

# The role of odor receptor protein Or22a in *Drosophila melanogaster* oviposition and the response spectra of chimeric receptor protein Or22a-b

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## Abstract

The fruit fly *Drosophila melanogaster* has a well-studied olfactory system. A crucial part of the olfactory system is the olfactory sensory neurons (OSNs). One type of these OSNs, Ab3a, expresses the Odor receptors (ORs) Or22a and Or22b. Or22a is known to play an important role for behaviors like oviposition, but no strong ligand has been found for Or22b. Recent studies have shown that *D. melanogaster* prefers to oviposit on marula fruit. The volatile in marula fruit that is believed to attract the flies is called ethyl isovalerate. A strain of *D. melanogaster* that expresses a chimeric olfactory receptor Or22a-b has also recently been found. We wanted to find out if the Ab3a neurons respond to ethyl isovalerate and how the expression of Or22a-b affects the response spectrum of the neuron. We also wanted to find out if Or22a is a key factor for the preference for oviposition on marula fruit in *D. melanogaster*. Therefore, we have performed single sensillum recordings (SSR) to find out if *D. melanogaster* responds to ethyl isovalerate, and if the expression of Or22a-b instead of Or22a and Or22b affects the response spectrum of the Ab3a neurons. To see if Or22a affects the oviposition choice of the flies, we have performed a behavioral assay. We found that the Ab3a neurons in *D. melanogaster* respond to ethyl isovalerate. The expression of Or22a-b does change the response spectra. The flies that express Or22a-b exhibit lower response to ethyl hexanoate, and higher response to ethyl isovalerate than flies that express Or22a and Or22b in the Ab3a neurons. In conclusion, we have found that the Ab3a neurons in *D. melanogaster* respond to ethyl isovalerate, and the responsible OR for this response is probably Or22a, since Or22b has no known function. Flies that carry a mutation in the *Or22a* gene exhibit a different response spectrum than wild type flies. Or22a is an important OR for the finding of oviposition sites.

## Introduction

The fruit fly *Drosophila melanogaster* is a common model organism in physiology and genetics. The fly is an excellent model organism for studies of the olfactory system because their nervous system is easy to access, and smaller compared to vertebrates. A long history of genetic research in *D. melanogaster* has also led to both understanding of the genome and techniques to manipulate the genetics of the fly (Stocker, 1994, Carlson, 1991).

Insects and vertebrates have a well-developed sense of smell. The diversity and magnitude of the olfactory system is enabled by the expression of many odor receptors (ORs).

*D. melanogaster* expresses just over 60 different ORs (Drosophila Odorant Receptor Nomenclature Committee, 2000, Montagné et al., 2015, Robertson et al., 2003), and some of them can bind many different ligands, i.e. odors (Hallem and Carlson, 2006). In the study presented in this article, the focus has been on the OR Or22a, one of many ORs that recognizes odors that are categorized in the chemical group of esters (Pelz et al., 2006).

## **Organization and physiology of the insect olfactory system**

The peripheral olfactory system of *D. melanogaster* is located on the third antennal segment, also called the funiculus, and on the maxillary palps, situated on the mouth parts. These sensory organs are densely covered by hair shaped structures called sensilla. There are four types of sensilla on the surface of the funiculus, defined by their morphology: basiconic sensilla, trichoid sensilla, coeloconic sensilla and intermediate sensilla and they are all olfactory (Venkatesh and Singh, 1983, Clyne et al., 1997, Stocker, 1994, Shanbhag et al., 1999). The only sensilla type of interest in this study is basiconic sensilla, and therefore trichoid and coeloconic sensilla will not be accounted for any further.

Basiconic sensilla can be divided into large, small and thin basiconic sensilla (Martin et al., 2013). In *D. melanogaster*, basiconic sensilla can be found on both the maxillary palp and the antenna. Because of their different locations and response spectra, the two are often separated in text by different abbreviations. Basiconic sensilla on the maxillary palp are abbreviated Pb, and antennal basiconic sensilla are abbreviated Ab (Venkatesh and Singh, 1983, de Bruyne, 1999, Hallem and Carlson, 2004).

