subsampled to 1182 reads per sample and Bray-Curtis dissimilarity was calculated and visualized with nonmetric multidimensional scaling (NMDS) using ggplot2 [124]. ADONIS was used to calculate the percent of variance attributed to known factors.

## Acknowledgements

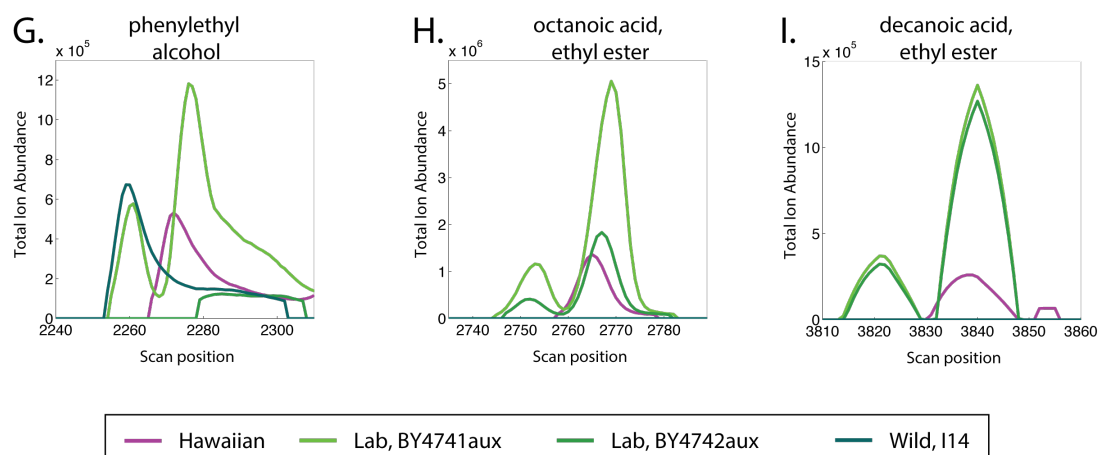
None of this would have been possible without fieldwork training from Patrick O'Grady. Rosie Gillespie generously allowed me to join the first Dimensions field expedition in the initial phases of this project. Don Price, Ken Kaneshiro, and Maxi Richmond provided valuable advice on rearing Hawaiian *Drosophila* in the laboratory. I am also grateful to Jessica Craft, Kulle Roy, and Brenda and Norman Quan for their assistance in field collections.

# Supporting Information

## SHORT CHAIN VOLATILES

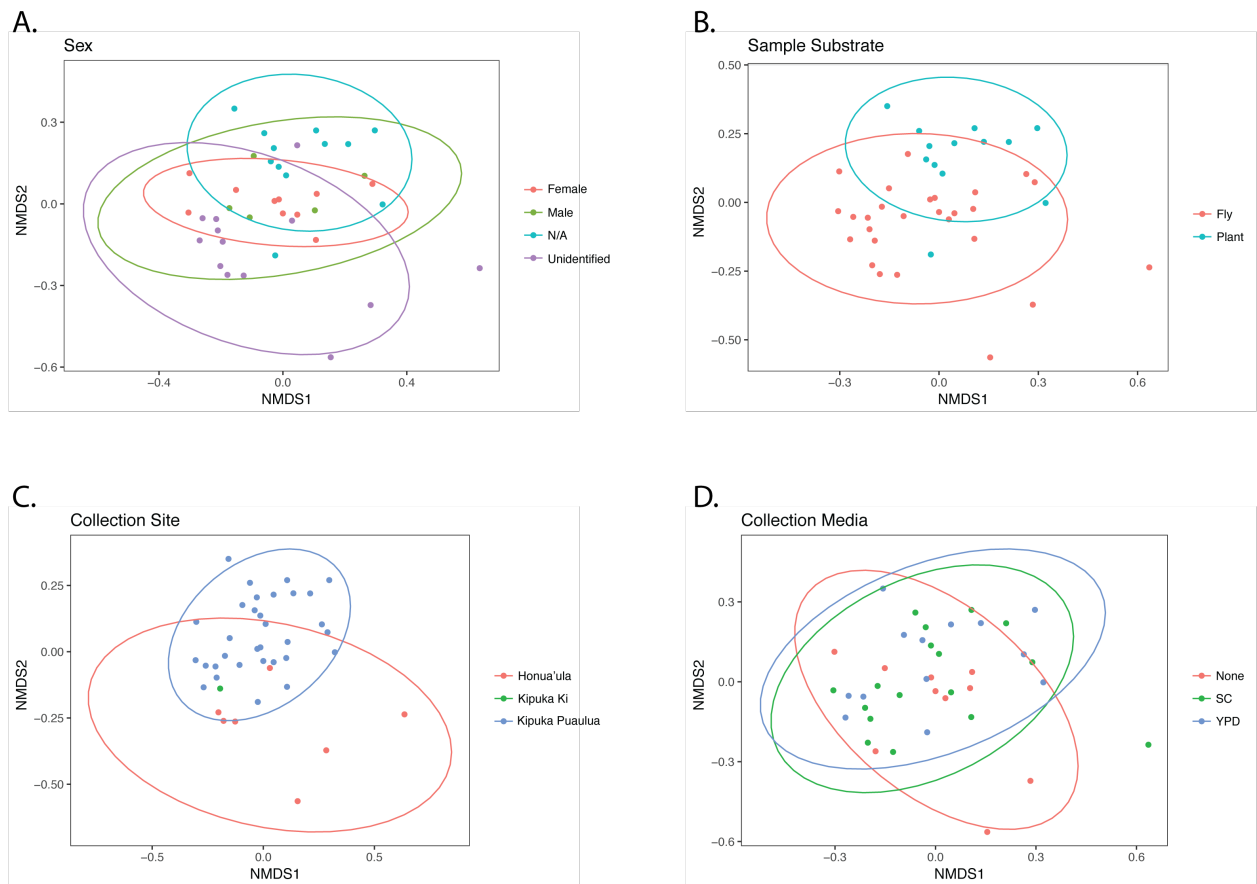


## LONG CHAIN VOLATILES



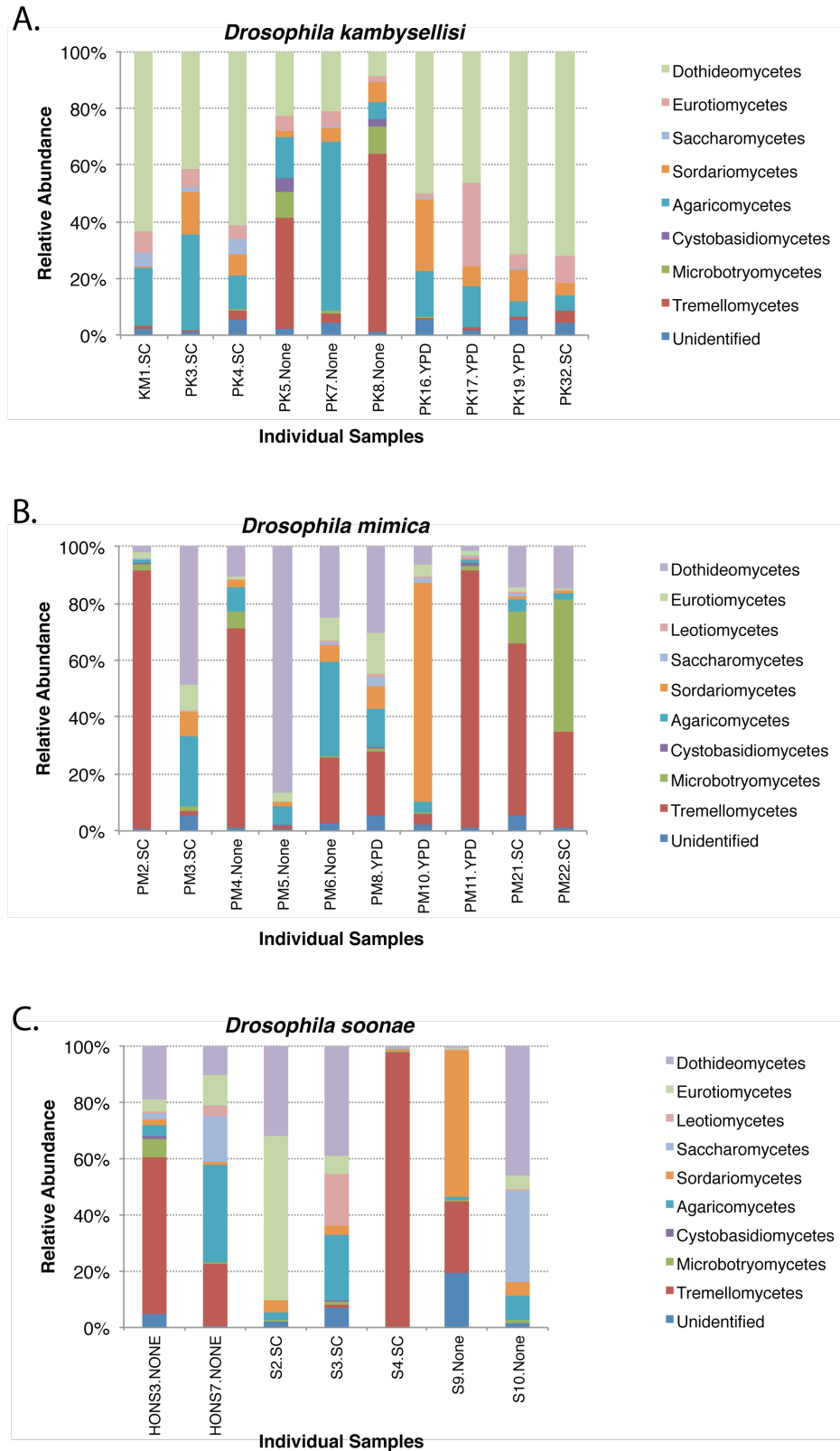
**Figure S4.1. Comparison of individual compounds produced by the *S. cerevisiae* strains in Figure 4.7.**

Each line represents the average of three to four GC-MS replicates for a given *S. cerevisiae* strain grown at 30°C for 22hrs on YPD media.



**Figure S4.2. Fungal Bray-Curtis dissimilarity NMDS of fungal communities by other categorical factors.**

Each sample was rarefied to 1082 sequences and is represented by a single point.  $R^2$  values in Table 4.2.



**Figure S4.3. Average, relative abundance of fungal classes associated with individual flies.** Average, relative abundances shown for fungal classes present in at least 1% of all samples within a single fly species. (A) *D. kambysellisi*, (B) *D. mimica*, and (C) *D. soonae*.

