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Fakultät für Biowissenschaften
Institut für Spezielle Zoologie und
Evolutionsbiologie mit Phyletischem Museum


# Deorphanisation of tobacco hawkmoth olfactory receptors using the empty neuron system of the vinegar fly

**Bachelorarbeit**

Zur Erlangung des Grades eines

Bachelor of Science

Vorgelegt von

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Table of contents

## Introduction

Among the many tools insects possess to maneuver through the environment, and make decisions to ensure their survival and the survival of their offspring, the detection of olfactory cues is of great importance. These cues allow them to find suitable sexual mates, appropriate sources of food, good nesting places, among many other things (Hallem et al 2004a, Nakagawa et al 2005, Raguso & Willis 2002). As an example, vinegar flies actively decide whether to approach a possible food source or mating partner, or whether or not to lay their eggs in a specific spot mostly based on olfactory cues (Haverkamp et al 2018). But vinegar flies are mostly confined to the specific world of rotting fruits, as feeding, mating and egg-laying is done here (Hansson & Stensmyr 2011); on the other hand, insects like moths find themselves in a much more complex and dynamic environment, and being able to detect a wider assortment of olfactory cues is essential (Couty et al 2006). The tobacco hawkmoth *Manduca sexta* (*M. sexta*), a moth belonging to the Sphingidae family, found through much of the American continent, has become one of the key model organisms for understanding diverse questions regarding evolution, ecology, and neuroethology. At present, we know that olfaction plays a big role in the detection of female emitted pheromones over large distances (Baker 1986), and we know how adaptive and sensitive they respond to small pheromone concentrations during flight (Dolzer et al 2003). Olfaction plays an important role during foraging as well, as *M. sexta* moths are nocturnal foragers, and are attracted to a wide range of plant-emitted volatiles (Bisch-Knaden et al 2018). Among the many different flowers *M. sexta* chooses as nectar sources, two have been identified as the ones from which the moths take the most pollen, jimsonweed *Datura wrightii* (*D. wrightii*) and Palmer's century plant *Agave palmeri* (*A. palmeri*) (Alarcon et al 2008)

Just like other insects, *M. sexta* possesses olfactory sensory neurons (OSNs) which sit in small hairs, so-called olfactory sensilla, on the antenna (Strausfeld & Hildebrand 1999). Olfactory receptors (ORs) are transmembrane proteins which activate ion channels once they bind to their ligand (Clyne et al 1999), and are expressed by the OSNs. In *M. sexta*, 73 types of ORs (MsexORs) have been identified through analyzing the genome data (Koenig et al 2015). The process of identifying the ligands that bind to an OR is called the deorphanisation of that receptor.

One way of deorphanising insect ORs is through the so-called “empty neuron system” in the vinegar fly *Drosophila melanogaster* (*D. melanogaster*), first introduced by Elissa

Hallem and team (Hallem et al 2004b). In this method, a *D. melanogaster* mutant is used, *Δhalo*, where two co-expressed ORs (DmelOR22a and DmelOR22b), are not expressed as the genes are not present (Dobritsa et al 2003). The OSNs expressing these ORs in wild type flies are housed in one specific type of sensilla (ab3 sensilla) on the antenna (Couto et al 2005).

With the help of the Gal4/UAS system it is possible to express ORs from other insects, e.g. moths in these "empty neurons" (De Fouchier et al 2017). The Gal4 gene found in the yeast *Saccharomyces cerevisiae* encodes a positive regulator of various genes (Laughon & Gesteland 1984). Gal4 regulates the transcription of GAL10 and GAL1 in the yeast by directly binding to four related 17 base pair sites located between the two (Giniger et al 1985). An Upstream Activating Sequence (UAS) is defined by these four sites, which is essential for the transcription of the GAL4 regulated genes (Duffy 2002). Using a Dmel *Δhalo*-Gal4 fly line and a MsexOR-UAS fly line allows for moth ORs to be expressed in the empty OSN. Then, single sensillum recordings (SSR) from the ab3 sensilla of the fly can be performed to deorphanise the transgenic moth receptor.

Except for MsexOR1, which detects the main pheromone compound bombykal (Wicher et al 2017) no MsexORs have been deorphanised so far. We choose the next interesting receptors to test based on a phylogenetic tree of lepidopteran ORs (Koenig et al 2015), where we found a *M. sexta*–specific cluster of closely related ORs (Figure 1). This recent gene duplication event indicate that these receptors might play an important role in the ecology of *M. sexta*. One of these receptors (MsexOR36) has already been expressed in the empty neuron system (Christian Klinner, Sascha Bucks, Ewald-Grosse-Wilde, unpublished), and was tested in my study with floral volatiles of *D. wrightii* and *A. palmeri*. I cloned three other receptors from the same cluster (MsexOR8, MsexOR33, MsexOR80), and three additional ORs that were chosen because of their female-biased expression on the antenna (MsexOR13, MsexOR15, MsexOR17, Figure 2). Molecular cloning of these six ORs now enables the injection of plasmid vectors into *Drosophila* embryos, and subsequently the deorphanisation of the ORs.

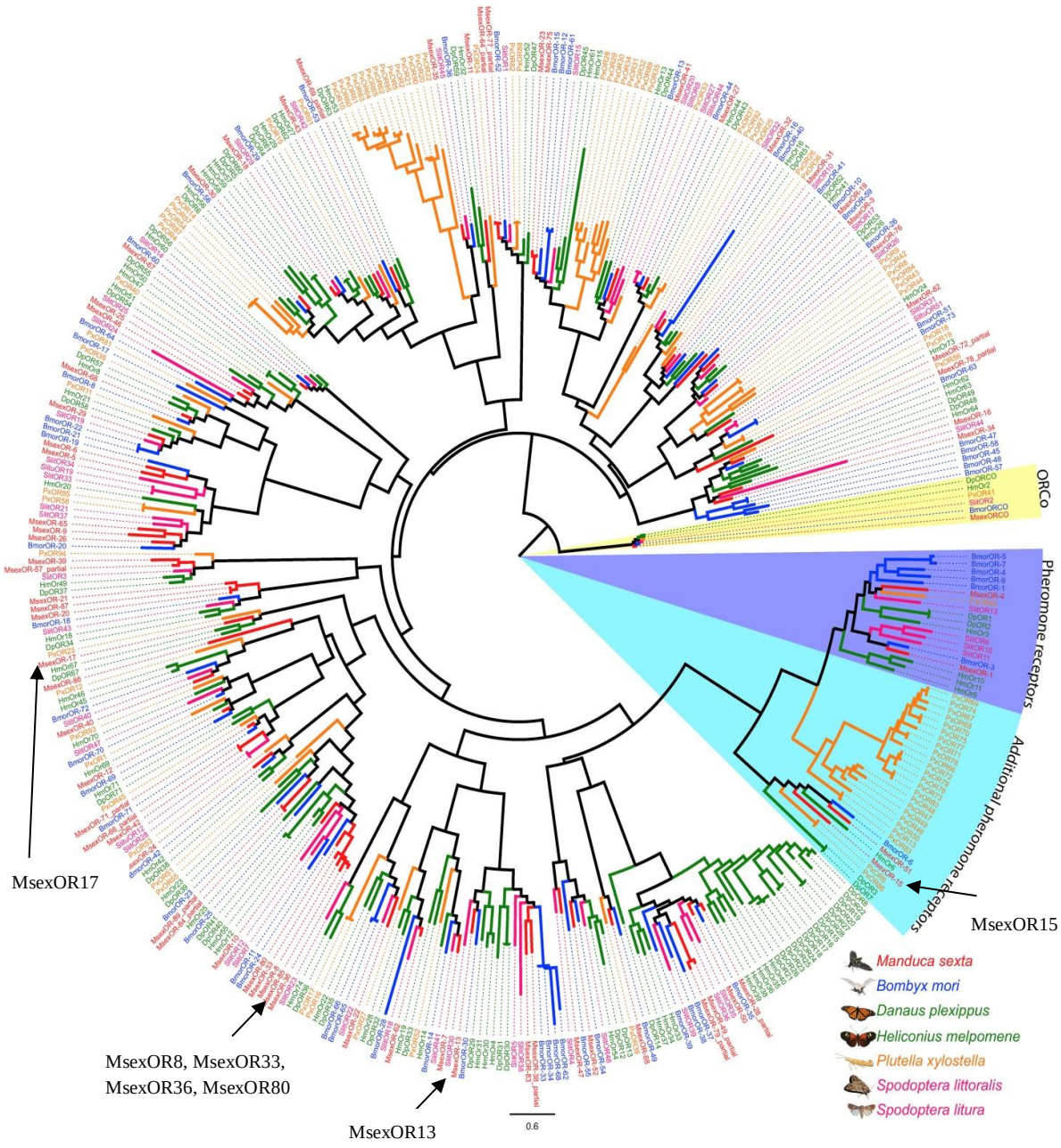


**Figure 1: from koenig 2015. Maximum likelihood phylogenetic tree of Lepidoptera ORs. Arrows show the position of MsexOR8, MsexOR13, MsexOR15, MsexOR17, MsexOR33, MsexOR36 and MsexOR80.**

Msex15 has been shown to be a female specific OR, and Msex08, Msex13, Msex17, and Msex36 have also been shown to have higher expression levels in female *M.sexta* moths (Koenig et al 2015). Msex33 is the only OR chosen that is slightly more expressed in male *M.*sexta. These reasons make these ORs interesting objects of studies for the understanding of sexual specific behavior, duplication of OR lineages, among other things. Due to time contraints and the nature of this work, however, only MsexOR36 was deorphanised.