Each basiconic sensilla is built up by the hair, sheath cells and olfactory sensory neurons (OSNs) (Venkatesh and Singh, 1983). How many OSNs a sensillum has depends on the type of sensillum. Large basiconic sensilla have 2 or 4 OSNs (de Bruyne et al., 2001, Stocker, 1994). This was discovered in electrophysiology experiments, where spontaneous activity could be observed. The activity, in form of action potentials, triggered from single sensilla can be visualized as spike amplitudes. When recording the spontaneous activity of large Ab sensilla, some Ab sensilla exhibited spike amplitudes that could be divided into two populations. The conclusion drawn from these results was that these sensilla contain two neurons. The neuron that exhibited the highest spike amplitude was decided to be referred to as the “A neuron” and the neuron that exhibited the smallest spike amplitude is referred to as the “B neuron”. Some sensilla exhibited spike amplitudes that could be divided into four populations. These sensilla were assumed to contain four neurons, decided to be referred to as the “A”, “B”, “C”, and “D” neurons based on the height of their spike amplitudes. The sensilla with two OSNs could be divided further into two types, based on which odors the OSNs in the sensillum responded to. In the first group of sensilla, the A neuron responded to ethyl acetate and the B neuron responded to hexanol. In the other group of sensilla, the A neuron responded to ethyl butyrate and pentyl acetate, and the B neuron responded to heptanone and hexanol. The sensilla with four OSNs were named Ab1. The one that responded to ethyl acetate and hexanol were named Ab2. Lastly, the one that responded to ethyl butyrate, pentyl acetate, heptanone and hexanol were named Ab3 (de Bruyne et al., 2001).

The response spectrum of a sensillum depends on which OR the OSNs of the sensillum express. (Hallem et al., 2004). Typically, each type of olfactory sensory neuron expresses one type of OR. However, there are exceptions (Goldman et al., 2005, Vossell et al., 1999, Ray et al., 2008). The axons of OSNs that express the same OR converge to form distinct glomeruli in a part of the deutocerebrum called the antennal lobe (Gao et al., 2000). These glomeruli constitute a map of odors detected by the sensilla in the antenna, and the information provided when action potentials travels from OSNs activated by an odor and activate a glomerulus can then be interpreted by higher centers in the brain (Rodrigues, 1988).

Here, the information is processed and affect memory, learning and decision-making (Tully and Quinn, 1985, Busto et al., 2010).

Odors that elicit high responses in the OSNs either attract or repulse the flies, which is an indication that *D. melanogaster* uses the olfactory sensory system to find food, but also to detect if the food is of suitable quality or not (Stensmyr et al., 2003). Further, it is known that genetic changes of the olfactory system can lead to changes in behavior in *Drosophila spp*, like attraction or repulsion to certain odors (Richgels and Rollmann, 2011).

### **Function of Or22a**

Or22a, the OR of interest in this study, is expressed in one of the two OSNs of the Ab3 sensillum, called Ab3a. Or22a was first found to respond to ethyl butyrate in electrophysiology experiments (Dobritsa et al., 2003). However, in 2006, ethyl hexanoate and methyl hexanoate proved to elicit the strongest response in Or22a and ethyl hexanoate is today considered the strongest ligand of Or22a (Pelz et al., 2006, Hallem and Carlson, 2006). In total, more than 25 compounds elicit easily detected responses in Or22a (<http://neuro.uni-konstanz.de/DoOR/default.html>). Or22b is co-expressed with Or22a in Ab3a, but no strong binding ligand has been found for Or22b (Dobritsa et al., 2003).

Esters detected by Or22a are typically found in fruit. Methyl hexanoate can be found in many fruits such as grapes, melon, raspberry, blackberry and plum ([https://pubchem.ncbi.nlm.nih.gov/compound/methyl\\_hexanoate#section=Top](https://pubchem.ncbi.nlm.nih.gov/compound/methyl_hexanoate#section=Top)). Ethyl hexanoate can also be found in many fruits. (<https://pubchem.ncbi.nlm.nih.gov/compound/31265>). Both methyl hexanoate and ethyl hexanoate are described to have a fruity odor, similar to pineapple ([http://gestis-en.itrust.de/nxt/gateway.dll/gestis\\_en/032110.xml?f=templates\\$fn=default.htm\\$3.0](http://gestis-en.itrust.de/nxt/gateway.dll/gestis_en/032110.xml?f=templates$fn=default.htm$3.0)). (<http://www.thegoodscentscompany.com/data/rw1008741.html>, Pelz et al., 2006)

### **D. melanogaster prefers marula fruit**

Oviposition is also influenced by olfactory stimuli. For instance, *D. melanogaster* prefers to lay eggs in citrus fruits. This is not only because the terpene limonene, an odor characteristic for *citrus spp*. acts as an oviposition attractant to the flies, but it also stimulates egg-laying. The receptor for limonene is Or19a (Dweck et al., 2013).

Recent studies performed with a two- choice assay has revealed that *D. melanogaster* prefers marula fruit over citrus as oviposition site. One of the most abundantly present volatile in marula fruit is an ester called ethyl isovalerate. The presence of ethyl isovalerate in marula fruit was found with the help of gas chromatography- mass spectroscopy (pers comm. Marcus Stensmyr. 16 November 2017). However, it was already known that ethyl isovalerate is present in marula fruit (Ekesi et al., 2016) Since ethyl isovalerate is abundant in marula but not in many other fruit, it was suspected that ethyl isovalerate is the main component in marula that attracts the flies and makes them choose to oviposit in marula instead of orange. A T- maze assay showed that *D. melanogaster* is attracted to ethyl isovalerate, and are more attracted to ethyl isovalerate than limonene (pers comm. Marcus Stensmyr. 16 November 2017).