**Table S4.1. Read quality filtering statistics.**

Quality filtering step	Total read count	Total OTUs	% of original reads retained
Raw sequences	1855754	--	--
After merging pairs	1498206	--	80.73%
After demultiplexing	1081385	--	58.27%
After quality filtering	1089106	--	58.69%
After chimera filtering	1087900	--	58.62%
OTU threshold (>0.001%)	1076549	1608	58.01%
Filter 'no blast hit'	817682	1128	44.06%
Filter negative control reads	578402	1070	31.17%
Final sequence count	578329	1049	31.16%

**Table S4.2. Media used in this chapter.**

Media name	Purpose	Volume	Recipe
<b>Wheeler-Clayton [126]</b>	Rearing Hawaiian <i>Drosophila</i>	Makes ~100 wide mouth vials	7.5g Special K, 11.25g Wheat Germ (Kretschmer), 3.75g Product 19, 37.5g yeast, 11.25g Hi-Protein baby cereal (Gerber), 101.25g of Gerber's banana baby food, 10.5g of agar (BD Bacto Agar), 1L of MilliQ in each, 4.875mL of 95% ethanol (Koptex, 200proof), and 4.875mL of propionic acid (Sigma). Boiled and poured into wide mouth <i>Drosophila</i> vials (wide mouth, K-resin, Genessee Scientific).
<b>5% YPD, liquid</b>	Liquid media for yeast collection	1L	20g Peptone (BD Bacto Peptone), 10g Yeast Extract (Amresco Yeast Extract, Bacteriological, Ultra Pure Grade), 50g Dextrose (Fisher Scientific Dextrose Anhydrous), and MilliQ water to 1L. Autoclaved and filter sterilized (Nalgene 75mm filter unit, 0.2aPES).
<b>5% SC, liquid</b>	Liquid media for yeast collection	1L	1.7g YNB without amino acids (BD Difco), 2g synthetic complete amino acid mixture (MP Biomedicals), 50g dextrose (Fisher Scientific Dextrose Anhydrous), and MilliQ water to 1L. Autoclaved and filter sterilized (Nalgene 75mm filter unit, 0.2aPES).
<b>5% YPD, agar</b>	Solid media for yeast collection and isolation	1L	20g Peptone (BD Bacto Peptone), 10g Yeast Extract (Amresco Yeast Extract, Bacteriological, Ultra Pure Grade), 50g Dextrose (Fisher Scientific Dextrose Anhydrous), 20g Agar (BD Bacto Agar), MilliQ water to 1L. Autoclaved and poured into 100 x 60mm petri dishes (Falcon).
<b>WLB</b>	Buffer for DNA extraction	-	2M Guanidinium thiocyanate (Fisher Scientific), 0.5 M EDTA (Fisher Scientific), 1.8% Tris base (Promega), 8% NaCl (Sigma Aldrich), 150mL of MilliQ water, and adjust to pH 8.5. Autoclaved and filter sterilized (Nalgene 75mm filter unit, 0.2aPES). (Will Ludington, personal communication).

# CHAPTER 5: Or22 allelic variation alone does not explain differences in discrimination of yeast-produced volatiles by *D. melanogaster*

*This chapter and work detailed therein was a joint effort by Allison Quan and Carolyn Elya. Note that the contents of this chapter, with some minor modifications, were previously published as:*

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## Abstract

Different lines of the fruit fly *Drosophila melanogaster* show variation in the ability to discriminate between volatiles produced by the yeast *Saccharomyces cerevisiae* under natural (nitrogen-limiting, YVN) or laboratory (sugar-limiting, YVL) conditions. Previous work in our laboratory uncovered a strong correlation between heightened sensitivity to YVN wild *D. melanogaster* lines that harbored a chimeric variant of the highly variable odorant receptor 22 (Or22) locus of *D. melanogaster*. We sought to determine if this trend held for an extended set of *D. melanogaster* lines, if observed variation within chimeric and non-chimeric lines could be explained by nucleotide polymorphisms and if replacing Or22 with a chimeric allele in a non-chimeric background could confer the enhanced ability to detect YVN. In parallel, we performed crosses of chimeric and non-chimeric fly lines and assayed the behavior of their progeny for enhanced sensitivity to YVN to assess the heritability of the Or22 locus. Ultimately, we found that, while the overall trend of increased sensitivity to YVN in chimeric lines persists, there are exceptions and variation that cannot be explained by sequence variation at the Or22 locus. In addition, we did not observe increased sensitivity for YVN upon replacing the Or22 allele in a non-chimeric line (OreR) with that from our most YVN-sensitive, chimeric line (ME). Though our results do not support our hypothesis that Or22 is the primary driver of sensitivity to YVN, Or22 remains an interesting locus in the context of fly-yeast ecology.

## Introduction

The fruit fly *Drosophila melanogaster* and the yeast *Saccharomyces cerevisiae* are close natural partners: flies require yeast for development and nutrition [23] and yeast depend on flies to be vectored to new substrates [29]. Flies can sense a variety of compounds that are produced by fermenting yeast via olfaction [59] and have demonstrated a preference to yeasted over non-yeasted fruit in the context of the laboratory [28]. Evidence to date suggests that chemical communication is the basis of the co-occurrence of flies and yeast in nature, but the specific components that mediate this molecular conversation are incompletely understood.



Olfactory sensing in *Drosophila* begins in the antenna and maxillary palp, the two main odor sensing organs in adult flies [46]. Both the antenna and maxillary palp are covered with sensory hairs (sensilla) which house one to four olfactory receptor neurons [51]. These neurons express transmembrane odorant receptors and project onto distinct glomeruli in the antennal lobe, the central olfactory processing center [51]. Olfaction is sensed when a volatile compound (odorant) diffuses into a sensillum and binds its cognate olfactory receptor [46] thereby eliciting a stimulus that is processed by the antennal lobe [51].

The *Drosophila* genome encodes 62 different olfactory receptors, each of which is expressed in a particular type of olfactory neuron either alone or in conjunction with up to two additional olfactory receptor types [59,67]. All neurons expressing a given olfactory receptor project onto the same glomerulus within the antennal lobe [51]. Extensive work has profiled the repertoire of each odorant receptor by recording responses of neurons ectopically-expressing olfactory receptors to a panel of 110 odors, revealing that *D. melanogaster* odorant receptors can detect a diverse set of organic compounds with varying sensitivity and response kinetics [59].

Previous work in our laboratory showed that the wild-type fly line Ral437 [179] can differentiate between volatiles produced by yeast under natural (nitrogen-limiting, YVN) or laboratory (sugar-limiting, YVL) conditions and that six volatile compounds mediate this attraction [34]. Three of these compounds, ethyl hexanoate, ethyl octanoate, and isoamyl acetate, are recognized by the same odorant receptor, Or22a [59,180]. Intriguingly, genomic comparison of *Scaptomyza flava*, an herbivorous drosophilid, and *D. melanogaster* found that Or22a is one of two olfactory receptors conserved among drosophilids but completely lost in *S. flava*, suggesting that Or22a plays a role in the fungivorous lifestyle of *D. melanogaster* [73].

In *D. melanogaster*, Or22a is one of two tandem copies of Or22 (the other copy being Or22b) present at the Or22 locus on chromosome 2L [181]. Both odorant receptors are expressed in basiconic sensilla of the ab3A olfactory neuron [58]. A tandem duplication of Or22 occurred in the *D. melanogaster* lineage prior to the divergence from *D. simulans* but after divergence from the *D. erecta* and *D. yakuba* lineage [181]. The Or22 locus is functionally variable between *Drosophila* species, indicating that it is a quickly evolving region and likely under selective pressure [64,69]. In *D. erecta*, Or22 has evolved to sense odors from the host plant *Pandanus* spp [70]. In *D. sechellia*, Or22a has specialized to detect odors that emanate from the host plant *Morinda citrifolia* while Or22b has decayed into a pseudogene [72].

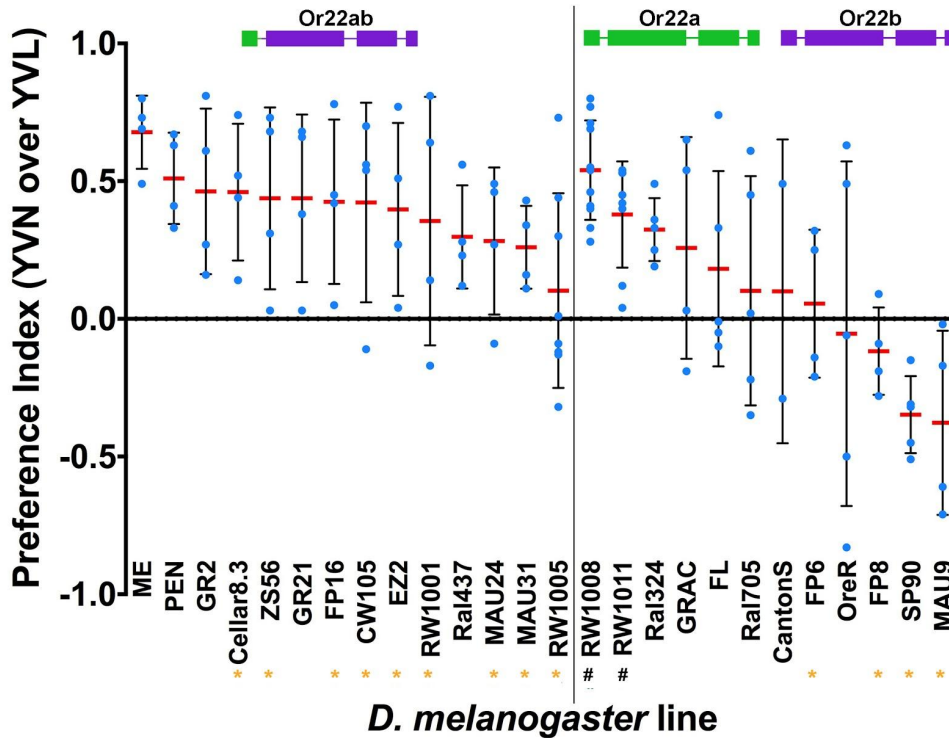
In addition to being highly variable between species, studies have observed significant sequence variability at the Or22 locus between different lines of *D. melanogaster* [181,182]. A set of *D. melanogaster* lines were found to segregate by two variants at the Or22 locus: one non-chimeric variant contained two copies of Or22, Or22a and Or22b, while the other contained a chimera (Or22ab) consisting of the first exon of Or22a fused to the last three exons of Or22b [181]. In addition to the length variants observed in *D. melanogaster* lines, some lines were also observed to possess an inversion on 2L whose breakpoint is just 0.7 Mb away from the Or22 locus; however, no association between the inversion and the length variant was observed [181]. Despite tolerating substantial variation, the Or22 locus has been implicated as a region undergoing positive selection in comparative population genetic studies of *D. melanogaster* in African and Europe [183]. In Australia, the presence of the length variants is clinal, where all southern lines were non-chimeric at the Or22 locus and almost all northern flies were chimeric [182]. These studies suggest that the Or22 locus has recently undergone positive selection with *D. melanogaster*, though the conferred benefit indicated by that selection is unknown [181].