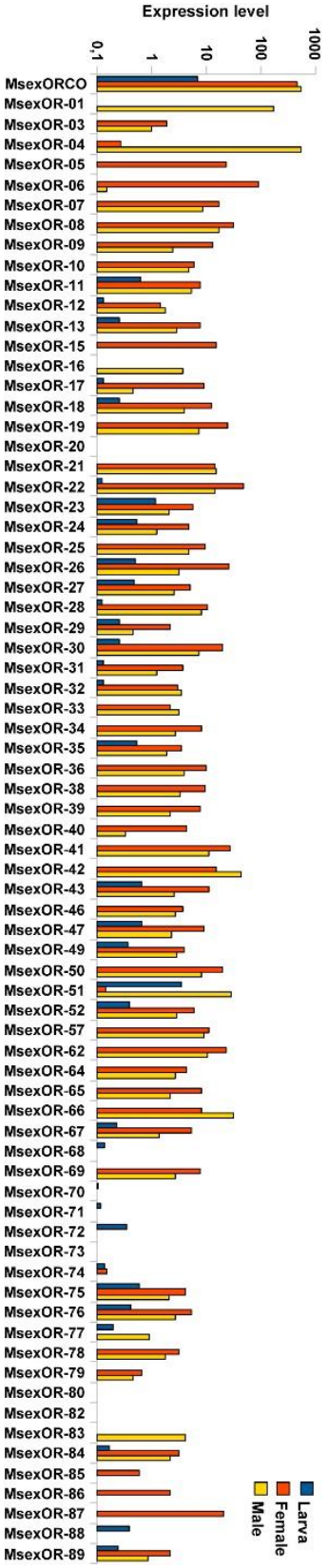


**Figure 2: from Koenig 2015. Expression levels of ORs in male, female, and larval *M.sexta***

## Materials and Methods

1 List of devices, kits and software

| Device/Kit/Software | Model/Name | Manufacturer |
|---|---|---|
| High speed homogenizer | TissueLyser LT | QIAGEN (Hilden, Germany) |
| Microvolume UV-Vis Spectrophotometer | NanoDrop™ One | Thermo Fisher Scientific Inc. (Waltham, Massachusetts, United States) |
| Thermomixer | Thermomixer Compact 5350 | Eppendorf AG (Hamburg, Germany) |
| Table top incubator | Novotron A182k | Infors AG (Bottmingen-Basel, Switzerland) |
| submarine type electrophoresis system | Mupid-exU | NIPPON Genetics Europe (Dueren, Germany) |
| DNA purification kit | NucleoBond® Xtra Midi EF | MACHEREY-NAGEL GmbH & Co. KG (Berlin, Germany) |
| Gel extraction kit | E.Z.N.A.® Gel Extraction Kit (V-spin) | Omega Bio-tek, Inc. (Norcross, Georgia, United States) |
| Microscope | Olympus BX51WI | Olympus Corporation (Tokyo, Japan) |
| Micromanipulator for ground electrode | MM-3 | NARISHIGE Group (Tokyo Japan) |
| Micromanipulator for reference electrode | DC-3 KS with Piezo Manipulator PM10 | Märzhäuser Wetzlar GmbH & Co. KG (Wetzlar, Germany) |
| Channel signal amplifier | IDAC-4 | Ockenfels SYNTECH GmbH (Kirchzarten, Germany) |
| PCR thermal cycler | GeneAmp PCR System 9700 | Thermo Fisher Scientific Inc. (Waltham, Massachusetts, United States) |
| Light source for gel analysis | BioVision Superbright Mode II 3000 | VWR International (Radnor, Pennsylvania, United States of America) |
| Software for DNA data analysis | Geneious Prime v.2019.1.3 | Biomatters Ltd (Auckland, New Zealand) |
| Software for restriction enzymes protocol | NEBcloner® v.1.3.13 | New England Biolabs (Ipswich, Massachusetts, United States) |
| Plasmid DNA isolation kit (from bacterial cultures) | E.Z.N.A.® Plasmid Mini Kit I, (V-spin) | Omega Bio-tek, Inc. (Norcross, Georgia, United States) |
| Software for electrophysiological acitivity | Autospike 3.9 | Ockenfels SYNTECH GmbH (Kirchzarten, Germany) |
| Software for significance test | GraphPad InStat version 3.10 | GraphPad Software Inc, (California, United States) |

2 List of chemicals

| Chemical | Manufacturer |
|---|---|
| pUAST.attB | Institute of Molecular Life Sciences WEST, Univversity of Zurich |
| Nuclease-Free Water | Thermo Fisher Scientific Inc. (Waltham, Massachusetts, United States) |
| One Shot™ TOP10 Chemically Competent E. coli | Thermo Fisher Scientific Inc. (Waltham, Massachusetts, United States) |
| S.O.C. Medium | Thermo Fisher Scientific Inc. (Waltham, Massachusetts, United States) |
| TAE buffer (0.04 M Tris, 0.04 M vinegar acid, 1 mM EDTA) | 0.04 M Tris, 0.04 M vinegar acid, 1 mM EDTA |
| Biozym LE Agarose | Biozym Scientific GmbH (Hessisch Oldendorf, Germany) |
| Ethidiumbromid ≥98 % | Carl Roth GmbH + Co. KG (Karlsruhe, Germany) |
| Gel Loading Dye, Purple (6X), no SDS | New England Biolabs (Ipswich, Massachusetts, United States) |
| Quick-Load® 1 kb Plus DNA Ladder | New England Biolabs (Ipswich, Massachusetts, United States) |
| T4 DNA Ligase Buffer (10X) | Thermo Fisher Scientific Inc. (Waltham, Massachusetts, United States) |
| T4 DNA Ligase (5 U/µL) | Thermo Fisher Scientific Inc. (Waltham, Massachusetts, United States) |
| Taq DNA Polymerase, recombinant (5 U/µL) | Thermo Fisher Scientific Inc. (Waltham, Massachusetts, United States) |
| 2-Heptanone | Sigma-Aldrich Corporation (St. Louis, Missouri United States) |
| Ethyl hexanoate | Sigma-Aldrich Corporation (St. Louis, Missouri United States) |
| Ethyl-3-hydroxy butyrate | Thermo Fisher Scientific Inc. (Waltham, Massachusetts, United States) |
| Dichloromethane 'Dichlormethan ROTISOLV® ≥99,9 %, Pestilyse® plus' | Carl Roth GmbH + Co. KG (Karlsruhe, Germany) |
| Hexane 'n-Hexan ROTISOLV® ≥95 %, GC Ultra Grade' | Carl Roth GmbH + Co. KG (Karlsruhe, Germany) |
| Restriction enzymes: KpnI-HF, XbaI, XhoI, NotI-HF | New England Biolabs (Ipswich, Massachusetts, United States) |
| 10x CutSmart ® Buffer | New England Biolabs (Ipswich, Massachusetts, United States) |
| Roti®-Mix PCR 3<br>10 mM (pro dNTP) dATP, dTTP, dGTP, dCTP | Carl Roth GmbH + Co. KG (Karlsruhe, Germany) |
| 10x CutSmart ® Buffer | New England Biolabs (Ipswich, Massachusetts, United States) |

| | |
|---|---|
| 10x CutSmart ® Buffer | New England Biolabs (Ipswich, Massachusetts, United States) |
| UAS and MsexOR primers | Eurofins Genomics Germany GmbH (Ebersberg, Germany) |
| Ampicillin 'Ampicillin Natriumsalz CELLPURE® ≥91 %' | Carl Roth GmbH + Co. KG (Karlsruhe, Germany) |
| Sodium chloride 'Natriumchlorid CELLPURE® ≥99,8 %' | Carl Roth GmbH + Co. KG (Karlsruhe, Germany) |
| Yeast extract 'Hefeextrakt pulv., für die Bakteriologie' | Carl Roth GmbH + Co. KG (Karlsruhe, Germany) |
| Trypton 'Trypton/Pepton aus Casein pankreatisch verdaut, für die Mikrobiologie' | Carl Roth GmbH + Co. KG (Karlsruhe, Germany) |
| 10xPCR Buffer Y | VWR International (Radnor, Pennsylvania, United States |
| Taq-Polymerase | VWR International (Radnor, Pennsylvania, United States |
| TRIS-Acetate 'TRIS-Acetat PUFFERAN® ≥98 % Tris-(hydroxymethyl)-aminomethan-acetat' | Carl Roth GmbH + Co. KG (Karlsruhe, Germany) |
| EDTA 'Ethylendiamin-tetraessigsäure ≥99 %, p.a., ACS' | Carl Roth GmbH + Co. KG (Karlsruhe, Germany) |

# 3 Molecular cloning of *Manduca sexta* ORs

## 3.1 Cloning of the receptors into the pCRII-Vector.

The following *M.sexta* ORs were obtained already inside the pCRII vector from the department of Neuroethology of the Max Planck Institute for Chemical Ecology: OR8, OR13, OR15, OR17, OR33, OR80.

## 3.2 Recloning of the receptors from the pCRII vector into the pUAST.attB vector

The cloning and transformation vector pUAST.attB, with the accession number EF362409 in GenBank, was used for the recloning. This plasmid recloning step is important because pUAST contains a donor sequence (attB) which will be injected into *D. melanogaster* embryos with a recipient sequence (attP) in their genome. In conjunction with the insertion of the appropriate integrase (φC31 integrase), this results in the site-specific insertion of the transgene into the attP site of the fly's genome (Fish et al 2007). pUAST.attB was placed into a 1.5 ml centrifuge tube and mixed with 50 µl RNase free water. The tube was placed inside a thermomixer at 950 RPM and 30°C for 10 minutes. Two µl of the plasmid

were mixed with 25 µl of chemically competent cells and left to rest for 30 minutes at room temperature. A heat shock was then induced inside the thermomixer at 42°C for 45 seconds and the tube was then left to rest on ice for two minutes. 250 µl of S.O.C. medium were added to the centrifuge tube and the cells were incubated for 1 h in a table top incubator shaker at 220 RPM and 37°C.

