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Faculty of Landscape Architecture, Horticulture and Crop Production Science

Effect of Co-expression of *Spodoptera* Olfactory Receptors on *Drosophila* Odour Perception

Title in Swedish:

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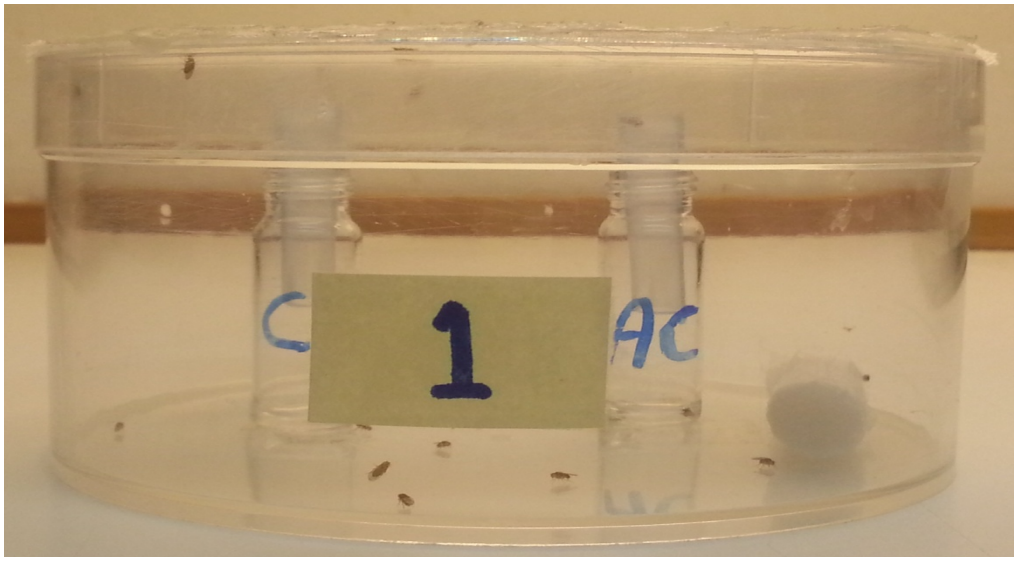

Hayder Muhammad Awais

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Effect of Co-expression of *Spodoptera* Olfactory Receptors on *Drosophila* Odour Perception

Samuttryck av *Spodoptera*-doftreceptorer hos *Drosophila*: effekter på flugornas doftuppfattning

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Table of contents

Abstract

Like humans, other animals are also able to smell and differentiate a large number of odours with great accuracy. Chemosensory signals play a vital role in locating host plants by insects. *Drosophila* has a well-developed olfactory system which can differentiate between different odours and recognize food, oviposition sites and potential mates. Studies of olfactory behaviour in invertebrates may aid in understanding the link between odour recognition and behaviour. Some selected olfactory receptors from *Spodoptera* (Slit-ORs) were co-expressed in *Drosophila* with its olfactory receptor Or92-a in the *Drosophila* ab1B neuron type by means of existing DmelOR92a-Gal4 and UAS-SlitOR lines. *Drosophila* olfactory receptor 92-a responses to acetoin. Acetoin is also detected by *Drosophila* olfactory receptor Or9-a which is located in ab8B neuron. Olfactory mediated behavioural responses of flies having modified olfactory systems were investigated to different odours, to determine which (if any) of the receptor modifications would change odour preference of flies. An olfactory attraction assay was employed for this purpose. In parallel, DNA sequences were cloned to prepare a transformation vector construct for stable expression of the Slit-ORs in DmelOR92-a neurons, containing the promoter sequence for *Drosophila* olfactory receptor Or92-a and selected olfactory receptor sequences from *Spodoptera.* The sequences were aimed to be cloned into a suitable *Drosophila* germ line transformation vector attB. There was no significant effect of co-expressing SlitOR in OR92-a neuron on the olfactory behaviour of *D. melanogaster* OR-a, OR-b, OR-c and OR6 receptors. Most strains were significantly attracted to acetoin, similar to wild type flies. In contrast, very few were attracted to the corresponding ligands for the Slit-ORs, regardless whether the *Spodoptera* OR was expressed or not.

1 Introduction

A majority of the known species of our planet are insect species and they are well adapted to different habitats which are the reason for their successful survival. Insects are beneficial for humans as they play an important role in pollination and they are harmful as well because they are disease carriers also which negatively affects the human life (Hill, 1997). Insects also need food for development and growth. They have to search the mates for reproduction as well, during these activities; they have the chances to meet predators (Bell, 1990).

Like humans, other animals are also able to smell and differentiate a large number of odours with great accuracy (Voshall et al., 2007). Different studies showed that insects are able to recognize their host plants by the mixtures of specific volatiles with specific ratios (Bruce et al., 2005; Riffell et al., 2009). Chemosensory signals play a vital role for locating the host plant by insects, including the fruit fly, *Drosophila melanogaster*, as well (Hildebrand and Shepherd, 1997). *Drosophila* has a well-developed olfactory system which can differentiate between different odours and recognize food, oviposition sites and potential mates. Olfactory sensory neurons (ORNs) of the fruit fly are primarily located on antenna and maxillary palp which are present on the head. Olfactory sensory neurons have odorant receptors expressed on the surface of their dendritic branches (Clyne et al., 1999). There are sensory hair on the antenna and maxillary palp that are called sensilla. The ORNs project their dendrites up into the sensilla. In *Drosophila,* there are 410 and 60 sensilla on antenna and maxillary palp respectively. Sensilla are divided into three classes called basiconic, trichoid and coeloconic. There are further two types of basiconic sensilla, large and small (Fig1). Large basiconic sensilla are located on the medial part of antenna while the three types of trichoid sensilla are located on medio-basal part of antenna. Coeloconic sensilla are present on the central part of antennae. Trichoid sensilla are called T1, T2, T3 and T4 (Couto et al., 2005) have one, two or three olfactory sensory neurons respectively. Basiconic sensilla have two to four neurons. Coleconic sensilla own two or three neurons (Couto et al., 2005). Flies also have the ability to taste by proboscis, legs wings and vaginal plate (Fig1).

