ORN activity patterns in *Drosophila melanogaster* larvae elicited by ecologically relevant odorants

A thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in Neuroscience and the Honors Program

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May, 2016
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entitled

ORN activity patterns in Drosophila melanogaster larvae elicited by ecologically relevant odorants

be accepted in partial fulfillment of the requirements for the degree of

BACHELOR OF SCIENCE, NEUROSCIENCE

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May, 2016
Abstract

Most insects locate their food source primarily through olfaction. In Drosophila larvae, attraction and repulsion to environmental odorants are based on the activity of only 21 olfactory receptor neurons (ORNs). While a considerable amount of information has been generated regarding the ORN responses of worms, flies, and mammals to odorants, much less is known about their role in driving behavioral output. This gap in knowledge prevents the development of reliable odor coding models that can elucidate general principles of information processing, as well as instruct effective solutions for insect control. In this study, we examined the hypothesis that ecologically relevant attractive or repulsive odorants elicit specific patterns of ORN activity in the Drosophila melanogaster larva. To measure attractive or repellent odorants, a simple two-choice behavioral paradigm was used to test the behavioral response of wild type Drosophila melanogaster larvae to 54 odorants selected from its ecological habitat. Using this behavioral screen, a panel of 10 odorants that elicited the strongest attractive or repulsive responses in larvae was identified. This panel of odorants was then used to assess the response patterns among the 21 larval ORNs. For this, we expressed each larval odorant receptor in an in vivo expression system, the “empty neuron” system, and measured neural responses using single unit electrical recordings. At the test concentration, the panel of strong behavioral determinants elicited both excitatory and inhibitory responses from a variety of larval odor receptors expressed in the empty neuron system. Further, many of these receptor-odorant combinations exhibited varying response dynamics. Overall, our preliminary evidence suggests that ecologically relevant odorants elicit specific patterns of ORN activity. This study is significant because conserved patterns of sensory neuron activity may instruct downstream olfactory coding of behavioral valence. By comparing amplitude, temporal dynamics, and distribution of all 21 ORN responses, we aim to identify conserved patterns among sensory neuron activity elicited by attractants and repellents. The results from this study have the potential to impact development of more reliable odor coding models as well as to transform existing methods of insect control.

Keywords: olfaction, Drosophila melanogaster, chemotaxis, sensory perception
Acknowledgments

First and foremost, I’d like to thank Dr. Dennis Mathew for his continued enthusiasm and encouragement with this project. He has been kind enough to support me from the beginning and has offered exceptional insight. I’d also like to thank my family and friends for their optimism and polite attention to any and all frustrations I’ve had during the course of this project. Additionally, I give special thanks to the Honor’s Program and the Office of Undergraduate Research for the academic and financial backing of this project, specifically through the Honors Undergraduate Research Award. Finally, I’d like to thank Dr. Tamara Valentine for her attention to detail and extremely helpful edits.
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Human disease detection, insect-borne disease transmission, and global crop loss can be investigated in a tiny organism, the fruit fly. *Drosophila melanogaster*, or the common fruit fly, offers an ideal system of experimentation. Fruit flies are genetically, physiologically, and behaviorally simple, making the fruit fly an ideal organism to study neural circuits, or networks of neural connections. One of the most simple known neural circuits in the fruit fly is the olfactory circuit, which takes chemical odors in the environment and relays information to parts of the brain that relate to olfaction. The sensory processing aspect of this remarkably straightforward circuit has not been fully discovered, and understanding how the fruit fly processes olfactory information can be useful for areas of neuroscience, agriculture, epidemiology, and medicine. This project evaluates the role of peripheral sensory neurons in the olfactory neural circuit of the fruit fly larvae to better understand odor coding.

**Background**

The conventional model of sensory information processing in any organism begins with an environmental cue such as a chemical odorant, temperature, sound, or light acting as a sensory input. The sensory input is received via sensory receptor neurons, also called first-order neurons or peripheral neurons, and sensory information is transferred to higher neural centers to be processed. Higher neural centers, consisting of higher-order neurons, then transmit the information to motor output neurons to produce a behavior response, such as movement (Figure 1; Kreher, Mathew, Kim, & Carlson, 2008). This paradigm for sensory processing can be applied to olfaction in the *Drosophila melanogaster*. 
Figure 1. Diagram depicting sensory processing model for *Drosophila* larvae. Commonly accepted paradigm of sensory processing is via input>processing>output. In the case of olfaction, the sensory input is a chemical odorant being processed by olfactory receptor neurons, transmitted to the larval brain for additional processing, which, in turn, results in a behavioral output.

In *Drosophila* olfaction, an environmental chemical odorant binds to an odor receptor (Or) to induce an electrical response in an olfactory receptor neuron (ORN). The ORN acts as the first-order neuron to transmit the odorant information to higher-order neurons in the fly brain. The brain then conducts sensory neural processing in areas such as the antennal lobe and mushroom body and exports information to motor output neurons; the fly exhibits a behavioral response (Kreher, Kwon, & Carlson, 2005).
This system of sensory processing is numerically simple in the *Drosophila melanogaster* larva. In olfactory studies, the third instar larva is often used over the adult fruit fly due to easy behavior assays and conserved olfactory mechanisms (for overview of the fruit fly life cycle, see Appendix 1). The larva has 25 olfactory receptors, which are G protein-coupled receptors known to be used in a multitude of biological systems, expressed in neurons of the dorsal organ (Clyne et al., 1999). The dorsal organ contains 21 first-order ORNs, each expressing the co-receptor Orco, and one to two additional Ors (Fishilevich et al., 2005; Kreher et al., 2005). When a chemical odorant is exposed to the dorsal organ, it binds to one or many Ors. Binding of an odorant to the odor receptor initiates a series of signal transduction steps, which convert the chemical signal into an electrical signal in the ORN. Each of the 21 ORNs synapses to one of 21 glomeruli in the larval antennal lobe. These 21 glomeruli are morphologically unique and always receive the axons, the part of the neuron that outputs signals, of the same corresponding ORN (Ramaekers et al., 2005). Therefore, there is very little redundancy in larval olfaction. Each ORN-glomeruli pair corresponds with a single projection neuron (PN) that synapses with higher-order neurons for neural processing (Figure 2; Kreher et al., 2005). The result of this sensory input circuit is a consistent, compact, and linear ratio of Ors to ORNs to glomeruli to PNs (1:1:1:1 ratio), resulting in a neural path that is ideal for studying odor coding.
Figure 2. Depiction of the larval olfactory circuit in the dorsal organ. The larval olfactory system is a linear, 1:1:1:1 ratio of olfactory receptors to olfactory receptor neurons to glomeruli to projection neurons, depicted here with two of the 21 of each. The simple and linear system allows for little redundancy and mechanism conservation.