### 3.3 pUAST.attB plasmid multiplication

250 ml of LB Agar medium and one ml of an Ampicillin solution (1g Ampicillin per 20 ml of double distilled water) were added to a 1l laboratory glass bottle (pUAST.attB contains an Ampicillin resistance gene). This mixture was then poured on petri dishes and left to harden. The cells were then poured and evenly distributed into one of these petri dishes and left to incubate for about 15-18h at 37°C. A colony was then picked up and transferred to a 0.2 ml PCR -tube with 50 µl of the LB-Ampicillin mixture. It was left to incubate for 1h at 37°C/225 RPM, then transferred to a 1 ml centrifuge tube with 1 ml LB-Ampicillin mixture and the incubated again for the same time and settings. The solution was then transferred into a 1l laboratory glass bottle filled with 100 ml LB medium and left to incubate for ~15 h with the same settings.

### 3.4 DNA purification

The NucleoBond® Xtra Midi EF kit was used for plasmid DNA purification. All the steps under the section 8.1 ‘High-copy plasmid purification’ were followed as detailed in the protocol and Buffer TE-EF was used at the end to dilute the resulting plasmid DNA pellet.

### 3.5 Restriction Enzyme Double Digestion

To decide which restriction enzymes would be used for each receptor the software Geneious Prime was used to see the ORs’ sequences and compare them with the restriction enzymes’ cutting sites contained in the pCRII and the pUAST.attB plasmids (figure A1 and A2). Using the NEBCloner online tool the chosen restriction enzymes (Table 1) were specified and the appropriate ingredients and volumes for the double digestion were obtained. Nine centrifuge tubes were filled with 1 µg of the respective DNA (six times for each OR and three times pUAS.attB, as there were three different restriction enzymes combinations), 5 µl 10x CutSmart ® Buffer, 1 µl of each of the two restriction enzyme and enough Nuclease free water to fill up to 50 µl. The tubes were incubated for 15 minutes at 37°C.

**Table 1: Restriction enzymes used for each OR**

| Olfactory receptor/vector | Restriction enzymes |
|---|---|
| MsexOR8 | KpnI, NotI |
| MsexOR13 | KpnI, XbaI |
| MsexOR15 | KpnI, XhoI |
| MsexOR17 | KpnI, NotI |
| MsexOR33 | KpnI, NotI |
| MsexOR80 | KpnI, NotI |

3.6 Agarose-Gel electrophoresis

1% gels were prepared by heating 150 ml TRIS-Acetate-EDTA (TAE) buffer and 1.5 g Agarose. To make the DNA visible 7.5 µl of ethidium bromide were added. The mixture was poured on containers with 13 wells and left to harden for 20 min. The gels were then submerged into electrophoresis systems filled with TAE buffer. The digested DNA samples were mixed with 10 µl of purple loading dye 6x and split into two wells each in the gel, 25 µl respectively. A well was loaded with 5 µl of 2-Log Ladder as a marker. Electrophoresis was performed for 25 minutes at 135 V.

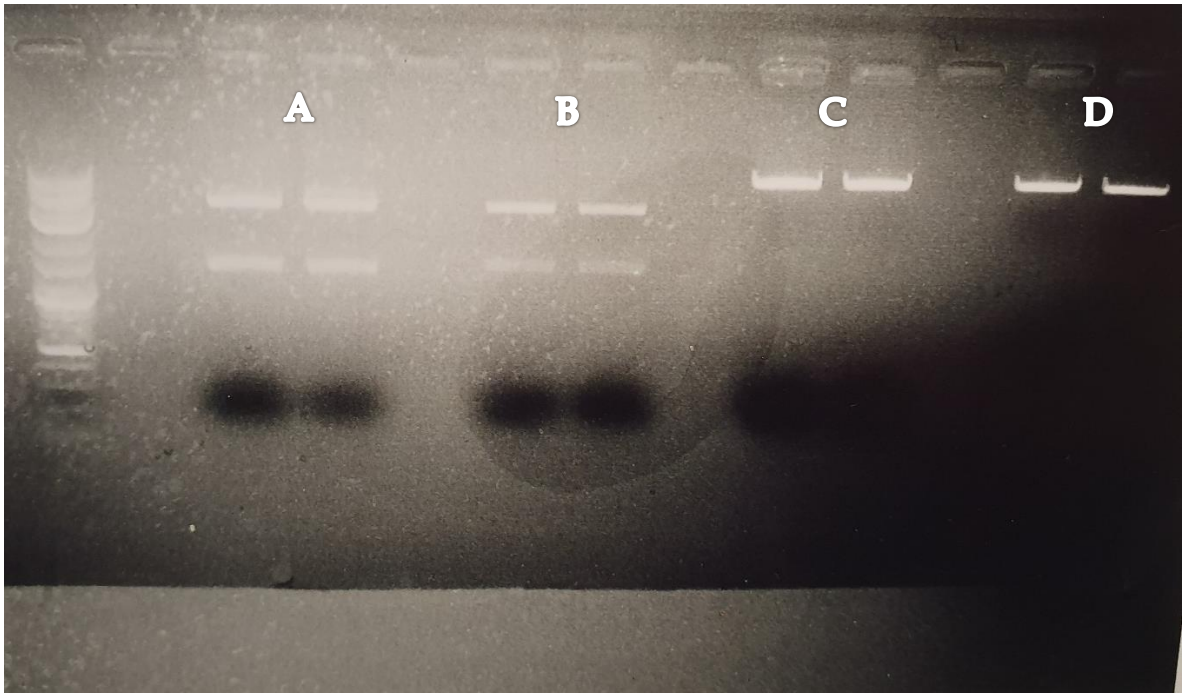


**Figure 3: Gel electrophoresis. A and B show MsexOR13 and MsexOR15 respectively. The upper bands are the remains of the pCRII plasmid, and the lower bands is the digested OR DNA, which travels farther, as it is smaller. C and D show the pUAST.attB plasmid.**

3.7 Gel extraction

A scalpel was used to excise the DNA from the gels, which could be seen with a source of blue light (figure 3). The A E.Z.N.A.® Gel Extraction Kit was used according to the manufacturer's instructions for the extraction of the DNA from the gels.

3.8 DNA insert ligation into vector DNA

The volume of 100 ng of the three extracted pUASattb samples was calculated and the same volume was taken from the ORs samples that matched each restriction enzymes combination. Six PCR tubes were used for each OR. 2 µl of 10x T4 DNA Ligase buffer, 1 U of T4 DNA Ligase and enough Nuclease-Free Water to fill up to 20 µl were added to the tubes. These were then left to incubate for 10 min at room temperature. Two µl of each tube were added to a new centrifuge tube with 25 µl chemically competent cells. These were then left 30 min in ice and then heat shocked for 45 s at 42°C and dropped in ice for 2 min. 250 µl S.O.C. medium were added and the cells were left to incubate for 1 h at 37°C/220 RPM. The cells were then poured into agar petri dishes and incubated for the night at 37°C.

3.9 Colony polymerase chain reaction (PCR)

Eight colonies were picked from each petri dish (48 in total) and were transferred into PCR tubes with 50 µl of the LB-Ampicillin medium. They were incubated for 1h at 37°/220

RPM. A master mix was made for the Colony-PCR composing of the following ingredients: 17.875 µl of Nuclease-Free Water, 2.5 µl 10xPCR Buffer Y, 0.5 µl dNTP Mix 10 mM each, 1 µl UAS forward primer, 1 µl UAS reverse primer, and 0.125 µl of Taq-Polymerase. These quantities were multiplied by forty-eight, and aliquoted in forty-eight PCR-tubes. 2 µl of each of the LB-Colony tubes were taken and added one at a time to the previously prepared PCR-tubes with the master mix. PCR was done in a thermal cycler with the following program:

1. One cycle at 94°C for 2 min,
2. 25 Cycles going from 94°C for 15 s, to 57°C for 30 s, and to 72°C for 100 s,
3. One cycle going from 72°C for 5 min.
4. One cycle at 4°C until samples removed.

### 3.10 Gel electrophoresis

Following the same procedure as with the first gel electrophoresis, the results of the PCR are loaded into gels with a 2-Log Ladder as the marker. Using a source of blue light, the colonies of each OR where the OR is being expressed where identified (figure 4), and two were chosen at random for sequencing (for MsexOR08 and MsexOR15 only one colony expressed the OR, and as such only one colony was prepared for sequencing).

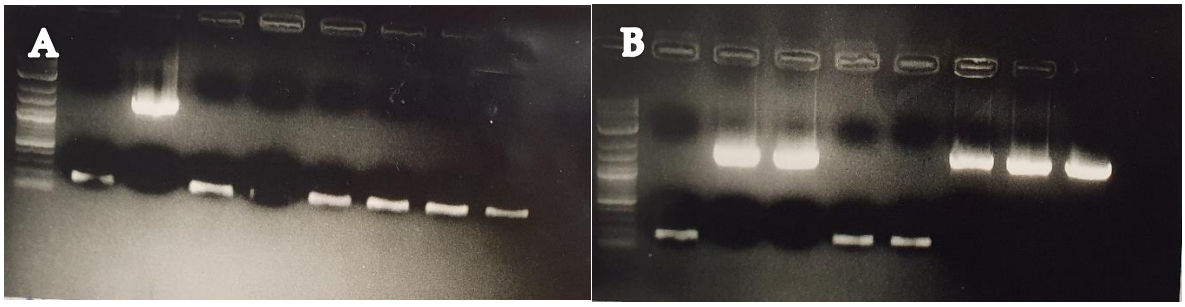


**Figure 4: Second gel electrophoresis. A Results for MsexOR8. Only colony number 2 expressed the OR, and as such only it was chosen for purification. B Results for Msex17. Five colonies expressed the OR, and two were chosen randomly.**

### 3.11 Plasmid DNA purification

From the colonies used for the PCR, the clones chosen after the gel electrophoresis (those where the OR was expressed) where transferred to new centrifuge tubes with 1 ml LB-Ampicillin medium and incubated for 1 h at 37°C/220 RPM. They were then transferred to 15 ml falcon tubes containing 5 ml LB-Ampicillin medium and left to incubate over night at 37°C/220 RPM. Using a plasmid DNA isolation kit, the plasmid DNA was isolated from the rest of the bacterial matter and its concentration was measured.