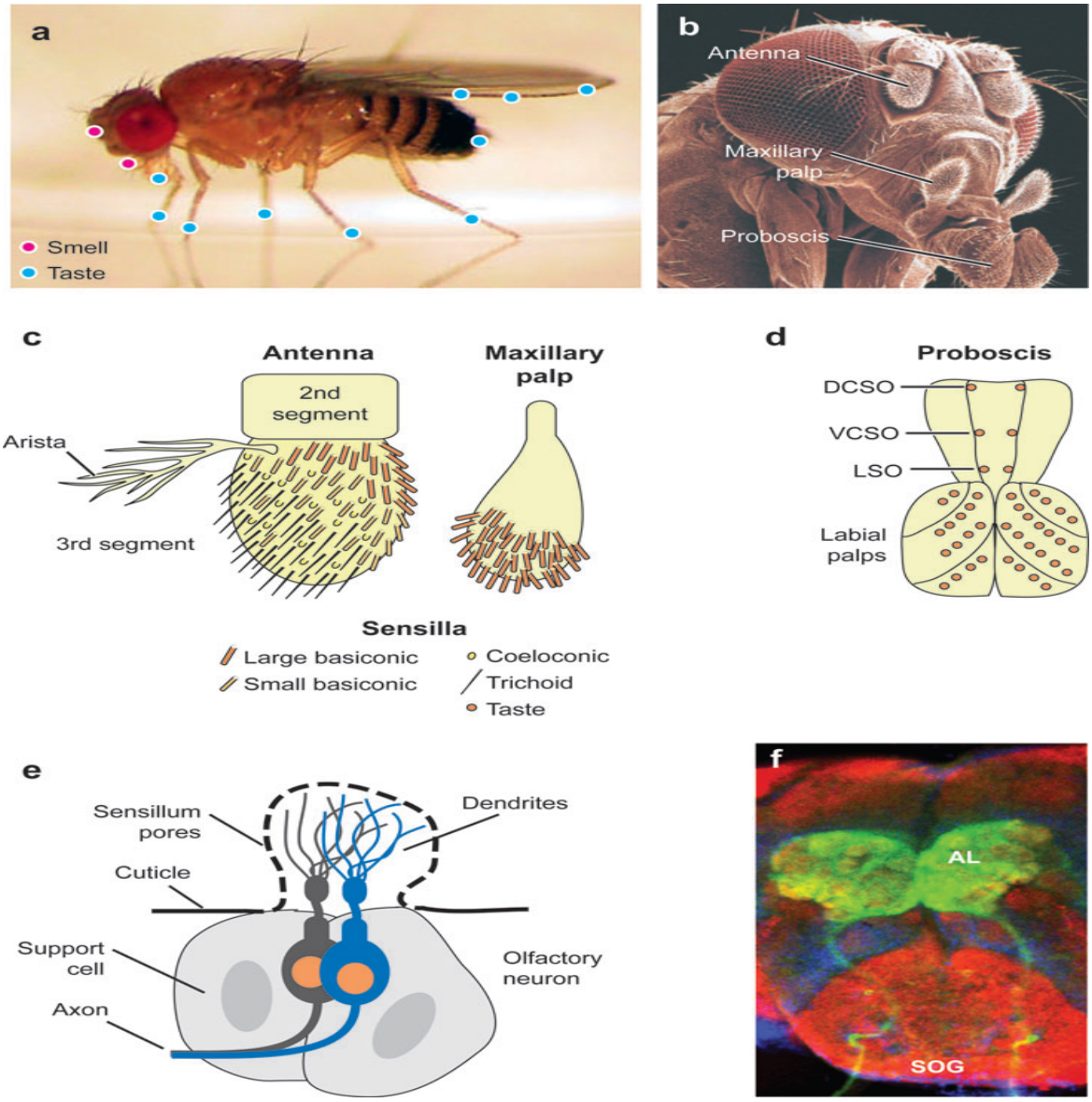

Fig1: *Drosophila* Olfactory system (Diagram Source Voshall and Stocker 2007).

When an odour molecule comes in contact with the odorant receptors, an electrochemical response is mediated (Bargmann, 2006). The odour molecules come in contact with the ORs, which are on the antenna or maxillary palp. Then there is electrical transformation through action potential of ORNs, and the message is first sent to the projection neurons from the antennal lobe and then the signal is passed to the higher brain centers including the mushroom body and lateral horn (fig 2).

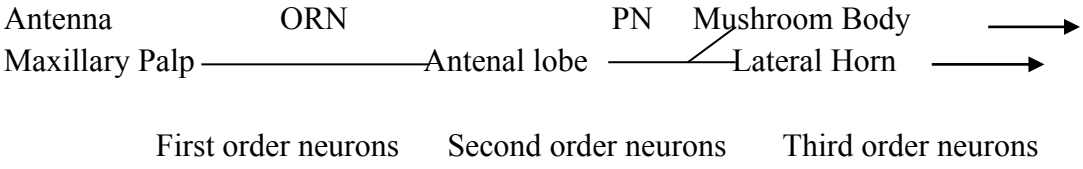

Fig 2: Schematic diagram of olfactory processing (Source: Tanka et al., 2004)

Studies of olfactory behaviour in invertebrates may aid in understanding the link between odour recognition and behaviour. Insects provide an attractive model system for studying the peripheral and central events in olfaction. Still, the relationship between odour input channels (olfactory sensory neuron types) and recognition of specific odours is only superficially known.

In the present project I will specifically study the influence of specific types of sensory neurons on insect odour perception. Are any individual types of sensory neurons essential for the perception of a defined class of odour molecules? If this is the case, can we "fool" the olfactory system by changing the response spectrum of these sensory neurons towards another type of odour molecule?

One chemical may often excite more than one type of receptor and vice versa (Dobritsa et al., 2003: Hallem et al. 2004, 2006). Nevertheless, many receptors appear to be evolved to detect certain key ligands with high sensitivity and specificity. Our aim was to express *Spodoptera* olfactory receptors OR-a, OR-b, OR-c (Larsson et al., *unpublished data*) and OR6 (Montagne et al., 2012) in the *Drosophila* ab1B neuron, with its native receptor 92-a, which responds to acetoin odour (a fermentation product) (Stensmyr et al., 2003; Couto et al., 2005). Or92-a was chosen to avoid the involvement of other neurons as it is expressed singly and in a few neurons not in the whole brain. Acetoin is also detected by *Drosophila* olfactory receptor Or9-a which is present in ab8B neuron (Hallem et al., 2004).

Or 92a is not the only receptor in *D. melanogaster* that responds to acetoin (Hallem et al., 2004), but it is likely to be an important input channel. The question is whether activating this single receptor alone would be sufficient for the flies to perceive the olfactory impression of acetoin? If this would be the case, we would expect an attraction response to any ligand known to activate *S. littoralis* ORs co-expressed with the Or92a receptors, if the flies perceive the two compounds as similar based on this shared input channel.

Olfactory receptor Or-a of *S. littoralis* responds to linalool, a naturally occurring terpene alcohol found in many flowers and spice plants. Or-b responds to beta ocimene while OR-c responds to eugenol (Unpublished data Larsson et al.,). *S. littoralis* employs receptor OR6, which responds to pheromone component (Z9, E12-14:OAc), for long-range sex pheromone attraction (Montagne et al., 2012).

1.1 Aims and/or formulation of objectives:

- Heterologous co-expression of receptors from the moth *S. littoralis* in defined olfactory neurons of *Drosophila*, together with their native receptors.
- Investigation of the behavioural response of the flies that have a modified olfactory system in the detection of different odours.
- Preparation of transgenic constructs with direct fusions of *Drosophila* promoters and *Spodoptera* receptor genes in order to obtain constitutive expression of selected *Spodoptera* receptors in *Drosophila*. For further investigations we aim to express optical imaging dyes in the brain, while heterologously expressing a SlitOR in the Or92a neuron. To avoid the interefecnce of the Gal4 system, we would choose to use a direct fusion construct for the SlitORs, thus freeing the brain for using the Gal4 system there (Ng et al., 2002; Wang et al., 2003).

1.2 Hypothesis

The olfactory mediated behaviour of *Drosophila* that co-expresses *Spodoptera* olfactory receptors with its native receptor 92-a, to the tested corresponding *Spodoptera* ligands should be similar to flies native response to acetoin based on this shared input channel.

Theoretical assumptions regarding shared input channels. A ligand may be detected by the combination of two or more olfactory receptors. The modification of one of these olfactory receptors may expand olfactory attraction towards a novel ligand based on this single input channel. Alternatively, this single input channel may not be sufficient, or there could be non-specific activation of the novel ligand on other receptor neurons, which may change the olfactory perception of the other animals.

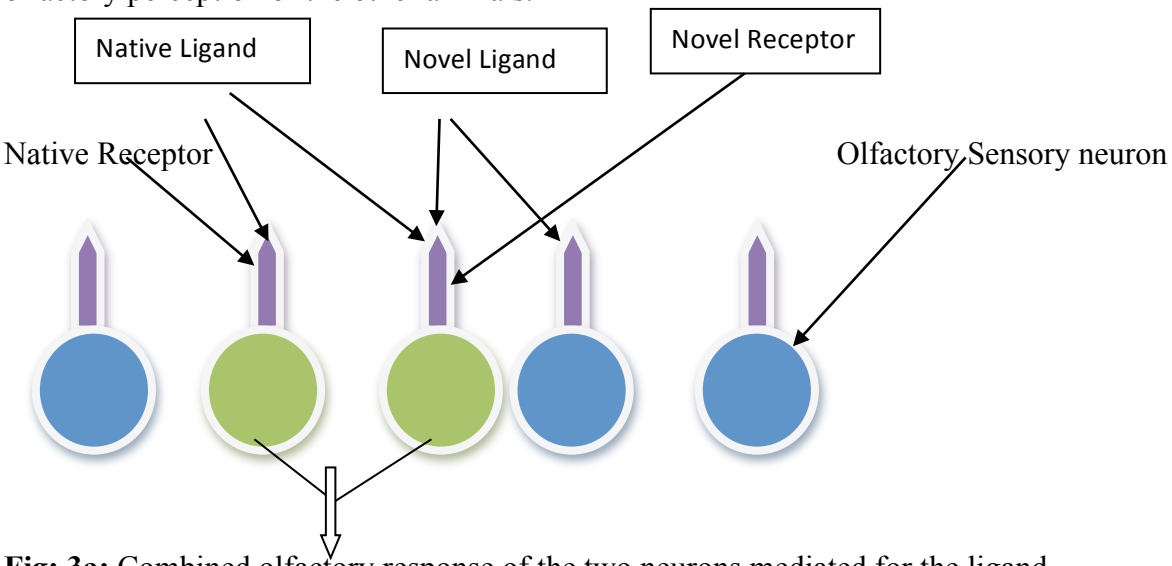

Fig: 3a: Combined olfactory response of the two neurons mediated for the ligand

On the basis of my hypothesis that a single input channel would be sufficient for attraction, I expect the olfactory behaviour to the tested compound similar to that of acetoin which is shown in a hypothetical figure (4) in terms of attraction index (A.I).