The simplicity of the larval olfactory neural physiology should not be confused with simplicity of function. The fruit fly larva is capable of responding to a large number of odorants with an array of different behaviors, likely by using a combinatorial code of ORN activation (Clyne et al., 1999). Experiments published in 2005 identified 25 Ors, of which 21 confer odorant responses, and identified the genes that encoded for the Ors, as well as for the confinement of odor receptors, excluding Orco, to single ORNs (Fishilevich et al., 2005; Kreher et al., 2005; Kreher et al., 2008). Such experiments demonstrated the limited diversity in larval Or expression, but, likewise, determined that each Or responds to binding chemicals with different breadths of responses, including tuning curves, activation, inhibition, and timing differences.

A previous study, which considered the activity of the entire repertoire of larval Ors, suggested that a small group of only five Ors is sufficient to predict 75% of larval olfactory behavior (Kreher et al., 2008). A similar study assessed a full panel of
physiological responses of ORNs to odorants and then assessed the behavior responses to 
the odorants that activated specific ORNs. The result was that some odorants strongly 
activated specific ORNs but did not have an equivalently robust behavioral response 
(Mathew et al., 2013). Multiple studies have found similar discrepancies between the 
strength of ORN activation and the strength of behavioral output (Fishilevich et al., 2005; 
Hallem & Carlson, 2006; Kreher et al., 2008). This discrepancy is the heart of my thesis 
project. Differences in ORN activation and behavioral output are not explained in the 
current literature. Understanding how behavior output to an odor relates to ORN 
activation would provide a better standard for ORN cross-activation and odor coding.

Most studies in larvae assessed odor coding by evaluating the electrophysiological 
response in ORNs to an odor, by measuring the electrical output resulting from 
chemically-activated ORNs, and then measuring the behavior response to that odor 
(Kreher et al., 2008; Mathew et al., 2013). To get a better understanding of the 
combination of ORNs that are responsible for strong behavioral responses, this project 
first aims to screen for odorants that elicit strong behavioral responses in larvae and then 
to determine the physiological responses elicited from larval ORNs by this panel of 
identified odorants. The rationale for starting with a strong behavioral response to an 
odorant and then assessing electrophysiological activation is that an odorant that induces 
a strong behavioral response may activate more than one ORN to generate behavior. This 
possible result also suggests that there is a level of cross-communication among 
individual ORNs in the olfactory circuit during odor coding. Such a systematic approach 
of understanding odor coding at the level of first-order neurons is crucial for developing 
more reliable models of olfactory information processing.
Significance of Research

The neural networks and molecular mechanisms found in *Drosophila melanogaster*, including the organization of odorant receptors and olfactory receptor neurons, are remarkably similar across most insects and other model organisms (Carey & Carlson, 2011). For example, 13 of the 25 larval Ors are also expressed in adult fruit flies, and the larval receptor Orco has direct homologues in mosquitoes, moths, mealworms, and the honey bee (Krieger et al., 2003; Larsson et al., 2004; Kreher et al., 2005). The method of odor coding used by larvae is similarly conserved across most adult fruit flies, other insects, and mammals (Clyne et al., 1999; Ressler, Sullivan, & Buck, 1994). Thus insect odor coding models can be applicable to more complex organisms. The *Drosophila melanogaster* larvae are therefore an excellent organism to conduct olfaction.

Worldwide, it is estimated that 18-35% of all food crops are lost to predatory insects (Oerke & Dehne, 2004; Pimentel, 1991). This global crop loss contributes to food insecurity experienced by 800 million people internationally, especially given that 60% of nutrients consumed worldwide come from only three major crops: wheat, rice, and maize (Pinstup-Anderson, Pandya-Lorch, & Rosegrant, 2001; Tilman, Cassman, Matson, Naylor, & Polasky, 2002). In Nevada, a large proportion of crops such as wheat and alfalfa are lost due to heavy predation and outbreaks of the Mormon cricket, *Anabrus simplex* (Macknet, Breazeale, Knight & Johnson, 2006). Like many insects, including moths, beetles, honey bees, and nematodes, the Mormon cricket has a remarkably similar olfactory circuit to *Drosophila*, allowing this research to have high relevance to the agricultural economy of the state of Nevada (Van Naters & Carlson, 2006). Studying insect olfactory behavior has significant implications for the possible prevention of crop
loss and thereby food shortages.