### 3.12 Sequencing of the ORs DNA

Two small PCR tubes were prepared for each sample, one with 0.5 µl of the UAS forward primer (table A2) and the other with 0.5 µl of the UAS reverse primer. 140 ng of plasmid were added to each tube and they were then filled with nuclease free water up to 6 µl. Sequencing was done in the Max Planck Institute for Chemical Ecology (Jena, Germany). Taking into consideration the average size of the ORs of *M. sexta*, ~1200 bps (Koenig et al 2015), a second sequencing was performed to ensure the quality of the copies remains high throughout the whole genes. This time internal primers were used, which directly bond to the ORs DNA (table A2).

### 3.13 Analysis of the sequencing results

With help of Geneious Prime the obtained DNA sequences were compared to the original MsexORs' sequences published by Koenig and team (Koenig et al 2015) to confirm the cloning has been correctly performed.

## 4 Empty Neuron System

### 4.1 Flies

Transgenic *D. melanogaster* flies were obtained from Bestgene Inc (Chino Hills, California, United States), which performed the pUAST.attB injection in the embrios. They were fed an artificial diet (Table A1) and were kept in an incubator with a constant temperature of 25°C, a humidity of 70% and 12h light. In order to obtain the final *D. melanogaster* lines which were used for the experiments a series of genetic crosses were performed. The same crossing scheme as described in (Gonzalez et al 2016) under the name 'Fly Crossing Scheme for the ab3A Empty Neuron System' was followed.

### 4.2 Single sensillum recording (SSR) preparation

Adult flies between 2 and 6 days after hatching were sorted out. Only females were taken, as they are significantly bigger than males, making them more suitable for SSR. An individual fly was then immobilized in the top end of a 20-200 µl, with its head on the smaller end and its legs and wings tucked in. The rest of the pipette tip was sliced with a razor blade making a small tube where the fly is fixed. The immobilized fly was then fixed on dental wax on top of a microscope slide, with its ventral side on top, and more wax was pushed on the rear end of the tube, to make the antenna of the fly stick out on the front side. A microscope cover slip was placed at the same height in front of the fly. A thinned-out glass capillary was

used to fix the antenna of the fly on top of the microscope cover slip, between the second and third segment.

Stimulation odors cartridges were prepared by dropping 10 µl of the desired odor on 1 $cm^2$ filter paper circles, which were previously inserted into Pasteur pipettes. These were covered with a 1ml pipette tip with wax at the end to prevent evaporation (figure 5).

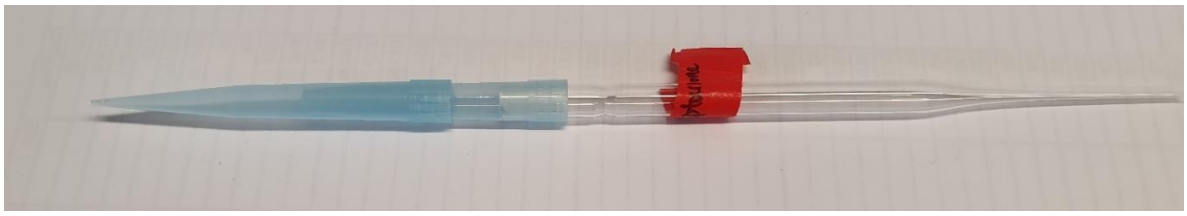

**Figure 5: Stimulation odor cartridge**

A microscope with a 50x magnification was used to locate the sensilla. Tungsten rods were used for the electrodes. The ground electrode pierced the left eye of the fly using a micromanipulator and the reference electrode pierced the ab3 sensillum using a second micromanipulator with an electrical manipulator attached to it for finer movement control (figure 6). A tube with a 7 mm diameter was placed about 1 cm away from the antenna delivering a constant 1.5 $lmin^{-1}$ flow of humidified, charcoal filtered air. Odor stimulations were achieved by placing the end of each Pasteur pipette in a small hole in the tube and then letting a 500 ms (0.6 l/min) pulse of air run through the filter paper and into the air flow provided by the tube.

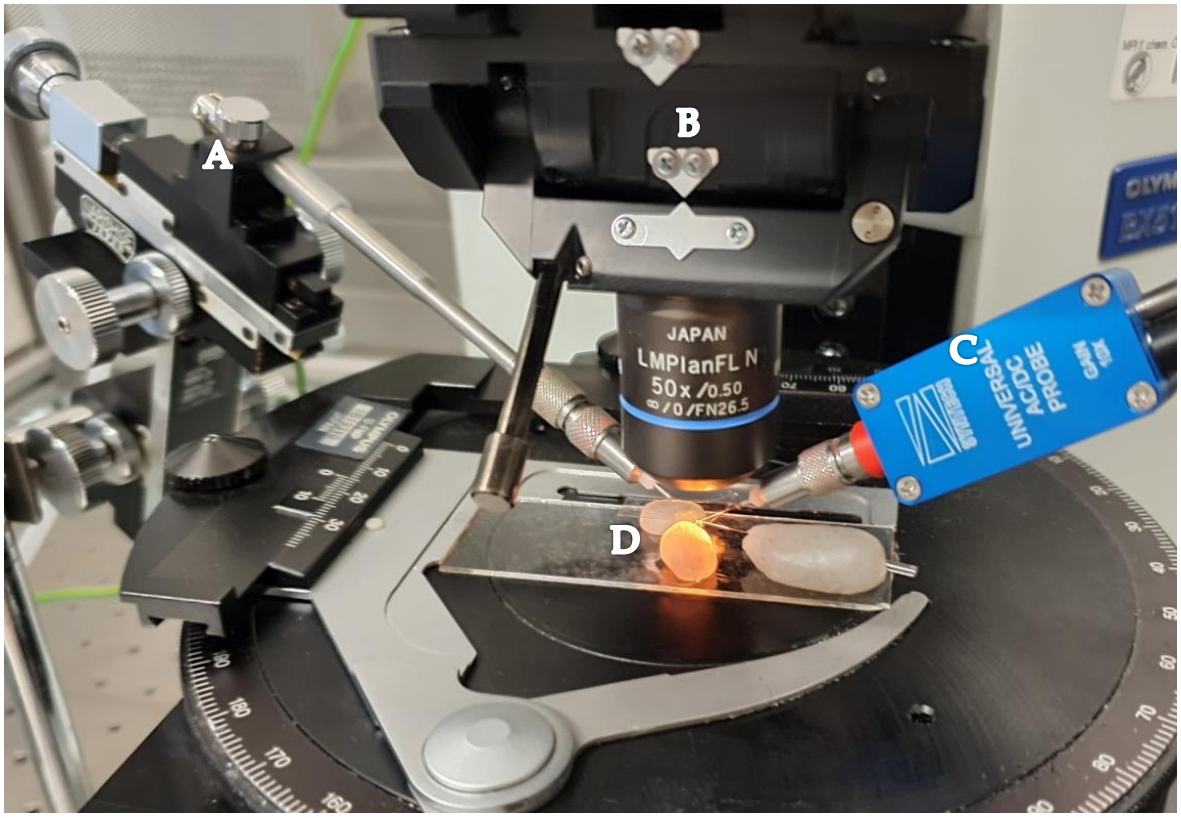


**Figure 6: Single sensillum recording (SSR) setup. A) ground electrode attached to MM-3 micromanipulator. B) BX51WI microscope with 50x magnification lens. (Olympus Corporation, Tokyo, Japan) C) Reference electrode attached to DC-3 KS micromanipulator. D) Fixated *D. melanogaster* on microscope slide.**

4.3 Floral headspace odors

The *A palmeri* and *D. wrightii* floral headspace was collected by Sonja Bisch-Knaden (unpublished) using a modified version of the method described in (Linz et al 2013). A custom-build mobile pump was used instead of the vacuum pump described, and the sample collection was done for 12 h from sunset to sunrise. The collection site was Santa Rita Experimental Range near Tucson, Arizona, US. The pure scents were diluted in Dichloromethane (DCM) to a concentration of 1:10, 1:100, and 1:1000.

4.4 Locating ab3 sensilla

If the D. melanogaster fixation has been done properly, a cluster of large basiconic sensilla can be spotted through the microscope (Figure 7, dark blue circles). Inside this cluster, ab3 sensilla can be found, as they belong to the large basiconic type (figure 8). With the aid of maps showing ab3 sensilla marked with green fluorescent proteins as markers (Lin & Potter 2015, Syed et al 2010), the target sensilla were poked with the recording electrode. If two distinct neuronal activities could be distinguished (the A and the B neuron in the case of ab3), diagnostic odors were used to determine if it was indeed the correct sensillum.,

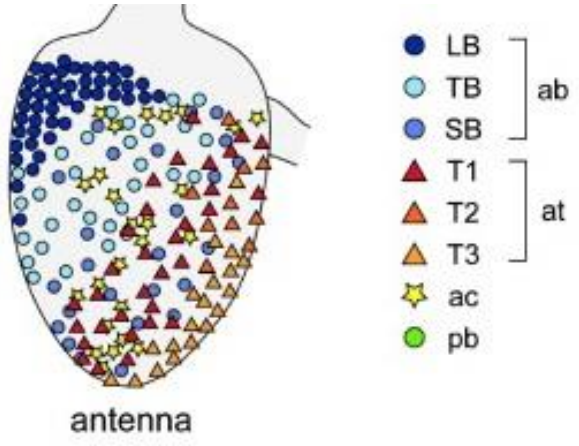


**Figure 7: Distribution of sensillum types on the fly antenna from (Couto et al 2005). The dark blue circles represent a compact cluster of large basiconic sensilla where the ab3 sensilla can be found.**

