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Evolution and Natural Selection of Olfactory Receptor Genes in Hawaiian *Drosophila*

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EVOLUTION AND NATURAL SELECTION OF OLFACTORY RECEPTOR GENES IN HAWAIIAN *DROSOPHILA*

By

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ABSTRACT

The olfactory system is a powerful tool for sensing countless odorants. In *Drosophila*, the olfactory system is critical for detecting food, finding mates, laying eggs, avoiding predators, and adapting to new environments. Understanding the olfactory system in *Drosophila* will advance our knowledge of sensory biology in various insects and vertebrates, including humans. Drosophila has been a valuable model for biology since the early 1900s, and the *Drosophila* melanogaster olfactory system is well-studied. The Hawaiian *Drosophila* represent approximately 1/3 of the world's *Drosophila*, however, there is limited research on Hawaiian *Drosophila* olfactory genes. We conducted a comparative analysis of olfactory receptor (OR) genes in four Hawaiian *Drosophila* and five non-Hawaiian *Drosophila* species. The four Hawaiian *Drosophila* (*Drosophila silvestris*, *Drosophila basisetae*, *Drosophila grimshawi*, and *Drosophila sproati*) were sequenced, assembled, and annotated, while five non-Hawaiian *Drosophila* species (*Drosophila melanogaster*, *Drosophila simulans*, *Drosophila yakuba*, *Drosophila virilis*, and *Drosophila mojavensis*) served as outgroup species. Notably, about 40 out of the 60 OR genes in *Drosophila melanogaster* were found to be conserved across most Hawaiian *Drosophila* species. Several genes experienced a high number of positive selection sites, including OR2a, Or46a, OR67a, OR69a, OR71a, OR85f, OR88, and OR92a, which are vital for various functions such as reproduction, oviposition, and detecting food sources and threats. No extreme negative selection was observed among the detected OR genes. There were some differences in OR gene expression between females and males and among different Hawaiian *Drosophila* species. The changes in OR gene sequences between Hawaiian *Drosophila* species and differential gene expression indicate that the olfactory system has evolved

differences in chemosensory responses between species and sexes. Our study enhances the comprehensive knowledge of sensory biology and the evolutionary patterns of olfactory receptors, providing valuable insights into the distinctive adaptations of Hawaiian *Drosophila*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Olfaction and olfactory receptors

Detection of smells is one of the most important senses for ensuring survival in many organisms. However, 5% of the human population is affected by anosmia (a disease that causes loss of smell) and 25% of elderly population (above 50) has presbyosmia which is age-related decline in sense of smell (Huttenbrink et al., 2013). These diseases not only impact your sense of smell but can also be indicative of early signs of neurodegenerative diseases such as Parkinson's, Alzheimer's disease, and depression. It was reported that the loss of smell during COVID-19 impacts about 60.5 percent of infected patients (Mitchell et al., 2023). Therefore, a better understanding of olfactory genes is important.

In the course of daily life, olfaction (sense of smell) is crucial in most animals' survival because it helps them to detect food, find mates, and be aware of dangers such as fire. It is also involved in predator-spotting, and mother and child recognition (Zarzo, 2007). In humans, olfaction can influence our emotions as well as social interactions (Sharma et al., 2019). Moreover, loss of smell is considered an early marker for neurodegenerative conditions such as Parkinson and Alzheimer's diseases (Godoy et al., 2015). And most recently, olfactory dysfunction affected "the hundreds of millions of cases seen in COVID-19" (Butowt et al., 2023). Similarly, the olfactory system in *Drosophila* is of vital importance for distinguishing volatile odorants around them and identifying food sources, mates, and appropriate sites for laying eggs (Vosshall, 2000). They detect these volatile chemicals using odorant-gated ion channels which include a co-receptor (ORCO) subunit and an olfactory receptor (OR) subunit (Del Marmol et al. 2021). ORCO is highly conserved among species and acts as an ion channel. It does not respond to odorants without the presence of ORs (Kleinheinz et al., 2023).

Drosophila melanogaster uses many different olfactory receptors (ORs) to detect volatile chemical substances (Khallaf et al. 2020).

Olfactory receptors (ORs), novel seven-transmembrane domain proteins, play key roles in the sense of smell in *Drosophila* (Smart, 2008). These receptors were identified in *Drosophila* by several groups of researchers in 1999 (Clyne et al. 1999b; Gao and Chess 1999; (Vosshall et al., 1999). Benton noted that a better understanding of these receptors could help with characterization of the olfactory system, including structural and physiological features in *Drosophila* as well as other animals (Benton, 2022). The study identified the process by which chemical signals from the odorants in the environments are converted into electrical signals in the antenna by ORs. Odorant-binding proteins (OBP) act as molecular carriers to deliver odorants to ORs that are induced in olfactory sensory neurons (OSNs) (Vieira et al., 2007). After that, the olfactory information is sent to the antennal lobes which is the first olfactory processing center (Depetris-Chauvin et al. 2005). The OSNs are housed in a sensilla of antennae and maxillary palps, which are main olfactory appendages in *Drosophila* (Auer et al. 2022). (Charro & Alcorta, 1994) proposed that the antennae oversee 90% of the olfactory information, and the maxillary palps mediate the rest. While there are four major types of sensilla, including basiconic, trichoid, intermediate and coeloconic (Lin & Potter, 2015), ORs are housed in basiconic and trichoid sensilla (Gomez-Diaz et al., 2018). Basiconic and trichoid sensilla are responsible for food odors and pheromones, respectively (Halem and Carson 2006; (Kurtovic et al., 2007). In addition, without a chemosensory system, specifically olfactory genes,*Drosophila nasuta* failed to have any successful copulations which directly affects adaptation and speciation (Chowdanayaka, 2023).

In *Drosophila melanogaster*, there are 60 genes and several pseudogenes in the OR protein family. While most of them are spread throughout the genome, some of them formed in groups (Robertson et al., 2003). In the OR protein family, 45 of them are induced in adults and 25 are in larval stage. The total of these genes is more than 60 because some ORs are induced in both stages while some are only present in one or the other (Couto et al. 2005). Orco, one of the OR genes, is co-induced with every member of the gene family. In the heteromeric complex, without Orco, ORs cannot function as normal and will be impaired because Orco enhances ORs flexibility and compensates for OR diversity (Butterwick et al., 2018).

Hawaiian *Drosophila*

For over a century, *Drosophila* has been a model organism because of its low cost, homology to human genetics as well as rapid generation time (Tolwinski, 2017); (Ugur, 2016). Specifically, *Drosophila* is an ideal model to study olfaction because its olfactory system is simpler compared to humans and olfactory function can be analyzed in vivo by measuring behavioral responses (Stocker, 1994);(Carlson, 1996). Moreover, it was proposed that olfaction in *Drosophila melanogaster* offers an efficient model to study sensory coding in order to give a better understanding of vertebrate and other insects' brains and sensory systems (Benton 2022).

Since the 1960s, Hawaiian *Drosophila*, in particular, has been a model system for evolutionary and ecological studies because of its diversity. In addition, it was proposed that Hawaiian *Drosophila* is an exceptional example of adaptive evolution because of its ability to invade new habitats. They came from an ancestral species that colonized Hawaii approximately 25 million years ago (Kaneshiro & Boake, 1987). They are also the largest and oldest lineage in the Hawaiian archipelago. There are approximately 1,000 species of Hawaiian Drosophilidae (O'Grady, 2018). They are saprophytic in plant species including fermenting leaves, fruits, and

bark (Carson & Kaneshiro, 1976). They are also known for their unique courtship displays that play an important role in sexual selection and diversity (Kaneshiro and Boake 1987).

Hawaiian *Drosophilids* consists of two main genera: *Scaptomyza* and *Drosophila,* which is predominant. They share the same ancestral species that colonized Hawaii about 25 million years ago (O'Connor et al., 2014). Hawaiian *Drosophila* are divided into five groups: picture-winged group, antopocerus-modified tarsus-ciliated tarsus (AMC) clade, modified mouthparts group, and the haleakalae group (Kambysellis et al., 1995; (Magnacca & Price, 2015). The picture-wing group has 120 known species (Magnacca &Price, 2012; Magnacca & Price 2015), most of them have unique pigmentation on their wings (Edwards et al. 2007) and are bark breeders (Montgomery 1975; (Magnacca, 2008).

Taxa in this study

In this study, four Hawaiian picture-wing *Drosophila* were used: *Drosophila grimshawi, Drosophila silvestris, Drosophila basisetae* and *Drosophila sproati.* I also included *Drosophila melanogaster, Drosophila simulans, Drosophila yakuba, Drosophila virilis* and *Drosophila majavensis* as outgroup species.

Drosophila grimshawi is a picture-winged species that occupies a majority of Hawaiian Islands including Kauai, Oahu, Maui, Lanai and Molokai. *Drosophila grimshawi* is a lek-forming Hawaiian *Drosophila* (Carson, 1970). In order to attract females, males produce chemical substances called pheromones to mark their presence on a surface in order to attract females (Spieth, 1986). Droney and Hock (1998) suggested that males that smeared their pheromone often had a higher mating success rate.

Drosophila silvestris is a part of the *planitibia* subgroup of the Hawaiian picture-winged species (Boake, 1995). They are found only on the Big Island (Templeton, 1977) and are more

tolerant to cold temperatures than heat stress (Uy, 2015). They are known for their unique dancing courtship displays, such as moving their wings up and down laterally. It was also proposed that females that slashed at the attracting males had a higher successful courtship rate (Boake and Hoikkala 1995). Moreover, *Drosophila silvestris* and *Drosophila heteroneura* are sympatric species that can produce hybrids in an appropriate lab setting (Val, 1977).

Drosophila basisetae belongs to the glabriapex subgroup of the Hawaiian picture-winged species (Edward et al. 2007; Magnacca & Price 2015). Even though they were discovered for a long time, little is known about this species. On the other hand, *Drosophila sproati* is a more common Hawaiian picture-winged species (Eldon et al. 2019). They are in a grimshawi group, occupy Hawaiian Island and use Araliaceae as a host plant. Similar to *Drosophila grimshawi*, they form leks for mating purposes (Magnacca, & Price 2015). Furthermore, their tolerance for heat stress is better than it is for cold environments (Uy et al. 2015).

Drosophila simulans and *Drosophila yakuba* are closely related to *Drosophila melanogaster*. All of them belong to the melanogaster subgroup (Chyb and Gompel 2013). Along with *Drosophila grimshawi*, *Drosophila simulans* and *Drosophila yakuba* were utilized in the famous Drosophila 12 Genomes Consortium study. Gegun and his colleagues (Begun et al., 2007) suggested that these three species share the same euchromatic DNA majorly. While *Drosophila simulans* is from South-West Africa (Lachaise, 2004), *Drosophila yakuba* is common in sub-Saharan Africa and has a symbiotic relationship with humans (Lachaise, 1988).