Previous work in our laboratory had demonstrated a strong correlation between sensitivity to YVN over YVL and the chimeric allele of Or22 using a trap-based olfactory assay [184]. We hypothesized that the chimeric variant of the Or22 locus confers a heightened sensitivity to differences in yeast volatile bouquets and consequently contributes to flies' ability to locate yeast in nature. Here, we sought to explore this hypothesis by expanding our behavioral set with wild, inbred lines, assaying the behavior of progeny of reciprocal crosses from this set, analyzing Or22 sequences for polymorphisms that co-varied with preference for YVN over YVL and, finally, use genome editing to swap allele types (chimeric for non-chimeric) in an otherwise identical genetic background.

## Results

### Expanding the Or22 behavioral panel shows enhanced sensitivity to YVN in chimeric Or22 lines, similar to the original set

We first sought to assay the behavior of additional wild-type, fly lines to ascertain if the correlation between Or22 allele and increased sensitivity to YVN held true in a larger group. In addition to the existing panel of 14 lines, we obtained ten additional fly lines from Africa and Australia (Table 5.1), determined their allele type at the Or22 locus and tested their sensitivity to YVN using a trap-based olfactory assay [34]. These additional lines behaved in a manner consistent with our hypothesis that the chimeric allele mediates increased sensitivity to YVN (Figure 5.1).

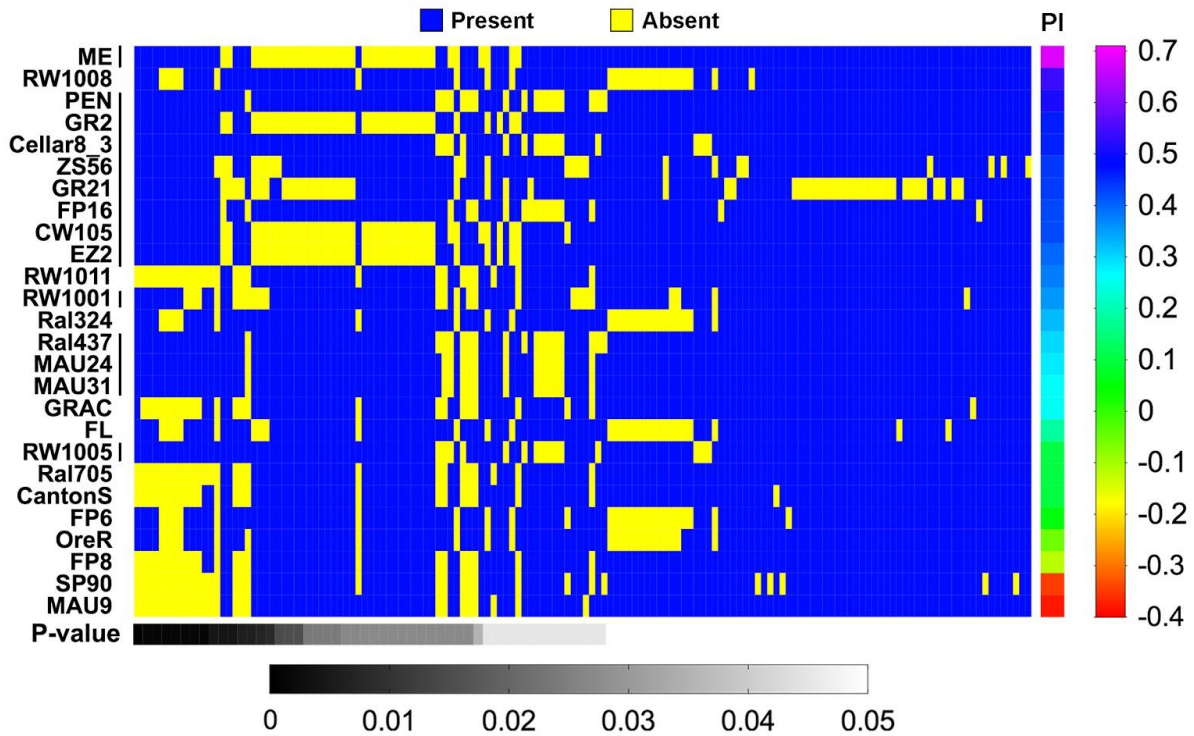


**Figure 5.1. Behavior of each fly line in our Or22 behavioral panel in olfactory trap assay.** Replicate behavioral experiments are plotted as blue dots; red lines indicate mean and black lines indicate standard deviation for all replicates. Positive preference index indicates a preference for yeast grown on limiting nitrogen over limiting sugar (i.e. preference for YVN); preference index of 0 indicates lack of sensitivity to YVN; negative preference index indicates preference for YVL. Lines to the left of the black vertical line have chimeric Or22 alleles (Or22ab); lines to the right have non-chimeric (Or22a and Or22b) alleles. Orange asterisks next to fly line indicate new additions to the behavioral panel. Black octothorpes next to fly line indicate that these were included in the original panel [184] but retested for this study.

#### Polymorphisms in Or22 locus weakly correlate with behavioral trends

Although our expanded behavioral panel showed the same general pattern of chimeric sensitivity to YVN, there was some variability in behavior between lines with the same Or22 length variant. To determine if this variation could be attributed to nucleotide variation at the Or22 locus, we set out to clone and sequence each Or22 locus present in our behavioral panel. Although this seemed like a straightforward task, it proved to be immensely challenging. This difficulty, in fact, had been the reason why no sequence information for these alleles was available prior to this study. While the chimeric alleles can be amplified, cloned and sequenced with relative ease, non-chimeric alleles require a particular set of atypical conditions during PCR and a very large amount of template (see Methods). Additionally, Sanger sequencing across the non-chimeric alleles required different primers than for sequencing chimeric alleles, probably due to mis-priming issues in the presence of the non-chimeric tandem duplication. After much effort, we were able to clone, Sanger sequence and assemble at least three Or22 amplicons from each fly line from which we generated a line consensus that we used to called nucleotide polymorphisms

(e.g. SNPs and indels). Analysis of these polymorphisms did not reveal patterns underlying sequence variants that consistently tracked with mean preference index for YVN (Figure 5.2).



**Figure 5.2. Sequence polymorphism analysis for 24 *D. melanogaster* lines in Or22 behavioral panel.**

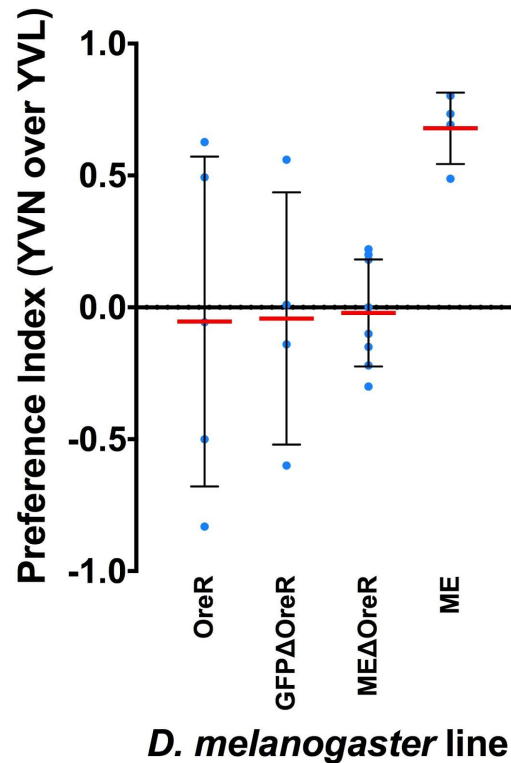
For each polymorphism, a t-test was performed between the set of preference indices for YVN (PI) for lines where the variant was present or absent. Polymorphisms are ranked by p-value (shown below heatmap). No significant correlation between variance across trials for a given line and polymorphisms were found (data not shown). Fly lines are ordered by preference index (highest at top). Mean preference index for each line is given on the right. Black lines to the right of strain names indicate strains with chimeric Or22 allele. All other strains are non-chimeric.

### Replacement of a non-chimeric with a chimeric Or22 allele does not confer sensitivity to YVN

In order to directly test our hypothesis that chimeric alleles drive sensitivity to YVN, we first took advantage of the empty neuron odorant receptor system established by the Carlson group to test odorant receptor function [58]. First, we cloned a chimeric allele (Ral437-Or22) into a vector under the control of UAS expression. Then, through a series of crosses, we generated flies with this UAS construct and GAL4 expression under the control of the Or22 promoter in an Or22 null background ( $\Delta$ halo, [58]). Unfortunately, the  $\Delta$ halo homozygotes were very sick and their health was not improved by expressing Or22 in our experimental animals. We were unable to generate sufficient numbers of animals for our behavioral assay and moved to adopt a different approach.

We next turned to the CRISPR-Cas9 gene-editing system and began implementing a two-step allelic replacement scheme (Figure S5.1). We opted to perform this swap in two steps rather than one due to the technical challenges we had encountered with amplification of the Or22 locus. For example, we were concerned that if we were to swap a non-chimeric allele with a chimeric allele, we would be able to robustly detect heterozygotes but would be unclear whether the non-chimeric allele was successfully removed when generating the homozygote. In the converse swapping experiment, we would have the opposite problem: detecting heterozygotes would be difficult due to the preferential amplification of the chimeric over the non-chimeric allele. In order to aid our detection of transformants, we designed the first step to replace the Or22 allele with a visible marker (beta-tubulin GFP cassette) so we could use visual screening to identify heterozygotes during the first round of replacement and homozygotes during the second.