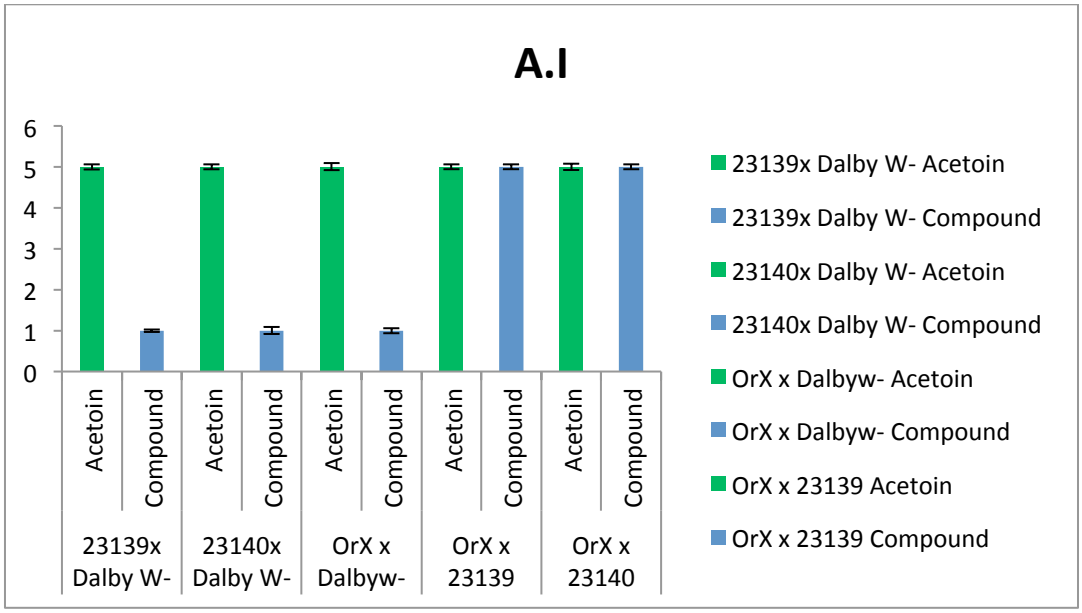

Fig: 4 Expected attraction index of fly lines based on the hypothesis

Olfactory mediated behaviour responses of each fly strain to each tested odour can be assessed independently by one-sample T-test determining whether the mean is significantly different from 0 (indicating significant attraction/repulsion). Within each strain, the mean responses to acetoin and to the corresponding signature odour for the SlitOR can also be compared by using a two-sample T-test.

2. Materials and Methods:

2.1 Experimental Organism

D. melanogaster was used as a model organism. Readily available mutants and genetic tools for research make *Drosophila* an advantageous model organism (Greenspan, 2004). Genetic manipulations can be carried out both in *Drosophila* cell culture (Bai et al., 2009) and in adult flies (Dzitoyeva et al., 2001). Short generation time (about 2 weeks) of *Drosophila* allows for rapid experiments within a short period of time.

2.2 Genetic tool used for the modification of *Drosophila* olfactory System:

UAS-Gal4 is a powerful gene expression technique that is used worldwide for the genetic modification of the *D. melanogaster*. For the induction of expression of *S. littoralis* ORs, males of Delta-Halo/Cyo; UAS-SlitOR(x) were mated with virgin females of Delta-Halo/Cyo; OR92a-Gal4. With the *Gal4-UAS* expression system, in F-1 generation, Delta-Halo/Delta-Halo; OR92a-Gal4/UAS-SlitOR(x), the promoter for the DmOR92a gene drives expression of the yeast Gal4 transcription factor, whereas *Gal4* binding to the *UAS* drives expression of the downstream transgene, SlitOR(x).

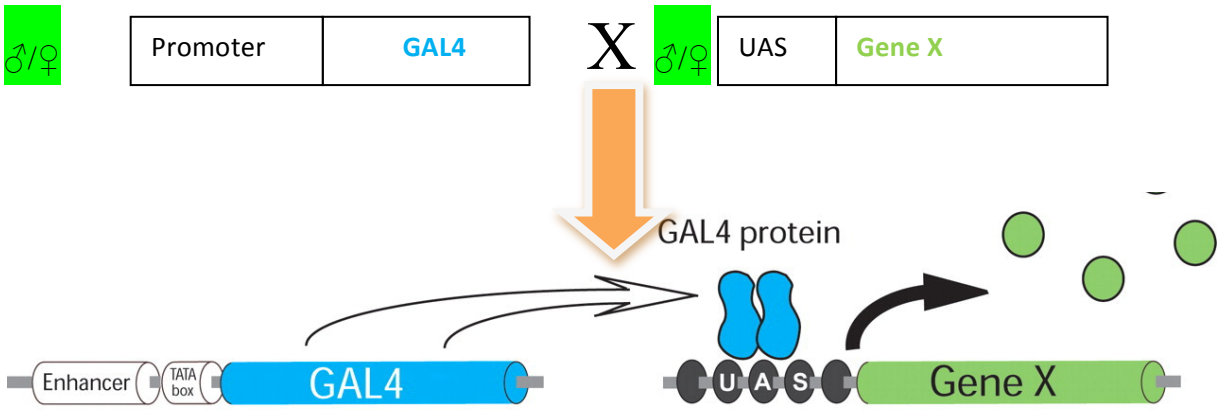

Fig 5: UAS/GAL4 Sysytem (Modified Image: Cold Spring Harb. Protoc; 2008;doi:10.1101/pdb.top49)

2.3 Fly lines

Transgenic lines were generated to make use of the Gal4/UAS system. Gal4 is a yeast transcription activation factor and UAS is upstream activation sequence, Gal4 exclusively binds to UAS to initiate the transcription of genes positioned downstream of the UAS binding site sequence.

We used the lines that were maintained in our lab, Chemical ecology group SLU, Alnarp campus, Sweden. White eyed Dalby-HL strain (Collected from outside environment of the

campus), made by repeated back-crosses of white mutant flies into a Dalby-HL (Ruebenbauer et al., 2008) line was used as control while the experimental lines were

(1) +/+; DOr92-a Gal4 (strain code 23139 from Blomington), in this strain the Gal4 is on the 3rd chromosome

(2) DOr92-a Gal4; +/+; (Strain code 23140 from Blomington), in this strain the Gal4 is on the 2nd chromosome.

(3) dHalo/cyo ; UAS-SlitOr-a/ SlitOr-a
(4) dHalo/cyo ; UAS-SlitOr-b/ SlitOr-b (SlitOrs; Alnarp strains)
(5) dHalo/cyo ; UAS-SlitOr-c/ SlitOr-c (dHalo/cyo; Dobritsa et al., 2003)
(6) dHalo/cyo ; UAS-SlitOR6/ SlitOR6