The two outgroup species that are phylogenetically related to Hawaiian *Drosophila* are *Drosophila virilis* and *Drosophila mojavensis*. These two species are known as *virilis–repleta* radiation. *Drosophila virili,* which is from the Holarctic region, breeds on sap flux and other tree decaying parts (Throckmorton, 1982). *Drosophila mojavensis* is a cactophilic species and found

in North America's deserts (Heed, 1978). Normally, most male flies move their wings to make songs during courtship and female flies stay silent and decide on a mate. However, both *Drosophila virili's* males and females perform a courtship duet by vibrating their wings (LaRue et al., 2015). On the other hand, *Drosophila mojavensis* females choose their mates by the smells produced by males. Interestingly, Khallaf and his colleagues (2020) showed that while OR65a enhances sexual receptivity in *Drosophila mojavensis* females, it makes females less attract to cVA which is a male-specific pheromone in *Drosophila melanogaster* (Sengupta, 2014).

Lek mating behavior between males and females

Hawaiian *Drosophila* is one of a few species that have lekking mating behavior. This happens when males gather to engage in competitive courtship rituals to attract females (Dossey et al. 2016). There are differences in the mating behavior between males and females. Males choose a lek site, gather around, and compete with each other while females visit the site to assess male courtship displays (Rathore, 2023). Males also release sex pheromones to attract females. Moreover, males do not provide parental care, and females find host plants for laying eggs and larval development (Shelly, 2018). These differences might contribute to the differences in OR gene expressions between males and females in Hawaiian Drosophila.

Adaptation in olfactory system

Animals adapt to new environments by modifying their behaviors, developing specialized organs and new genetic variation. Since many Hawaiian *Drosophila* live in highly humid and diverse habitats (average of 70% humidity), they have developed several methods to adapt such as unique courtship rituals and colorful wing patterns. Humidity also affects olfaction in animals. For example, the shape of the sensilla, which is an important part of the olfactory system in

Drosophila and other insects, is changed by the humidity level (Li et al. 2022). In addition, it was indicated that species that live in different environments will have different distributions of olfactory receptors which allow them to have optimal adaptation to their living environments (Tesileanu et al., 2019). Another study showed that high humidity also improves olfactory sensitivity in humans (Kuehn et al., 2008). A group of neuroscientists from Northwestern university also discovered a sensory map that Drosophila use to navigate humidity and temperature in their brains (Frank et al. 2017). Therefore, it is reasonable to predict that Hawaiian *Drosophila*'s olfactory system changes to adapt to their living environment.

HYPOTHESES AND OBJECTIVES

1) **Gene annotation and extraction**

   I hypothesize that a majority of OR genes in *Drosophila melanogaster* will be present in and homologous to the OR genes in Hawaiian *Drosophila*. Additionally, I anticipate that Hawaiian *Drosophila* will share OR genes that are more similar to the OR genes in other closely related species such as *Drosophila virilis* and *Drosophila mojavensis*.

2) **Natural selection of olfactory receptors genes in the available Hawaiian *Drosophila* compared with to the non-Hawaiian *Drosophila* species.**

   I hypothesize that certain olfactory genes, potentially crucial for adaptation, food finding, and sexual selection in Hawaiian *Drosophila*, will exhibit positive selection that cause changes in the amino acid sequence of the proteins produced by these genes. This positive selection may be due to the changes in breeding sites on hostplants and alterations in pheromones used in mating.

3) **Gene expression of olfactory receptor genes in some picture-winged Hawaiian *Drosophila*.**

   I hypothesize that as lekking species, there will be differences in olfactory gene expression between females and males due to their distinct mating behaviors. Males are tasked with selecting lek sites, releasing sex pheromones, and engaging in competitive courtship to attract females. In contrast, females are likely to rely on olfaction to find courting male lek areas, choose mates, and detect suitable host plants for choosing oviposition sites and laying eggs.

METHODS AND MATERIALS

Specimen collection

Drosophila picture-wing flies native to Hawaii were captured using conventional sponge baits. These baits were enriched with fermented banana paste and sprayed with fermented mushroom solution before being suspended at a height of 1-2 meters above the ground. The specific collection locations for each species are listed in Table 1. Specimens were collected from the sponges with large plastic vials and transferred to glass vials and transported to University of Hawaii, Hilo for identification and preserved in 90% EtOH stored in -20 °C. The specimens were then shipped to the University of Nevada, Las Vegas for longer term storage at -80 °C. For genome sequencing the specimens were shipped to Stanford University to the laboratory of Dimtri Petrov and the sequencing and genome assembly conducted by Benard Kim. The nanopore sequencing was done with males of each species to allow for sequencing of the Y chromosome. All specimens were collected under permit number FHM16-393.

Other Hawaiian and non-Hawaiian *Drosophila* species

In addition to the three Hawaiian Drosophila specimens collected in-house, the analysis incorporated *Drosophila grimshawi*. I also utilized *Drosophila mojavensis*, *Drosophila virilis* as well as *Drosophila melanogaster* and its closely related species *Drosophila simulans* and *Drosophila yakuba* as an outgroup in the analysis. The genome annotations of these species are publicly available on NCBI (Table 2).

Laboratory methods

DNA sequencing

DNA data from all Hawaiian *Drosophila* species were obtained from Dr. Bernard Kim (Stanford University). Long reads were generated from MinION, a portable and pocket-sized

Oxford Nanopore sequencer. Using ONT 1D ligation kit approach, a high molecular weight genomic DNA (gDNA) extraction was performed on each sample. The sequencing library was prepared with ONT Ligation Sequencing Kit (SQK-LSK109) protocol. For detailed protocol, please see **dx.doi.org/10.17504/protocols.io.bdfqi3mw**. Short reads were sequenced by Illumina NovaSeq to perform 2x150bp whole-genome sequencing. The genomes are high quality and complete, with an average contig N50 of 10.5 Mb and greater than 97% BUSCO completeness (Kim et al., 2022).

RNA sequencing

RNA extractions for all samples were performed at Price Lab, University of Nevada, Las Vegas. Prior to the extraction, flies from all samples were placed into tubes with liquid N2 to freeze. We utilized a Quick-RNA Tissue/Insect kit from Zymo Research to extract RNA from the whole body and larvae for some species (See Table 3 for RNA-Seq sample information). RNA extractions were sent to Genomics Acquisition and Analysis Core at UNLV for library preparation using NextSeq 500/550 v2.5 sequencing reagent kits. All samples were sequenced from Illumina NextSeq 550 sequencer with maximum read length is 150bp.

Bioinformatics analyses

Genome annotation

I utilized BRAKER3 (Gabriel et al., 2023), the newest version of BRAKER to annotate the assemblies obtained from Kim et al. 2021. BRAKER3 is a homology-based annotation that uses both transcript and protein evidence. In-house RNA-Sequencing data and published protein evidence from the closely related *Drosophila melanogaster* (https://www.uniprot.org/proteomes/UP000000803) were used as homology-based evidence. In

addition, BRAKER3 also used GeneMark-ETP (Bruna et al., 2023), AUGUSTUS (Stanke & Waack 2003), and TSEBRA (Gabriel, 2021) for ab initio gene prediction.

Gene extraction

Hmmsearch function of HMMER3 (Eddy 2011) was used to perform homology searches for OR genes in all of *Drosophila* species in the study against an OR HMM profile of 23000 OR proteins in 1000 species that is available on Pfam database (https://www.ebi.ac.uk/interpro/entry/pfam/PF02949/taxonomy/uniprot/#sunburst). The E-value threshold was set to be 0.01. After that, I used BLAST-p (Camacho C., 2008) to confirm all hits produced by HMMER3 were the correct OR genes.

I validated the extracted genes from Hawaiian *Drosophila* by visualizing them on JBrowser. Additionally, I inspected their counterparts in *Drosophila melanogaster* on JBrowser and recorded the names of the neighboring genes on both sides. Subsequently, I conducted BLAST searches to compare the left and right neighbor genes of the Hawaiian *Drosophila* species with those of *Drosophila melanogaster* to identify potential matches.

Natural selection

PAML (Yang, 1997) and Hyphy (Kosakovsky Pond et al., 2020) were utilized to detect natural selection of OR genes in these species. I used PAML to perform branch-site model analyses which allows for the positive selection in a few sites on the lineages. For each OR gene, I ran it with both alternative and null models. Alternative model assumes that the foreground branch has experienced positive selection ($\omega > 1$) at specific sites, while the background branches have not. In the control file for this model, runmode = 0, seqtype = 1, CodonFreq = 2, ndata = 1, model = 2, NSsites = 2, fix_omega = 0 and omega = .5. On the other side, null model states that all sites in the gene have been evolving under neutral pressure **(ω =1)** across all

branches of the tree. The control file of null model is similar to the alternative model except for, fix_omega = 1 and omega = 1. After that, I obtained lnL for both models by running command grep lnL. A Chi-Square test was performed to determine if the Chi-square values are significant.

In addition, I utilized FEL (Fixed Effects Likelihood) test in Hyphy to perform site model analyses in order to identify sites under purifying selections. Default setting was applied while running this.

Species tree

The species tree in this analysis was provided by Dr. Anton Suvorov from Virgina Tech university. DNA sequence alignment was done with MAFFT using the --auto method. The multiple sequence alignments were consolidated to construct a supermatrix. Subsequently, a maximum likelihood phylogenetic tree was deduced from this supermatrix, also known as a concatenated alignment, using IQ-TREE v1.6.5. The supermatrix was treated as a unified partition during this process. Employing the GTR+I+G substitution model in IQ-TREE was imperative, as using any other substitution model might not necessarily enhance the accuracy of tree topology estimation. To assess the support for each node in the resultant tree, three distinct reliability measures were employed. This included conducting 1,000 ultrafast bootstrap (UFBoot) replicates, along with an additional approximate likelihood ratio test incorporating the nonparametric Shimodaira–Hasegawa correction (SH-aLRT), and a Bayesian-like transformation of aLRT.Differential Expression Analysis.

Differential gene expression analysis

First, I used Fastp (Chen et al., 2018) to perform adapter trimming and quality filtering for the RNA-Seq data produced in-house. Next, Fastqc (Fastqc) was used to check the quality of

the data. The trimmed data were aligned by STAR (Dobin et al. 2013) with annotated genomes for each species. After that, I used FeatureCounts (Liao, 2013) to get the gene counts from the aligned data. Lastly, I used DESeq, EnhancedVolcano, ggplot2 packages in R to get differential gene expression, PCA plots, volcano plots and heatmaps. First, the differential expression analysis was performed between females and males for 3 Hawaiian Drosophila species. Subsequently, I conducted pairwise differential expression analysis, comparing *Drosophila grimshawi* to *Drosophila basisetae, Drosophila grimshawi* to *Drosophila silvestris, Drosophila grimshawi* to *Drosophila sproati, Drosophila silvestris* to *Drosophila basisetae, Drosophila silvestris* to *Drosophila sproati,* and *Drosophila basisetae to Drosophila sproati,* respectively.