It was desired to find a receptor for ethyl isovalerate. Ethyl isovalerate is an ester, thus its receptor should be one that is known to respond to esters. Or22a was judged a good candidate, because it responds to esters (Pelz et al., 2006) and has been shown to play an important role

in oviposition in other fruit fly species. In *D. melanogaster*'s close relative *Drosophila sechellia*, the homolog to Or22a exhibits a much stronger response to methyl hexanoate than Or22a in *D. melanogaster*. The change in olfactory response spectrum of Or22a is reflected in the oviposition behavior. *D. melanogaster* is a generalist, but *D. sechellia* oviposit exclusively on morinda fruit (Dekker et al., 2005). A similar phenomenon can be found when comparing oviposition of *D. melanogaster* to another close relative, *Drosophila erecta*. *D. erecta* oviposits on pine screw cones, and SSR experiments from Ab3a neurons in *D. erecta* show an increased sensitivity towards 3- methyl-2-butenyl acetate, an odor found in abundance in pine screw cones, compared to other *Drosophila spp.* It was also found that 3- methyl-2-butenyl acetate stimulates oviposition in *D. erecta*, but the same effect could not be seen in *D. melanogaster* (Linz et al., 2013).

Or22a was tested as a potential receptor for ethyl isovalerate by expressing the calcium sensor GCaMP in the DM2 glomerulus (the glomerulus connected to the Ab3a neurons) (Couto et al., 2005). Increased fluorescence was observed in the DM2 glomerulus when the fly was exposed to ethyl isovalerate, which indicates that a receptor expressed in Ab3a neurons respond to ethyl isovalerate (pers comm. Marcus Stensmyr. 16 November 2017). Since Or22b has been speculated to be an evolutionary rest (Dobritsa et al., 2003), the assumption is that the response is elicited by Or22a. Therefore, in this study, the response to ethyl isovalerate was further investigated with a single sensillum recording (SSR) experiments.

Recently, a strain of *D. melanogaster* with a chimeric chemoreceptor gene of *Or22a* and *Or22b*, which here will be called Or22a/b was found in Rwanda (Aguadé, 2009). Since Or22a is used to detect odors present in fruits, and *D. melanogaster* oviposits in fruit, and since the response spectrum of Or22a has been shown to be important for egg- laying, Or22a in *D. melanogaster* can be assumed to have an essential role in finding oviposition sites. Thus, the expression of the Or22a-b chimeric protein could potentially change the response spectrum of Or22a and thus possibly the preference of oviposition sites. Consequently, the response to ethyl isovalerate and ethyl hexanoate of Ab3a of the Rwanda flies (RG18N) was also tested in SSR experiments, to investigate any difference between strains expressing Or22a and strains expressing Or22a-b.

To test the effect of ethyl isovalerate on oviposition, a two-choice assay was performed with flies where the function of Or22a had been knocked out and compared with flies where Or22a was intact.

This study has confirmed that the Ab3a neurons respond to ethyl isovalerate. Most interestingly, it was also found that in flies expressing Or22a/b, the Ab3a neurons respond even stronger to ethyl isovalerate than to ethyl hexanoate, the odor that has been considered the strongest ligand to Or22a since 2006. It also seems like Or22a play an important role in choosing oviposition site in *D. melanogaster*.

## Materials and methods

### Single sensilla recording

#### Materials

**Flies:** For the Single sensilla recording (SSR), we used two different strains of *D. melanogaster*. The common lab strain Canton-S (CS), acquired from New York university, and the Rwanda native strain, Rwanda Gkongoro (RG18N), which express the chimeric Or22a-b protein. The RG18N- flies had been acquired from a banana trap in the Gkongoro area in Rwanda. When used in the experiment, the flies were 6-10 days old, and had been kept at room temperature. Both strains had been fed a molasse, cornmeal and yeast based medium.

**Chemicals:** Ethyl isovalerate, ethyl hexanoate and other odors used in the experiment came from Sigma Aldrich and had 98% purity. The chemicals were used in  $10^{-7}$   $10^{-5}$  and  $10^{-3}$  mineral oil dilutions. The mineral oil was also from sigma Aldrich. All chemicals were kept at room temperature before use.

#### Methods

We used SSR to investigate the difference in response of the *D. melanogaster* strains Canton-S (CS) and Rwanda Gkongoro flies (RG18N) to ethyl hexanoate and ethyl isovalerate. The method has been described many times before (Pellegrino et al., 2010, Clyne et al., 1997, Mankin et al., 1987).

#### Mounting of the fly

To be able to perform the SSR, the fly must be immovable. To fix the fly, we trapped it in a 200  $\mu$ l pipette tip. This was done by using a piece of airline tubing with a cut 1000  $\mu$ l pipette tip attached to one of the openings. We then used the tubing to pick up a fly from the vial by inserting the pipette tip and sucking from the other end of the airline tubing.