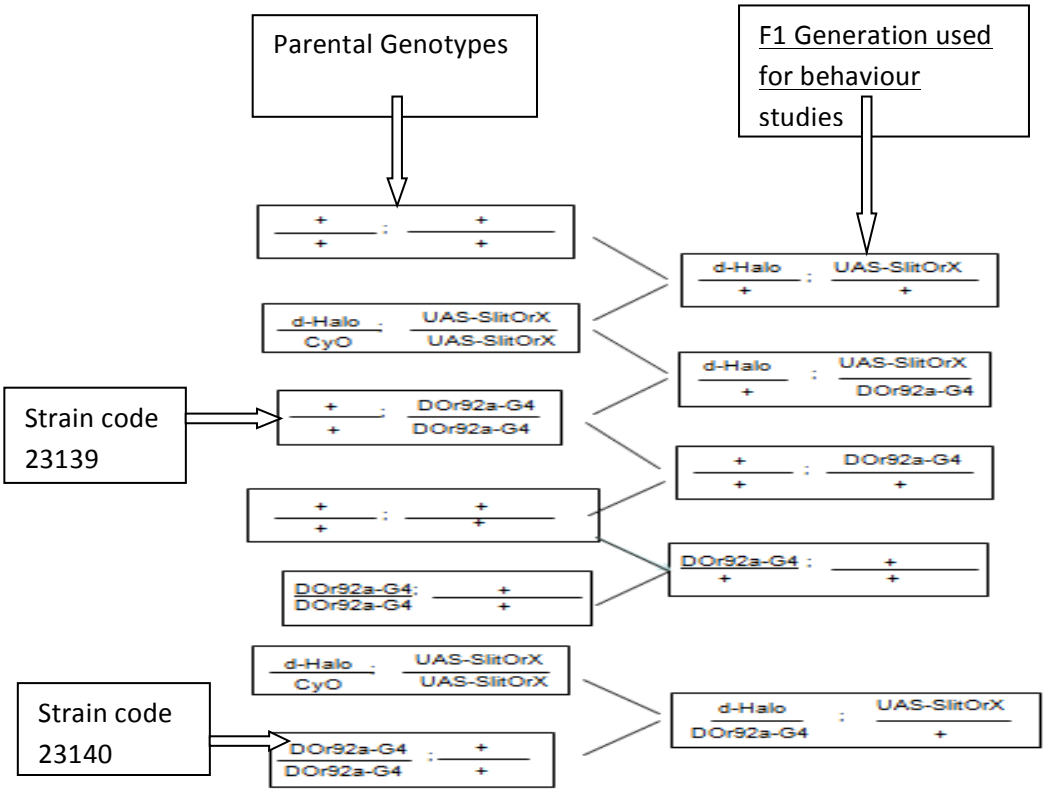

Fig 6: An overview of the genetic crosses of *Spodoptera* ORs(X) wild type and experimental strains

DOR: *Drosophila* olfactory receptor, SlitOR: *Spodoptera* Olfactory receptor, UAS: Upstream activation sequence: G4: Gal4 which is transcription activator factor from yeast, d-Halo: a deletion that is not relevant here as it is used in a heterozygous state: CYO: Balancer Chromosome with curly wings phenotypic marker (wild type condition is straight wings), +: wild type chromosome, Strain code 23139: in this strain the Gal4 is on the 3rd chromosome, Strain code 23140, in this strain the Gal4 is on the 2nd chromosome

The F1 generations of the crosses carried out were subjected to behavioural studies. The flies were exposed to different types of odours for a certain time period and their response was scored. A choice assay was employed for this purpose (described below). As a result we expect e.g. a wild type response (attraction) to the acetoin odour (a fermentation product) normally detected by the fly receptor Or92a.

2.5 Rearing conditions

Flies were fed with the standard laboratory diet. All the lines were maintained at 25 ^{o}C with 12:12 dark: light cycles with relative humidity 60%.

2.6 Exposure of flies to Acetoin

Drosophila olfactory receptor 92-a responds to acetoin (Stensmyr et al., 2003). To increase the behavioural attraction of flies towards acetoin, all lines were exposed to 1% acetoin and 1% sucrose during the day (Simpson and White 1990: Colomb, et al., 2009). Then all fly lines were starved overnight and odour attraction assay was carried out. The set up used for this purpose is shown in the following figure 7.

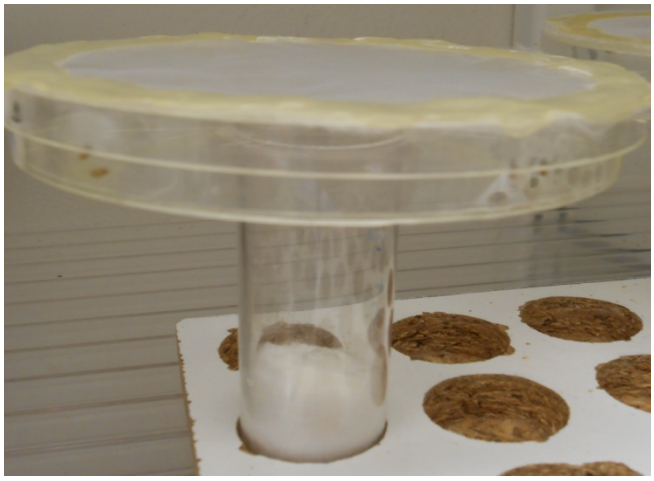

Fig 7: Set up for training of flies for acetoin

We have adopted a modified method for the fly olfactory training as used by Burke and Waddell (2011).

2.7 Odour attraction assay

The flies were kept in the round container (110 mm diameter and 60 mm height made of plastic material) with the test compound (0.1%) and the blank for 24 hours at 25^{O}C with 12:12 dark: light cycles with relative humidity 60%. Triton 0.01% was added in vials containing test solution and blank also. After 24 hours the numbers of flies drowned in the test compound or

in the blank were counted. Then attraction index (A.I) was calculated by using the following equation

A.I= (Treatment – Control)/ Total flies

Behavioural responses of different strains to acetoin ("native" control response odour) and the signature odour for each respective heterologously expressed SlitOR were assessed independently against water controls, and presented as mean A.I. from several replicates. About 32% flies died until the completion of the experiment (24 hours period). Two types of statistical comparisons were made, using Minitab 15. Responses of each strain to each odour was assessed independently by a one-sample T-test determining whether mean was significantly different from 0 (indicating significant attraction/repulsion). Within each strain, the mean responses to acetoin and to the corresponding signature odour for the SlitOR were also compared using a two-sample T-test.

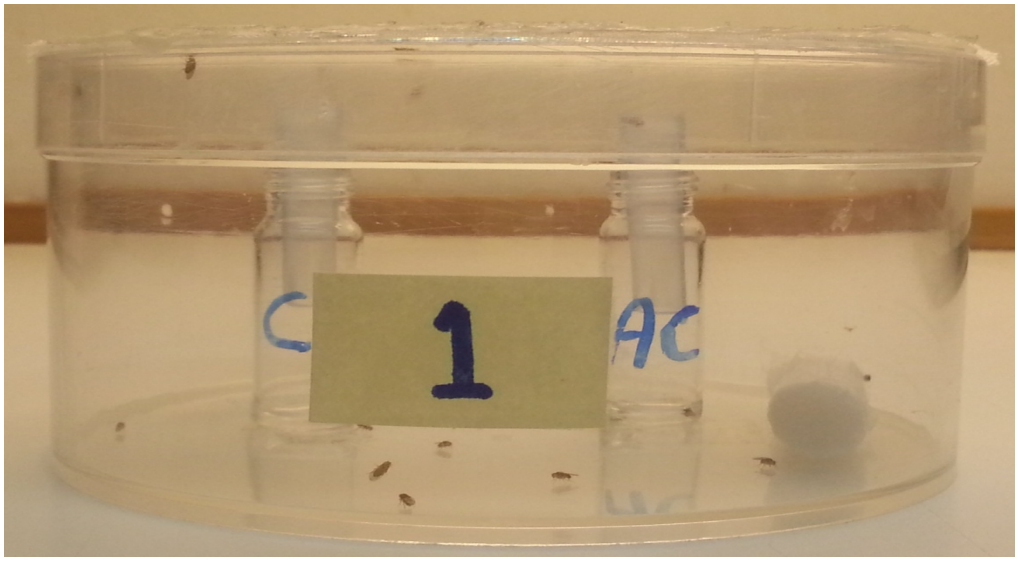

Fig 8: The set-up for the odour attraction assay

2.8 Preparation of constructs

I sought to prepare different vector constructs with the aim of achieving a permanently modified experimental olfactory system in *Drosophila*. A transformation vector construct containing the promoter sequence for *Drosophila* olfactory receptor Or92-a and selected olfactory receptors sequences from *Spodoptera* was prepared from both organisms and the sequences were aimed to be cloned into a suitable *Drosophila* germ line transformation vector attB (Bischof et al., 2007). A future aim would be for final constructs to be injected in the

Drosophila embryos for purposes of stable genomic transformation, outside the scope of the present project.

We have adopted a kit method called infusion cloning technique (Provided by clone tech), to polymerize the *Drosophila* 92-a Promoter containing overlapping sequence of *Spodoptera* ORs a, b, c and 6 individually, to the OR(X) sequences, which also include overlapping sequence from the 92a-Promoter. After PCR amplification the corresponding fragments were gel purified and the attB injection plasmid was digested. Then, attempts were made to fuse these fragments and the plasmid together in a single step reaction.