RESULTS

Genome annotation

Using BRAKER3, I annotated *Drosophila silvestris, Drosophila basisetae and Drosophila sproati,* which are not available publicly. The total of genes of *Drosophila silvestris, Drosophila basisetae and Drosophila sproati* are the following: 17033, 15975 and 22012, respectively. The total NCBI's annotated protein-coding genes for *Drosophila melanogaster* is 13,962 and *Drosophila grimshawi* is 13780.

OR genes extraction

The number of OR genes in all species of interest extracted by HMMER against an insect OR HMM profile is shown in Table 4. The counts of OR genes in *Drosophila melanogaster, Drosophila simulans* and *Drosophila yakuba* were 60, 58 and 57, respectively. Furthermore, *Drosophila virilis* had 42 OR genes and *Drosophila mojavensis* had 41 genes. The Hawaiian *Drosophila* species (*Drosophila grimshawi, Drosophila sproati, Drosophila basisetae, and Drosophila silvestris*) also exhibited a similar number of OR genes, with 41, 39, 40, and 40, in the same order. Furthermore, out of the 60 OR genes present in Drosophila melanogaster, approximately 40 were found to be conserved across most of the Hawaiian Drosophila species examined in this study. These genes were also available for the two species more closely related to the Hawaiian Drosophila, *Drosophila virilis and Drosophila mojavensis* and the other species in the *melanogaster* subgroup (Table 5).

Natural selection

Positive selection in the OR genes was identified in multiple sites across different branches within the Hawaiian *Drosophila* lineages using the branch-site model. In Table 6, 22 OR genes in *Drosophila silvestris*, 10 in *Drosophila grimshawi*, 21 in *Drosophila sproati*, and 16

in *Drosophila basisetae* have undergone positive selection. From the genes that underwent positive selection in *Drosophila silvestris*, OR2a, OR46a, OR67a and OR92a have a high number of PSS (>100)**.** Additionally, OR2a, OR46a and OR69a in *Drosophila grimshawi* exhibit a substantial quantity of sites under positive selection (> 100). In *Drosophila sproati*, OR85f and OR88a display a significant number of sites experiencing positive selection (more than 100 sites). And in *Drosophila basisetae*, no OR gene has more than 100 PSS (less than 25 sites).

Using a site model, negative selection was detected in several OR genes within the Hawaiian *Drosophila* species. Even though all of OR genes have undergone negative selection, the number of negatively selected sites are not as significant as the positive sites. The following OR genes have more than 10 negatively selected sites: OR9a, OR24a, OR30a, OR33c, OR42b, OR43a, OR46a, OR63a, OR71a, OR85c and OR88a.

Differential gene expression

In *Drosophila grimshawi*, genes that were highly induced in females compared to males are OR43a, OR49a, OR56a, OR83a, OR85c and OR94a. In contrast, only OR9a was more highly induced in males (Figure 6). In *Drosophila basisetae*, OR19a, OR2a, OR30a, OR35a, OR42b, OR43a, OR45b, OR46a, OR47b, OR49a, OR49b, OR56a, OR59a, OR59b, OR63a, OR67a, OR67c, OR67d, OR69a ,OR71a, OR74a, OR82a, OR83a, OR83c, OR85c, OR85e, OR85f, OR88a, OR92a, OR94b, OR9a and ORCO genes were highly induced in females compared to males, and no genes were significantly more highly induced in males. In addition, genes that were more highly induced in *Drosophila silvestris* females compared to males are OR2a, OR30a, OR33c, OR35a, OR42b, OR43a, OR49a, OR59a, OR59b, OR67a, OR67b, OR67c, OR67d, OR74a, OR82a, OR83a, OR85c, OR85f and ORCO. Only the OR9a gene was over-induced in males.

When comparing *Drosophila grimshawi* to *Drosophila basisetae* (females only) , genes that were upregulated in *Drosophila grimshawi* are OR2a, OR42a, OR42b, OR74a and OR94a. And genes that were highly induced in *Drosophila basisetae* are OR9a, OR19a, OR45b, OR46a, OR67a, OR67b, OR69a, OR85f and ORCO. In the comparison between *Drosophila grimshawi* and *Drosophila silvestris*, I observed an upregulation of genes including OR42a, OR42b, and OR46a in *Drosophila grimshawi*. Conversely, *Drosophila silvestris* exhibited elevated expression of genes, such as OR2a, OR9a, OR22c, OR35a, OR59a, OR59b, OR74a, OR85c, OR85f, OR92a, and ORCO.

In the comparison between females of *Drosophila grimshawi* and *Drosophila sproati*, the genes upregulated in *Drosophila grimshawi* were OR2a, OR42a, OR42b, OR43a, OR46a, OR74a, OR82a, OR83a, and OR94a. On the other hand, *Drosophila sproati* exhibited higher expression levels for genes including OR2a, OR9a, OR45b, OR59a, OR67c, OR67d, OR71a, OR83c, OR85f, OR92a, and ORCO. When comparing female *Drosophila silvestris* to *Drosophila basisetae*, there was a distinct gene expression profile with *Drosophila silvestris* displaying upregulated genes OR2a, OR19a, OR22c, OR35a, OR42b, OR49a, OR59a, OR59b, OR67a, OR69a, OR74a and OR85f. In contrast, *Drosophila basisetae* females exhibited elevated gene expression levels for OR43a, OR45b, OR46a and OR82a.

In the comparison between females of *Drosophila silvestris* and *Drosophila sproati*, the result showed that OR19a, OR22c, OR35a, OR42b, OR46a, OR49a, OR59b, OR67a, OR69a, OR74a, OR82a, OR83a, OR85f and ORCO genes were highly induced in *Drosophila silvestris* females while OR9a, OR42a, OR45b, OR59a, OR67c, OR71a and OR92a were upregulated in *Drosophila sproati* females. When comparing *Drosophila basisetae* and *Drosophila sproati*, the findings revealed that *Drosophila basisetae* displayed high expression levels in genes including

OR19a, OR24a, OR43a, OR46a, OR49a, OR56a, OR67a, OR69a, OR74a, OR82a, OR83a and OR83c. Conversely, *Drosophila sproati* exhibited upregulated expression in genes such as OR2a, OR22c, OR42b, OR59a, OR67c, OR67d, OR71a, OR85c, OR92a and OR94b.

DISCUSSION

Gene annotation

With improved technology, the rate of new genome sequencing has increased dramatically. Hence, genome annotation plays an important role in deciphering these sequences and understanding their functions and structures. The gene annotations produced by BRAKER for *Drosophila silvestris* (17,033 genes), *Drosophila basisetae* (15,975 genes), and *Drosophila sproati* (22,012 genes) surpass the number of genes annotated in Drosophila melanogaster (13,962 genes) and *Drosophila grimshawi* (13,780 genes) by the NCBI. This could be explained by the differences in the genome annotation pipelines between NCBI and BRAKER3. While NCBI involves manual curation and a step-by-step process, BRAKER3 is an automated genome annotation pipeline. NCBI uses Gnomon as a gene model prediction tool while BRAKER3 uses GeneMark-ETP, AUGUSTUS, and TSEBRA. According to Gabriel et al. 2023, BRAKER3 predictions demonstrated superior sensitivity and specificity in gene and transcript-level accuracy compared to other genome annotation pipelines. Also, BRAKER3 yields precise results for novel genomes with no close species that have a publicly available and reliable annotation yet. This is relevant to my project because the four Hawaiian *Drosophila* genomes used in this study are novel or previously unannotated genomes. Last but not least, BRAKER3 exhibits significantly faster processing times and demands less memory when executed on a supercomputer compared to other methods.

On the other hand, despite employing identical annotation parameters across all Hawaiian *Drosophila* genomes, *Drosophila sproati* exhibits significantly higher numbers of annotated genes. This difference may be attributed to variations in genome quality and assembly completeness. While all three Hawaiian *Drosophila* assemblies of interest were sourced from Dr.

Bernard Kim at the Petrov Lab, Stanford University, it's worth noting that only the assembly of *Drosophila sproati* has been published and subjected to a thorough assessment of genome completeness with contig N50 of 8Mb and 99.4% BUSCO completeness (Kim et al. 2021).

OR genes extraction

Table 4 reveals that Drosophila melanogaster had a comparable number of OR genes to *Drosophila simulans* and *Drosophila yakuba*. In contrast, *Drosophila virilis* and *Drosophila mojavensis* possess a similar number of OR genes to the Hawaiian *Drosophila* species, which include *Drosophila grimshawi, Drosophila sproati, Drosophila basisetae,* and *Drosophila silvestris*. The consistent number of OR genes among phylogenetically related species is evident in this study. *Drosophila melanogaster, Drosophila simulans,* and *Drosophila yakuba*, known for their close relationship (Figure 1), exhibited a logical and expected progression in the similarity of OR gene numbers. Likewise, *Drosophila virilis, Drosophila mojavensis*, and the Hawaiian *Drosophila* species (*Drosophila grimshawi, Drosophila sproati, Drosophila basisetae, Drosophila silvestris*) shared a common phylogenetic history (Figure 1), explaining the observed consistency in OR gene numbers across these species.

In addition, of the 60 OR genes in *Drosophila melanogaster*, approximately 40 were identified as conserved in the majority of the Hawaiian *Drosophila* species analyzed in this study. These genes were also prevalent in closely related species, such as *Drosophila virilis* and *Drosophila mojavensis*, as well as within the *melanogaster* subgroup, including *Drosophila simulans* and *Drosophila yakuba* (Table 5).

Natural selection

Natural selection is a general process that operates within populations and can lead to the adaptation of species to new environments and the diversification of genes and traits between

species. In this study, there were a few genes that showed a significant amount of PSS (more than 100 sites) which might suggest their important roles in some Hawaiian *Drosophila*. In both *Drosophila silvestris* and *Drosophila grimshawi*, OR2a and OR46a have a high number of positive sites. OR2a function is still not well-known while OR46a is known for oviposition site aversion (Mansourian et al., 2016) and sensitivity to both male and female extracts (Goes van Naters, 2014). In *Drosophila grimshawi*, genes that showed a high number of positive selection sites are OR69a and OR71a. Functionally, OR69a has been shown to be involved in dialect training during communal living and has a dual affinity for both sex and food odorants (Kacsoh et al., 2019). And OR71a helps *Drosophila* to detect hydroxycinnamic acids (HCAs) which are a part of some fruits' defense mechanism to protect them from being eaten by *Drosophila*. It also helps the flies to "induce positive chemotaxis, oviposition, and increased feeding" (Dweck et al. 2015).

In addition, two genes that had a significant number of positive sites in *Drosophila silvestris* were OR67a and OR 92a. OR67a is involved in the behavioral responses to lactone, organic acids, aldehydes, ketones, aromatics, alcohols, and esters (Hallem & Carlson, 2006). Chihara et al. (2014) suggested that OR92a is responsive to food-related odors. Moreover, OR92a is one of the neurons that activate caspase activity in the antennal lobe. DEVDase, which is an enzyme involved in apoptosis, in OR92a can cause a decrease in *Drosophila*'s instinctive attraction behavior when they get older.