We trapped the fly in the 200  $\mu$ l pipette tip by pacing the 200  $\mu$ l at the narrow end of the 1000  $\mu$ l pipette tip and blowing hard into the airline tubing. The fly always ends up with the head towards the narrow end of the pipette tip. Under a stereo microscope (Zeiss Stemi 350), we cut off the narrow end of the pipette tip with a razor blade close to the head of the fly. By blowing the airline tubing again, the fly will be pushed towards the opening of the narrow end of the pipette tip. When the fly had ended up in a position where the antennae and part of the eyes were sticking out from the opening, we cut off the wide end of the pipette tip close to the back part of the fly. To avoid the fly backing out, we inserted some dental wax behind the fly in the pipette tip.

We then placed the fly on a piece of dental wax on top of a microscope slide. It was positioned on its back with the head pointing towards a cover slip also placed on the microscope slide with some dental wax.

We made a glass electrode from a glass capillary in a vertical puller. We then used this glass capillary to keep the antenna fixed by folding the antenna down to be placed on top of the cover slip, and then pressing down the glass electrode between the second and third antennal segment.

To perform the recording, we placed the microscope slide under a light microscope (Nikon eclipse).

### **Sharpening of electrodes**

One recording electrode and one reference electrode, both made of tungsten, are needed to perform SSR. Before the recording, the electrodes must be sharpened. We did this electrolytically by inserting the electrode into a solution of nitrogen dioxide, and then running a current through the solution.

### **Odor delivery**

We diluted the odors to  $10^{-7}$   $10^{-5}$  and  $10^{-3}$  dilution in mineral oil. Then, we pipetted 10  $\mu$ l of each dilution onto separate pieces of filter paper and put the filter paper inside a glass Pasteur pipette. To deliver the odor to the fly under the microscope, we connected the Pasteur pipette at the large opening to an air pump and inserted the small opening in a hole of a glass tube leading to the microscope slide with the fly. The odor can then be puffed through the glass tube with the help of a pedal connected to the air pump.

### **Recording**

To first find the antenna, we used 40x magnification. With the help of a manually operated micromanipulator, we inserted the reference electrode into the eye of the fly.

To find an appropriate sensillum to insert the recording electrode into, we used 900x magnification. We used a motor- controlled micromanipulator to operate the recording electrode.

When an appropriate sensilla is found, spontaneous activity can be detected. To distinguish Ab3 sensilla from Ab1, we exhaled onto the fly, to observe the response to carbon dioxide. If any change in activity was detected, we ruled out the possibility of it being an Ab3 sensillum. To distinguish Ab3 from Ab2, we tested the response to ethyl 3-hydroxybutyrate and 2-heptanone. If the sensillum responded to ethyl 3-hydroxybutyrate and 2-heptanone it was ruled out to be Ab2. To distinguish Ab3 from Ab1 and Ab2, we also took the spontaneous activity in consideration, since different sensilla exhibit different spontaneous activity patterns (de Bruyne et al. 2001).

If a sensillum did not respond to CO<sub>2</sub>, ethyl 3-hydroxybutyrate or 2-heptanone, but did respond to ethyl hexanoate, we assumed it to be an Ab3 sensillum.

The signal from the recording was amplified 1000x and filed onto a PC. We used Autospike software for visualization and analysis.

### **Analysis**

We define the response as difference in spike amplitude frequency before and after stimuli. The signal was recorded for 12 s, and the stimuli was given for 0.5 s. The spike amplitudes were counted 0.5s before the stimulus, and 0.5 s during stimulus. We counted the difference in spike amplitude by subtracting the number of spike amplitudes before stimulation from the number of spike amplitudes after stimulation and multiplied it by two, to get the response in amplitudes per second, hertz (Hz).

### **Behavioural assay**

#### **Materials**

Flies: Transgenic CS *D. melanogaster* that expresses tetanus toxin in the ab3a neuron was used. These flies were obtained by crossing transgenic flies UAS-Or22a with Or22a-Gal4.

The UAS-Or22a strain expresses an UAS<sub>GAL</sub> element containing a gene expressing tetanus toxin light chain and the Or22a-Gal4 strain express GAL4 in Ab3a. Neither of the parental strains will express tetanus toxin in the Ab3a neurons, because Or22a-Gal4 lack the tetanus toxin gene and UAS-Or22a need GAL4 to express the tetanus toxin gene. However, the offspring, Or22a- TNT, both have the tetanus toxin gene and express GAL4 in the Ab3a neurons. The toxin prevents synaptic transmission. Thus, the Ab3a neurons in the Or22a- TNT strain cannot transfer its signal to the projection neurons in the glomeruli. This is a way to knock-out the function of the Ab3a neurons (Sweeney et al., 1995). The flies were 6- 10 days old when used in the assay and had been fed a molasse, cornmeal and yeast based medium.