In the first round of replacement of a non-chimeric allele (OreR) with our place holder cassette, we learned that our visible marker was not the reliable indicator of transformation that we hoped it would be. Though we expected global GFP expression in our transformed heterozygotes, we observed a weak symmetric GFP signal in the thorax and abdomen (Figure S5.2). This led us to identify some heterozygotes which were confirmed by non-lethal genotyping. Consistent with our expectations, the YVN sensitivity of the resultant homozygotes from these transformants phenocopied the parental line (Figure 5.3). We continued with our second round of replacement to swap in a chimeric allele (ME) in the place of our visible marker, obtained homozygotes (ME $\Delta$ OreR) and assayed their behavior (Figure 5.3). Our ME $\Delta$ OreR flies did not show an increased preference for YVN and so did not support our hypothesis.



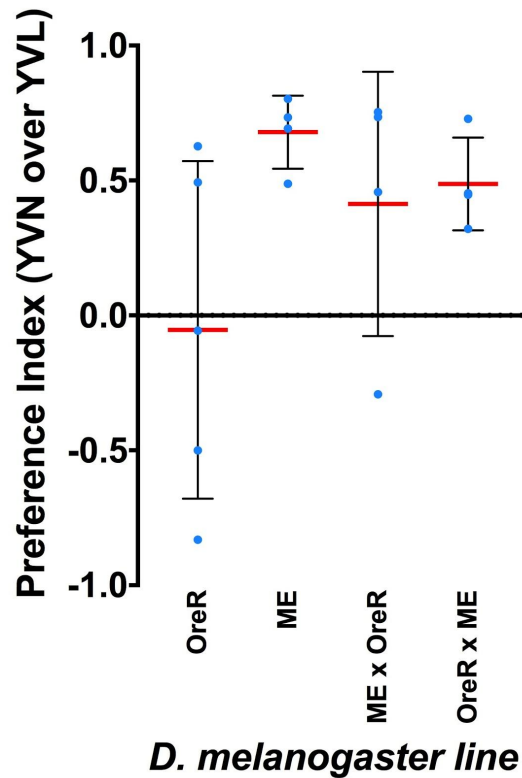
**Figure 5.3. Behavior of donor lines (OreR and ME), intermediate (GFPΔOreR) and swapped line (MEΔOreR) in olfactory trap assay.**

Replicate behavioral experiments are plotted as blue dots; red lines indicate mean and black lines indicate standard deviation for all replicates. Positive or preference index indicates a preference for YVN; preference index of 0 indicates lack of sensitivity to YVN; negative preference index indicates preference for YVL.

Crosses between chimeric and non-chimeric lines do not show a consistent inheritance pattern.

In parallel with functional studies, we performed crosses between chimeric and non-chimeric fly lines to determine the heritability of the Or22 locus with respect to behavioral sensitivity for YVN. We first crossed two fly lines with consistent, yet strikingly different behavioral responses to YVN over YVL. The OreR fly line, homozygous for the non-chimeric allele of Or22, has no behavioral preference for yeast grown on YVN or YVL while the ME fly line, homozygous for the chimeric allele, exhibits a strong preference for YVN (Figure 5.1).

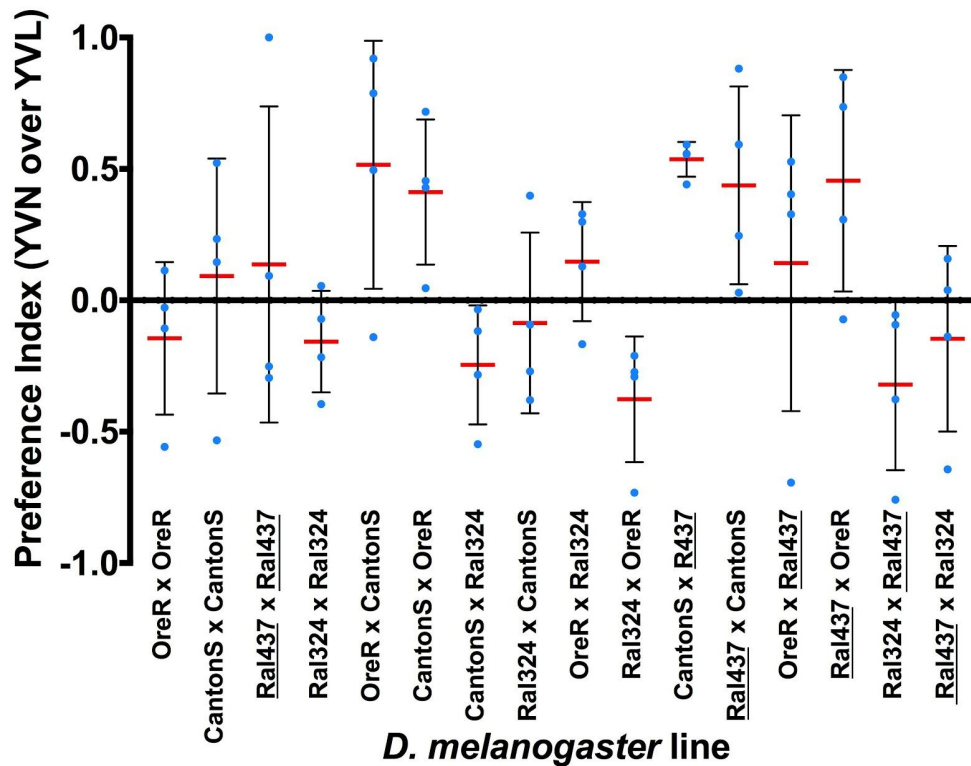
The OreR x ME cross was performed in both directions (i.e. one cross used an OreR virgin female and ME male; the other an OreR male and ME virgin female) and progeny were assayed for sensitivity for YVN. We found that the progeny of these crosses yielded inconsistent behavioral responses depending on the directionality of the cross (Figure 5.4). When ME virgin females were crossed to OreR males, the progeny preferred YVN over YVL, phenocopying the ME chimeric parental line. However, when OreR females were crossed to ME males, the F1s showed exhibited an intermediate phenotype.



**Figure 5.4. Behavioral preference for YVN over YVL for F1 crosses between ME and OreR fly lines in comparison to parental behavior.**

Replicate behavioral experiments are plotted as blue dots; red lines indicate mean and black lines indicate standard deviation for all replicates. Positive or preference index indicates a preference for YVN; preference index of 0 indicates lack of sensitivity to YVN; negative preference index indicates preference for YVL. Virgin females used in each cross are listed first.

The directional inconsistencies of this cross suggest that the genetics underlying the sensitivity for YVN may be sex linked. Based on our hypothesis, we did not expect a sex-linked inheritance pattern because the Or22 locus is located on chromosome 2L in *Drosophila melanogaster*. To confirm these results, we conducted additional crosses between chimeric and non-chimeric lines by crossing the chimeric Ral437 line to three different non-chimeric lines, OreR, CantonS, and Ral324. As a control, we also crossed the three non-chimeric lines to each other. The F1 progeny from each of these crosses were assayed for sensitivity to YVN (Figure 5.5). Again, the observed behaviors of these flies were inconsistent with our hypothesis that the Or22 allele is responsible for mediating sensitivity to YVN.



**Figure 5.5. Behavioral preference for YVN over YVL for F1 crosses between one chimeric fly line and three non-chimeric fly lines.**

Replicate behavioral experiments are plotted as blue dots; red lines indicate mean and black lines indicate standard deviation for all replicates. Positive or preference index indicates a preference for YVN; preference index of 0 indicates lack of sensitivity to YVN; negative preference index indicates preference for YVL. Virgin females for each cross are listed first. The only chimeric line tested here is Ral437, which is denoted as chimeric by a black underline.

Within this set, we were particularly puzzled by the outcome of the Ral437 x Ral437 control cross. Previously, we had found that Ral437 preferred YVN over YVL (Figure 5.1), but in this experiment Ral437 x Ral437 F1s showed no preference at all. We later determined that this inconsistency was a result of our Ral437 stock having passed through a population bottleneck between the time of these assays (for various reasons, the total number of adults in our Ral437 stock plummeted after the initial assays; all progeny of the second were considerably more inbred than previously). After genotyping the Or22 locus of the stocks before and after Ral437 bottleneck, we found that the original Ral437 stock was actually heterozygous at the Or22 locus. Most flies carried the chimeric allele but the non-chimeric allele was present and maintained at low abundance. During the bottleneck, the non-chimeric allele became over-represented, thus shifting the allele frequencies of the Ral437 stock from chimeric to non-chimeric. We believe that this explains the weaker preference for YVN over YVL in the original Ral437 fly line (Figure 5.1) and the complete loss of preference in our subsequent cross experiment (Figure 5.5). Ultimately, we were unable to clarify the heritability of the Or22 locus from these data. At face value, these crosses suggest that the Or22 locus does not or is not the only locus underlying behavioral sensitivity for YVN.



## Discussion

Though we initially observed a strong correlation between increased preference to YVN and a chimeric variant of Or22 and this correlation held when expanding the number of fly lines examined, our additional follow up experiments were inconsistent with our hypothesis that chimeric Or22 alleles confer heightened sensitivity to YVN. It is notoriously difficult to link a single gene to a behavioral phenotype due to the complicated nature of behavior. Still, we hope that that data presented in this work will contribute to the efforts in the field of behavioral genetics. While it is still possible that the Or22 locus is involved in sensitivity to YVN, we offer some alternative hypotheses and additional experiments to further investigate the variation in this behavior in wild *Drosophila* lines.

### Possible epistatic interactions between polymorphisms within the Or22 locus

Though it is possible that there are epistatic interactions between polymorphisms within the Or22 locus that could significantly correlate with preference index, we postponed these analyses until we learned the outcome of the functional experiment, reasoning that if replacement of a non-chimeric Or22 allele with a chimeric one did not result in the expected behavior, these analyses would be irrelevant. As this turned out to be the case, these analyses were never performed. Still, even with the results of our functional experiment, we cannot completely discount the possibility of epistatic interactions between Or22 and another gene or genes (see below).

### Multiple loci may mediate sensitivity to YVN in *D. melanogaster*

At this juncture, it is clear that Or22 alone does not explain the variation in sensitivity to YVN in *D. melanogaster* lines. Given the sequence variation at this locus, it still seems possible that Or22 is in some way involved in attraction to yeast, though at this point we do not understand the role it plays. Given the complexity in chemical signaling between yeast and flies, it seems more likely that the molecular basis for this attraction in flies lies not in one gene but in the combined or epistatic effects of many. This hypothesis would be best addressed by taking advantage of the Raleigh line collection, a set of recently established, iso-female *D. melanogaster* lines [179]. As all of these lines have been sequenced, it would be feasible to find a subset of flies that vary in their response to YVN and perform a genome-wide association study to determine sequence polymorphisms that correlate with this preference.