Furthermore, OR85f and OR88a genes in *Drosophila sproati* have a greater number of PSS. Specifically, OR85f is known to help *Drosophila* detect and avoid their main parasitoid enemies, *Leptopilina* wasps (Ebrahim et al. 2015). And OR88a responds to both male and female extracts. Specifically, it responded to a rubbing from the genital region of males but it did not

respond to *cis*-vaccenyl acetate (Wdv 2014) which is a male pheromone that reduces reproductive motivation of other males but enhances that in females so that there is a higher chance for mating success. OR88a also acts as an aggregation pheromone to lure both males and females to the gathering spot (Ejima 2015). Even though there are a few genes that experienced positive selection in *Drosophila basisetae*, none of them had an abundant amount of positive selection sites. Taken together, the OR genes subject to extensive positive selection appear to be crucial for various biological functions, including reproduction, oviposition, as well as the detection of food sources and potential threats.

Differential gene expression

Females vs males

When comparing gene expression between males and females, OR9a was highly induced in both *Drosophila grimshawi* and *Drosophila silvestris* males. Or9a responds to acetoin (Dweck et al. 2015) which is found naturally in some fruits and produced by the fermentation process (Mohd Yusoff et al., 2017). A study about mating and food deprivation in *Drosophila melanogaster* males (Wang, 2014) mentioned that it is crucial for male *Drosophila melanogaster* to promptly discover decomposing fruits as a source of sustenance. To accomplish this, males rely predominantly on volatile scents to detect rotting fruits. Additionally, the exclusive presence of the OR9a gene in male Drosophila suggests its essential role in guiding Hawaiian *Drosophila* males as they choose lek sites, engage in courtship displays to outcompete rivals, or release pheromones to attract potential mates. However, more studies are needed to confirm this suggestion.

On the other hand, there are several genes that are overinduced in Hawaiian *Drosophila* females. Both OR49a and OR83a are highly induced in all three Hawaiian *Drosophila*

(Drosophila grimshawi, Drosophila basisetae and Drosophila silvestris). OR49a is known for detecting actinidine and nepetalactol which are secreted by *Leptopilina* wasps, their parasitoid enemies (Shimma et al. 2015) This could be helpful for them to avoid the wasps when females try to find oviposition sites to lay eggs. OR83a responses to pentanol, ethyl acetate, and propyl acetate which are fruit odors (Information, 2023). This gene might be useful for them to detect their food sources or egg-laying sites. In *Drosophila basisetae* and *Drosophila silvestris* females, Orco, OR2a, OR30a, OR42b, OR59a, OR59b, OR67a, OR67c, OR67d, OR74a, OR82a and OR85f have a high level of expression. And genes that are overly induced in *Drosophila grimshawi* and *Drosophila silvestris* females are OR43a and OR85c compared to *Drosophila basisetae*. Moreover, OR56a is induced more in females of *Drosophila grimshawi* and *Drosophila basisetae* compared to *Drosophila silvestris*.

Even though there is still limited knowledge about highly induced ORCO in *Drosophila* females, David et al. 2023 reported that highly induced ORCO plays an important role in maintaining healthy embryonic development and hatching in female *Aedes aegypti* mosquitoes. This suggested that the ORCO gene might also be crucial for Hawaiian *Drosophila* to have a sustained fertility. OR30a and OR43a respond to indole which is produced by plants, fungi and bacteria. It was indicated that indole contributes to oviposition site selection in Anopheles gambiae mosquitoes (Blackwell & Johnson, 2000). OR42b has a few biological functions. It directly responds to humidity changes (Li et al., 2022). Along with OR94b, OR42b is involved in food-related odors detection and caspase activity (Chihara et al. 2014). While OR59a can detect odors from some species of *Annona*, a genus of flowering plants in the pawpaw/sugar apple family (Maia et al., 2012), OR59b has been shown to responded to N,N-Diethyl-meta-toluamide (DEET), the most widely used insect repellent worldwide (Pellegrino et al., 2011).

Furthermore, OR67d is responsive to acute responses to *cis*-vaccenyl acetate (Wvd 2014) which is a male pheromone that boosts female reproductive motivation and enhances the likelihood of successful copulation (Ejima 2015). Elevated expression of this gene in female Hawaiian *Drosophila* could potentially aid them in locating and identifying potential mates. OR74a gene in *Drosophila melanogaster*'s larvae quickly responds to butanol, octanol, 2-heptanone, and propyl acetate (Grillet, 2016).While OR82a can detect geranyl acetate (Schlief & Wilson, 2007), OR85c responds to 3-octanol and *2*-heptanone (Mathew et al., 2013; (Auer et al., 2020). And OR56a activates geosmin which is a microbial odorant that prevents flies from harmful microbes (Stensmyr et al., 2012). In short, these highly induced OR genes in Hawaiian *Drosophila* females serve a variety of functions, from reproduction and mate location to responses to environmental cues and the detection of specific odors.

Among Hawaiian Drosophila species

When comparing OR gene expression between *Drosophila grimshawi* and the other three species, OR42a and OR42b were consistently overinduced in *Drosophila grimshawi*. While OR42a has been reported to respond to several chemicals such as butanol, ethyl acetate, propyl acetate, pentyl acetate and pyrazines (Montague et al., 2011; Hoare et al. 2011), OR42b is responsible for several functions including humidity change detection as mentioned previously (Li et al. 2022).

Among four Hawaiian *Drosophila*, one gene that exhibited high expression in *Drosophila basisetae* was OR46a. This gene is responsive to both male and female extracts (Wdv 2014) and is associated with oviposition site aversion behavior (Mansourian et al. 2016). In addition, the following genes were highly induced in *Drosophila silvestris*: OR22c, OR35a, OR59b, Or74a

and OR85f. These genes detected a number of odorants such as alcohol. esters, insect repellent and wasp odor (Table 9).

Furthermore, when comparing *Drosophila sproati* and the rest of Hawaiian *Drosophila* in this study, OR59a, OR67c and OR71a, which are responsive food-odors (Table 5), were ones that experienced high gene expression. These results suggest that some OR genes may have species-specific functions and each Hawaiian Drosophila species may rely on specific sets of OR genes to adapt to their respective environments. Nevertheless, further analyses involving a broader range of species are necessary to validate this hypothesis.

CONCLUSION

In conclusion, the olfactory system in *Drosophila* plays a fundamental role in their survival and adaptation, including the detection of food sources and mates. Understanding the genetic aspects of olfaction in these unique Hawaiian species has significant implications for enhancing our knowledge of sensory biology and physiology in insects and even vertebrates, including humans.

In this study, approximately 40 out of the 60 OR genes found in Drosophila melanogaster were conserved in Hawaiian *Drosophila*. Furthermore, we identified several OR genes experiencing positive selection, with genes like OR2a, OR46a, OR67a, OR69a, OR71a, OR85f, OR88, and OR92a standing out due to a substantial number of PSS. These genes are responsible for a range of biological functions, including reproduction, oviposition, and the detection of food sources and potential threats. Conversely, none of the identified OR genes exhibited extreme negative selection.

Additionally, our research discovered the differences in OR gene expression patterns between females and males and among several Hawaiian *Drosophila* species. These findings indicate that distinct sets of OR genes are essential for the functioning of females and males, given their unique mating behaviors. Also, some OR genes might be species-specific, and some Hawaiian Drosophila species likely depend on distinct sets of OR genes to successfully adapt to their unique environments.

Overall, our study contributes to the broader understanding of sensory biology and the evolutionary dynamics of olfactory receptors, offering insights into the unique adaptations of Hawaiian Drosophila.

APPENDIX: TABLES & FIGURES

Table 1. Specimens used in the analysis with collection information.

Species name	Island	Locality
Drosophila silvestris	Hawaii	Pu'U Maka' Ala Natural Area Reserve 19.523780,-155.296730
Drosophila sproati	Oahu	Tom's trail 19.574513,-155.216191
Drosophila basisetae	Hawaii	ʻŌlaʻa Forest Reserve 19.457250,-155.248972

Table 2. Genome assembly information of other *Drosophila* species.

	Species name	Link	Contig N50	BUSCO completeness
Hawaiian *Drosophila*	*Drosophila grimshawi*	https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000005155.2/	91.2 kb	99.7%
Other *Drosophila*	*Drosophila mojavensis*	https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_018153725.1/	121.5 kb	99.6%
	Drosophila virilis	https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_003285735.1/	8.7 Mb	99.8%
	Drosophila melanogaster	https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000001215.4/	21.5 Mb	N/A
	Drosophila simulans	https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_016746395.2/	22.3 Mb	99.8%
	Drosophila yakuba	https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_016746365.2/	18.7 Mb	99.8%

Table 3. Sample information for RNA-Sequencing of *Drosophila silvestris*, *Drosophila basisetae* and *Drosophila sproati*.

Sample Number	Species Name	Sex	Sample Description
1	*Drosophila silvestris*	female	2 whole body females, 3-4 weeks old, eclosed 11-18 Nov 2022
2	*Drosophila silvestris*	female	2 whole body females, 3-4 weeks old, eclosed 11-18 Nov 2022
3	*Drosophila silvestris*	female	2 whole body females, 3-4 weeks old, eclosed 11-18 Nov 2022
4	*Drosophila silvestris*	male	2 whole body males, 3-4 weeks old, eclosed 11-18 Nov 2022
5	*Drosophila silvestris*	male	2 whole body males, 3-4 weeks old, eclosed 11-18 Nov 2022
6	*Drosophila silvestris*	male	2 whole body males, 3-4 weeks old, eclosed 11-18 Nov 2022
8	*Drosophila basisetae*	female	2 whole body females, 3-4 weeks old, eclosed 11-18 Nov 2022
9	*Drosophila basisetae*	female	2 whole body females, 3-4 weeks old, eclosed 11-18 Nov 2022
10	*Drosophila basisetae*	female	2 whole body females, 3-4 weeks old, eclosed 11-18 Nov 2022
11	*Drosophila basisetae*	male	2 whole body males, 3-4 weeks old, eclosed 11-18 Nov 2022
12	*Drosophila basisetae*	male	2 whole body males, 3--4 weeks old, eclosed 11-18 Nov 2022
13	*Drosophila basisetae*	male	2 whole body males, 3-4 weeks old, eclosed 11-18 Nov 2022
116	*Drosophila sproati*	female	1 whole body female, 21 weeks old, eclosed 25 July 2023
117	*Drosophila sproati*	female	1 whole body female, 21 weeks old, eclosed 25 July 2023
119	*Drosophila sproati*	female	1 whole body female, 21 weeks old, eclosed 25 July 2023

Table 4. Number of OR genes extracted by HMMER.

Species	Number of OR genes
Drosophila melanogaster	60

Drosophila simulans	58
Drosophila yakuba	57
Drosophila virilis	42
Drosophila mojavensis	41
Drosophila grimshawi	41
Drosophila silvestris	39
Drosophila sproati	40
Drosophila basisetae	40

Table 5. OR genes found in species of interest.