Beyond crop loss, insect olfactory systems have significant impacts on human disease transmission. Mosquito bites alone, specifically those of *Anopheles gambiae* and *Aedes aegypti*, are responsible for the spread of life-threatening diseases such as malaria, dengue, yellow fever, West Nile virus, Zika virus, lymphatic filariasis, and Japanese encephalitis (WHO, 2016). Per the World Health Organization (2016), these vector-borne diseases account for 17% of all infectious disease and cause 1-3 million deaths per year. Specifically, malaria causes more than 600,000 deaths per year, most of whom are children under 5 years old (WHO, 2016; Snow, Guerra, Noor, Myint, & Hay, 2005). Several studies have shown that mosquitoes locate their vector hosts through olfactory cues emitted by humans (Pates, Takken, Stuke, & Curtis, 2001; Smallengage, Qui, van Loon, & Takken, 2005). Given the lethal worldwide effect of mosquito predation, understanding olfaction has significant implications for disease transmission prevention (Van Naters & Carlson, 2006). The *Drosophila* olfactory system offers an easy, highly conserved mechanism to study mosquito olfaction. A study published in *Nature* in 2004 used the similarity between olfactory systems of fruit flies and mosquitoes to successfully express the female *A. gambiae* olfactory receptor protein AgOr1 in an adult *Drosophila*, offering avenues of future mosquito olfactory research (Hallem, Fox, Zwiebel, & Carlson, 2004c). The larval olfactory circuit is advantageous for studying olfaction as it has only 21 Ors, compared to 62 Or types in adult fruit flies (Clyne et al., 1999), ~400 Or types in humans (Zozulya, Echeverri, & Nguyen, 2001), and ~1,000 Or types in dogs (Quignon et al., 2005).

Understanding of the olfactory neural circuit, even in invertebrates, has
implications in human disease detection. Particularly, difficulty with odor discrimination and detection is a known early symptom of Alzheimer’s disease, and degree of deficit has been linked to the severity of dementia (Doty, Reyes, & Gregor, 1987; Murphy, Gilmore, Seery, Salmon, & Lasker, 1990). Further studies suggest that olfactory deficiencies may precede the onset of dementia in Alzheimer’s, and may correlate with risk of developing Parkinson’s disease (Devanand et al., 2015; Ross et al., 2008). Additionally, olfactory deficits have been consistently shown to correlate with schizophrenia and panic disorders, with severity of deficit positively linked to duration of illness (Moberg et al., 1997; Kopala & Good, 1996). The connection of olfactory deficits and other neural deficits, including correlation with severity and future onset, suggests that olfactory neurons are prone to the same pathology indicative of neurodegenerative disorders (Hawkes, 2003). Therefore, improved understanding of olfactory neural circuits has significant application to human neurodegenerative disorders and disease detection.

**Objectives and Hypothesis**

The overall goal of this research is to determine the contributions of olfactory receptor neurons to odor coding. In this context, the specific objective of this thesis research is to reveal the combinations of ORN activity that result in a strong attractive or repulsive response in the *Drosophila* larva. Accordingly, we hypothesize that odorants that elicit strong behavioral responses from the larva also elicit responses from specific combinations of ORNs. The rationale for this hypothesis is that some ORNs appear to be activated by attractants and inhibited by repellents, while other ORNs appear to be inhibited by attractants and activated by repellents (Hallem, Ho, & Carlson, 2004a;
Kreher et al., 2008).

To test our overall hypothesis and achieve the intended goals of the project, we propose the following two specific aims;

**Aim 1:** Identify strong attractant and repellent odorants. For this Aim, we will test a panel of approximately 50 odorants and identify the 10 strongest attractant and repellent odorants to fruit fly larva.

**Aim 2:** Determine the pattern of ORN activity elicited by the strongest attractants and repellents. For this Aim, we will test the response of each of the 19 larval odor receptors likely to elicit a response against the panel of 10 strongest attractants and repellents. We will then develop a heat map of ORN activity to search for patterns of activation that instruct strong attraction or strong repulsion.

The outcomes of this research would be a significant contribution to our understanding of sensory neuron activity to instruct attractive and repulsive behavior in animals.

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**Materials and Methods**

**Drosophila Stocks**

All behavioral experiments were performed on third instar larvae of either sex. A Canton-S (CS) line was used as the wild type line in behavioral experiments. Electrophysiological recordings were obtained from flies of either sex of the genotype $w;\Delta halo/\Delta halo;Or22a-GAL4/UAS-Or$. This genotype was obtained as F1 progeny from a cross between the genotype: $w;\Delta halo/\Delta halo;Or22a-GAL4$ flies and the genotype: $w;\Delta halo/\Delta halo;UAS-Or$. Different variations of the latter genotype were used to express
nineteen different larval Or in the adult antennal neuron (“empty neuron”) that lacks Or22a (Hallem, Ho, & Carlson, 2004b; Kreher et al., 2008; Mathew et al., 2008).

Odorants and Other Reagents

Odorants used in these studies were obtained at the highest purity available (see Appendix 2. List of 54 odorants by functional group and RI; ≥ 98% purity, Sigma-Aldrich Inc. St. Louis, MO). Each of them were diluted in paraffin oil (Sigma-Aldrich Inc. St. Louis, MO) for all studies. High purity Agarose (Apex Bioresearch product purchased from Genesee Scientific Inc.) gel was used to prepare the crawling surface for larvae during chemotaxis behavior experiments. 6mm filter discs (GE-Whatman) used in the behavior assays were purchased from VWR Inc.

Behavioral screen

Two-choice assay was conducted as described previously (Kreher et al., 2008; McKenna, Monte, Helfand, Woodard, & Carlson, 1989). 25µL of an odorant diluted to 10⁻² in paraffin oil (vol:vol) was added to a filter disc on one side of a 9-cm Petri dish and 25µL of the diluent (paraffin oil) was added to a filter disc on the opposite side. 35-50 third instar larvae were then placed in the center of the dish and allowed to migrate for five minutes in dark conditions, as larvae exhibit negative phototaxis (Sawin-McCormack, Sokolowski, & Campos, 1995). After five minutes, the number of larvae on each side of the plate were counted and the Response Index (RI) was calculated. The RI is an accurate measure of behavioral chemotaxis and is calculated by RI=(S-C)/(S+C), where S is the number of larvae on the half of the plate containing the experimental odorant and C is the number of larvae on the control side (Kreher et al., 2008). The RI for each experimental odorant was averaged across 10 trials (n=10). The resulting values
range from +1 to -1, where +1 indicates that all larvae migrated to the odorant side, whereas -1 indicates that all larvae migrated to the control side, or away from the odorant side. Therefore, a high RI, close to +1, implies a strongly attractant chemical odorant, and a low RI, close to -1, implies a strongly repellant chemical odorant.