### Is the yeast attraction phenotype robust enough?

However, before such a study is performed, it should be considered whether the behavioral differences in the chosen panel of fly lines are consistent enough from generation to generation to make this feasible. While the number of replicate behavioral assays run was certainly appropriate for measuring previous phenotypes, [34] it may need to be increased for subsequent experiments in this line of inquiry. It is possible that different testing conditions (e.g. yeast strains) could reveal more robust behavioral differences between these lines. As is the case with many other behavioral assays, this it is certainly not the only set of conditions that could be used.

### MEΔOreR Or22 locus exhibits aberrant amplification behavior

Despite the confirmation of transformants through non-lethal PCR screening during the second round of replacement, PCR genotyping of the final homozygotes gave unexpectedly small amplification products (Figure S5.3). Sequencing these products and those from the screening

steps prior revealed the expected sequence, with the caveat that, in the non-lethal genotyping amplicons, the sequences became heterozygous about half way through. We are hard-pressed to explain why, by all apparent measures, these animals appear to be our desired transformants and yet show this unexpected PCR phenotype. Though we believe that our transformants have the correct genotypes, we thought this was an important caveat that needs to be explored for future work on this project.

Finally, it is possible that the effects of the non-chimeric Or22 allele replacement in our functional experiment are masked by other behavioral deficiencies or phenotypes in the OreR background. OreR is a common lab fly line and its decades-long maintenance in the laboratory under unnatural conditions may have selected for behavioral phenotypes that are ecologically irrelevant or potentially conflicting with the behaviors tested here. If another Or22 allele replacement experiment such as the one described above was repeated, we suggest using a more recently established non-chimeric background line.

### Concluding thoughts

Given the importance of yeast to *D. melanogaster*, the variation in preference towards yeasts grown under different conditions that we observed in fly lines collected from around the world is likely to have ecological relevance. Understanding the basis of this variation can only improve our understanding of the complex relationship between flies and yeast and on a more general scale, how behavior is encoded in the genome. The fly behaviors underlying this relationship are likely to have multiple components, many of which can be controlled under laboratory conditions but unfortunately, never completely. These caveats are what makes studying behavior challenging and why we know so little about the genetics encoding natural behaviors. In the spirit of science, we hope that this data, although subject to the complexities of behavioral phenotypes, will still be informative and productive in generating new questions in the field.

## Acknowledgements

We'd like to thank our high school volunteers Addie Norgaard and Julie Deng for collecting behavior data for the Australian fly lines and for helping to virgin for our reciprocal crosses. We're also grateful to the Begun lab for providing many of our fly lines, to the Carlson lab for providing flies and protocols for the empty neuron experiment and to Dr. Aguadé for sharing her Or22 amplification advice. This research was supported by MBE's Investigator Award from the Howard Hughes Medical Institute as well as CE's and AQ's National Science Foundation Graduate Research Fellowships.

## Materials and Methods

### Fly stocks

All *Drosophila melanogaster* lines used in behavioral panel are shown in Table 5.1. Additional lines used were Attp64 (BestGene) and w;  $\Delta$ halo/CyO; Or22a-GAL4/TM3 (J.R. Carlson, personal communication). All lines were reared on medium from UC Berkeley's Koshland fly kitchen (0.68% agar, 6.68% cornmeal, 2.7% yeast, 1.6% sucrose, 0.75% sodium tartrate tetrahydrate, 5.6 mM CaCl<sub>2</sub>, 8.2% molasses, 0.09% tegosept, 0.77% ethanol, 0.46% propionic

acid) supplemented with activated dry yeast pellets at 25C on a 12:12 photoperiod unless otherwise stated.

**Table 5.1. *D. melanogaster* lines used in behavioral panel.**

<i>D. melanogaster</i> line	Collection site	Or22 genotype	Source <sup>^</sup>
CantonS	Canton, OH, USA	Non-chimeric	Eisen laboratory stock [185]
OreR	Roseburg, Oregon, USA	Non-chimeric	Eisen laboratory stock [185]
Ral437	Raleigh, NC, USA	Chimeric	[179]
Ral324	Raleigh, NC, USA	Non-chimeric	[179]
Ral705	Raleigh, NC, USA	Non-chimeric	[179]
GRAC	Crete, Greece	Non-chimeric	
GR2	Crete, Greece	Chimeric	
GR21	Crete, Greece	Chimeric	
RW1001*	Cupertino, CA, USA	Chimeric	
RW1005*	Cupertino, CA, USA	Chimeric	
RW1008	Cupertino, CA, USA	Non-chimeric	
RW1011	Cupertino, CA, USA	Non-chimeric	
Cellar8.3*	Healdsburg, CA	Chimeric	
ME	Bowdoin, ME, USA	Chimeric	[186,187]
PEN	Media, PA, USA	Chimeric	[186,187]
FL	Homestead, FL, USA	Non-chimeric	[186,187]
MAU9*	Rockhampton, AU	Non-chimeric	[188] <sup>#</sup>
MAU24*	Rockhampton, AU	Chimeric	[188] <sup>#</sup>
MAU31*	Rockhampton, AU	Chimeric	[188] <sup>#</sup>
FP6*	Sydney, AU	Non-chimeric	[188] <sup>#</sup>
FP8*	Sydney, AU	Non-chimeric	[188] <sup>#</sup>
FP16*	Sydney, AU	Chimeric	[188] <sup>#</sup>
CW105*	Mbengwi, Cameroon	Chimeric	[183] <sup>#</sup>
EZ2*	Ziway, Ethiopia	Chimeric	[183] <sup>#</sup>
SP90*	Phalaborwa, South Africa	Non-chimeric	[183] <sup>#</sup>

ZS56*	Sengwa, Zembabwe	Chimeric	[183] <sup>#</sup>
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\*Fly lines that were added to initial set examined by [184].

^blank indicates lines that were collected and established as inbred, isofemale lines for this study

<sup>#</sup> Provided by the Begun laboratory (UC Davis)

### Olfactory behavior assay

The behavior assays in this study were performed as described in [34] Briefly, *Drosophila melanogaster* lines were raised at room temperature (21-23C) on Koshland diet. Newly eclosed flies were pushed onto new food daily and aged at room temperature for at least four days under ambient lighting conditions (i.e. adjacent to a window) before being used in behavior assays.

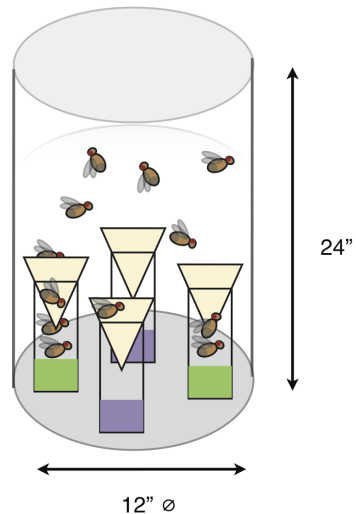
The day prior to the start of the behavior assay, the *Saccharomyces cerevisiae* strain, I14 [165], was plated onto either YVN or YVL media (Table 5.2) and grown at 30C for 22 hours. Two plates for each media type were streaked out per arena.

**Table 5.2. Yeast media recipes used in behavior assays.**

Media*	Recipe
YVN	1.7g YNB without amino acids and ammonium sulfate (BD Difco), 2.0g SC amino acid mixture (MP Biomedicals), 50g dextrose (BD Difco), 20g agar (BD Difco), MilliQ water to 1L
YVL	6.7g YNB without amino acids (BD Difco), 50g dextrose (BD Difco), 20g agar (BD Difco), MilliQ water to 1L

\*One liter batches were made every one to two weeks and poured into 60 x 10mm petri dishes [34].

The following day, grown plates were removed from the incubator, fitted with a custom, 3D printed lid, and secured with Parafilm. Lids were fitted with a 50mL conical centrifuge tube (Falcon) with the end removed and covered in mesh. A funnel was fashioned from 150mm filter paper (Whatman, Grade 1) and a 5mm hole snipped off the tip. This funnel was used to top the centrifuge tube and secured with tape. Two traps for each media type were placed into behavior arenas (*Drosophila* population cages, 24" x 12" clear acrylic cylinders, TAP plastics) fitted with netting (Genesse Scientific) as shown in Figure 5.6. All possible orientations of YVN and YVL plates within were tested to control for environmental effects (e.g. attractivity to light).



**Figure 5.6. Schematic of behavior assay per [34].**

*Saccharomyces cerevisiae*, strain I14 [165], was grown on either YVN or YVL media and used to bait custom made traps. One hundred and twenty adult *Drosophila* (4-10 days old) of mixed sex were allowed to choose between traps over an 18 hour period. Preference was quantified by the number of flies in each trap at the end of the assay.

One hundred and twenty 4-10 day old mixtures of male and female *Drosophila melanogaster* were anesthetized with CO<sub>2</sub> and allowed to recover on Koshland diet for 2 hours before being used in behavior assays. Flies were introduced into behavior arenas at 3pm and allowed survey traps. After 18 hours, traps were removed from the arena and the number of flies in each trap were counted, sexed and recorded. Flies were discarded after counting so flies were only used in behavior assays once. A preference index was calculated from the number of flies in each trap as follows:

For **A** = total number of flies in YVN traps

For **B** = total number of flies in YVL traps

Preference Index =  $(A - B)/(A + B)$

#### Genomic DNA extraction from behavior panel fly lines

For each line, three females were pooled in a single DNA extraction using either the QIAamp DNA Micro (QIAGEN) following the manufacturer's instructions for the isolation of genomic DNA from less than 10 mg tissue or the PureGene Tissue kit (Gentra). Concentration of each DNA sample was quantified using the Qubit High Sensitivity dsDNA kit.