Group		**Hawaiian *Drosophila***		***repleta***		***virilis***		***melanogaster***	
	Drosophila basisetae	***Drosophila grimshawi***	***Drosophila silvestris***	***Drosophila sproati***	***Drosophila mojavensis***	***Drosophila virilis***	***Drosophila melanogaster***	***Drosophila simulans***	***Drosophila yakuba***
OR1a					✓	✓	✓	✓	✓
OR2a	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR7a							✓	✓	✓
OR9a	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR10a	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR13a		✓			✓	✓	✓	✓	✓
OR19a	✓	✓		✓		✓	✓		

OR19b					✓		✓	✓	✓
OR22a							✓	✓	✓
OR22b							✓	✓	
OR22c	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR23a						✓	✓	✓	✓
OR24a	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR30a	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR33a							✓	✓	✓
OR33b							✓	✓	✓
OR33c	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR35a	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR42a	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR42b	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR43a	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR43b							✓	✓	✓
OR45a							✓	✓	✓
OR45b	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR46a	✓	✓	✓	✓	✓	✓	✓		✓
OR47a					✓	✓	✓	✓	✓
OR47b	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR49a	✓	✓	✓	✓		✓	✓	✓	✓
OR49b	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR56a	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR59a	✓	✓	✓	✓	✓	✓	✓	✓	✓

OR59b	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR59c							✓	✓	✓
OR63a	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR65a							✓	✓	✓
OR65b					✓		✓	✓	✓
OR65c							✓	✓	✓
OR67a	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR67b	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR67c	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR67d	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR69a	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR71a	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR74a	✓	✓	✓	✓			✓	✓	✓
OR82a	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR83a	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR83c	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR85a							✓	✓	✓
OR85b							✓	✓	✓
OR85c	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR85d	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR85e	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR85f	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR88a	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR92a	✓	✓	✓	✓	✓	✓	✓	✓	✓

OR94a	✓	✓	✓	✓	✓		✓	✓	✓
OR94b	✓	✓	✓	✓	✓	✓	✓	✓	✓
OR98a					✓		✓	✓	✓
OR98b							✓	✓	✓
ORCO	✓	✓	✓	✓	✓	✓	✓	✓	✓

Table 6.High positive selection sites in some Hawaiian *Drosophila* using branch-site model (LRT: Likelihood ratio test; PSS: Positive selection sites)

Genes	***Drosophila silvestris***		***Drosophila grimshawi***		***Drosophila sproati***		***Drosophila basisetae***	
OR2a	PSS	LRT	PSS	LRT	PSS	LRT	PSS	LRT
	121	61.9	121	3.9	5	24		
OR46a	PSS	LRT	PSS	LRT	PSS	LRT	PSS	LRT
	254	25.3	254	5.9	6	24.5		
OR67a	PSS	LRT	PSS	LRT	PSS	LRT	PSS	LRT
	151	13	24	37.9				
OR69a	PSS	LRT	PSS	LRT	PSS	LRT	PSS	LRT
			303	7.6	7	13.4		
OR71a	PSS	LRT	PSS	LRT	PSS	LRT	PSS	LRT
	13	30.9	300	4.3	2	10.6	4	17.8
OR85f	PSS	LRT	PSS	LRT	PSS	LRT	PSS	LRT
					216	12.4		
OR88a	PSS	LRT	PSS	LRT	PSS	LRT	PSS	LRT
	22	21.2			310	13.7	24	26.4
OR92a	PSS	LRT	PSS	LRT	PSS	LRT	PSS	LRT
	254	10.6						

Table 7. Negative selection sites in Hawaiian *Drosophila* using site model.

Genes	Negatively selected sites	Average LRT	Average p-value
ORCO	3	3.7	0.05
OR2a	3	4.7	0.06
OR9a	13	5.8	0.04
OR10a	3	4.5	0.04
OR22c	8	4.3	0.04
OR24a	15	4.3	0.05
OR30a	11	4.7	0.04
OR33c	12	4.6	0.05
OR35a	4	4.3	0.04
OR42a	2	5.6	0.03
OR42b	11	4.3	0.05
OR43a	13	5.0	0.04
OR45b	10	4.1	0.06
OR46a	13	4.3	0.05
OR47b	9	5.0	0.04
OR49a	2	3.9	0.06
OR49b	6	4.1	0.05
OR56a	1	**3.1**	**0.07**
OR59a	9	4.4	0.04
OR59b	4	3.4	0.06

OR63a	13	4.7	0.04
OR67a	10	5.1	0.04
OR67b	5	4.2	0.04
OR67c	7	4.0	0.05
OR67d	6	5.2	0.04
OR69a	9	4.3	0.05
OR71a	15	5.0	0.04
OR74a	2	3.1	0.08
OR82a	10	5.3	0.04
OR83a	7	5.1	0.04
OR83c	1	**3.9**	**0.05**
OR85c	13	4.8	0.04
OR85d	8	5.0	0.04
OR85e	7	3.6	0.06
OR85f	8	9.8	0.03
OR88a	14	4.3	0.06
OR92a	2	2.8	0.09
OR94a	7	5.0	0.03
OR94b	3	6.3	0.03

Table 8. Known functions for OR genes in *Drosophila melanogaster*.

Genes	Functions	Sources
ORCO	- Serves dual role as a chaperone for cell surface expression of the OR/Orco complex as well as being integral to the function of the olfactory receptor complex.	Flybase
OR2a	- Codes for a chemoreceptor with multiple transmembrane domains, facilitating the detection and response to volatile chemicals.	Flybase
OR9a	- Detect acetoin	Functional loss of yeast detectors parallels transition to herbivory - PMC
OR10a	- Responds to esters	Uniprot
OR22c	- Responds to structurally related aromatic odorants	Molecular determinants of odorant receptor function in insects - PMC
OR24a	- Responds to methyl phenyl sulfide. - Responds to structurally related aromatic odorants.	Functional diversity among sensory receptors in a Drosophila olfactory circuit | PNAS Molecular determinants of odorant receptor function in insects - PMC

OR30a	- Responds to indoles	Functional diversity among sensory receptors in a Drosophila olfactory circuit | PNAS Identification and functional characterization of olfactory indolergic receptors in Drosophila melanogaster - ScienceDirect
OR33c	- Drives expression of GFP (green fluorescent protein) - Responses to E2-hexenal	Coexpression of Two Functional Odor Receptors in One Neuron - ScienceDirect
OR35a	- Responds to alcohols and acetates.	Functional diversity among sensory receptors in a Drosophila olfactory circuit | PNAS Uniprot
OR42a	- Responds to alcohols and acetates.	Uniprot Mechanisms of odor receptor gene choice in Drosophila Functional diversity among sensory receptors in a Drosophila olfactory circuit | PNAS
OR42b	- Directly respond to humidity changes	Humidity response in Drosophila olfactory sensory neurons

	- Mediating innate attraction to food-related odors - Caspase activation in Or42b and Or92a neurons is responsible for altering animal behavior during normal aging	requires the mechanosensitive channel TMEM63 - PMC Caspase Inhibition in Select Olfactory Neurons Restores Innate Attraction Behavior in Aged Drosophila | PLOS Genetics
OR43a	- Promotes functional reconstitution of odor-evoked signaling in sensory neurons that normally respond only to carbon dioxide.	Identification and functional characterization of olfactory indolergic receptors in Drosophila melanogaster - ScienceDirect
OR45 b	- Responds to anisole	Uniprot
OR46a	- Sensitive to both male and female extracts - Necessary for oviposition site aversion	Drosophila Pheromones - Neurobiology of Chemical Communication - NCBI Bookshelf Fecal-Derived Phenol Induces Egg-Laying Aversion in Drosophila

OR47b	- Detect a pheromone secreted by both males and females. - Plays an important role in sociosexual interactions since its enhances courtship in a pheromone-dependent manner.	Receptors and neurons for fly odors in Drosophila Uniprot
OR49a	- Wasp odors actinidine and nepetalactol	Drosophila Avoids Parasitoids by Sensing Their Semiochemicals via a Dedicated Olfactory Circuit - PMC
OR49b	- Sensitive to indoles - Elicites strong excitatory responses all contain a benzene ring	Identification and functional characterization of olfactory indolergic receptors in Drosophila melanogaster - ScienceDirect Coding of Odors by a Receptor Repertoire: Cell
OR56a	- Specific receptor for geosmin, a microbial odorant that constitutes an ecologically relevant stimulus that alerts flies to the presence of harmful microbes and induces avoidance behavior.	Uniprot

OR59a	- Respond to 4-methyl-5-vinylthiazole	Functional diversity among sensory receptors in a Drosophila olfactory circuit | PNAS
OR59b	- Respond to N,N-Diethyl-meta-toluamide (DEET), the most widely used insect repellent worldwide	Uniprot
OR63a	- Responds to butyl acetate, isoamyl acetate, and hexanoic acid	Uniprot
OR67a	- Responds to apple, lactone, organic acids, aldehydes, ketones, aromatics, alcohols, and esters	Coding of odors by a receptor repertoire
OR67b	- Responds to alcohols and other chemicals	Functional diversity among sensory receptors in a Drosophila olfactory circuit | PNAS Uniprot
OR67c	- Respond to food odors	https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0013389
OR67d	- Acute responses to *cis*-vaccenyl acetate which is in male pheromone.	Drosophila Pheromones - Neurobiology of Chemical Communication - NCBI Bookshelf

	- Does not respond to virgin female extracts. - Detection of male-specific pheromones	Pleiotropic actions of the male pheromone cis-vaccenyl acetate in Drosophila melanogaster - PMC Receptors and neurons for fly odors in Drosophila
OR69a	- Involves in dialect training during communal living - Has a dual affinity for both sex and food odorants	Neural circuitry of social learning in Drosophila requires multiple inputs to facilitate inter-species communication PMC
OR71a	- Detects hydroxycinnamic acids (HCAs) - Induces positive chemotaxis, oviposition, and increased feeding	Olfactory proxy detection of dietary antioxidants in Drosophila
OR74a	- Responds to butanol, octanol, anisole, 2-heptanone, and propyl acetate	The peripheral olfactory code in Drosophila larvae contains temporal information and is robust over multiple timescales - PMC Uniprot
OR82a	- Reponses to geranyl acetate	Olfactory Processing and Behavior Downstream from Highly Selective Receptor Neurons - PMC

OR83a	- Responds to pentanol, ethyl acetate, and propyl acetate	Uniprot
OR83c	- Mediates farnesol-induced attractionbehavior	Farnesol-Detecting Olfactory Neurons in Drosophila - PMC
OR85c	- Responses to 3-octanol and *2*-heptanone	Functional diversity among sensory receptors in a Drosophila olfactory circuit | PNAS Olfactory receptor and circuit evolution promote host specialisation - PMC
OR85 d	- Responds to the yeast metabolites	Evolution of herbivory in Drosophilidae linked to loss of behaviors, antennal responses, odorant receptors, and ancestral diet
OR85e	- Responds to fenchone and some other odorants	Coexpression of Two Functional Odor Receptors in One Neuron - ScienceDirect
OR85f	- Detects the wasp odors actinidine and nepetalactol	Drosophila Avoids Parasitoids by Sensing Their Semiochemicals via a Dedicated Olfactory Circuit | PLOS Biology