To account for the effects of paraffin oil on response index, mean RI for paraffin oil was subtracted from each experimental odorant. To control for lingering location and odorant distribution effects, the side containing the experimental odorant dilution was altered with every trial. To control for circadian rhythm effects on geotaxis (Mazzoni, Desplan, & Blau, 2005), all trials were performed between 12:00PM and 5:00PM. As larvae are susceptible to environmental humidity conditions (Dillon, Wang, Garrity, & Huey, 2009), humidity was maintained at above 45%. Additionally, in order to exclude possible developmental effects on olfaction and mobility, only third instar larvae, aged 6 days from fertilization, were used for all trials.

**Electrophysiology**

Using the empty neuron system with a GAL4-UAS driver specific for each of the 19 larval Ors, electrophysiological recordings were conducted in response to each of the 10 odorants selected from the screening. To do take electrical recordings, a microscopic electrode was placed in a right-sided ab3A antennal sensilla of the adult fly, with a reference electrode placed in the left eye. The sensilla was then exposed to a 0.5 second stimulus, at 5.9ml/s with 24ml/s airstream, of either a control or experimental odorant.

For electrophysiological analysis, 50 μL of an odorant diluted to $10^{-2}$ in paraffin oil (vol:vol) was placed on Whatman 13-mm filter paper discs and inserted in Pasteur pipettes. These cartridges were prepared shortly before odor presentation and were never
Action potentials of the ORNs in a sensillum were recorded by placing an electrode through the sensillum wall into contact with the lymph that bathes the dendrites. The antennal surface was observed at 500x magnification, which allowed individual sensilla to be clearly resolved, through an Olympus BX51WI microscope. For the recording electrode, a tungsten electrode was used. For the reference electrode a glass capillary with the tip drawn to <1 μm diameter was filled with E&B solution (Kaisdling & Thorson, 1980) and slipped over an AgCl-coated silver wire. Signal from the recording electrode was led into a $10^{12} \Omega$ input impedance amplifier (IsoDam8A, WPI, Sarasota, FL), fed through a 100 Hz high-pass filter into Syntech IDAC-4 digitizer associated with the AutoSpike software (Syntech, Kirchzarten, Germany). Action potential spikes in response to two controls, spontaneous measure and paraffin oil, and 10 experimental odorants were recorded using AUTOSPIKE 3.0.

Action potential, measured as spikes/second, were averaged across all trials ($n=9$) with each fly limited to three trials. Only female flies aged 3-4 days from eclosion used. Recordings were analyzed offline using AUTOSPIKE 3.0 software. Only traces in which the activity of the different neurons in a sensillum could be separated on the basis of impulse amplitude. Responses of all neurons were quantified from a count of the number of impulses during the 0.5 s stimulus period.

To further assess neuron activation, mean spontaneous activation and mean activation due to paraffin oil diluent were subtracted from the mean spikes per second for each odorant/receptor pair. MATLAB routine was used to convert mean neuron activation to the format of a heat map as done previously (Mathew et al., 2013). To qualify activation and inhibition neural responses, excitatory odorant-ORN activation
response is defined as ≥100 spikes/s, while inhibitory response is classified as <50% of the spontaneous firing rate (Kreher et al., 2008). For temporal dynamic evaluation, MATLAB routine was used to graphically represent mean spikes/s over full 10 second recording period.

**Statistical Analysis**

**Statistics:** Statistical analyses were performed using IBM SPSS Statistics (IBM Corp., Armonk, NY). For assessment of correlation between chemical functional group and odorant RI, a non-parametric Kruskal-Wallis one-way ANOVA was conducted, given that RI values failed tests of normality.

**Results**

**Selection of odorants that are ecologically relevant for the Drosophila melanogaster larva**

In order to select odorants most likely to elicit a strong behavior response in *Drosophila* larvae, chemicals for behavior screen were selected based on their origin in the larva’s habitat, as well as chemicals that are known to elicit a previously strong response (refer to Appendix 2. List of 54 odorants by functional group and RI; Larsson et al., 2004; Fishilevich et al., 2005; Mathew et al., 2013). Initial selection included 10 odorants which previous studies found to be strong attractants to *Drosophila* larvae in the same two-choice response index. Ecologically relevant odorants were selected based on their origin in fruits known to be common as egg-laying sites for *Drosophila*, including bananas, apples, mango, and citrus fruits. An additional three odorants were included for their origin as a byproduct of fermentation given that *Drosophila* are attracted to
fermenting alcohols.

**Larvae show a range of behavioral responses to ecologically relevant odorants**

We have postulated that strong attractive odorants to *Drosophila* are chemicals derived from ecologically relevant substances, such as fruits and fermentation byproducts. Attractiveness of odorants to larvae was evaluated using the behavioral model of the two-choice assay.

Briefly, the two-choice assay is based on the premise that larvae migrate toward an attractive experimental odorant or away from a repulsive odorant, which can be quantitatively measured (Rodrigues, 1980; Khurana, Abubaker, & Siddiqi, 2009). Using this common model, two choice assays were conducted with each of the 54 chemical odorants. Two odorant discs were placed at opposite ends of the dish and loaded with $10^{-2}$ odorant dilution in paraffin oil and paraffin oil control (Figure 3).