Fruit: Marula fruit were collected in Matopos national park, Zimbabwe and oranges purchased from local supermarkets in Lund, Sweden.

## Methods

We performed a behavioral assay to investigate the importance of Or22a regarding attraction and oviposition in marula fruit. The procedure has previously been described (Liu et al., 2017, Joseph et al, 2009). A small plastic petri dish was filled with the same medium as previously used for incubation of the flies. One half was covered with finely chiseled marula fruit and the other part with similarly cut orange. A plastic flask with air holes was put upside down on top of the petri dish to keep the flies trapped. 20 mated female flies were added to each petri dish. The flies were incubated in room temperature and a dark room for 24 h. The assay was performed with the parental strains UAS-Or22a and Or22a-Gal4 (for control) and the offspring strain, Or22a- TNT.

## Statistical analysis

After 24 h, we counted the eggs on each side of the petri dish. We used an unpaired t-test to analyze the result.

## Results

### Single Sensilla Recording

When we measured the response of the Ab3a neurons in CS- flies to ethyl hexanoate, the flies exhibited a response similar in frequency as seen before (Hallem and Carlson 2006.). For instance, when using the highest concentration ( $10^{-3}$  dilution), we measured an average response of 218 spikes/s. However, when we tested the same odor with the same dilution on the RG18N- flies, the response was significantly lower, with an average of 114 spikes/s (figure 1).

When exposing the flies to ethyl isovalerate, we detected a response in the Ab3a neurons in both strains. The CS- flies responded with an average of 120 spikes/s to ethyl isovalerate in  $10^{-3}$  dilution. In the RG18N-flies, the response to the same odor with the same dilution was notably higher, with an average of 218.667 spikes/s (Figure 2). When we compared the response of Ab3a neurons of the RG18N- flies to ethyl hexanoate and ethyl isovalerate, the response to ethyl isovalerate was much stronger. The response to ethyl isovalerate in RG18N- flies were similar in frequency to the response to ethyl hexanoate in CS-flies, and not significantly higher. Further, the response to ethyl isovalerate in CS-flies were similar in frequency to the response to ethyl hexanoate in the RG18N- flies. This trend is consistent for all concentrations we used. Thus, there is a shift in the response spectrum of the RG18N- flies, which carry the chimeric Or22a/b protein, where ethyl isovalerate has taken the place of

ethyl hexanoate as the stronger ligand, and the response to ethyl hexanoate has decreased and taken the place corresponding to the response to ethyl isovalerate in the CS- flies.

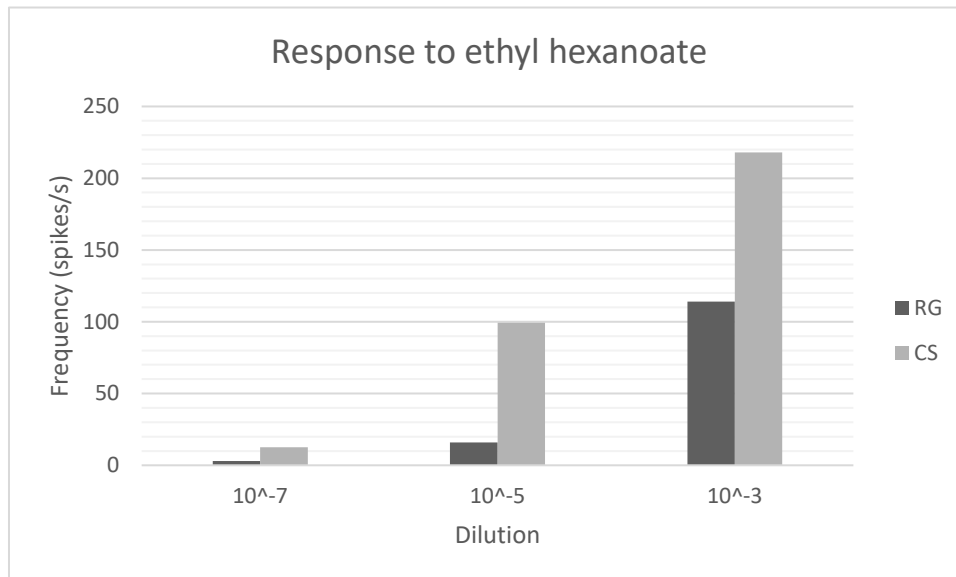


Figure 1: Respons of RG18N and CS-flies to ethyl hexanoate. CS- flies' response is significantly higher than the RG18N flies to all dilutions of ethyl hexanoate. \*The mean of CS- flies' response frequency for  $10^{-7}$  dilution is calculated from 2 measurements whereas the other two dilutions for CS, and all three dilutions for RG18N, are based on 3 measurements.