OR88a	- Sensitive to both male and female extracts - Respondsto a rubbing from the genital region of males but it did not respond to *cis*-vaccenyl acetate (which is a male pheromone)	Drosophila Pheromones - Neurobiology of Chemical Communication - NCBI Bookshelf Pleiotropic actions of the male pheromone cis-vaccenyl acetate in Drosophila melanogaster - PMC
OR92a	- Mediates innate attraction to food-related odors. - Caspase activation in Or92a neurons is responsible for altering animal behavior during normal aging	Caspase Inhibition in Select Olfactory Neurons Restores Innate Attraction Behavior in Aged Drosophila | PLOS Genetics
OR94a **OR94b**	- Affects larval behavioral response to 2-methoxyphenyl acetate	Functional diversity among sensory receptors in a Drosophila olfactory circuit | PNAS

Figure 1. Phylogeny of Drosophila species (Powell, 1997)

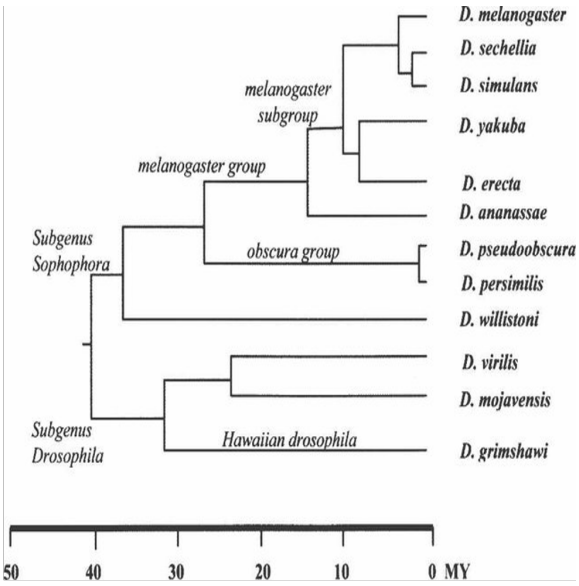


Figure 2.Positive selection sites in Hawaiian *Drosophila* identified using program CODEML. Genes with numerical labels represent those exhibiting over 100 positive selection sites.

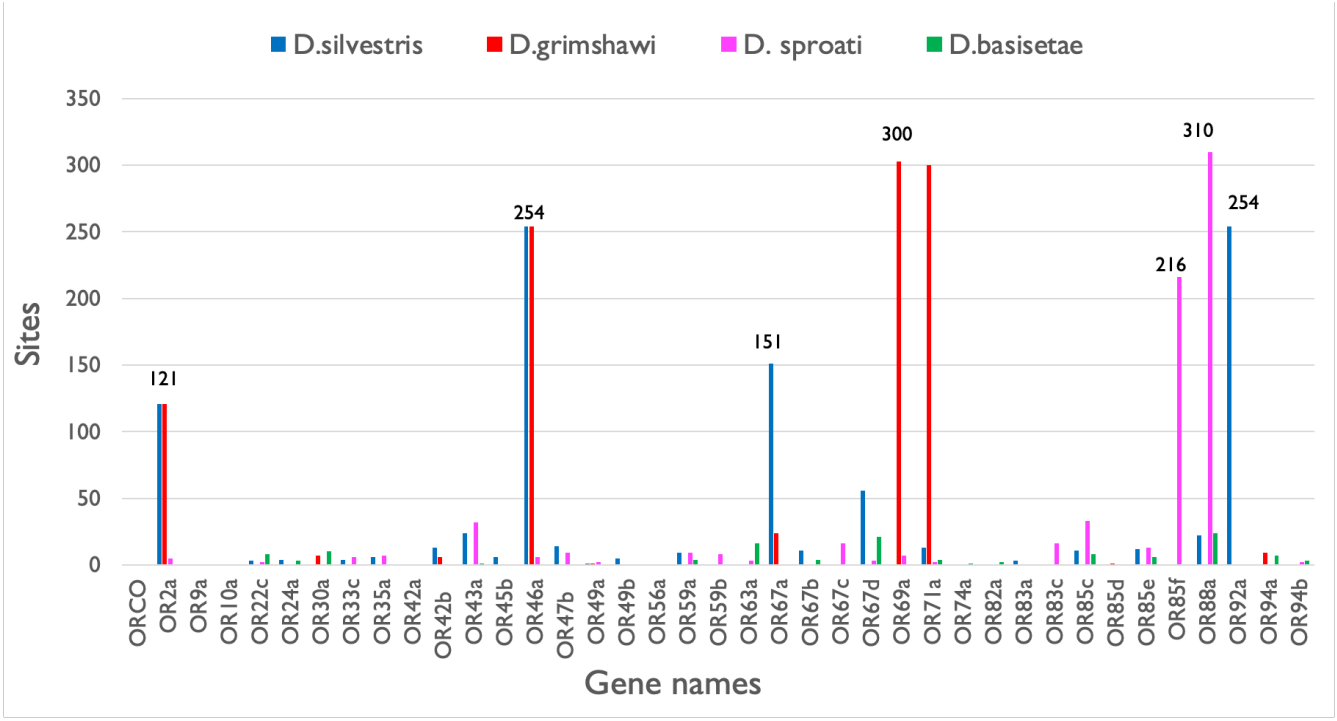


Figure 3. Negative selection sites in Hawaiian *Drosophila* identified using program Hyphy.

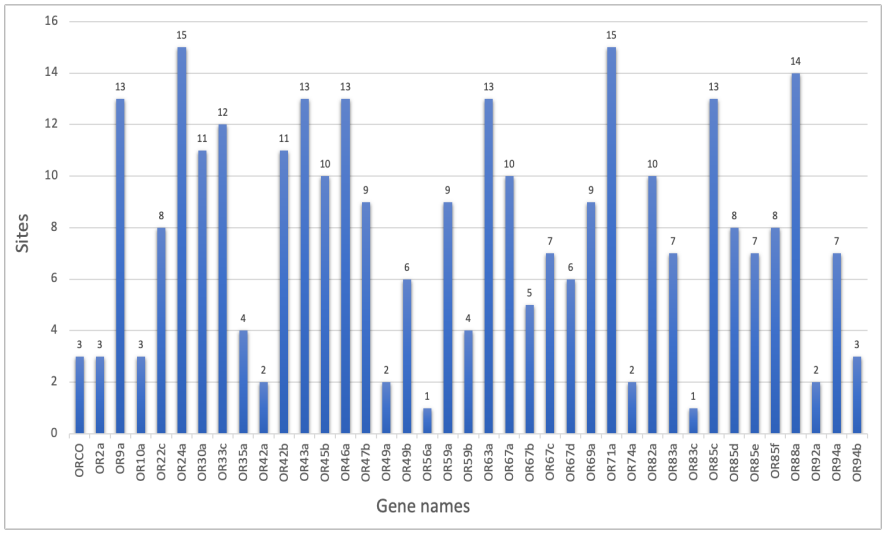


Figure 4. OR gene expression between males and females in Drosophila *grimshawi*. Genes with red labels exhibit upregulation in females, while genes with blue labels display higher fold changes/induction in males.

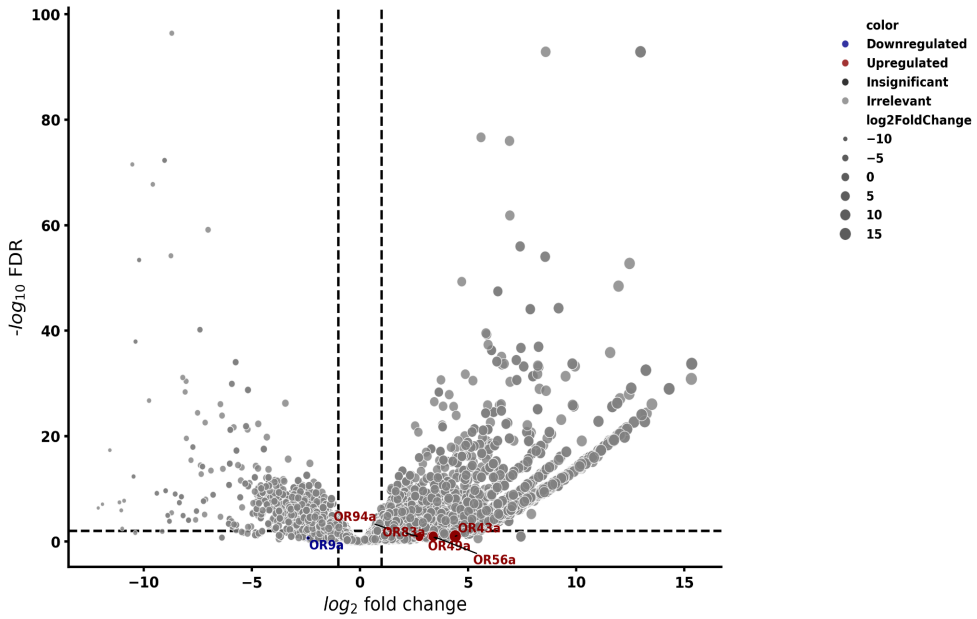


Figure 5. OR gene expression between females and males in *Drosophila basisetae*. Genes with red labels exhibit upregulation in females, while genes with blue labels display higher induction in males.