**Figure 3. Two-choice behavioral assay to measure odor attractiveness.** 35-50 larvae are placed in the center of the petri dish and allowed 5 minutes to move toward or away from the experimental odorant. After five minutes, larvae are counted on each side of the plate and the Response Index (RI) is calculated as a measure of odorant attractiveness.
Ten trials were conducted for each odorant, totaling a minimum of 540 trials, not including control experiments of paraffin oil v. paraffin oil. Displayed in Figure 4, ordered from highest (top/left) to lowest (bottom/right) response index (RI). The strongest attractants included odorants known to have a previously strong larval response, such as propyl butyrate (RI=0.578±0.046), and several odorants with sources in banana, apple, and mango, such as 1-hexanol (0.427±0.075), propyl acetate (0.390±0.064), and ethyl butyrate (0.378±0.045). A significant odorant from this screen includes pentanal (-0.409±0.077), a previously unidentified strong repellent of larvae. This novel repellent is surprising given its presence in both apple and mango. Additionally, no previous strong repellents have been identified for larvae using a two-choice screen, making pentanal the first known repellent of *Drosophila* larvae. The next most repellent odorants, 1-pentanol (-0.145±0.084) and toluene (-0.130±0.068), elicit a much weaker repellent response compared to pentanal. Several odorants produced an insignificant behavior response, despite ecological relevance, such as ethanol (0.045±0.048). The highlighted odorants were selected for further assessment as strong inducers of behavior. Of the 54 odorants screen, 10 odorants were selected for electrophysiological assessment. Figure 5 lists these 10 odorants as well as their RI ranking, RI, functional group, and chemical structure. These 10 odorants were selected as they include those that elicited the top 7 attractive average RI (+0.578 to +0.349) and the top 3 repulsive average RI (-0.129 to -0.409).
Figure 4. Mean response indices of third-instar larvae to 54 ecologically relevant odorants. Using the two-choice behavioral paradigm, larvae were given the choice between an experimental odorant and paraffin oil for 5 minutes, after which larvae on each side were counted. Response index was averaged over all trials (n=10) and mean response to diluent, paraffin oil, was subtracted. If turned counterclockwise, the x-axis shows mean responses of 54 odorants, error bars represent the standard error of the mean.
Figure 5. Ten odorants from 54 odorant panel selected for strong behavioral response. These 10 odorants include the seven most attractive odorants (green) and the three most repellent odorants (red) selected to conduct electrophysiology on, with the intent of determining which ORNs are activated by these odorants.

**Esters elicit highest mean attractive responses, aldehydes elicit the highest mean repellent responses**

Given the similar origin of chemically attractant odorants, we postulated that the chemical composition, specifically functional group, may be relevant for larval RI. To assess this possibility, statistical analysis was performed to assess any correlation between RI and functional group of the chemical odorant. For full 54 odorant panel, response index appears to be correlated to functional group of the odorant (Figure 6). Given that RI distribution across multiple odorants did not pass tests of normality, a
nonparametric Kruskal-Wallis Test was conducted. Functional group and RI appear to be strongly correlated across all functional groups ($X^2=17.237$, df=5, Asymp. Sig.=0.004). Esters, such as propyl butyrate (0.578±0.046) and propyl acetate (0.390±0.064), appear more likely to elicit a strong attractive response, in addition to alcohols and ketones, such as 1-hexanol (0.427±0.075) and 4-hexen-3-one (0.349±0.060). In comparison, aldehydes, such as pentanal (-0.409±0.077) and acetaldehyde (-0.078±0.057), may be more likely to elicit a repellent response in larvae.

**Figure 6.** Mean response index for all 54 odorants grouped by chemical functional group. Statistical analysis confirms that mean response indices are strongly correlated to functional group via Kruskal-Wallis nonparametric one-way ANOVA. Esters, ketones, and alcohols result in higher mean response indices while aldehydes result in more negative mean response indices.
**Strong attractant and repellents elicit physiological responses from different larval odor receptors**

Following odorant screening, 10 odorants were selected for electrophysiological assessment. We hypothesized that the strongest attractants and the strongest repellent elicit conserved patterns of ORN activity. Based on data from other studies, we speculated that attractants were more likely to activate Or42a and Or85c, while repellents were more likely to activate Or7a and Or82a. To assess this hypothesis, electrophysiology was conducted on all 10 behaviorally relevant odorants. The purpose of electrophysiology is to determine which receptors are binding odorants to transmit information to higher order neurons. Receptor activation leads to the firing of an action potential, or electrical signal, in that neuron, which can be measured with single-unit electrophysiology. In this experimental paradigm, genetic tools are used to express larval olfactory receptors in place of an adult olfactory receptor in an ‘empty neuron’ system (Dobritsa, van der Goes van Naters, Warr, Steinbrecht, & Carlson, 2003; Hallem et al., 2004a). In this genetically constructed system, an Or22a-GAL4 driver is paired with a UAS promoter sequence specific for one of the 21 larval Ors on the third chromosome. When coupled with a Δhalo deletion mutant, which removes the adult Or22a without removing the Or22a promoter, the result is ectopic expression of a larval Or in place of adult Or22a in the ab3A antennal neuron (genetic expression diagrammed in Figure 7). Or2a and Or49a were eliminated from the screening as an extensive previous assay found no response to any of 479 broadly selected odorants (Mathew et al., 2013).
Figure 7. Diagram of empty neuron system. Genetic system to express a larval olfactory receptor in the ab3A adult neuron. In this system, a Δhalo mutant deletes the adult Or22a receptor expressing gene. Then, the GAL4 Or22a promoter is paired with a UAS OrX gene, where OrX is the gene to express one of 19 primary larval receptors in place of Or22a. Figure adapted from Hallem, Ho, & Carlson, 2004b.

Briefly, electrical recordings of from each of the Ors were recorded from the right-sided ab3A neuron by pinning the antenna of a genotypically correct adult female fruit fly. A reference electrode was placed in the left eye and a recording electrode was placed in an ab3 sensilla on the left antenna (Figure 8). Electrical response of the ORN was measured in spikes/second.