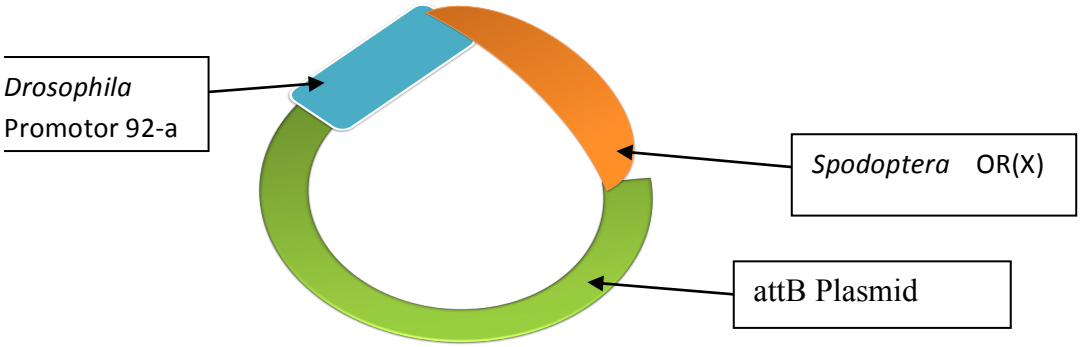

Fig:9 Schematic strategy for the fusion of fragments in the attB Plasmid

The primers were designed with primer 3 soft ware. The sequence of the primers is as follows

OR92aF – TTAACCCTTAAGGTTACCCAACTTTGACACCAAATGCAAGGGTAA

OR92a-aR - GCTGTTTGATCGGCTGACAGAATAAGTCTACCACTTGTGTAAAGCACC

OR92a6R - GCTGTTTGATCGGCTGACAGAATAAGTCTACCCAAATTTTTTCAAAGAA

OR92a-bR – GCTGTTTGATCGGCTGACAGAATAAGTCTACTTAAGCAAAGAAGTCTC

OR92a-cR - GCTGTTTGATCGGCTGACAGAATAAGTCTACCTTCTATTACATCGTAT

OR-aF - GACTGTCTTATTCAGATGGTGAACACATTTCGTGG

OR6F – GACTGTCTTATTCAGATGGGTTTAAAAAAGTTTCTT

OR-bF – GACTGTCTTATTCAGATGAATTCGTTTCTTCAGAG

OR-cF – GACTGTCTTATTCAGATGGAAGATAATGTAGCATA

OR4RattB - GGCCGCAGATCTGTTAACCTAAACTTTAAATATAGTGACAAAC

OR6RattB - GGCCGCAGATCTGTTAACTCAAATGCTGCGTAGGAA

OR29RattB – GGCCGCAGATCTGTTAACCTACACTTTGTTGAGGAAATAA

OR31RattB – GGCCGCAGATCTGTTAACCTAGCGATTCAAGAACGTAAACAATG

Blue is the sequence overlap for plasmid, whereas red is HPA digest site overlapped Greens are complementary overlaps.

2.8.1 Polymerization of Fragments

Polymerase Chain Reaction (PCR) was carried out for the synthesis of following fragments

I. attB-92aF----------OR92a-aR
II. attB-92aF----------OR92a6R
III. attB-92aF----------OR92a-bR
IV. attB-92aF----------OR92a-cR

The composition of master mix for the PCR

1.	Water	18.75µl
2.	Advantage 2 Buffer 10X	2.50 µl
3.	DNTPs 10mM	0.50 µl
4.	Advantage 2 Taq polymerase 1U	0.25 µl
5.	gDNA	1.00 µl
6.	Forward primer 10µM	1.00 µl
7.	Reverse primer 10µM	1.00 µl

Running Conditions for the reaction

1. Melting temp. 94^0C for 2 min.
2. { Melting temp. 94^0C for 30 S
Annealing Temp. 55^0C 30S
Extension 68^0C for 90S } 32 cycles
3. Final Extension 68^0C for 10min

2.8.2

Another PCR was carried out for the synthesis of the following fragments

I. 92a-OR-aF----------OR-a-RattB
II. 92a-OR6F----------OR6RattB
III. 92a-OR-b-F---------OR-b-RattB
IV. 92a-OR-c-F---------OR-c-RattB

Master Mix

1.	Water	18.75µl
2.	Advantage 2 Buffer 10X	2.50µl
3.	DNTPs 10mM	0.50µl
4.	Advantage 2 Taq polymerase 1U	0.25µl
5.	cDNA	1.00µl
6.	F-Primer 10µM	1.00µl
7.	R-Primer 10µM	1.00µl

PCR running conditions

1. Melting temp. 94^0C for 2 min.
2. Melting temp. 94^0C for 30S
Annealing temp. 55^0C for 30S
Extension 68^0C for 3 min } 32 cycles
3. Final extension 68^0C for 10min

2.8.3 Purification of the PCR products

The PCR products were run on the gel for confirmation of the success of the experiment; the running conditions for the gel electrophoresis were 100 volts for 45min. After electrophoresis, the bands on the gel were cut and purified as described. The gel bands were weighed and gel binding solution was added at volume equal to the mass of the excised gel: the gel then was melted in binding solution at 60^0C for 15 minutes. The melted contents were transferred into the spin column and centrifuged at max speed for 1 min and the flow through was discarded. After that, 700 µl membrane wash solution was added and centrifuged at max speed for 1min and the flow through was discarded. Then 500 µl membrane wash solution was added and centrifuged at max speed for 3min and the flow through was discarded. The next step was just spinning without any reagent to remove any excess wash buffer. Finally the DNA was eluted with water by spinning for 1 min at maximum speed (kit method provided by Clone tech, USA) and then the DNA was quantified by nanodrop method. The purified product was again run on the gel for the confirmation of required product.

2.8.4 Digestion and purification of plasmid (attB)

The plasmid was linearized by digestion with the HPA-1enzyme. The components of the reaction were mixed and incubated at 37^0C for 3 hours followed by incubation at 65^0C for 20 min to stop the reaction. The components of the reaction mixture are

(I). Undigested plasmid (attB)	5 µl
(II). 10 X buffer	2 µl
(III). HPA1	1 µl
(IV). Water	12 µl
Total Volume	20 µl

Afterwards, purification of the digested plasmid was done by the same procedure adopted for the purification of previously synthesized PCR products.

2.8.5 Infusion of fragments

The components for the infusion reactions are as follows

1. Infusion enzyme mix 4 µl
2. Digested plasmid 2 µl
3. Fragment X having full OR sequence and promoter 92-a sequence as a smaller portion 5 µl
4. Fragment X having promoter 92-a as a major portion 5 µl
5. Water 4 µl

The reaction mixture was incubated at 50^0C for 15 min.

2.8.6 Transformation by heat shock method

For transformation TOP10 invitrogen chemically competent *E.coli* cells were used provided by Clonetech, USA. The cells were thawed on ice and 2.5 µl of fused fragments were added to the 25 µl competent cells and were given heat shock at for 30S at 42^0C.

2.8.7 Cell culturing

250µl SOC media (Provided by New England, Biolabs) was then added to the transformation mixtures and the cells were incubated at 37^0C for 1 hour on the shaker- incubator maintained

at 225Rpm. Petri plates containing LB-Ampicillin media were preheated at 37^0C for 30 minutes. Then the cells were inoculated on the Petri plates and were grown over night.

2.8.8 Diagnostic Colony PCR

After overnight growth, individual bacterial colonies were picked and added to the eppendorf tubes contacting 50µl of the LB-Ampicilin media and were grown for 2hrs at 37^0C on the shaker-incubator maintained at 225 Rpm.

Then 2 µl of the cell cultures were used for the diagnostic PCR. The components of the reaction mixtures are

(I). Cell culture	2.00 µl
(II). Dream taq. Buffer 10X	2.50 µl
(III). Dream Taq. Enzyme	0.25 µl
(IV). dNTPs 10mM	0.50 µl
(V). Primer F 10µM	1.00 µl
(VI). Primer R 10 µM	1.00 µl
(VII). Water	17.75 µl

Running Conditions

1. Melting temp.95^0C for 2 min.
2. { Melting temp. 95^0C for 30S; Annealing 54 ^{0}C for 30S; Extension 72 ^{0}C for 1min and 20S } 32 Cycles
3. Final Extension 72 ^{0}C for 5 min

2.9 Alternative method for the preparation of constructs

We performed an alternative method for the preparation of constructs. The incorporation of the promoter 92-a and the other ORs was done in two steps rather than in a single step. There

are no changes for the rest of procedures of digestion, purification, transformation cell culturing and colony PCRs etc. The following primers were used

For EcoRI Flanked Dmel. OR92-a Promoter

Forward Primer TATGAATTCCAACTTTGACACCAAATGCAAGGGTAA

Reverse Primer ATTGAATTCGCTGTTTGATCGGCTGACAGAATAAGTC

For NotI Flanked Slit OR-a gene

Forward Primer TATGCGGCCGCATGGTGAACACATTTCGTGG

Reverse Primer ATTGCGGCCGCCTAAACTTTAAATATAGTGACAAAC

For NotI Flanked SlitOR-c gene

Forward Primer TATGCGGCCGCATGGAAGATAATGTAGCATACT

Reverse Primer ATTGCGGCCGCCTAGCGATTCAAGAACGTAAACAATG

Note: Red sequences are the enzymatic cut sites, greens are the OR sequences and the underline sequences are the random structural elements.