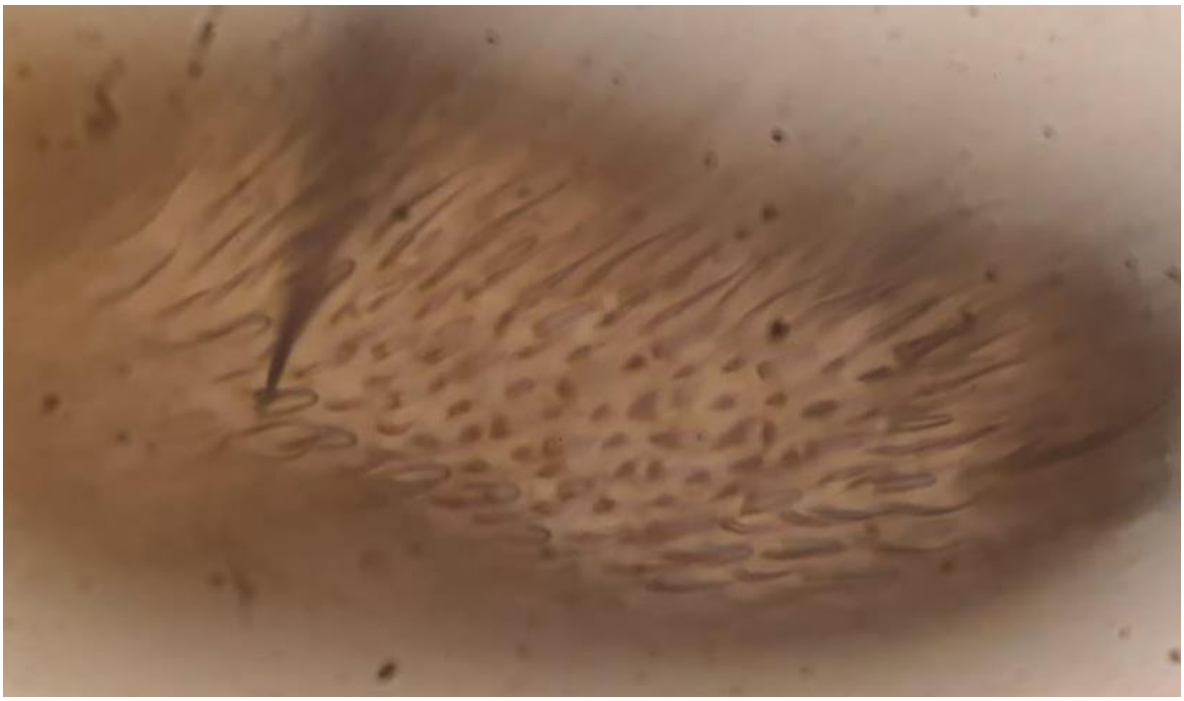

**Figure 8: *D. melanogaster* antenna with a large basiconic sensillum pierced by the reference electrode. Image taken through the lens of the BX51WI microscope (Olympus Corporation, Tokyo, Japan)**

4.5 Diagnostic odors

Diagnostic odors were used to identify the Ab3 sensilla in the fly, as morphology alone or location in the antenna is not enough. Dilutions of 1:1000 in hexane were used for this purpose. The following odors were used:

1) 2-heptanone to confirm the DmelOR85b expressed in the B neurons (Swarup et al 2011).

2) Ethyl hexanoate to confirm the absence of the DmelOR22a, which would otherwise be expressed in the wildtype and react strongly (Pelz et al 2006).
3) Ethyl-3-hydroxy butyrate to rule out ab2 sensilla, which like ab3 also house two types of OSNs, and can by morphology and location be mistaken for ab3 (Stensmyr et al 2003).

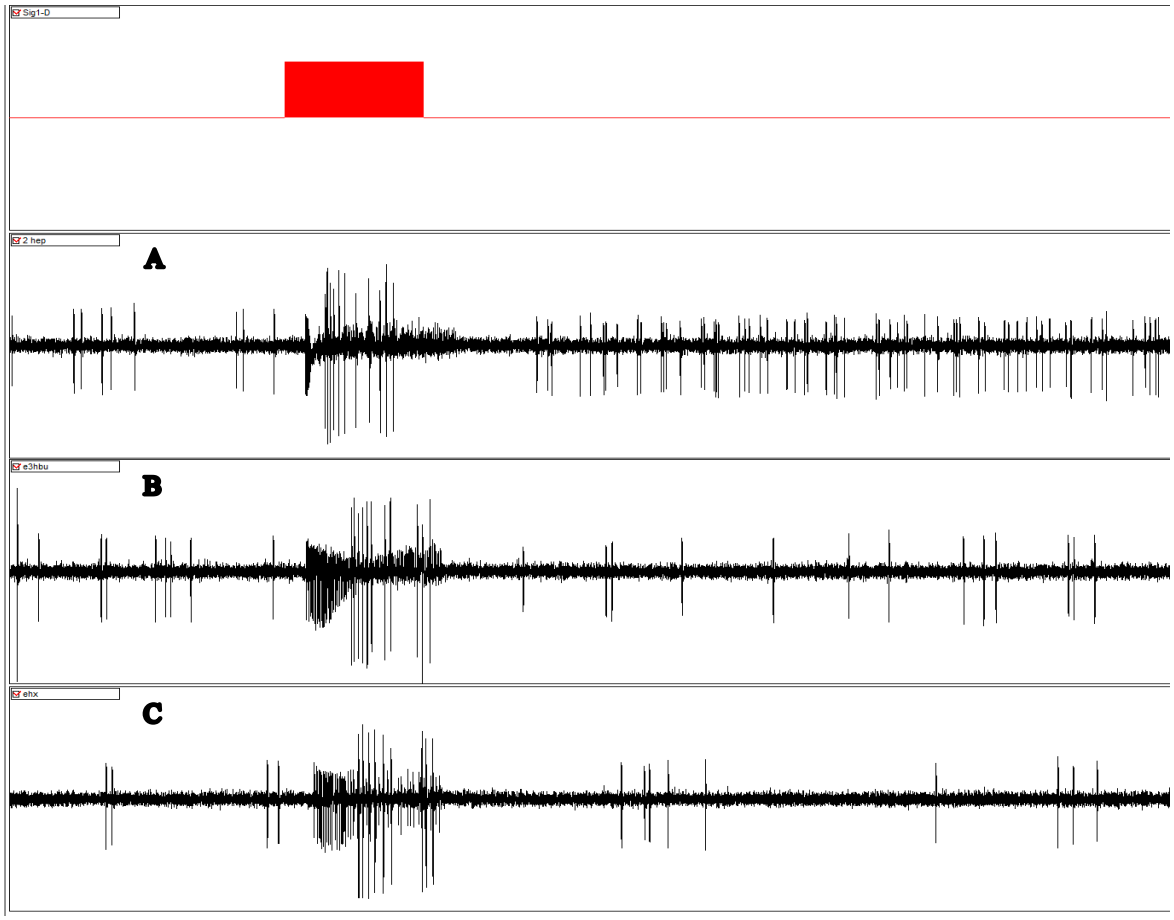


**Figure 9: Identification examples of an ab3 sensillum expressing MsexOR36 in D. melanogaster. The red bar indicates the duration of odor stimulus. A) After stimulation with 2-heptanone, an increase in the electrophysiological activity of the B neuron (smaller spike amplitude) can be observed. B) After stimulation with ethyl hexanoate, the A neuron (larger spike amplitude) does not respond as DmelOR22a is absent. C) After stimulation with ethyl-3-hydroxy butyrate, the diagnostic odor for ab2 sensilla, there is also no response from the A neuron.**

### 4.6 Recordings

Recordings were done using the software Autospike 3.9 which received information from the electrodes through a 4-channel signal amplifier. The software recorded the spikes

in electrophysiological activity 0.5 s before the odor sample was puffed through the tube and 0.5 s after it. Eleven flies in total were recorded. The diagnostic odors were always puffed at first (figure 9). The *A. palmeri* and *D. wrightii* odors where puffed in alternating order each time, but always starting with the lowest concentration first. In order to discard the reaction that MsexOR36 might have to the solvent alone, pure DCM activity was recorded once before both plant scents and a second time after the plant scents' activity was recorded. Between every pulse/recording there was an interval of about 40-60 s. Odor cartridges were used a maximum of three times before they were discarded, and new ones were prepared.

The raw data was processed with Autospike 3.9. To distinguish the electrophysiological activity from the A neuron and the B neuron, a threshold value was determined for each recording. Spikes with a higher electrical potential than the threshold were assigned to belong to the A neuron. This assignment was possible due to the clear difference in electrical potential [V] of both OSNs, as the A neuron had a higher electrical potential than the B neuron. Then in order to remove the effect of spontaneous activity inside the neuron, the number of spikes in electrophysiological activity of the A neuron recorded 0.5 s before the pumping of each odor was subtracted from the number of spikes recorded 0.5 s after the pumping. The data was multiplied by two to account for a whole second, and the averaged DCM spike count done was subtracted from all the *A. palmeri* and *D. wrightii* readings.

4.7 Significance Test

This data was then processed with GraphPad InStat. Considering the small sample size and the inability to determine if the data is normally distributed, a non-parametric Wilcoxon signed rank test was performed to test the significance of the odors used, with a significance level of 0.05.

## Results

### 5 Cloned ORs

The first aim of this study was to clone several olfactory receptors of *M. sexta* in order to enable a future deorphanisation in heterologous expression systems. The ORs which were cloned were MsexOR8, MsexOR13, MsexOR15, MsexOR17, MsexOR33 and MsexOR80. After a comparison with the DNA sequences published by Koenig (Koenig et al 2015) the obtained clones can be identified as the intended olfactory receptor. There were no nucleotide replacements, additions or deletions that could have resulted in the coding of different amino acids in the cloned ORs (figure 10 and 11).