Figure 8. Electrophysiology recordings. In order to record the electrical signal from an ORN expressing a larval odorant receptor via the empty neuron system, an adult fly with the GAL4-UAS genotype, is pinned with only the antenna and eye exposed. A reference electrode is placed in the left eye, and a recording electrode is placed in the ab3A neuron expressing the larval Or. Electrical signals are recorded and analyzed in response to odorant exposure. ab3A neuron diagram adapted from Shanbhag, Muller, & Steinbrecht, 1999.
Results of ORN activation obtained from electrophysiological experiments can be displayed as a heat map, as demonstrated in Figure 9, in which each colored rectangle represents the average spikes/second of the receptor listed on the x-axis in response to the odorant listed on the y-axis (See Appendix 3 for raw numerical values). We hypothesized that most attractants would strongly activate specific olfactory receptors or repress specific receptors to generate attractive behavior. This does not appear to be the case as some attractants such as 2-hexanol moderately activate several ORNs but does not strongly activation any specific one. Alternatively, the strongest attractant identified in the behavior screen, propyl butyrate, appears to repress many ORNs and does not strongly activate any ORNs. These data would indicate that attractive odorants may generate behavior in first order neurons via a method of additive activation of multiple ORNs. Additive activation may not be necessary for all attractant odorants though, as Or33b, Or74a, and Or85c are activated by 4 of 7 attractant odorants, which suggests that these receptors may play a role in attractant behavior. When assessing repellent odorants, Or7a does not respond to most attractant odorants or is significantly repressed by them, but is moderately activated by two repellent odorants, 1-pentanol and pentanal. As a result, Or7a could be significantly responsible for repellent behavior.
Figure 9. Heat map visualizing ORN activation in response to 10 behaviorally relevant odorants. Using electrophysiological recordings and genetic systems, activation of an ORN expressing each of 19 larval Ors in response to 10 odorants is visualized in form of a heat map. Each value represents the average ORN response to exposure of an odorant for 0.5s measured in spikes/second ($n=9$). Chemical odorants are listed with greatest attractiveness at the top (green dots) and most repellent at the bottom (red dots). Some Ors, such as Or33b and 74a appear to generally be activated by attractants such as ethyl butyrate, while other Ors, such as Or7a appear to be generally repressed by attractant odorants and activated by repellent odorants.

The strongest attractants and repellents elicit characteristic response patterns among larval Ors

A major aim of this project was to identify patterns of activation or repression of ORNs that are conserved across attractant or repellent odorants. To assess this goal, we established a threshold value of >50 spikes/sec for activation. Repression threshold was defined as spikes/s that are less than half of the spontaneous firing rate for that ORN. Using these threshold values for activation and repression, an alternate heat map was
developed to examine any patterns conserved between attractants and repellents (Figure 10). Toulene, the weakest repellent used for electrophysiology, appears to significantly repress the most ORNs and only significantly activates Or30a, which is not activated by any other odorants. Interestingly, the strongest attractant identified for larvae, propyl butyrate, does not appear to activate any olfactory receptors at this concentration, but appears to repress multiple olfactory receptors such as Or7a, Or22c, Or45b, and Or67b. This could indicate that strong activation of an ORN is not necessary to produce a behavior response. However, other attractants appear to activate at least 1-2 olfactory receptors. For example, propyl acetate activates both Or47a and Or85c, which have been previously indicated as attractive olfactory receptors, while ethyl butyrate instead activates Or33b and Or85c. These characteristic responses indicate that each attractant and repellent instructs larval behavior with different patterns of ORN activity.
We hypothesized that patterns of olfactory receptor responses may be similar among attractants or repellents to indicate responsibility for larval behavior. While characteristic responses appear likely for individual odorants, we were unable to identify any conserved patterns of activation or repression. While a conserved pattern may be revealed with alternate threshold values or analyses, no identifiable pattern indicates that larval behavior may be instructed by other components of the olfactory neural network.
Patterns of response dynamics elicited by the strongest attractants and repellents

Activation and repulsion as measured for a heat map does not necessarily convey complete information for ORN activity in response to an odorant. A heat map assessment shows only activation or repression for the 1 second following stimulus odorant exposure, but does not show information for the full 10 seconds of recording, which may hold important information. To determine if additional information is available, visualization of all 10 seconds of recording was completed for select Or-odorant pairs. For example, Or22c clearly responds to propyl butyrate with ORN repression, as demonstrated by both temporal dynamics and heat map. However, the heat map shows significant activation of Or7a in response to pentanal, while temporal assessment shows said strong activation followed by long term repression compared to spontaneous readings. Figure 11 shows activation of the ab3A neuron expressing Or33b and Or7a in response to ethyl butyrate and pentanal, respectively. Activation is seen by an increase number of larger spikes compared to neuron activation at rest. In the near future, response dynamics of several odorant-receptor combinations will be considered using a method similar to Mathew et al. These differences in activation patterns may reveal additional conserved patterns of activation or repression for attractants and repellents.
Figure 11. Time-based assessment of ORN activation for two Ors. Temporal assessment of Or7a and Or35a to pentanal, a behavioral repellent. Pentanal exposed to Or7a shows strong activation followed by repression, while exposure to Or35a shows strong activation followed by slow decline to spontaneous levels. Spontaneous responses to both Ors visualized below pentanal exposure spikes.

Discussion

Major conclusions

The major conclusion of our study is that the strongest attractive or repulsive odorants do not elicit conserved patterns of activity among the repertoire of sensory neurons of the Drosophila larva. This conclusion is based on the following experimental evidence. First, using a behavioral screen, we identified a sub-panel of strong attractants and repellents from a larger panel of 54 odorants that are considered to be ecologically relevant to Drosophila melanogaster. Next, using electrophysiology techniques, we
tested the response of 19 of the 21 Drosophila larval odor receptors to the panel of strong attractants and repellents. Finally, using a series of data analysis strategies, we demonstrated that although each strong attractant or repellent elicits a characteristic pattern of activity among the panel of odor receptors, no conserved patterns were obvious. Collectively, our experimental evidence strongly supports the overall concept that a conserved patterns of sensory neuron activity is not required to determine behavioral valence.