2.9.1 Polymerization of Fragments

PCR I

Polymerase Chain Reaction (PCR) was carried out for the synthesis of Slit OR-a and OR-c

The composition of master mix for the PCR

(I). Water	15.75µl
(II). Advantage 2 Buffer 10X	2.50 µl
(III). DNTPs 10mM	0.50 µl
(IV). Advantage 2 Taq polymerase 1U	0.25 µl
(V). gDNA	1.00 µl
(VI). Forward primer 10µM	2.50 µl
(VII). Reverse primer 10µM	2.50 µ

Running Conditions for the reaction

1. Melting temp. 94^0C for 2 min.
2. { Melting temp. 94^0C for 30 S; Annealing Temp. 55^0C 30S; Extension 68^0C for 90S } 32 cycles
3. Final Extension 68^0C for 10min

PCR II

Polymerase Chain Reaction (PCR) was carried out for the synthesis of promoter OR92-a from *Drosophila*

The composition of master mix for the PCR

(I). Water	15.75µl
(II). Advantage 2 Buffer 10X	2.50 µl
(III). DNTPs 10mM	0.50 µl
(IV). Advantage 2 Taq polymerase 1U	0.25 µl
(V). cDNA	1.00 µl
(VI). Forward primer 10µM	2.50 µl
(VII). Reverse primer 10µM	2.50 µl

Running Conditions for the reaction

1. Melting temp. 94^0C for 2 min.
2. { Melting temp. 94^0C for 30 S; Annealing Temp. 55^0C 30S; Extension 68^0C for 90S } 32 cycles
3. Final Extension 68^0C for 10min

2.9.2 Purification of the PCR products

The PCR products were run on the gel for confirmation of the success of the experiment; the running conditions for the gel electrophoresis were 100 volts for 45min. After electrophoresis,

the bands on the gel were cut and purified as described. The gel bands were weighed and gel binding solution was added at volume equal to the mass of the excised gel: the gel then was melted in binding solution at 60^0C for 15 minutes. The melted contents were transferred into the spin column and centrifuged at max speed (13000 rpm) for 1 min and the flow through was discarded. After that 700 µl membrane wash solution was added and centrifuged at max speed for 1min and the flow through was discarded. Then 500 µl membrane wash solution was added and centrifuged at max speed for 3min and the flow through was discarded (which was collected in the collection tube). The next step was just spinning without any reagent to remove any excess wash buffer. Finally the DNA was eluted with water by spinning for 1 min at maximum speed (13000 rpm) and then the DNA was quantified by nanodrop method. The purified product was again run on the gel for the confirmation of required product.

2.9.3 Digestion of Plasmid attB

2.9.3.1 Components of the reaction for Digestion of Plasmid attB

(I). Undigested plasmid (attB)	2 µl
(II). Buffer 10X	2 µl
(III). Not.1	1 µl
(IV). BSA	10X
(IV). Water	12 µl
Total Volume	20 µl

2.9.3.2 Digestion of ORs (a and b)

Components of the reaction

(I). OR-a/b	35 µl
(II). Buffer 10X	5 µl
(III). Not.1	5µl
(IV). BSA 10X	5µl
Total Volume	50 µl

2.9.3.3 Ligation of fragments

The components for the infusion reactions are as follows

1. T4 DNA ligase 0.50 µl
2. attB digested plasmid Not. I 2.0 µl
3. OR-a/b digest of Not. I 4.0 µl
4. T4 DNA ligase buffer 1.0 µl
5. Water 2.50 µl

The reaction mixture was incubated at 22^0C for 1hour.

2.9.3.4 Transformation by heat shock method

For transformation TOP10 invitrogen chemically competent *E.coli* cells were used provided by Clonetech. The cells were thawed on ice and 2.5 µl of fused fragments were added to the 25 µl competent cells and were given heat shock at for 30S at 42^0C.

2.9.3.5 Cell culturing

250µl SOC media was then added to the transformation mixtures and the cells were incubated at 37^0C for 1 hour on the shaker- incubator maintained at 225Rpm. Petri plates containing LB-Ampiciline media were preheated at 37^0C for 30 minutes. Then the cells were inoculated on the Petri plates and were grown over night.

2.9.3.6 Diagnostic Colony PCR

After overnight growth, individual bacterial colonies were picked and added to the ependorf tubes contacting 50µl of the LB-Ampicilin media and were grown for 2hrs at 37^0C for 1 hour on the shaker-incubator maintained at 225 Rpm.

Then 2 µl of the cell cultures were used for the diagnostic PCR. The components of the reaction mixtures are

(I). Cell culture 2.00 µl

(II). Dream taq. Buffer 10X 2.50 µl

(III). Dream Taq. Enzyme 0.25 µl

(IV). dNTPs 10mM 0.50 µl

(V). Primer F (10μM) 1.00 μl

(VI). Primer R (10 μM) 1.00 μl

(VII). Water 17.75 μl

Running Conditions

1. Melting temp.95^{0}C for 2 min.
2. Melting temp. 95^{0}C for 30S
   Annealing 54 ^{0}C for 30S
   Extension 72 ^{0}C for 1min and 20S
   } 32 Cycles
3. Final Extension 72 ^{0}C for 5 min

3. Results

3.1 Behavioural Studies

There was little overall effect on the olfactory behaviour of *Drosophila* to the tested compounds on the basis of shared input channel of OR92-a co-expressed with the other receptors of interest from *S. littoralis*.

Most strains were significantly attracted to acetoin, as expected because this compound is a known attractant for *Drosophila*. In contrast, very few were attracted to the corresponding ligands for the Slit-ORs, regardless whether the *Spodoptera* OR was expressed or not. There was no consistent case of attraction to Slit-OR ligand odours only in the presence of Slit-OR expression.

3.1.1 Response to linalool:

The attraction indices of the fly lines are shown in Figure 10. Neither of the lines displayed any significant attraction to linalool, regardless whether they expressed the linalool-responding SlitOR-a or not. However, there was a consistent difference among the fly strains in their response to acetoin. All three control lines were significantly attracted to acetoin, whereas both strains expressing SlitOR-a in the acetoin-responding Or92a ORNs were not.