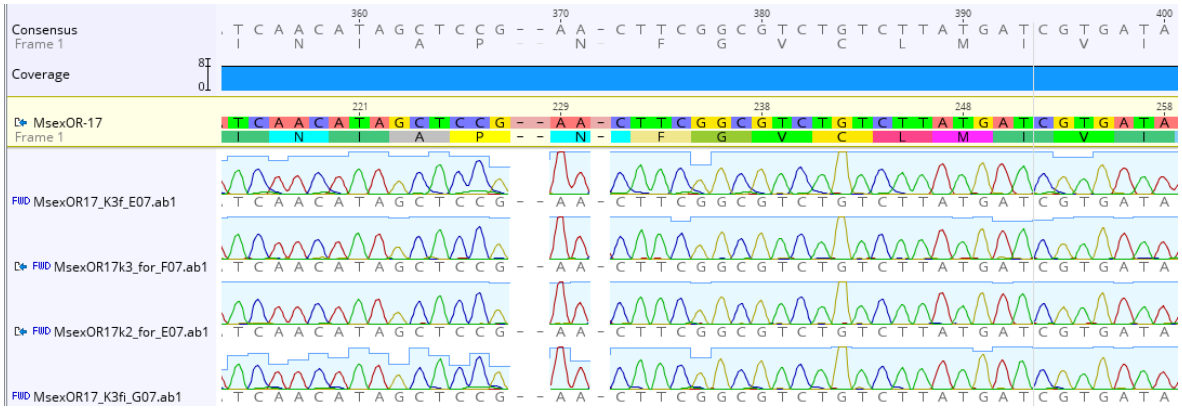


**Figure 10: Cloned MsexOR17 sequences compared to the reference Geneious Prime. The figure shows the OR sequenced with the forward primers. The positions from the base pairs number 355 to 400 are shown, which match the reference (named consensus here) one to one.**

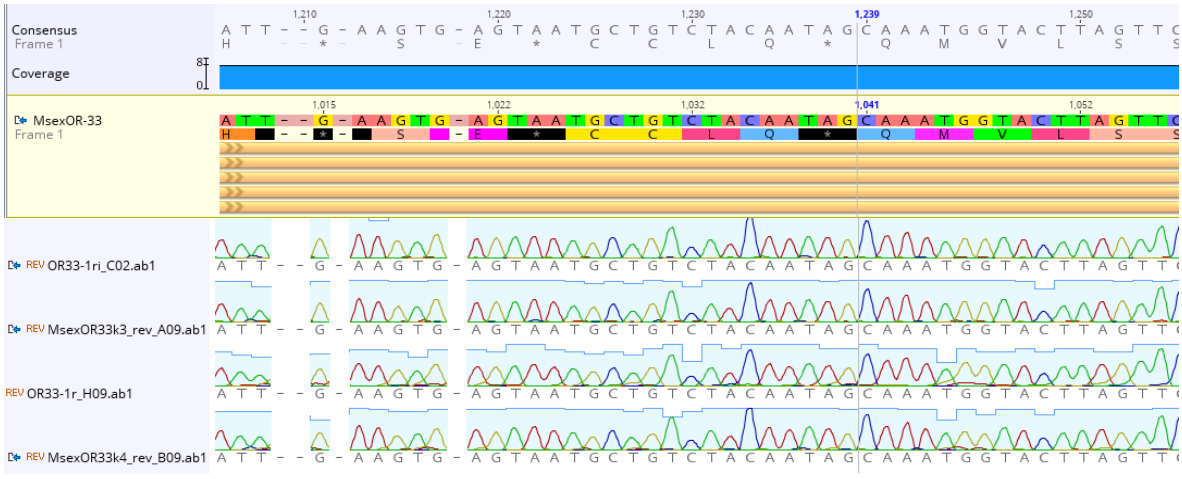


**Figure 11: Cloned MsexOR33 sequences compared to the reference in Geneious Prime. The OR here was sequenced with the reverse primers. The positions from the base pair number 1210 to 1250 are shown, which match the reference (here named consensus) one to one.**

## 6 Single sensillum recordings

The second aim of this study was to use the floral headspace volatiles of Palmer's century plant and jimson weed to deorphanise the olfactory receptor MsexOR36 of the tobacco hawkmoth. From the different concentrations used, only the pure concentration of both flowers and a 1:10 dilution of *A. palmeri* elicited significant responses within the ab3 sensillum (table 2). Figure 12 and 13 show two examples of the recorded spikes. A boxplot was made to visualize the responses of all different concentrations of the floral headspaces (figure 14).

**Table 2: Net number of spikes per second after stimulation with the headspace of A. palmeri and D. wrightii. (-) indicates that this stimulation was not done; and p values from the non-parametric Wilcoxon signed rank test of the different concentrations.**

| | *A. palmeri* | | | | *D. wrightii* | | | |
|---|---|---|---|---|---|---|---|---|
| | 1:1000 | 1:100 | 1:10 | pure | 1:1000 | 1:100 | 1:10 | pure |
| Fly 1 | (-) | -3 | 11 | 25 | (-) | -2 | -1 | 13 |
| Fly 2 | 2 | 6 | 7 | 10 | 8 | -3 | -4 | -11 |
| Fly 3 | 0 | 0 | 22 | 15 | 0 | 0 | 3 | 5 |
| Fly 4 | -16 | -6 | -3 | 13 | -6 | -14 | -4 | -1 |
| Fly 5 | 0 | 0 | 0 | 24 | 0 | 0 | 0 | 13 |
| Fly 6 | 0 | 0 | 0 | 23 | 0 | 0 | 3 | 13 |
| Fly 7 | 0 | 0 | 12 | 16 | 0 | 0 | 7 | 19 |
| Fly 8 | 0 | 0 | 7 | 35 | 0 | 0 | 0 | 14 |
| Fly 9 | 0 | 0 | 4 | 30 | 0 | 0 | 4 | 14 |
| Fly 10 | 0 | 0 | 0 | 15 | 0 | 0 | 0 | 6 |
| Fly 11 | (-) | (-) | (-) | (-) | 0 | 22 | 10 | 6 |
| p= | >0.999 | 0.750 | 0.031 | 0.002 | >0.999 | 0.875 | 0.383 | 0.014 |

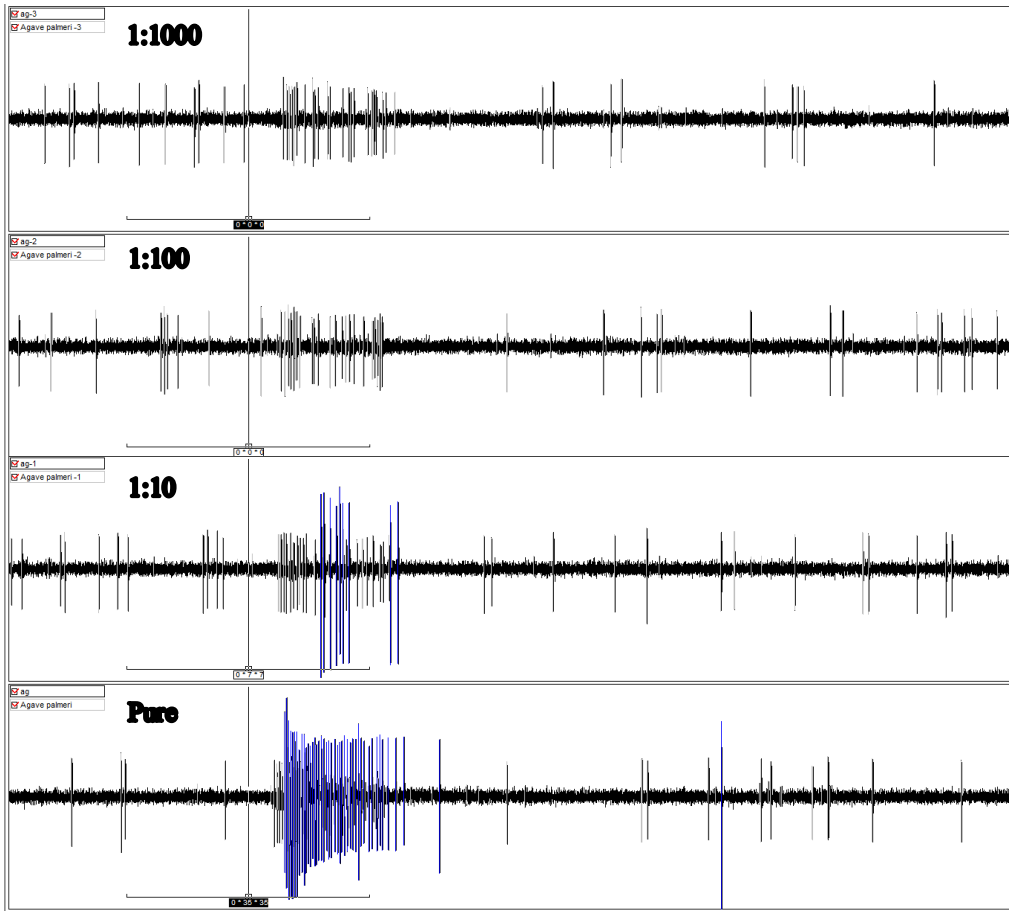


**Figure 12: Single sensillum recordings from transgenic flies after stimulation with the floral headspace of *A. palmeri* (Fly number 8). The blue lines represent the electrophysiological spikes in activity of the A neuron.**