#### Cloning Or22 alleles via TOPO TA

Or22 alleles were cloned by amplification with GoTaq mastermix (Promega) using 240 ng of template gDNA, and 400 nM each o2F and o2R (Table 5.3) in a 50 uL reaction. Reactions were cycled using a specialized thermocycler protocol (M. Aguadé, personal communication): an initial melting step of 94 C for 3 min followed by 35 cycles of 96 C for 10 seconds, 55 C for 10 seconds, 65 C for 4.5 min, then a final polymerase elongation step of 65 C for 7 min. Expected bands were excised from 1% agarose gels after running at 100V and gel purified using the QIAquick Gel Extraction (QIAGEN) kit eluting in 30 uL of buffer EB. Adenosine tails were

added to these fragments in anticipation of TOPO TA (Invitrogen) cloning with 5U Taq polymerase (NEB), 280 uM dNTPs in 1x standard Taq buffer (NEB) for 20 minutes at 72C. A-tailed products were then immediately cloned into TOPO TA 2.1 vector using manufacturer's instructions. Fresh TOPO TA reactions were drop-dialyzed on 0.025 um membrane (Millipore) floated in a 100x15 mm petri dish with sterile DI water (~25 mL) for 15 minutes at RT. Drop-dialyzed TOPO TA reactions were then transformed into DH5alpha *E. coli* via electroporation, rescued immediately with room temperature SOC and outgrown 15 min at 37 C with 180 rpm shaking before plating all cells pre-warmed LB + carbencillin (100 ug/mL) agar plates. Plates were incubated overnight at 37 C. Colonies were picked and dissolved into 5 uL of LB + kanamycin (50 ug/mL) in 96-well plates. One uL of cell suspensions were then used to template 20 uL colony PCR reactions with GoTaq mastermix (Promega) using primers o2F and o2R (800 nM each) with the following thermocycler settings: initial melt at 95C for 5 min followed by 35 rounds of 95 C for 30 seconds, 51 C for 30 seconds and 72 C for 2.5 min, then a final extension step at 72 C for 10 min. Positive hits were those that gave a 2.5 kb bands when run on a 1% agarose gel. Up to five colonies for each fly line were grown overnight in LB + kanamycin (50 ug/mL) and plasmids were extracted via MiniPrep (QIAGEN).

#### Cloning Or22 alleles via pUC19 Gibson assembly

Or22 alleles were cloned by amplification with GoTaq mastermix (Promega) using 240 ng of template gDNA, and 400 nM each o2F-pUC19 and o2R-pUC19 (Table 5.3) in a 50 uL reaction. Reactions were cycled using a specialized thermocycler protocol (Montserrat Aguadé, personal communication): an initial melting step of 94C for 3 min followed by 35 cycles of 96C for 10 seconds, 55C for 10 seconds, 65C for 4.5 min, then a final polymerase elongation step of 65C for 7 min. PUC19 backbone was amplified from pUC19 (Invitrogen) using pUC19-PCR-F1 and pUC19-PCR-R1 (500 nM each, Table 5.3) with Q5 High-Fidelity DNA Polymerase (NEB) with the following conditions: 98C for 30 sec followed by 30 rounds of 98C for 10 sec, 62C for 30 sec then 72 for 1 min, finishing with 72C for 2 min. Expected bands were excised from 1% agarose gels after running at 100V and gel purified using the QIAquick Gel Extraction (QIAGEN) kit eluting in 30 uL of buffer EB. Or22 bands were mixed with pUC19 backbone and assembled with NEBuilder HiFi DNA Assembly (NEB) incubating 1 hour at 50C but otherwise following manufacturer's instructions. Gibson reactions were then dialyzed and transformed into DH5alpha *E. coli*; transformants were screened and plasmid was extracted as with TOPO TA cloning with the difference that only LB + carbencillin (100 ug/mL) agar plates were used for selection.

**Table 5.3. All primers used in present study.**

Primer name	Sequence	Source*
o2F	TAACACCGCCAATGGTCAAC	[181]
o2R	TCTTGCTGTTGACCCATCTC	[181]
o3F	GGGTGGAAGAGTTTTGAA	[181]
o2F-pUC19	TTGTA AAAACGACGGCCAGTGTAACACCGCCAATGGTCAA C	
o2R-pUC19	CTATGACCATGATTACGCCATCTTGCTGTTGACCCATCTC	
pUC19-PCR-F1	CACTGGCCGTCGTTTTACAA	
pUC19-PCR-R1	TGGCGTAATCATGGTCATAG	
o4F	GAGAGAATACAAGGGAAATG	[181]
o4R	CATTTCCCTTGATTCTCTCACA	[181]
Or22_long_3	GATTGATGACGGTAAGTCCTTTT	
Or22b_1	CAACTTTCGTGACATTGTTG	[69]
Or22b_P	TTGAAACTTTTCTGCCAAG	[69]
Or22b_J	CAGGAAGGACGGAAGATGAG	[69]
Or22_long_3-flip	AAAAGGACTTACCGTCATCAATC	
Or22_sl_5	AAACAAAGCCACGGACAAG	
Or22a_E	AACGTCTCCATGGACACGTC	[69]
Or22a_G-flip	GTGCATTCCGGATCATCGAT	[69]
Or22-pWALIUM-F	GGAATTGGGAATTCGCAAGCTGAAATGTAACCTGC	
Or22-pWALIUM-R	GAACTAGTTTGCTCTAGAGTGCGAAAGAGACAACTG	
pWALIUM-F	TCTAGAGCAAACCTAGTTCTG	
pWALIUM-R	TGCGAATTCCCAATTCCC	
pUAST-MCS-F1	AGCGCAGCTGAACAAGCTA	
pUAST-MCS-R1	TGTCCAATTATGTCACACCACA	
5'-out-pCFD4-F2	TATATAGGAAAGATATCCGGGTGAACTTCGAAAGGCAAT GATATTGGGCGTTTTAGAGCTAGAAATAGCAAG	
3'-out-pCFD4-R2	ATTTTAACTTGCTATTTCTAGCTCTAAAACACCATTGATT GATGATGAGCGACGTTAAATTGAAAATAGGTC	

pCFD4-seq	GACACAGCGCGTACGTCCTTCG	[189]
Or22-5'flank-pUC19-F1	TTGTAAAACGACGGCCAGTGGTGCCTACCAATCCATTTG	
GFP-HR-5'-R	CCGCAGGTTACATTTTCAGCTGAACACGCCCAATATCATT GC	
Or22-Ral437-NEB-F1	AATTCATTTCAGCTGAAATGTAACCTGC	
Or22-Ral437-NEB-R1	GTTGACCCATCTCCAATCTCACCCATGC	
GFP HR tubP-F	TTCAGCTGAAATGTAACCTGCGGTGGCCACACTGCGGCC ATCG	
GFP HR tubP-R	CCTCGCCCTTGCTCACCATAACAACAAACTGTCCGC	
GFP-HR-GFP-F	GCGGACAGTTTGTGTTGTATGGTGAGCAAGGGCGAGG	
GFP-HR-GFP-SV40-R	ACCCATCTCCAATCTCACCCAGGTTACTTGTACAGCTCGT CC	
GFP-HR-3'-F2	CCTGGGTGAGATTGGAGATGGGTGCTCATCATCAATCAA TGGTGTGCTAGC	
Or22-3'flank-pUC19-R2	CTATGACCATGATTACGCCAAAAACAAGCCCAGTTGATG GCG	
5'-in-pCFD4-R	ATTTTAACTTGCTATTTCTAGCTCTAAAACCAGGTTACAT TTCAGCTGAACGACGTTAAATTGAAAATAGGTC	
3'-in-pCFD4-F	TATATAGGAAAGATATCCGGGTGAACTTCGACCCATCTC CAATCTCACCCGTTTTAGAGCTAGAAATAGCAAG	
5' PAM F	GCCGCATATTTTTCACGAGT	
GFP-5'PAM-R	GCTGAACTTGTGGCCGTTTA	
Or22-5'PAM-R	TCTTGTCCGTGGCTTTGTTT	
3' PAM R	CGAAGGGAGTGCGATGTAGT	
SV40-3'PAM-F	CCACACCTCCCCCTGAAC	
Or22-3'PAM-F	GTGGTCTGGGGTAGGAGACA	
CNE-Or22-R2	GCAGCTGACTGAAACCACAA	
o1F	CTGAAGTCGGGTTGTCTTGGTATTT	[181]
Or22-b2	CACTATTGTAACCACAGTAAAG	[69]
Or22-CNE-long-R2	CTTGCGGAAAGAACGAAAAG	

\*If no source is specified the oligo was designed in this study.



### Sequencing and assembling Or22 loci for each fly line in behavioral panel

Chimeric Or22 alleles were Sanger sequenced with six primers (o2F o2R, Or22\_long\_3, Or22b\_1, Or22b\_P, Or22b\_J) and non-chimeric Or22 alleles were Sanger sequenced with 10 primers (o2F, o2R, o3F, o4F, o4R, Or22\_long\_3-flip, Or22\_sl\_5, Or22b\_1, Or22a\_E, Or22a\_G-flip) using third party services (ELIM, Barker Hall Sequencing facility) (Table 5.3). Loci were assembled from these sequences using SeqMan Pro (DNASTAR Lasergene v.10) after lowering signal threshold to 2 and manually checking and resolving any disagreements between reads. A consensus for each line was assembled by aligning at least three individual clones for a given fly line in SeqMan Pro.

### Polymorphism analysis for Or22 sequences

The consensus sequence for each line were aligned to the Or22 genomic reference using Geneious (version 5.1.7); chimeric sequences were split at the first intron in order to achieve alignment of the entire locus. Indels and SNPs were called manually for each consensus compared to the consensus reference of all sequenced Or22 loci to generate a presence/absence matrix of all observed polymorphisms in our set of sequenced Or22 loci. T-tests comparing the set of preference indices or variances (data not shown for latter) for all lines possessing versus lacking a given allele were performed using the stats library in Python. Data were plotted with Prism 7 (GraphPad).

### Empty neuron ( $\Delta$ halo) experiment

UAS-Or22Ral437 was generated by cloning the open reading frame of Ral437 Or22 downstream of the 5x UAS in pWALIUM10 (M.R. Stadler, personal communication). To do this, Or22 was amplified from Ral437 Or22 in TOPO TA vector (Invitrogen) using primers Or22-pWALIUM-F and Or22-pWALIUM-R (500 nM each, Table 5.3) and pWALIUM backbone was amplified from pWALIUM using primers pWALIUM-PCR-F and pWALIUM-PCR-R (500 nM each, Table 5.3) with Q5 High-Fidelity DNA Polymerase (NEB) with the following conditions: 98C for 30 sec followed by 30 rounds of 98C for 10 sec, 62C for 30 sec then 72 for 45 sec (Or22) or 3:15 min (pWALIUM), finishing with 72C for 2 min. The resultant products were gel-purified, Gibson assembled (NEBuilder HiFi DNA Assembly Master Mix, NEB) transformed into chemically-competent DH5alpha *E. coli* (NEB #C2987) and selected for on LB + carbencillin (100 ug/mL) agar plates. Plasmid was extracted from 2-4 transformant clones (QIAGEN miniprep) and sequenced with pUAST-MCS-F1 and pUAST-MCS-R1 (Table 5.3) to confirm proper insertion had taken place. Plasmid was extracted from a verified clone (QIAGEN midiprep) and quantified using the Qubit HS dsDNA kit (Thermo Fisher Scientific). This plasmid was injected into AttP64 flies in the presence of PhiC31 recombinase and progeny were backcrossed, screened and balanced with TM3,Sb (BestGene). These w; +; UAS-Ral437Or22/TM3,Sb flies were crossed according the scheme from [58]. First, UAS-Ral437Or22/TM3,Sb were crossed to w;  $\Delta$ halo/Cyo; Or22a-GAL4/TM3 to generate w;  $\Delta$ halo/+; UAS-Ral437Or22/TM3 and w; CyO/+; UAS-Ral437Or22/TM3 progeny. These progeny were crossed to generate w;  $\Delta$ halo/Cyo; UAS-Ral437Or22/TM3 which were crossed back to w;  $\Delta$ halo/Cyo; Or22a-GAL4/TM3 to generate w;  $\Delta$ halo/ $\Delta$ halo; UAS-Ral437Or22/Or22a-GAL4 flies.