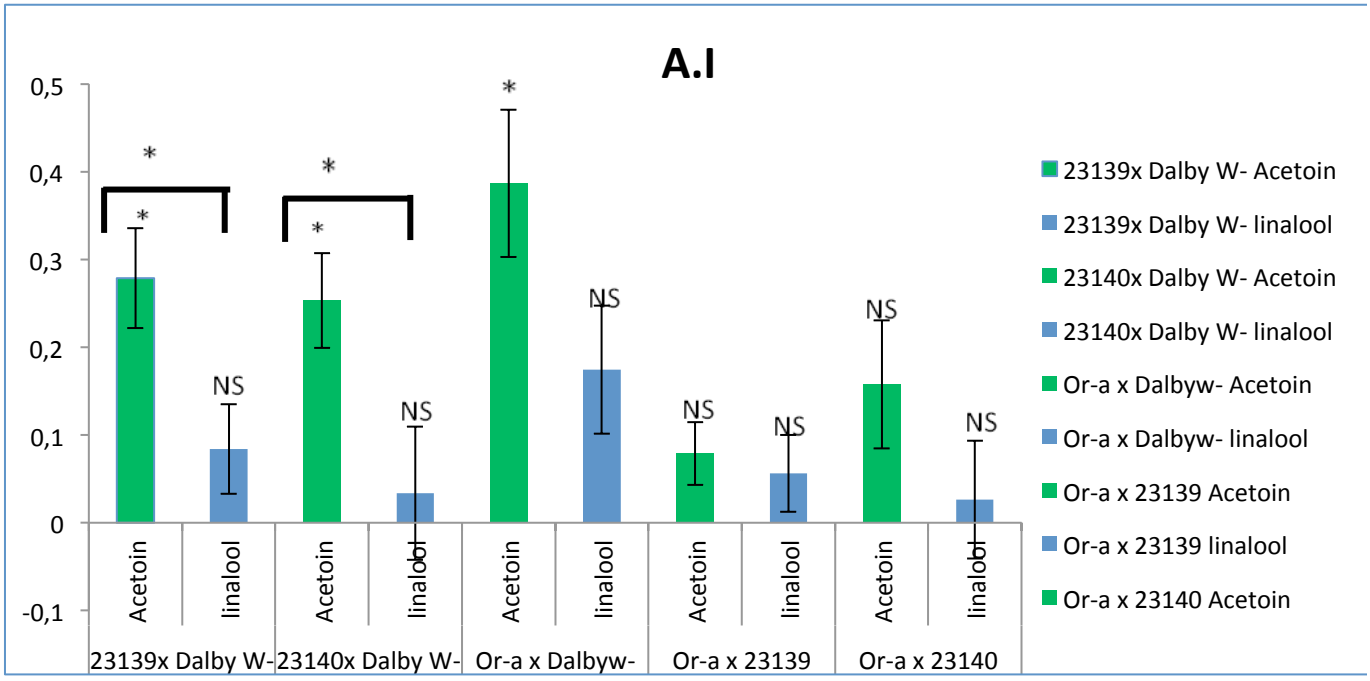

Fig10: The Attraction index (A.I) of the fly lines to Acetoin and Linalool: NS- Non significant; *- significant against zero ⌐¬ : Comparison between acetoin and the test compound of the same genotype, which are significantly different from each other

3.1.2 Response to beta ocimene:

Responses to beta-ocimene were different than to all other *Spodoptera* related odours in that some fly strains displayed significant attraction to this odour. However, there were no consistent differences between SlitOR-b-expressing lines and control lines that would indicate that attraction was specifically mediated by SlitOR-b expression. The Or-b-expressing line Or-b x 23140 displayed significant attraction to beta-ocimene, but not to acetoin, whereas attraction to beta-ocimene in the other Or-b-expressing line Or-b x 23139 was near significant, and significant to acetoin. However, the control line Or-b x Dalby W^- also displayed significant attraction to beta-ocimene, possibly suggesting a general contribution from the genetic background of the Or-b parental line rather than specifically from Or-b-expression in Ab1B (Or92a) neurons. From these results it can be concluded that there is no unambiguous effect on the olfaction of *D. melanogaster* when the beta-ocimene receptor from *S. littoralis* is incorporated.

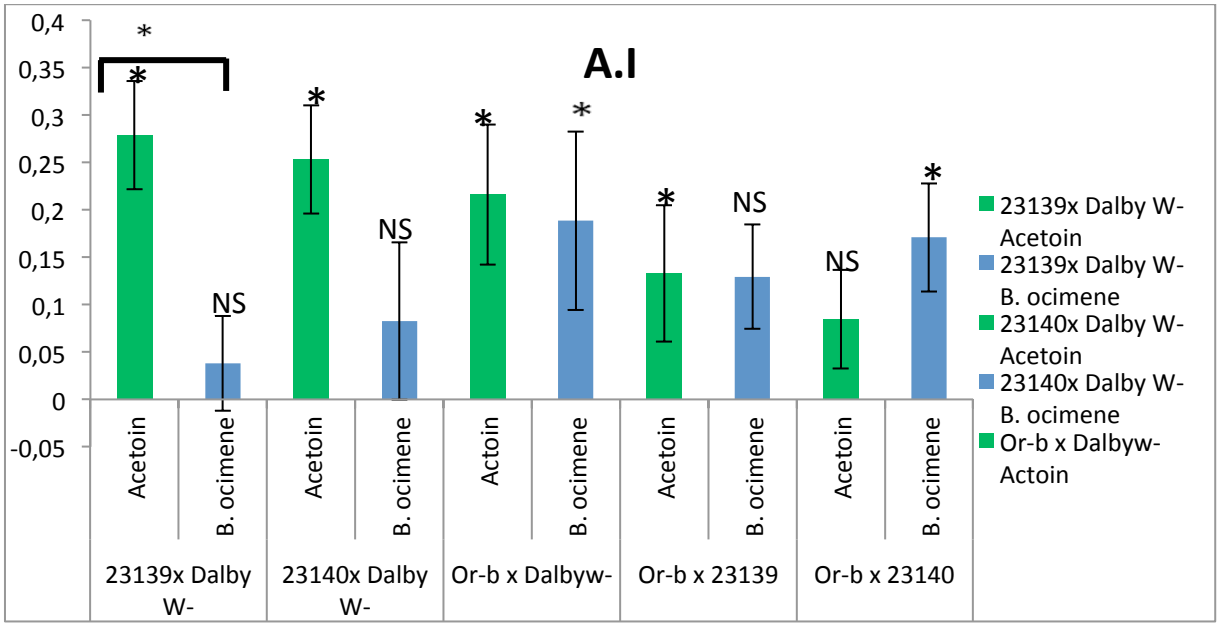

Fig 11: A.I towards Acetoin Beta ocimene: NS; Non significant, *; significant against zero, ⌐¬ : Comparison between acetoin and the test compound of the same genotype, which are significantly different from each other

3.1.3 Response to Eugenol:

The olfactory response in-terms of attraction indices of the fly lines are shown in Figure 12. All the control and experimental lines were significantly attracted towards acetoin. There was no significant attraction towards eugenol by any of the fly line. However, the response spectrum to acetion was different. These results indicate that there is no definite effect on the

olfaction of *D. melanogaster* when the eugenol receptor from *S. littoralis* was co-expressed with 92-a receptor in the defined olfactory neurons of *Drosophila*, together with their native receptors.

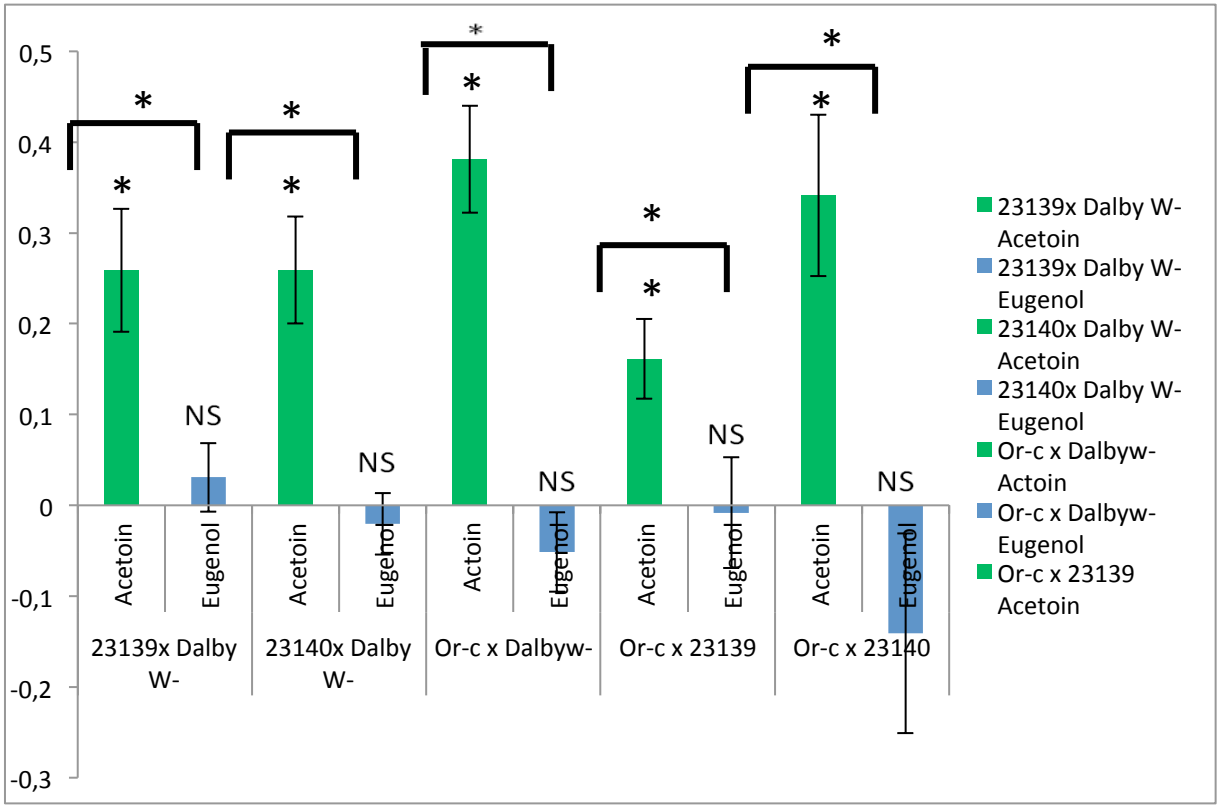

Figure 12: Behaviour response of fly lines to Acetoin and Eugenol: NS; Non significant, *; significant against zero, ⌐¬ : Comparison between acetoin and the test compound of the same genotype, which are significantly different from each other

3.1.4 Response to Pheromone (z914OAc)

Results for the response to pheromone (z914OAc) are compared in the figure 13, in-terms of attraction indices. All the lines responded significantly to acetoin. There was no attraction to the pheromone component (z914OAc dissolved in hexane, adsorbed 2 µl on a filter disc which was immersed in the blank solution in the vial) by any of the lines. So, there is no explicit effect on the olfaction of *D. melanogaster* when pheromone receptor from *S. littoralis* was incorporated.