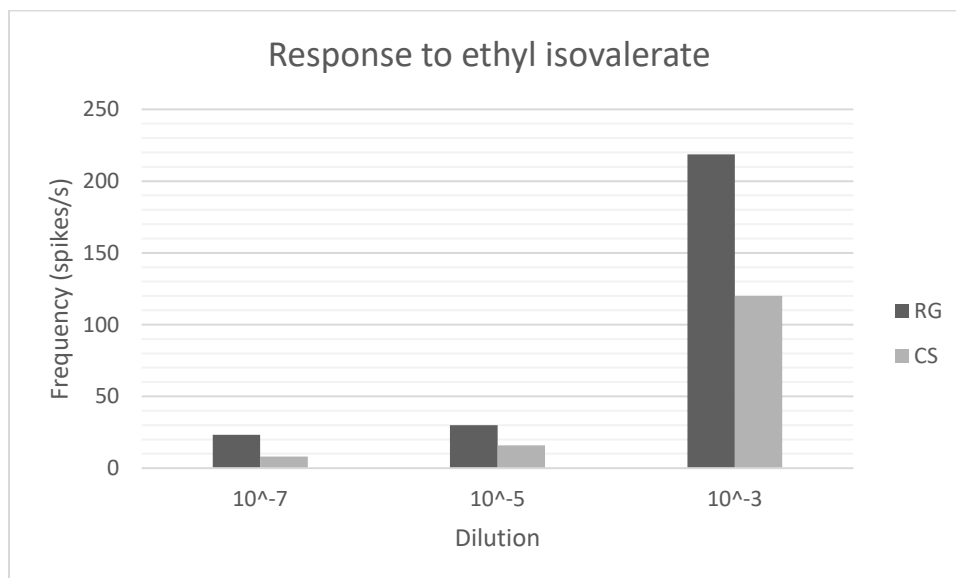


Figure 2: Respons of RG18N and CS-flies to ethyl isovalerate. RG18N- flies' response is significantly higher than the CS flies to ethyl isovalerate. \*The mean of CS- flies' response frequency for  $10^{-7}$  dilution is calculated from 2 measurements whereas the other two dilutions for CS, and all three dilutions for RG18N, are based on 3 measurements.

### Behavioral assay

We have performed a behavioral assay to investigate the importance of Or22a regarding attraction to and oviposition on marula fruit. The p- value for the Or22a- TNT strain is 0.6773, which is statistically insignificant when using significance level of 0.05 ( $p > 0.05$ ) (figure 3). For the one of the parental strain UAS-Or22a, the p-value is 0.0457, which is

statistically significant ( $p < 0.05$ ) (figure 4). For the other parental strain, Or22a-Gal4, the p-value is 0.0007, which is highly statistically significant ( $p < 0.5$ ) (figure 5). In conclusion, the offspring strain, Or22a- TNT, lays eggs randomly, with no significant preference for marula fruit. However, both parental strains, the control groups, exhibit a significant preference for marula fruit.

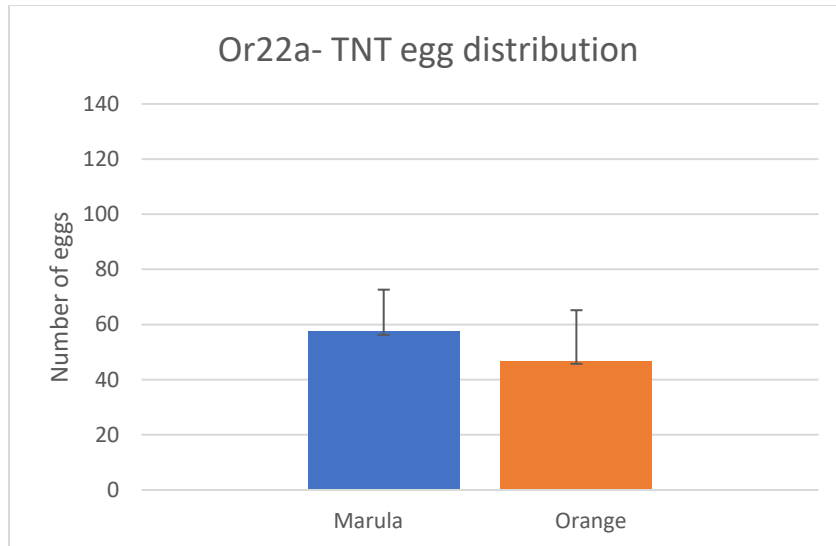


Figure 3: Distribution of eggs laid by Or22a-TNT between marula fruit and orange. Mean for marula = 57.25 and mean for orange = 46.75 Error bars indicate standard error (SE). SE marula = 15.39142. SE orange = 18.44078.