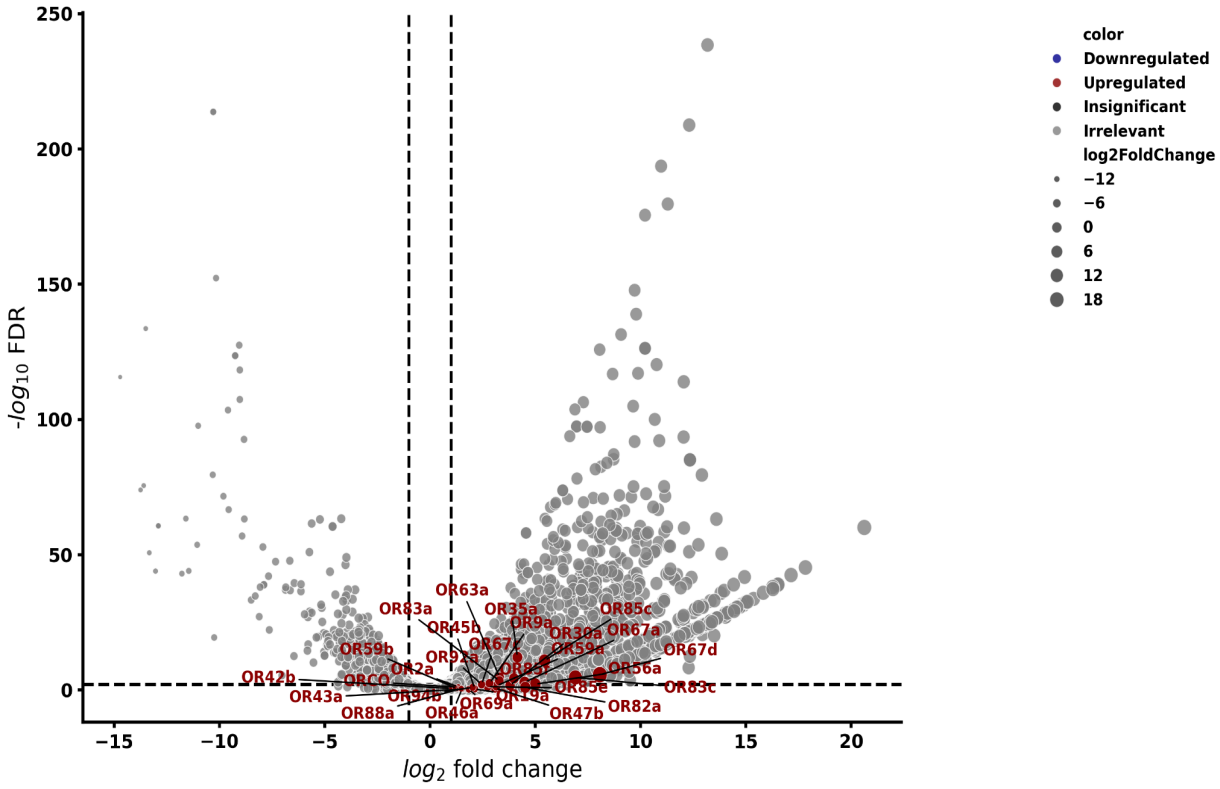


Figure 6. OR gene expression between females and males in *Drosophila silvestris*. Genes with red labels exhibit upregulation in females, while genes with blue labels display high induction in males.

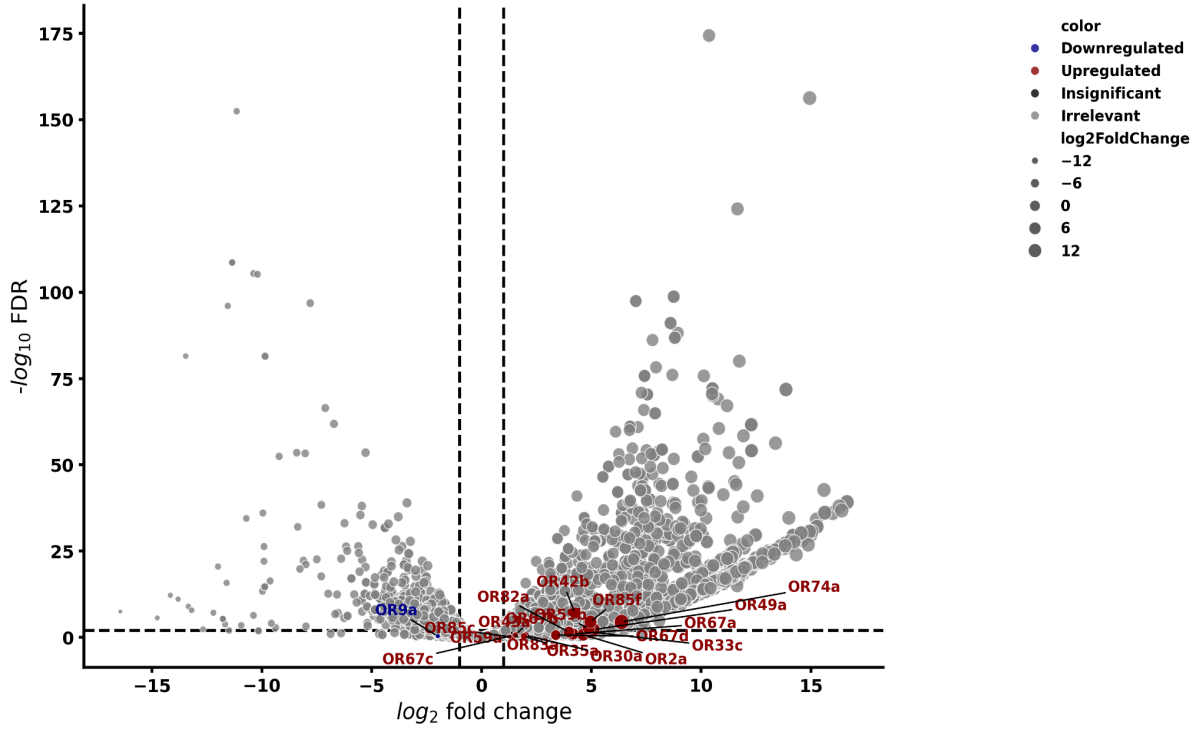


Figure 7. OR gene expression between *Drosophila basisetae* and *Drosophila sproati*. Genes labeled in red demonstrate increased expression in *Drosophila basisetae*, whereas those marked in blue indicate elevated expression in *Drosophila sproati*.

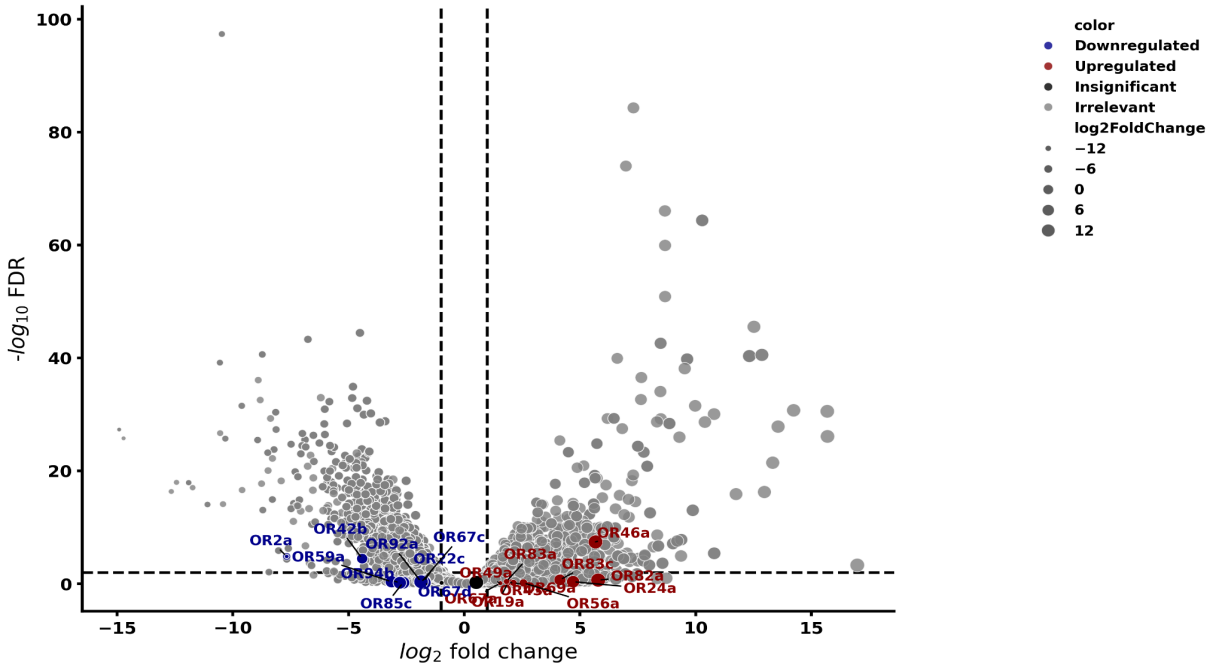


Figure 8. OR gene expression between *Drosophila silvestris* and *Drosophila sproati* (females only). Genes labeled in red demonstrate increased expression in *Drosophila silvestris*, whereas those marked in blue indicate elevated expression in *Drosophila sproati*.

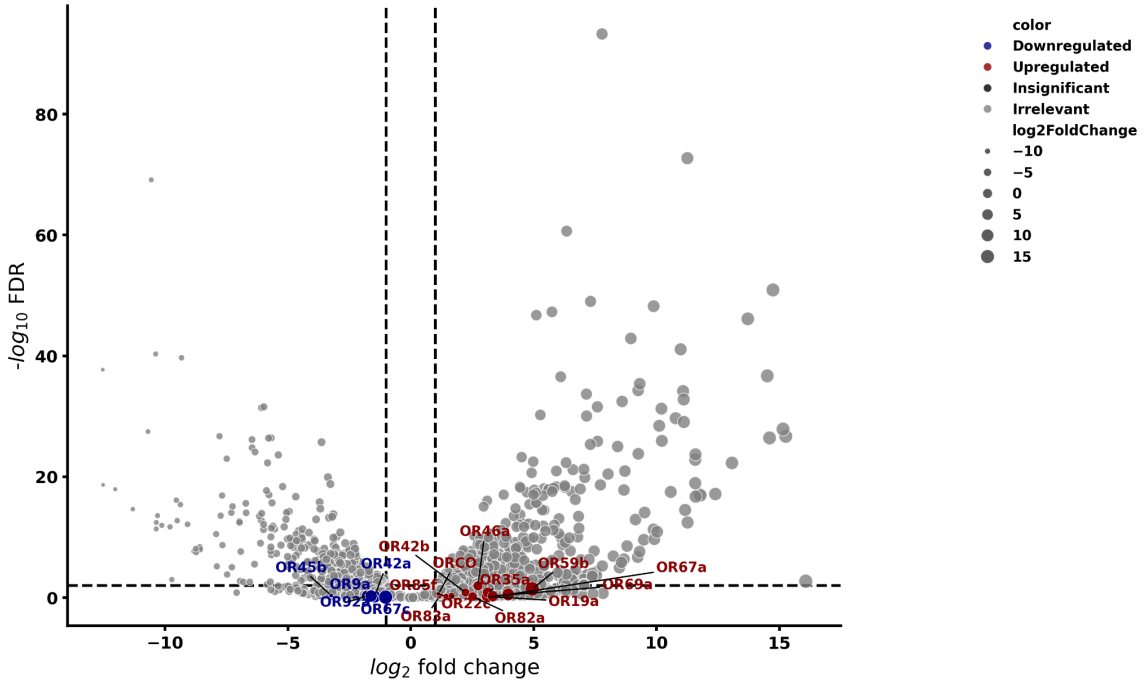


Figure 9. OR gene expression between *Drosophila silvestris* and *Drosophila basisetae* (females only). Genes labeled in red demonstrate increased expression in *Drosophila silvestris*, whereas those marked in blue indicate elevated expression in *Drosophila basisetae*.