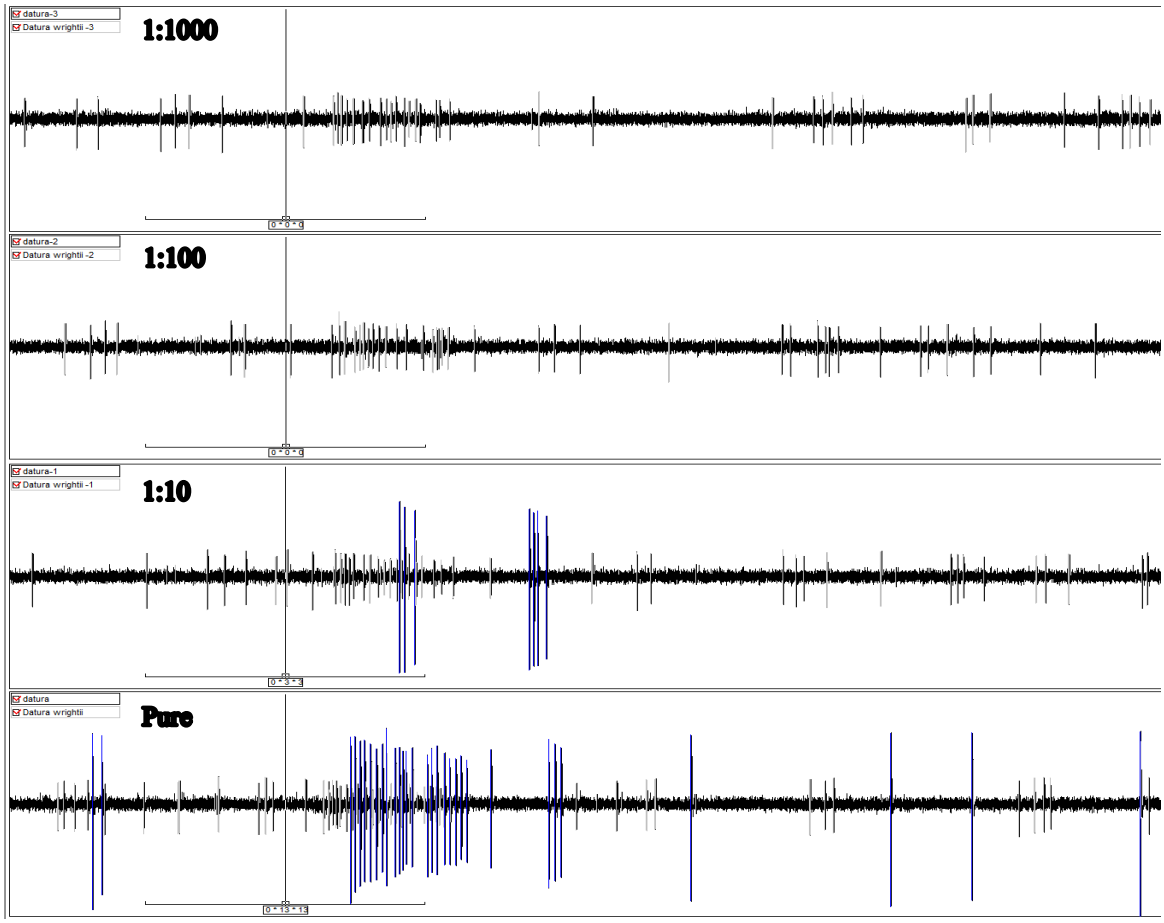


**Figure 13 Single sensillum recordings from transgenic flies after stimulation with the floral headspace of *D. wrightii* (Fly number 6).**

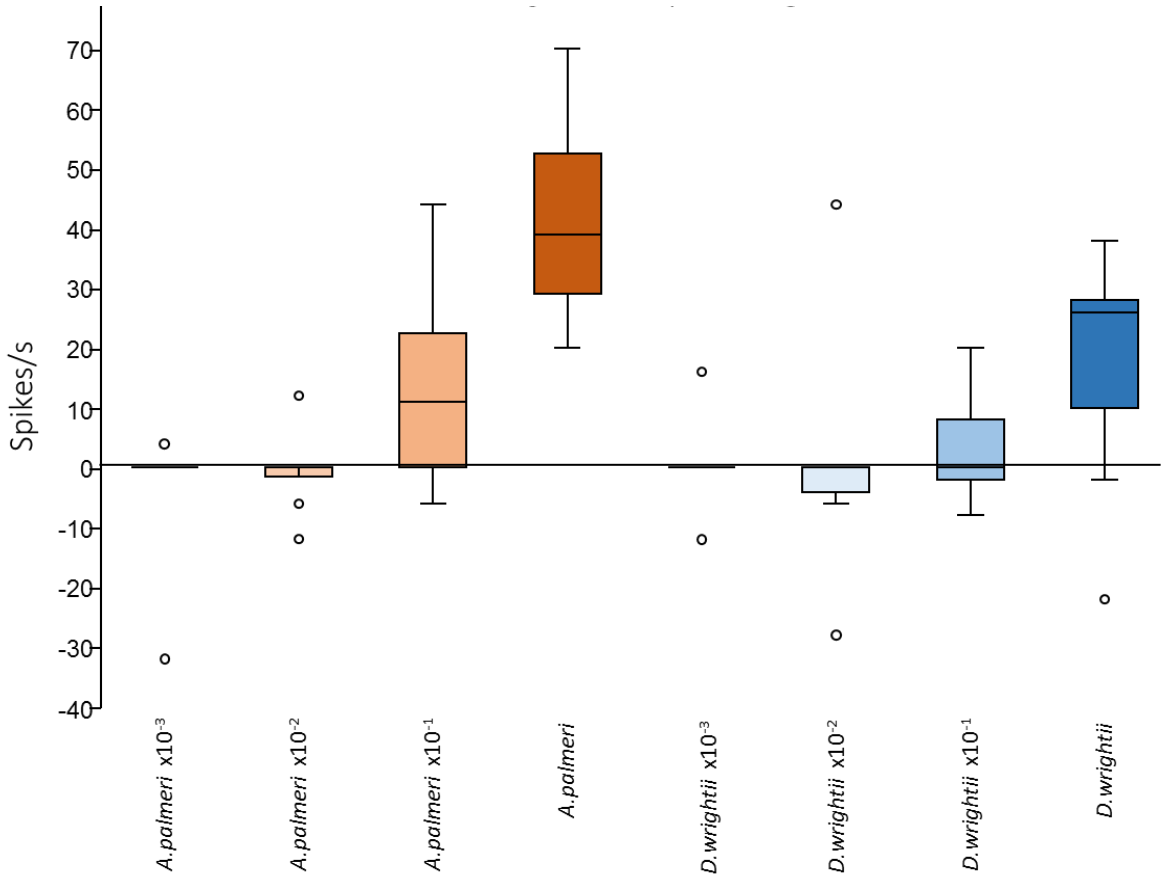


**Figure 14: Response of MsexOR36 expressed in the empty neuron system of the fly towards stimulations with the headspace of *A. palmeri* and *D. wrightii* at different concentrations. Box plots depict the net median number of spikes/s (horizontal line in the box) and the 25th and 75th percentiles (upper and lower margins of the box) together with minimum and maximum values (whiskers), and outliers (circles).**

**Discussion**

With the successful cloning of the six *M. sexta* ORs (MsexOR8, MsexOR13, MsexOR15, MsexOR17, MsexOR33, and MsexOR80) a window of opportunities is open. As performed with MsexOR36, the deorphanisation of these receptors using the empty neuron system of the fly will be the next step. Three of the ORs cloned in this study (MsexOR8, MsexOR33, MsexOR80) are in the same duplication clade as MsexOR36. These receptors might, therefore, respond to chemically similar compounds as MsexOR36, and might also play a role in finding nectar sources. The other cloned ORs have a higher expression in the female antenna than in the male antenna and as such might be important in female-specific behaviors like oviposition (MsexOR13, MsexOR17). As MsexOR15 is located in a cluster with additional pheromone receptors, this receptor might be involved for example in the avoidance of courting with males from other moth species. Therefore, it would be interesting to test these ORs with hostplant-specific odorants (MsexOR13, MsexOR17), or male pheromones (MsexOR15), respectively.

Regarding MsexOR36, the electrophysiological data shows an almost two times stronger response towards stimulation with floral headspace from *A. palmeri* than from *D. wrightii*. This could very well translate to *M. sexta* having a higher affinity to *A. palmeri* flowers. This data also seems to agree with the results of Alarcon (Alarcon et al 2008). He and his team studied the composition of the pollen load found on the proboscis of *M. sexta* in its habitat in southern Arizona. These are roughly 75% *A. palmeri* pollen, 20% *D. wrightii* pollen, and 5% pollen from other flowers (Alarcon et al 2008). From these results, it is safe to say that MsexOR36 plays a relevant role in the detection of *A. palmeri* and *D. wrightii* during foraging. How big the role of this receptor is cannot be answered solely with these results, however. Until all *M. sexta'*s ORs have been tested with these two floral scents and other odorants, we cannot assess how big the impact of MsexOR36 is for the detection of possible food sources. Noteworthy is also to mention *M. sexta*'s range is vast, as it can be found in Central America well into subtropical South America (Kingsolver et al 2012, Singer & Cocucci 1997). It would be interesting to analyze the pollen load on the proboscis of *M. sexta* populations that live in parts the rest of the continent, where *A. palmeri* and *D. wrightii* don't grow natively. These studies will reveal which plant species serve as predominant nectar sources in other habitats. It will be interesting to test the floral headspace of those plants in future deorphanisation experiments. As mentioned before, MsexOR36 is closely related to MsexOR8, MsexOR33, and MsexOR80. Supported by the results of Msex36, it

could be possible that these other three receptors also take a part in the detection of flower volatiles, specifically of *A. palmeri* and *D. wrightii*. Testing them with both flower scents, as well as with other flower volatiles would be an compelling approach in understanding the nature of gene duplication within receptor lineages. A comparison with the broadly-tuned *Bombyx mori* odorant receptor OR24 (Tanaka et al 2009) would also be of high interest, as it is closely related to the *M. sexta* cluster mentioned above, and could also help in understanding why gene duplications take place.

It is important to mention possible error sources that could have affected the SSR data collection. During the puffing of the odors to the sensilla, it was sometimes difficult to avoid moving the whole setup slightly. This was sometimes reflected in the ab3a neuron showing a strong rise in activity, which was not seen before the moving of the setup. After some minutes, the activity returned to a similar state as previously, and the recordings then proceeded, but it's not possible to say that this could not have altered the recorded activity. Worth mentioning as well is the challenging task of fixing the *D. melanogaster* flies. In almost all eleven recorded flies it was not possible to completely immobilize the antenna, as this resulted in the antenna getting damaged. Therefore, the antenna always had a slight quiver, which sometimes resulted in the electrode popping out of the sensillum and the sensillum having to be poked again, or sometimes a new ab3 sensillum had to be found. To what extent this affected the recordings is not known and should be taken into consideration. However, the empty neuron system of the vinegar fly seems to be a very useful tool to deorphanise moth odorant receptors, and leaves the door open for many different types of approaches in the understanding of moths, and all other insects for that matter.