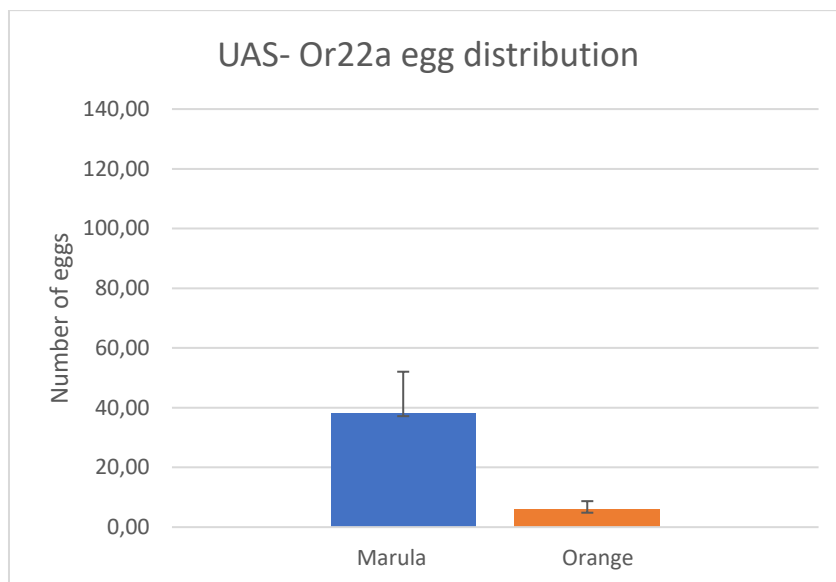


Figure 4: Distribution of eggs laid by UAS-Or22a between marula fruit and orange. Mean for marula = 38.17 and mean for orange = 5.83 Error bars indicate standard error (SE). SE marula = 13.88. SE orange = 2.87.

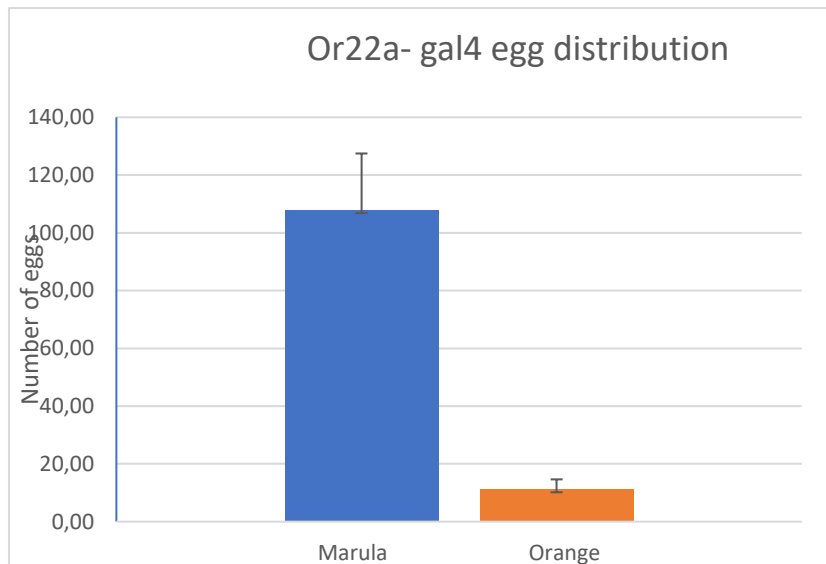


Figure 5: Distribution of eggs laid by Or22a-gal4 between marula fruit and orange. Mean for marula = 107.83 and mean for orange = 11.17 Error bars indicate standard error (SE). SE marula = 19.64. SE orange = 3.47.

## Discussion

With the use of SSR, we have found that the Ab3a neuron *D. melanogaster* responds to ethyl isovalerate. We also found differences in the response to ethyl isovalerate and ethyl hexanoate between the CS and the RG18N strains. Interestingly Ab3a neurons in RG18N flies respond stronger to ethyl isovalerate than Ab3a neurons in CS flies. Even more intriguingly, in RG18N flies, ethyl isovalerate elicits an even stronger response in Ab3a neurons than ethyl hexanoate, the odor that has been considered the strongest ligand of Or22a since 2006 (Pelz et al., 2006, Hallem and Carlson, 2006).

The result from our behavior assay indicate that it is Or22a that is responsible for the preference of marula fruit.

In CS flies, the Ab3a neurons express Or22a and Or22b. In RG18N- flies, the Ab3a neurons express Or22a-b. No ligand for Or22b has been found and it has been speculated to be an evolutionary rest (Dobritsa et al., 2003). Since no ligand has been found for Or22b it can be assumed that ethyl isovalerate is the ligand of Or22a. Although unlikely, the possibility that ethyl isovalerate is the ligand of Or22b cannot be ruled out only from the results in this study. Nonetheless, it can be concluded that the expression of Or22a-b instead of Or22a and Or22b results in a decreased response to ethyl hexanoate. Further, Or22a-b responds stronger to ethyl isovalerate than Or22a or Or22b.