**Conclusions in the context of available literature**

The outcome of this project contributes to the overall understanding of odor coding in the field of Drosophila larvae olfaction. Specifically, this study confirms the functional group significance for larval behavioral responses. Additionally, our evidence supports the combinatorial code of odor coding for attractive and repellent odorants, given that there does not appear to be a definitive pattern of activation or repression. This changes our understanding of odor coding and allows the field to move forward in assessing other aspects of the olfactory system, such as projection neuron activation, and how it may contribute to behavioral predictions. Advancement in this area brings up the ability for better odor coding models and predictive models for other systems.

Furthermore, we have identified strong attractant and repellent odorants for larvae, a previously unidentified strong repellent, and the associated olfactory receptor activity patterns, providing new information for the understanding of larval odor coding.

Parts of the present study have been conducted by previous researchers, with fairly similar results. Thus far, the most significant discrepancy in our findings relate to 1-pentanol. This odorant has been studied in two previous experiments and is previously
noted to be highly variable in eliciting a behavioral response (Kreher et al., 2008). This would explain differences in response index, which was positive in recent experiments (Mathew et al., 2013), but exhibits a negative response index in this study. This variability may also help explain differences in ORN activation to 1-pentanol, wherein this study finds 1-pentanol to activate Or35a at ~50 spikes/sec at a concentration of $10^{-2}$ compared to the previously assessed ~200 spikes/second at a concentration of $10^{-4}$.

Differences in ORN activation to previous studies may also be inherent to the nature of ORN activation. A 2007 study found that ORN activation is significantly more difficult to reproduce than projection neuron activation (Bhandawat, Olsen, Gouwens, Schlief, & Wilson, 2007). These discrepancies may be linked to inherent variability of the system.

We find that the strongest attractive odorants are more likely to be esters, alcohols, or ketones, while the strongest repellent odorants were most likely to be aldehydes. While the significance for this is not clear, it is known that Drosophila larvae thrive on rotting or fermenting parts of fruits and plants that are particularly enriched in esters and alcohols (Christenson & Foote, 1960). We have also identified a novel repellent, pentanal, of the Drosophila larvae, the first strong ecologically relevant repellent yet identified for larvae.

In this study, we find that the strongest attractant, propyl butyrate, does not strongly activate any olfactory receptor. This suggests that activation of an olfactory receptor alone may not be necessary to produce an attractive behavior. Such a conclusion is contrary to what is currently accepted in the field (Hallem et al., 2006; Kreher et al., 2008; Mathew et al., 2013).
Limitations of the present study

We acknowledge limitations with certain conceptual and experimental approaches in this study. Given limitations in time, for both behavioral assays and electrophysiology assays, only one experimental odorant concentration was used. As previous literature suggests influences of odorant concentration is influential in both the two-choice assay, and electrophysiology, additional dilutions would provide more information on specific ORN activation for the most robust response index (Hallem et al., 2004a; Kreher et al., 2008; Mathew et al., 2013). For a more complete view of ORN response necessary for behavior, ORN activation by more behaviorally relevant odorants should be tested. To confirm the necessity of ORN activation for behavioral responses, selective ablation of ORNs which are activated or repressed by an odorant should be tested in a two-choice assay. Furthermore, ORN activation assessment would likely benefit from calcium-imaging confirmation and assessment of corresponding projection neuron activation. Without these supplementary examinations, a complete examination of odor coding in response to attractants cannot be stated definitively.

We also acknowledge limitations in certain conclusions of our study. While it is clear that we could not observe any patterns of activity among the firing of sensory neuron, we have not considered the temporal dynamics of their firing. Nor have we considered variations of stimulus dynamics in this study. We acknowledge that an odorant is presented as an odor gradient in the behavior assay while it is presented as a 500 millisecond puff in the electrophysiology assay. Although commonly used as a stimulus method in insect and worm olfaction studies (Fernandez-Grandon et al., 2015; Monte et al., 1989; Rodrigues & Siddiqi, 1978; Spathe et al., 2013), different odor
gradients could elicit different levels of odor adaptation that could complicate results. We note that, unlike an adult fly, a fly larva that is normally found immersed in its natural food source has to navigate a mixture of odor gradients. Thus, use of odor gradients in our behavior assays has ecological relevance. While it was convenient to test similar concentrations and gradient strengths for all odorants in this study, it was more difficult to compensate for differences in physicochemical properties of the odorants, such that, for each odorant, an equivalent number of molecules reached the larval dorsal organ (Andersson, Schlyter, Hill, & Dekker, 2012; Martelli, Carlson, & Emonet, 2013). Thus, our results describe responses to standard dilutions of odorants and not to defined number of odorant molecules accessible to each ORN. With recent advances in optogenetic techniques, it would be possible to precisely activate only single ORNs and also control for the strength and duration of neuronal stimuli (Hernandez-Nunez et al., 2015).

Our study was restricted to the first order sensory neurons of the Drosophila larva. A lack of an obvious pattern among this set of neurons do not necessarily indicate a lack of activation pattern in the second order sensory neurons. Further investigations would be required to make this determination.