**Conclusion/ Zusammenfassung**

Insects rely profoundly on olfactory cues to navigate through their environment. The tobacco hawkmoth is no exception, as it relies on detecting different chemical signals for foraging, mating, and other behaviors. This study focuses on the deorphanisation of a tobacco hawkmoth odorant receptor (MsexOR36) using the empty neuron system of the vinegar fly. The headspace volatiles of the flowers of two plants known to be valuable nectar sources for these moths, jimson weed and palmer's century plant, were chosen for this purpose. Furthermore, six other odorant receptors were cloned and prepared for future deorphanisation (MsexOR8, MsexOR13, MsexOR15, MsexOR17, MsexOR33, and MSexOR80). MsexOR36 showed a significance response to both pure flower headspace volatiles, as well as a significant response to a 1:10 dilution of palmer's century plant. This result indicates that MsexOR36 plays a role in the detection of food sources for the tobacco hawkmoth, especially with palmer's century plant. A further deorphanisation of other odorant receptors could help answer the question of how big this role is.

Insekten sind auf olfaktorische Reize tiefgreifend angewiesen, um durch die Umgebung zu navigieren. Der Tabakschwärmer stellt keine Ausnahme dar, da er sich auch stark an das Aufspüren chemischer Signale für die Nahrungssuche, Paarung, und andere Verhalten verlässt. Diese Studie legt den Schwerpunkt auf die Deorphanisation eines olfaktorischen Rezeptors des Tabakschwärmers (MsexOR36) unter Verwendung des ‚empty neuron system' der schwarzbäuchigen Fruchtfliege. Die ‚headspace volatiles' der Blüten zweier Pflanzen, die als wertvolle Nektarquelle dieser Motten bekannt sind, ‚Palmer's century plant' und Wrights Stechapfel, wurden zu diesem Zweck ausgewählt. Darüber hinaus sechs weiteren olfaktorische Rezeptoren wurden kloniert (MsexOR8, MsexOR13, MsexOR15, MsexOR17, MsexOR33, and MSexOR80) und für zukünftige Deorphanisation vorbereitet. MsexOR36 hat eine bedeutende Antwort auf die pure ‚headspace volatiles' beider Blüten gewiesen, sowie eine bedeutende Antwort auf eine Verdünnung 1:10 von ‚Palmer's century plant'. Dieses Ergebnis deutet darauf hin, dass MsexOR36 eine Rolle bei der Aufspürung von Nahrungsquellen beim Tabakschwärmer spielt, insbesondere bei der Aufspürung von ‚Palmer's century plant. Die Deorphanisation weiterer olfaktorischer Rezeptoren kann bei der Aufklärung der Bedeutung dieser Rolle beitragen.

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## Appendix

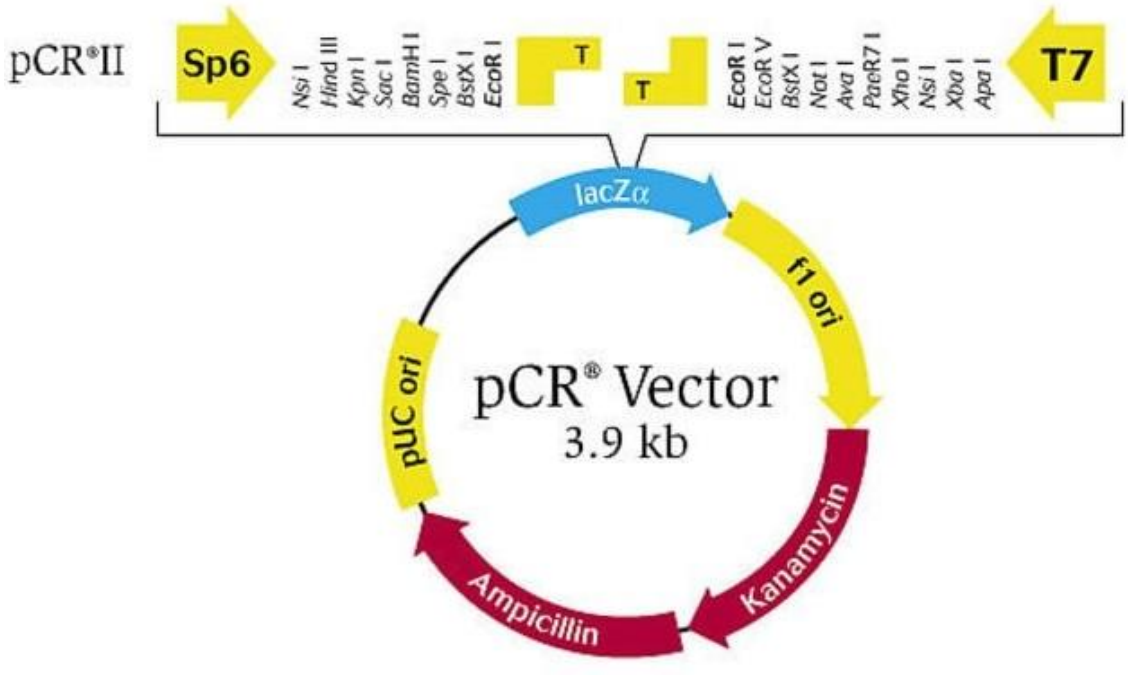


**Figure A1: pCR®II vector with the restriction enzyme sites. Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)**

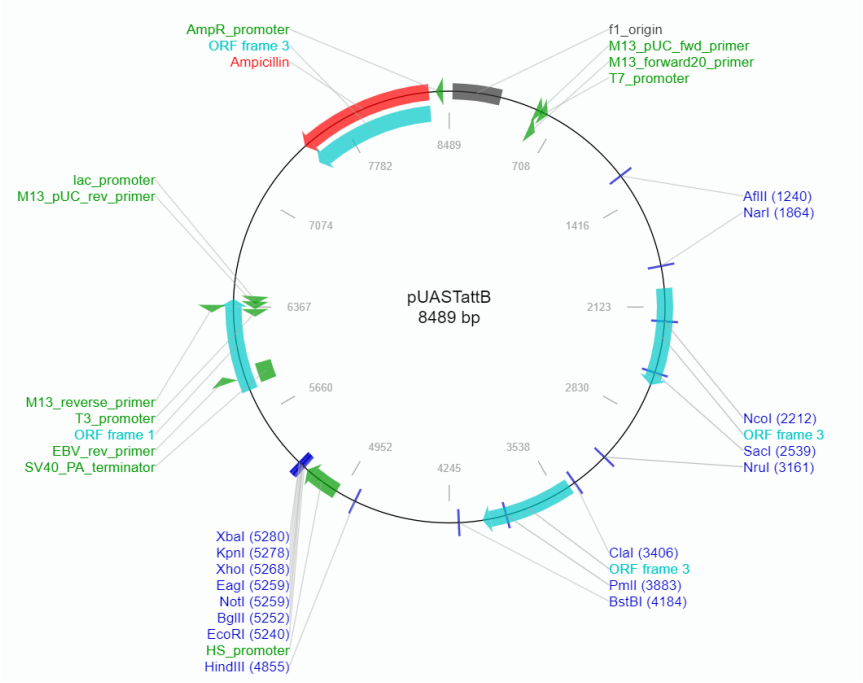


**Figure A2: pUASTattB vector showing the resctriction enzyme sites. Image belongs to Addgene, Inc. (Cambridge, Massachusetts, USA)**

**Table A1: Recipe fed to *D. melanogaster* flies**

| | | 500ml |
|---|---|---|
| treacle | g | 59 |
| brewer`s yeast | g | 5.4 |
| hot water | ml | 101 |
| agar | g | 2.1 |
| cold water | ml | 135 |
| Polenta | g | 47 |
| fill up with hot water | ml | 135 |
| flush out with hot water | ml | 34 |
| cold water | ml | 54 |
| propionic acid | ml | 1.2 |
| Nipagin 30% | ml | 1.65 |

**Table A2: Sequence of primers**

| Olfactory receptor/vector | forward primer | reverse primer |
|---|---|---|
| MsexOR8 | 5'-TTGCTGTGCTCAAAATTGCTAAAAAGAC-3' | 5'-ATGTTTCGATAGACATTCAAAATTGCTTG-3' |
| MsexOR13 | 5'-CGGAATAGACGTACCTCAAAAAAT-3' | 5'-ATAAGTGTGCAAGATTGTAGTCAC-3' |
| MsexOR15 | CTTCCGATCTAGCGGTACGTTGTA-3' | 5'-GCGAATTACTATGCAGCTAGCTCG-3' |
| MsexOR17 | 5'-TGATCAGACGCGTTTTCTAAAATGT-3' | 5'-GTATTTATCAATAAATTATCCGATACAGT-3' |
| MsexOR33 | 5'-AGAGGAGTCACTTTGGTAAAAAGAC-3' | 5'-ATGTTTCGATGATATTCAAATTGCTTG-3' |
| MsexOR80 | 5'-CGATGTCTGATCTGATGTTTGACCAAT-3' | 5'-TCTCAGTCAACTTATCACTCTCGAGG-3' |
| pUAST.attB | 5’-AACTACTGAAATAATCTGCCAAGAAGT-3’ | 5’-CCTTAGAGCTTTAAATCTCTGTAGG-3’ |

**Selbständigkeitserklärung**

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe, alle Ausführungen, die anderen Schriften wörtlich oder sinngemäß entnommen wurden, kenntlich gemacht sind und die Arbeit in gleicher oder ähnlicher Fassung noch nicht Bestandteil einer Studien- oder Prüfungsleistung war.

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