A possible consequence of RG18N- flies responding stronger to ethyl isovalerate than CS- flies could be that RG18N- flies are more attracted to fruits that contain ethyl isovalerate, like marula fruit, than CS-flies. Furthermore, since CS-flies respond stronger to ethyl hexanoate than RG18N- flies, CS-flies should be more attracted to fruits that contain ethyl hexanoate than RG18N- flies. It is already known that *D. melanogaster* prefer marula over other fruit. However, since ethyl hexanoate is found in many fruits, and ethyl isovalerate is rarer, one could speculate that RG18N- flies might be more selective on where to oviposit than the CS- flies.

This shift from generalism to specialism has been found when comparing species to each other. In a study where the olfactory gene expression of *D. melanogaster*, *D. sechellia*, and *D. simulans* was compared, it was indicated that differences in gene expression can lead to differences in host. It was also concluded that the same differences can affect if the species is a generalist or specialist. It was speculated that it is the upregulation and gain-of-function of certain OR and odor binding protein (OBP) genes that has resulted in *D. sechellia* becoming a specialist. Interestingly, one of the genes of increased expression in *D. sechellia* compared to *D. simulans* and *D. melanogaster* is Or22a (Kopp et al., 2012). If changes in olfactory gene expression can lead to changes of host plant between species, changes within species could reasonably lead to changes of host as well. Although RG18N- flies do not exhibit an upregulation of *Or22a*, the affinity of Or22a to ethyl isovalerate is increased, which means that it could result in stronger specialization of RG18N- flies compared to CS-flies.

In the future, the most interesting aspect to investigate would be how the RG18N- flies and CS- flies differ in their host selection. Maybe the RG18N- flies are more selective and maybe choose not to lay eggs in many fruits that CS flies do lay eggs in. It could be that RG18N- flies that are native to Rwanda, were also marula fruit grow wild, react stronger to ethyl isovalerate because marula fruit is their main host. Unlike RG18N, CS-flies are pandemic, and the reason for this could be that they are strongly attracted to ethyl hexanoate, and therefore oviposit in almost any fruit, even though they prefer marula. That geographically separated strains of *D. melanogaster* could have different hosts or varying in their selectiveness is not completely unlikely. For example, *D. melanogaster* relative *Drosophila mojavensis* oviposit on cacti. The four populations of *D. mojavensis* are geographically isolated from each other. In two- choice assays like ours it turned out that all four populations preferred their own host over other alternatives. Thus, which oviposition site they choose is not just a consequence of which cacti that are available in the environment, but probably genetic (Date et al., 2017).

Another important thing to remember is that the odors present in the fruit is not the only factor that affect attraction and oviposition. In the experiments with *D. mojavensis*, it turned out that microorganisms on the cacti affect which volatiles the cactus emit and influence the attraction of *D. mojavensis* (Date et al., 2017). This could of course also be something that has affected our result. The marula fruit and oranges came from different places. Thus, their microflora should differ. Earlier experiments have shown that *D. mojavensis* are little attracted to sterile fruit (Date et al., 2013).

Our results from the behavioral assay indicate that Ab3a neurons play an important role in the preference for marula fruit as oviposition site. Since Or22b has no known function we assume that it is the Or22a that is of importance for the marula fruit preference. Next step should be to see how the Or22a-b protein affect the oviposition, and if flies that express Or22a-b are more selective towards marula fruit than the CS flies.

In an artificial selection study, where the response of virgin female and male *D. melanogaster* to four different odors was tested, genes responsible for variation in feeding behavior, guided by olfaction, were found. The study also showed how the attraction to odors, which in turn affect the feeding behavior, can change in a population over time (Brown et al, 2017). . Therefore, it would be interesting to perform a similar experiment with focus on oviposition. If changes in genes important for odor-guided behaviors can lead to changes in food

consumption, changes like the one that has occurred in RG18N that express Or22a-b, could most likely change the oviposition behavior.

It would give us a broader picture over which ORs are important for oviposition, how the expression of ORs varies in different populations and which OR seem to be most important for which host. We know for example that Or19a is important for the preference for citrus, because it binds limonene, and that Or22a is important for the preference for marula fruit, because it binds ethyl isovalerate. We don't know why the response of Or22a seems more important than the response of Or19a, since the flies prefer marula over orange, it would be interesting to see if there are there any genetic changes that would lead to preference for limonene over ethyl isovalerate.

Also, since RG18N is a wild strain, polymorphism in this strain should naturally occur. How polymorphism affect odor-guided behavior could also be a thing to investigate, to see how big the variation is, and how much it affects the behavior.

We have found a new ligand for Or22a, ethyl isovalerate. We have also found that Or22a is important for the oviposition behavior. Lastly, we have shown that Or22a-b has another response spectrum than Or22a. This has lead to further understanding of the *D. melanogaster* olfactory system. However, with more research this could also lead to further understanding of *Drosophila* evolution. For instance, our result indicates that Or22a probably is important in oviposition in *D. melanogaster*, just like in *D. erecta* and *D. sechellia*.

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