### CRISPR-Cas9 Or22 allele replacement

First and second round CRISPR targets were selected using <http://tools.flycrispr.molbio.wisc.edu/targetFinder/> (Table 5.4). Primers 5'-out-pCFD4-F2 and 3'-

out-pCFD4-R2 or 5'-in-CFD-R and 3'-in-CFD-F were used to clone both sgRNAs into pCFD4 for the first or second round of CRISPR editing, respectively, per <http://www.crisprflydesign.org/> [189]. Constructs were verified by Sanger sequencing with pCFD4-seq [189]. To generate a homologous recombination template plasmid for the first round of replacement, five fragments (pUC19 backbone (pUC19-PCR-F1 and pUC19-PCR-R1, one kilobase 5' upstream of OreR Or22 locus (Or22-5'flank-pUC19-F1 and GFP-HR-5'-R), beta-tubulin promoter from OreR (GFP HR tubP-F and GFP HR tubP -R), GFP with SV40 3' UTR from pGREEN-Pelican (GFP-HR-GFP-F and GFP-HR-GFP-SV40-R) and one kilobase 3' downstream of OreR Or22 locus (GFP-HR-3'-F2 and Or22-3'flank-pUC19-R2) were amplified with Q5 High-Fidelity DNA Polymerase (NEB) using 500 nM each forward and reverse primer (Table 5.3) with the following conditions: 98C for 30 sec followed by 30 rounds of 98C for 10 sec, 62C for 30 sec then 72 for 2 min, finishing with 72C for 2 min. Fragments were assembled using NEBuilder HiFi DNA Assembly Master Mix (NEB), transformed into DH5alpha electrocompetent cells and plated on LB + carbencillin (100 ug/mL). Plasmid was isolated from 2-4 transformants and sequenced with primers M13F, M13R, o2F and CNE-Or22-R2 (ELIM) to confirm assembly (Table 5.3).

**Table 5.4. CRISPR targets for Or22 allelic replacement.**

	5' target*	3' target*
<b>Round I (out)</b>	GAAAGGCAATGATATTGGGCGGG	GCTCATCATCAATCAATGGTGGG
<b>Round II (in)</b>	TTCAGCTGAAATGTAACCTGCGG	CCTGGGTGAGATTGGAGATGGGT

\*PAM sites are underlined

Plasmid from a sequence-verified clone was prepared (QIAGEN Midiprep) and quantified using the Qubit HS dsDNA kit (Thermo Fisher Scientific). OreR flies were co-injected with pHsp70-Cas9, pCFD4 containing the two synthetic guides for round I editing (outer CRISPR targets) and the GFP homologous recombination donor (Rainbow Transgenics). Injected animals were individually backcrossed to OreR; progeny were screened using a compound fluorescence microscope and by extracting DNA from pools of 50 animals from each cross (VDRC stock center protocol "Good quality *Drosophila* genomic DNA extraction") then amplifying the 5' and 3' PAMs using a cocktail of primers 5' PAM F, GFP-5'PAM-R, Or22-5'PAM-R or primers 3' PAM R, SV40-3'PAM-F, Or22-3'PAM-F, respectively (Table 5.3), at a total final concentration of 1 uM for each forward and reverse primer(s) with GoTaq 2x mastermix (Promega) with the following thermocycling conditions: 95C for 5 min followed by 35 iterations of 95C for 30 seconds, 61C for 30 seconds then 72C for 30 sec then 72C for an additional 10 minutes. Sibling virgins from "hit" founder crosses were screened by non-lethal genotyping using each 5' PAM (5' PAM F, GFP-5'PAM-R, Or22-5'PAM-R) and 3' PAM (3' PAM R, SV40-3'PAM-F, Or22-3'PAM-F) primer cocktails per [190]. Heterozygotes were crossed and progeny screened as above to identify homozygotes. Homozygotes were crossed, progeny were screened as above and genomic DNA from two batches of three females each was extracted with the QIAamp Micro kit (QIAGEN) then PCR genotyped and Sanger sequenced using three sets of primers to confirm homogeneity: 5' PAM (5' PAM F, GFP-5'PAM-R, Or22-5'PAM-R), 3' PAM (3' PAM R, SV40-3'PAM-F, Or22-3'PAM-F) and whole locus (5' PAM F, 5'PAM-R) PAM primer sets and 5'PAM/3'PAM. Sibling flies were propagated as GFPΔOreR.

An analogous process was used for the second round of editing; this time pCFD4 contained the synthetic guides for round II (inner) CRISPR targets and the donor plasmid contained the Or22 allele from ME sandwiched between 5' and 3' Or22 flanking regions (assembled identically to the first round homologous donor template instead using four fragments (pUC19 backbone (pUC19-PCR-F1 and pUC19-PCR-R1, one kilobase 5' upstream of OreR Or22 locus (Or22-5'flank-pUC19-F1 and GFP-HR-5'-R), ME Or22 locus (Or22-Ral437-NEB-F1 and Or22-Ral437-NEB-R1) and one kilobase 3' downstream of OreR Or22 locus (GFP-HR-3'-F2 and Or22-3'flank-pUC19-R2) (Table 5.3)). These constructs and pHsp70-Cas9 were co-injected into GFP $\Delta$ OreR (Rainbow Transgenics). Injected animals were individually back-crossed to GFP $\Delta$ OreR then screened and homozygosed as above to establish line ME $\Delta$ OreR.

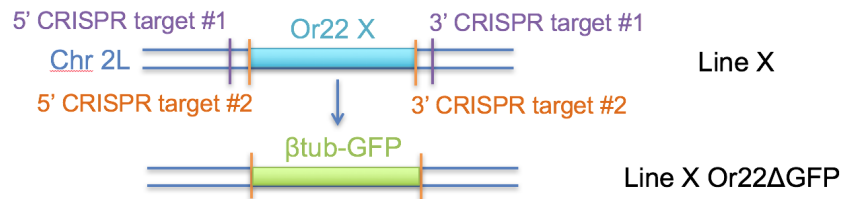
#### Chimeric and non-chimeric crosses

Fly lines were raised at 25C and virgins and males were collected twice a day. After five days, virgins were confirmed. Five females of a single fly line were crossed to five males of another line. Three replicates of each cross were set up and crosses were performed in both directions. As a control, virgins and males of parental fly lines were collected and crossed in parallel. F1 progeny were collected and aged for behavior assay.

## Supporting Information

### CRISPR round I

Replace Or22 in line X with dominant visible marker ( $\beta$ tub-GFP)