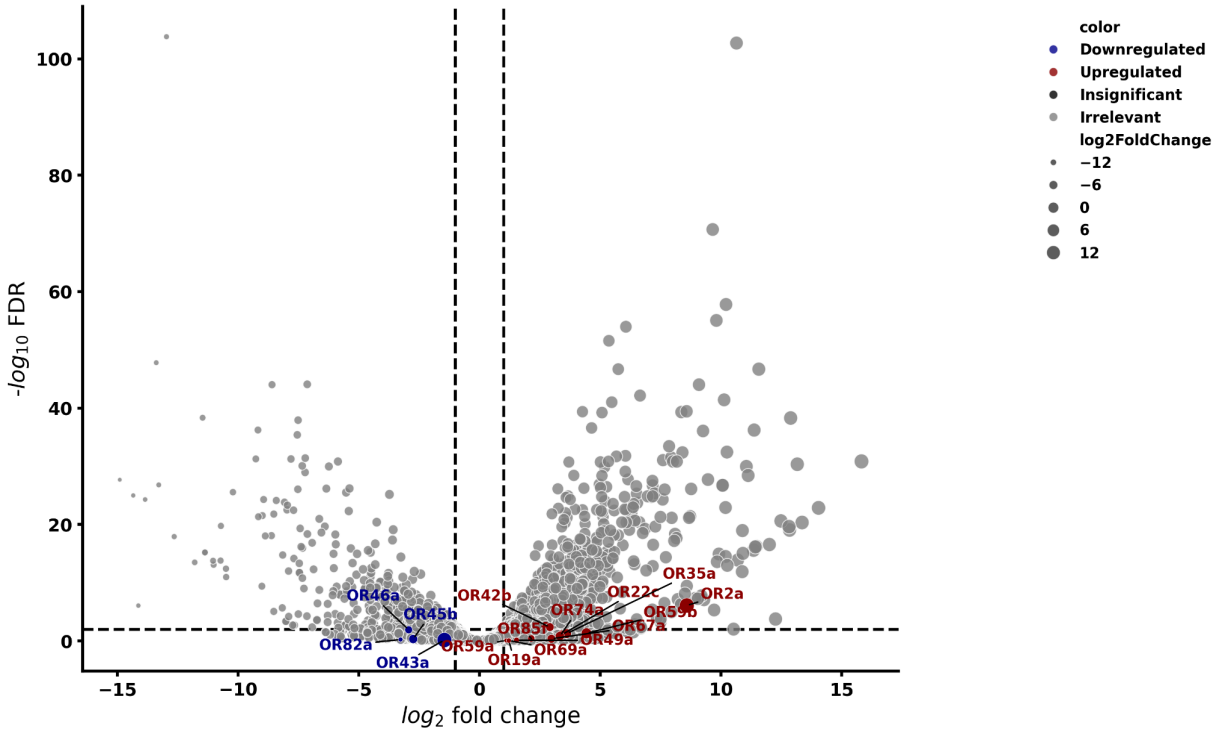


Figure 10.OR gene expression between *Drosophila grimshawi* and *Drosophila basisetae* (females only). Genes labeled in red demonstrate increased expression in *Drosophila grimshawi* and those marked in blue show high expression in *Drosophila basisetae*.

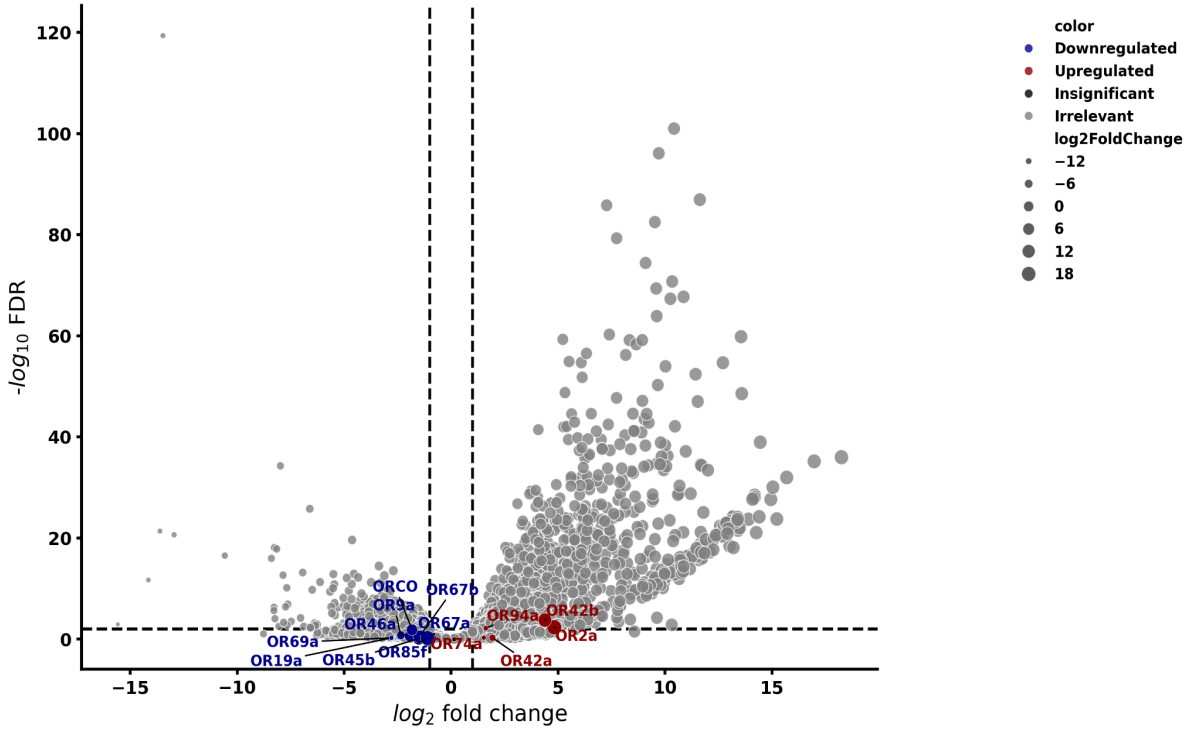


Figure 11. OR gene expression between *Drosophila grimshawi* and *Drosophila silvestris* (females only). Genes labeled in red demonstrate increased expression in *Drosophila grimshawi*, whereas those marked in blue indicate elevated expression in *Drosophila silvestris*.

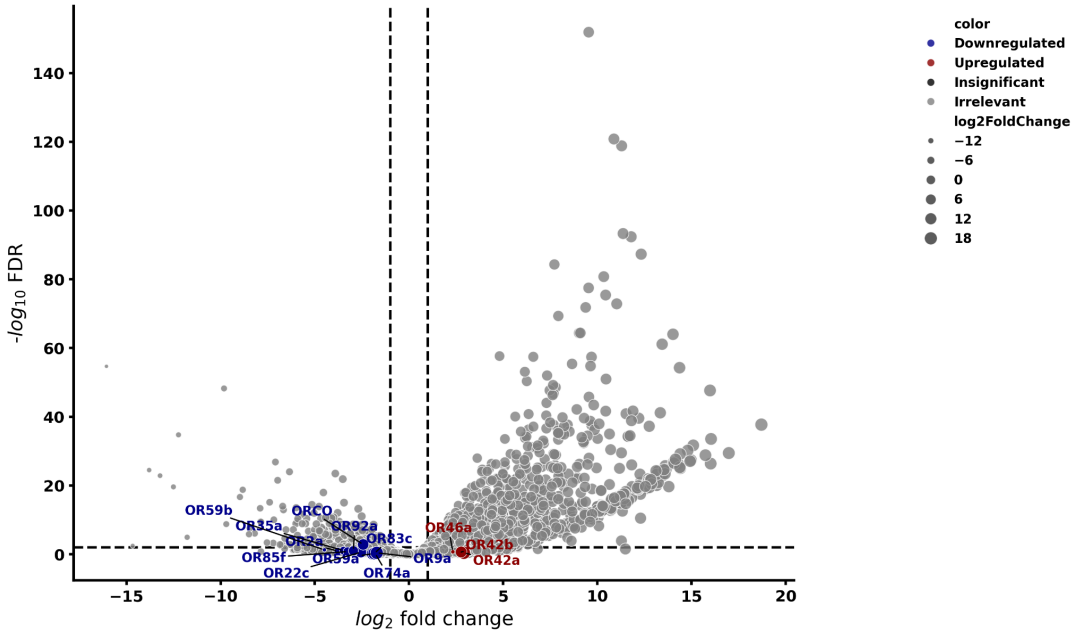
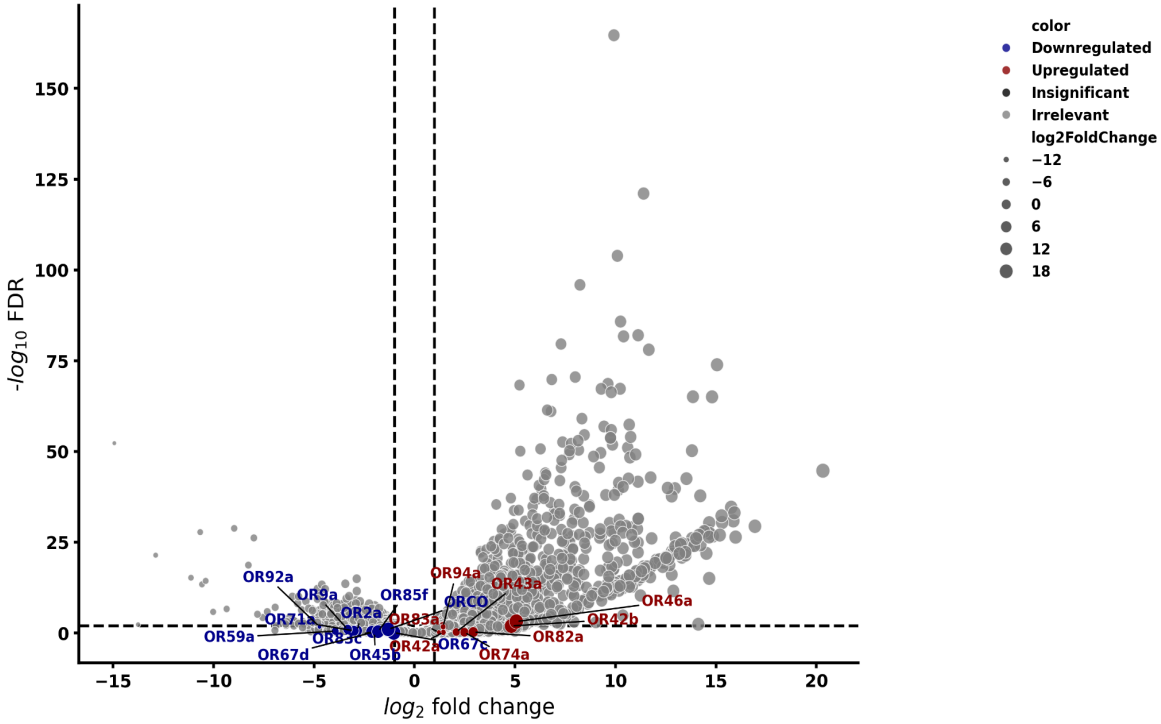


Figure 12. OR gene expression between *Drosophila grimshawi* and *Drosophila sproati* (females only). Genes labeled in red demonstrate increased expression in *Drosophila grimshawi* and blue dots display highly induced genes in *Drosophila sproati*.



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