**Final conclusions**

Within its ecological niche, a larva has to navigate multiple odor gradients to reach high quality food sources. Odorants in the larva’s environment activate one or more of its ORNs. Overall, our results suggest that a conserved firing pattern among first order sensory neurons in the Drosophila larva is not required to determine behavioral valence. Our analyses of activity patterns among larval sensory neurons have implications for improving existing models of odor coding and for elucidating how insect vectors of
disease locate their human or plant hosts.
References


http://dx.doi.org/10.1212/WNL.0000000000001132


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Quignon, P., Giraud, M., Rimbault, M., Lavigne, P., Tacher, S., Morin, E., ... & Galibert,


doi:10.1093/chemse/bji010


doi: 10.1371/journal.pone.0077135


Appendices

Appendix 1. Life cycle of the fruit fly, *Drosophila melanogaster*. Olfactory circuit research focuses on the third instar larva, which has easily studied behavior and a simple olfactory neural circuit.
Appendix 2. List of 54 odorants grouped by RI ranking and functional groups. All 54 odorants were selected for screening due to their likely ecological relevance to the *Drosophila* larva, or if they were known previously to induce a behavioral response. Left hand side groups odorants by functional group and within functional group, by decreasing response index.

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<th>RI ranking</th>
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<th>Larval Behavior Relevance</th>
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<td><strong>Alcohols</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1-hexanol</td>
<td>Banana, apple, mango$^{1,3,4}$</td>
</tr>
<tr>
<td>5</td>
<td>2-hexanol</td>
<td>Previous larval response$^6$</td>
</tr>
<tr>
<td>9</td>
<td>2-pentanol</td>
<td>Banana$^1$</td>
</tr>
<tr>
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<td>1-heptanol</td>
<td>Mango$^4$</td>
</tr>
<tr>
<td>19</td>
<td>1-octen-3-ol</td>
<td>Banana, mango$^{1,3}$</td>
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<tr>
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<td>3-octanol</td>
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</tr>
<tr>
<td>29</td>
<td>1-butanol</td>
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<td>35</td>
<td>1-propanol</td>
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</tr>
<tr>
<td>36</td>
<td>ethanol</td>
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</tr>
<tr>
<td>39</td>
<td>(-)-linalool</td>
<td>Ripening fruit, mango$^{2,4}$</td>
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<td>41</td>
<td>3-hexanol</td>
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</tr>
<tr>
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<td>methanol</td>
<td>Previous larval response$^6$</td>
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<td><strong>Aldehydes</strong></td>
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<td>benzaldehyde</td>
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<tr>
<td>46</td>
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<tr>
<td>47</td>
<td>octanal</td>
<td>Apple, mango$^{3,4}$</td>
</tr>
<tr>
<td>49</td>
<td>acetaldehyde</td>
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<tr>
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<td>pentanal</td>
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<td><strong>Esters</strong></td>
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</tr>
<tr>
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</tr>
<tr>
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<td>butyl acetate</td>
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<td>butyl butyrate</td>
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<td>ethyl 3-propionate</td>
<td>Pineapples, kiwis, strawberries$^7$</td>
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<td>isoamyl acetate</td>
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<td>21</td>
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</tr>
<tr>
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<td>pentyl acetate</td>
<td>Apple$^3$</td>
</tr>
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</tr>
<tr>
<td>28</td>
<td>methyl benzoate</td>
<td>Mango⁴</td>
</tr>
<tr>
<td>31</td>
<td>hexyl butyrate</td>
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</tr>
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<td>34</td>
<td>methyl hexanoate</td>
<td>Mango⁴</td>
</tr>
<tr>
<td>51</td>
<td>octyl acetate</td>
<td>Mango, oranges, grapefruits, and other citrus⁴</td>
</tr>
<tr>
<td>50</td>
<td>anisole</td>
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**Ketones**

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<tr>
<td>6</td>
<td>4-hexen-3-one</td>
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<td>8</td>
<td>2-heptanone</td>
<td>Mango⁴</td>
</tr>
<tr>
<td>13</td>
<td>3-octanone</td>
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</tr>
<tr>
<td>15</td>
<td>2-octanone</td>
<td>Mango⁴</td>
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<tr>
<td>16</td>
<td>2,3-butanedione</td>
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<tr>
<td>20</td>
<td>acetophenone</td>
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<td>24</td>
<td>2-butanone</td>
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<tr>
<td>32</td>
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<td>33</td>
<td>cyclohexanone</td>
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<td>40</td>
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**Terpene-derived**

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<td>37</td>
<td>L-carvone</td>
<td>Smell of spearmint, mango⁴</td>
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<tr>
<td>43</td>
<td>α-terpinene</td>
<td>Cardamon oil, synthetic flavoring, mango⁴</td>
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<tr>
<td>44</td>
<td>s-limonene</td>
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</tr>
<tr>
<td>48</td>
<td>r-limonene</td>
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**Other Functional Groups**

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<tr>
<td>23</td>
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<td>ethylbenzene (aromatic)</td>
<td>Mango⁴</td>
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<tr>
<td>26</td>
<td>acetic acid (carboxylic)</td>
<td>Active ingredient in vinegar; byproduct of fermentation¹⁰</td>
</tr>
<tr>
<td>52</td>
<td>toluene (aromatic)</td>
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## Odorant Activation of Larval Receptors

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<tr>
<th>Odorant</th>
<th>7a</th>
<th>13a</th>
<th>22c</th>
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<th>30a</th>
<th>33b</th>
<th>35a</th>
<th>42a</th>
<th>42b</th>
<th>45a</th>
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<td>6±5</td>
<td>10±6</td>
<td>18±5</td>
<td>16±4</td>
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<td>1±3</td>
<td>33±13</td>
<td>20±6</td>
<td>23±4</td>
<td>42±11</td>
<td>8±5</td>
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<td>14±7</td>
<td>37±15</td>
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<td>8±4</td>
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<td>1-pentanol</td>
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<td>21±14</td>
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<td>63±13</td>
<td>10±7</td>
<td>42±10</td>
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<td>6±6</td>
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<td>42±18</td>
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### Additional Odorant Data

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<th>74a</th>
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### Appendix 3. ORN Activation of Each Larval Or from All 10 Tested Odorants

Heat map is derived from spikes/second averaged over 9 trials. Heat map color values are derived from this set of numerical data, extracted in spikes/second from electrophysiological recordings.