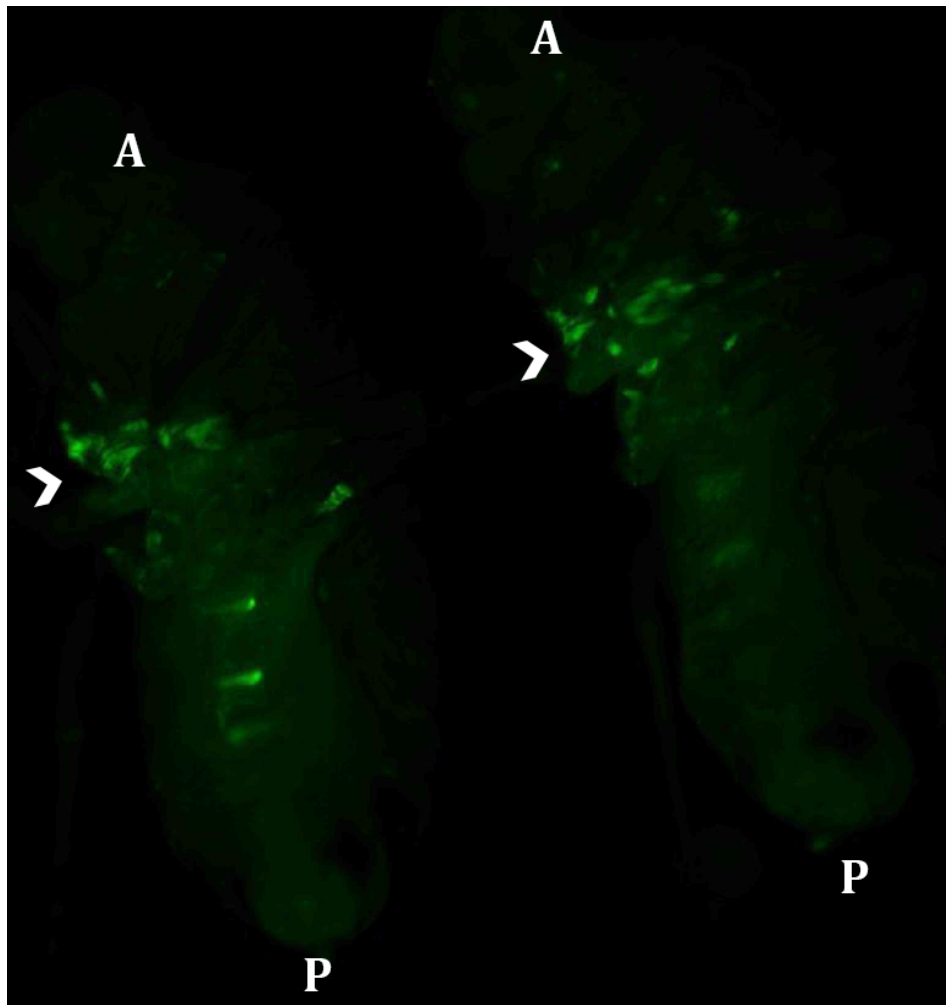
### CRISPR round II

Replace marker with Or22 sequence from line Y



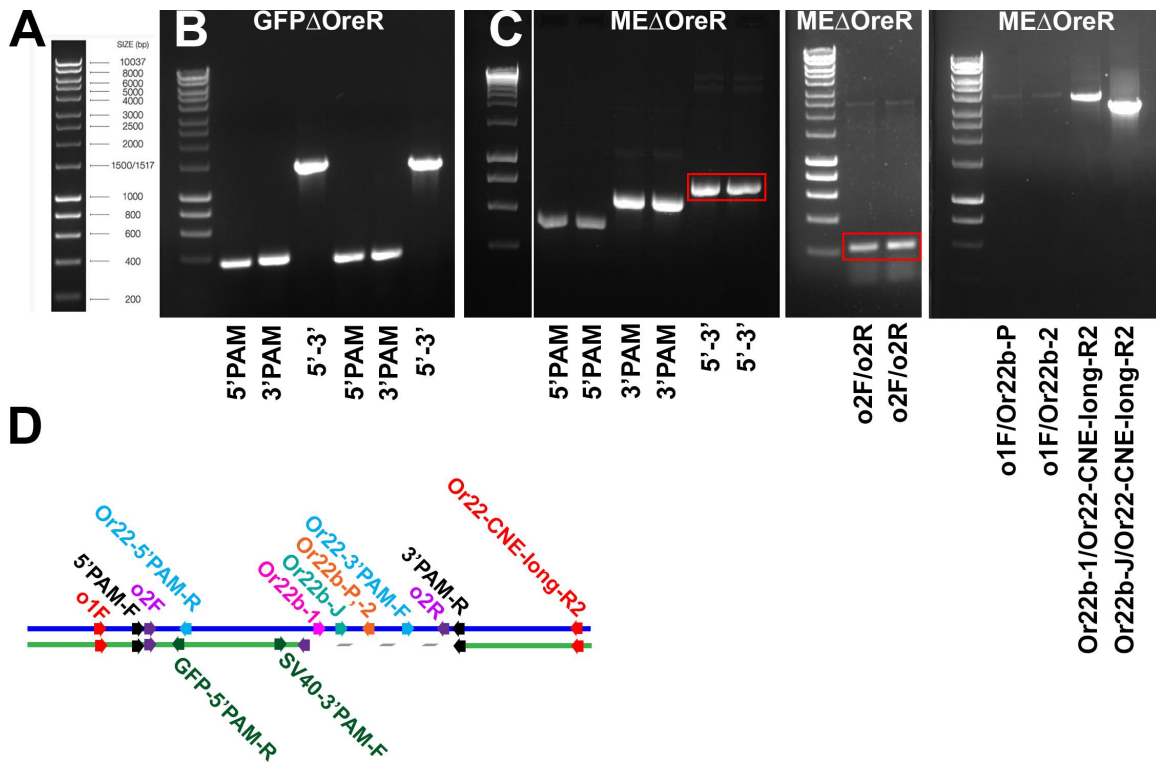
### Figure S5.1. Two step Or22 allelic replacement scheme using CRISPR-Cas9 genome editing.

In the first round of CRISPR editing, embryos of line X are injected with a plasmid expressing both 5' and 3' CRISPR #1 target synthetic guide RNAs (sgRNAs), a plasmid expressing Cas9 and a plasmid bearing a dominant visible marker ( $\beta$ -tubulin promoter GFP cassette) flanked by one kb sequences lying upstream and downstream, respectively, of the cut sites to template homologous recombination. The homologous template has mutated PAM sites for the #1 guides, so the resultant recombinant product will not have usable PAM sites. In the second round of CRISPR editing, embryos of LineX Or22 $\Delta$ GFP are injected with a plasmid expressing both 5' and 3' CRISPR #2 target synthetic guide RNAs (sgRNAs), a plasmid expressing Cas9 and a plasmid bearing an alternative allele of Or22 flanked by one kb sequences lying upstream and downstream, respectively, of the cut sites to template homologous recombination. The homologous template has mutated PAM sites for the #1 guides, so the resultant recombinant product will not have usable PAM sites.



**Figure S5.2. GFP expression pattern in two B-tubulin GFP cassette heterozygotes (+;+/Or22ΔGFP;+) as observed from ventral side.**

White arrowhead indicates ventral thorax where GFP expression can be observed under legs. GFP expression is also observed in ventral abdomen as three short, parallel stripes. A = anterior; P = posterior.



**Figure S5.3. Aberrant amplification behavior of ME $\Delta$ OreR Or22 locus.**

A) Hyperladder 1 kb (Bioline) used for B) and C). B) PCR behavior of GFP $\Delta$ OreR with 5' PAM cocktail (5'PAM-F, Or22-5'PAM'-R and GFP-5'PAM-R), 3' PAM cocktail (3'PAM-R, Or22-3'PAM-F and SV40-3'PAM-F) or 5'PAM-F and 3'PAM-R (Table 5.3). First three lanes after ladder are one DNA prep from three GFP $\Delta$ OreR females; second three lanes are a second DNA prep from another three GFP $\Delta$ OreR females. C) PCR behavior of ME $\Delta$ OreR as in B) as well as primer pairs o2F/o2R, o1F/Or22b-P, o1F/Or22b-2, Or22b-1/Or22-CNE-long-R2 and Or22b-J/Or22-CNE-long-R2 (Table 5.3.3). For two leftmost gels, adjacent lanes with same primer pairs are templated by two different DNA preps, each from three ME $\Delta$ OreR females. In rightmost gel, only one of these DNA preps is used to template all reactions. Red boxed indicate bands of unexpected sizes from these reactions. D) Relative positions and orientations of primers in ME (blue) and GFP-SV40 (green) constructs. Note that primers marked in red are outside of the 5' and 3' homology flanks used in the donor plasmid. Diagram is not precisely to scale.

# CHAPTER 6: Concluding thoughts and future directions

*“It appears that we simultaneously know both a great deal and not very much at all about mutualism.”*

- Bronstein, 1994 [191]

## The emerging narrative of the fly-yeast mutualism

The partnership between flies and yeast has been appreciated by fly biologists for decades [18,22] and reviewed in [19], but relative to *Drosophila* gut bacteria interactions, the fly-yeast mutualism has been underinvestigated [87]. While overall impact of gut-associated bacteria in *Drosophila* remains open to debate [177,192], the importance of yeast in the *Drosophila* lifecycle cannot be overlooked. *Drosophila* are clear vectors of yeast [89] and dietary yeast is essential for proper *Drosophila* larval development [24].

Much of the contemporary work on the fly-yeast mutualism is predicated on the simple, yet definitive, observation that yeast volatiles, not fruit volatiles, mediate *Drosophila* attraction [28]. Since then, the body of literature examining the fly-yeast mutualism, including this dissertation, has grown in both volume and detail. Many yeasts can serve as adequate mutualistic partners to *Drosophila*, provided that they produce attractants that facilitate their initial interaction (Chapter 2 and 3). The ability to attract *Drosophila* is a conserved trait in yeasts and the metabolic pathways producing volatile attractants are genetically regulated [34,39,111]. Yeast and the fermentation metabolites produced by yeast greatly enhance the attractiveness of bacterial co-cultures, demonstrating that *Drosophila* behavior is strongly influenced by yeast-specific volatiles [63].

*Drosophila* odorant receptors are fine-tuned to detect these compounds and slight changes in their concentrations. Final behavioral outputs are modulated by the overall context in which volatiles are presented (Chapter 3), including the volatiles produced as a result of interactions between yeasts and their microbial neighbors. Heritable changes in *Drosophila* behaviors toward yeast volatiles are likely driven by modifications in odorant receptor sensitivity. There are clear examples of this in *Drosophila* species with very specific host plant specializations. However, even *Drosophila melanogaster* and *Drosophila suzukii*, two *Drosophila* species utilizing similar fruit substrates at different ripening stages, exhibit different olfactory neuron responses and behavioral outputs in response to the volatile profiles of different yeast species, suggesting coevolution between each *Drosophila* species and the yeasts residing on substrates at particular times [62]. Given the species diversity, habitat range, and host of genetic tools available in both *Drosophila* and yeasts, the natural fly-yeast mutualism continues to be a useful model for studying mutualistic interactions.

## Plasticity in mutualistic interactions

The flexibility on both sides of the fly-yeast mutualism is remarkable despite how important it is for the fitness of both partners. A diversity of yeast species share the ability to produce volatile compounds that are attractive to *Drosophila* (Chapter 2 and 3). Consequently, a large majority of *Drosophila* odorant receptors are dedicated to detecting these volatiles [59]. While their mutualism is nonspecific, both flies and yeast have evolved broadly conserved mechanisms and fine-tuned behaviors for ensuring a close association with each other.

Extreme specificity is not a rule of mutualisms. In fact, most mutualisms, such as the *Drosophila*-yeast mutualism detailed in this work, are nonspecific [8,193]. Theoretically, natural selection can favor and support guild-guild mutualisms (interactions where several species can act as a partner) because less specific interactions increase risk spreading [8,109]. Mutualisms among free living species will tend to coevolve towards multi-species webs and, if successful, are repeated across diverse ecosystems [110]. Clearly many yeast species have converged upon traits that attract *Drosophila*. Reciprocally, *Drosophila* can use almost all of these yeast species as nutrition sources. These, loose, but evolutionarily conserved, mutualisms are the ones that can provide the most information about the parameters constraining these types of interactions.

## Mutualisms are evolutionarily dynamic

Mutualisms exist on an evolutionary continuum of specialization because all mutualisms, even specialized ones, are inherently unstable, as each partner is constantly evolving to benefit more from the interaction [8,110]. This occurs in the fly-yeast mutualism, as I observed yeast species that are very attractive to *Drosophila* but do not support larval development as well as other, less attractive, yeast species.

Long-term persistence of a mutualism requires adaptability to transient fluctuations over time and space [8]. Mutualistic flexibility is necessary for both flies and yeast, which are subject to seasonal fluctuations in temperature and feeding substrates in their habitats. Local hotspots for coevolution, such as the winery microhabitats in Chapter 2 and the kipukas in Chapter 4, can also create opportunities for certain fly populations to evolve differing degrees of specificity to particular yeast species [110]. These geographical mosaics also allow local populations to maintain polymorphisms that would otherwise be driven to fixation [110]. Indeed, extensive genotyping of the Or22 locus in Chapter 5 revealed that fly populations inhabiting winery microhabitats harbor both Or22 alleles while other clinal populations are fixed for one allele or the other. Together, these principles explain why flies and yeast exhibit specific associations in winery microhabitats but are able to utilize many species as partners. As we continue to characterize the general principles governing mutualistic interactions, it is imperative that they are viewed as evolutionarily dynamic.



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