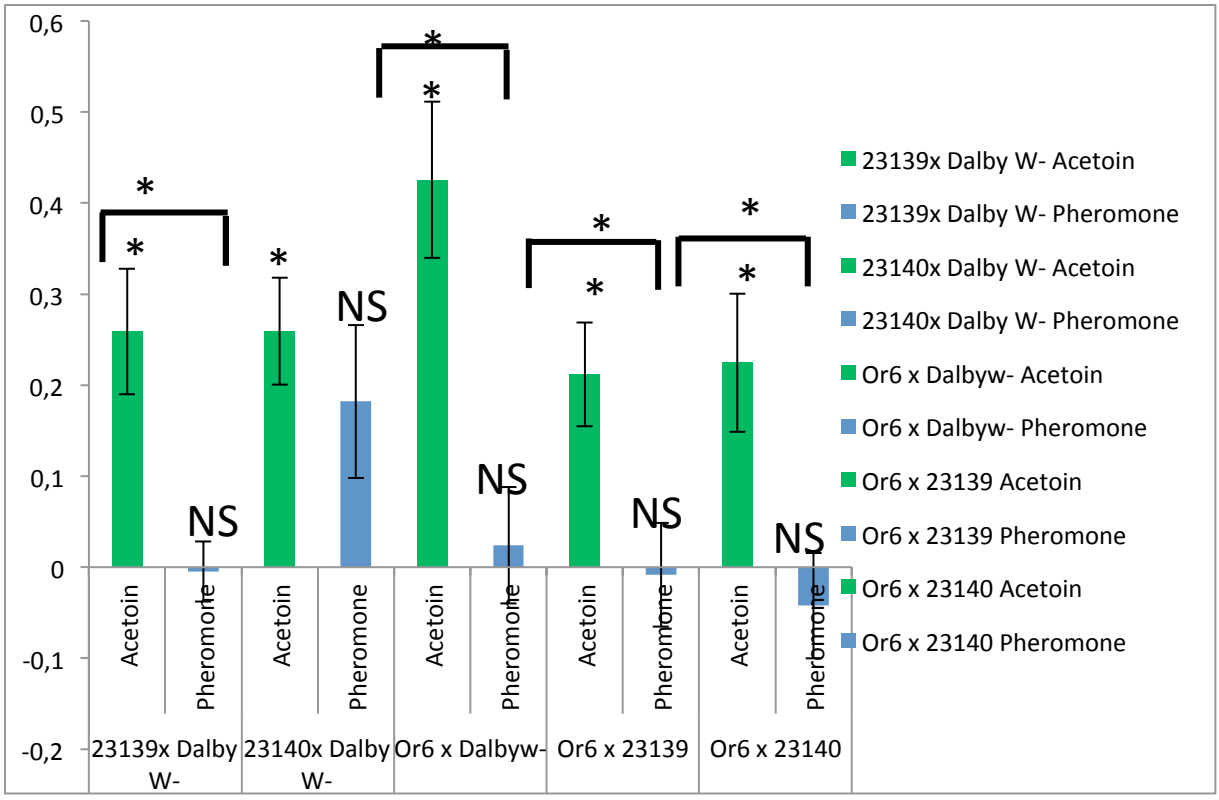

Figure 13: Response of fly lines measured in-terms of attraction index (A.I) to Acetoin and pheromone: NS; Non significant, *; significant against zero, ⌐¬ : Comparison between acetoin and the test compound of the same genotype, which are significantly different from each other

So it can be conclude from the results, there is no effect on *Drosophila* olfactory behaviour in case of manipulation of Slit-OR-6.

3.2 Molecular studies

3.2 Single step infusion method

3.2.1 Results for the PCR for the synthesis of fragments

The gel image after PCR of all the fragments is shown below (fig:14). The fragments OR-a and OR-c were successfully amplified while there was no product formation for OR6 and OR-b.

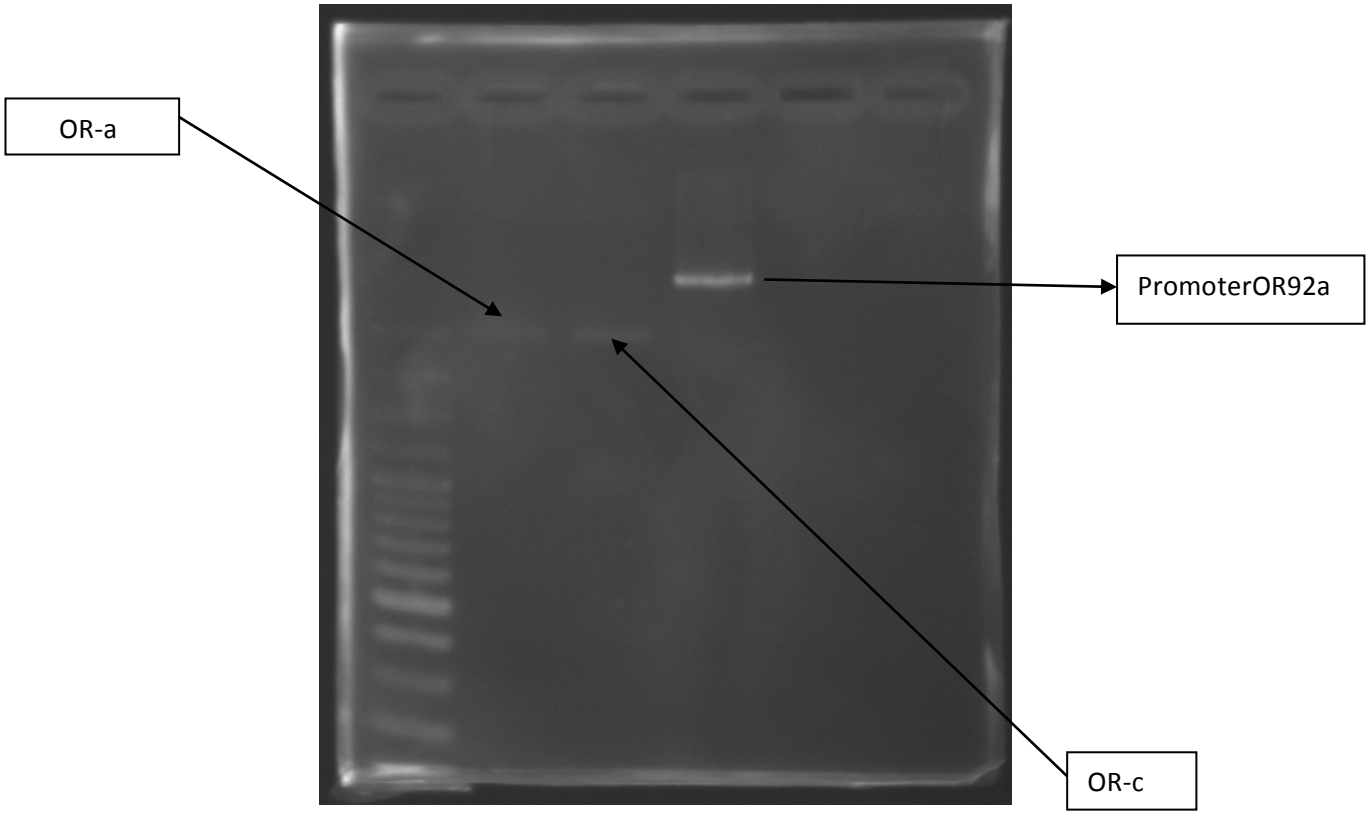

Fig 14: Gel image of the PCR products (purified) of the fragments

3.2.2 Cell culturing

There was growth of *E.Coli* colonies which were transformed by product of infusion reaction attB-(92-a)-OR-a and attB-(92-a)-OR-c by heat shock method, grown in separate plates and it is to be confirmed by the colony PCR.

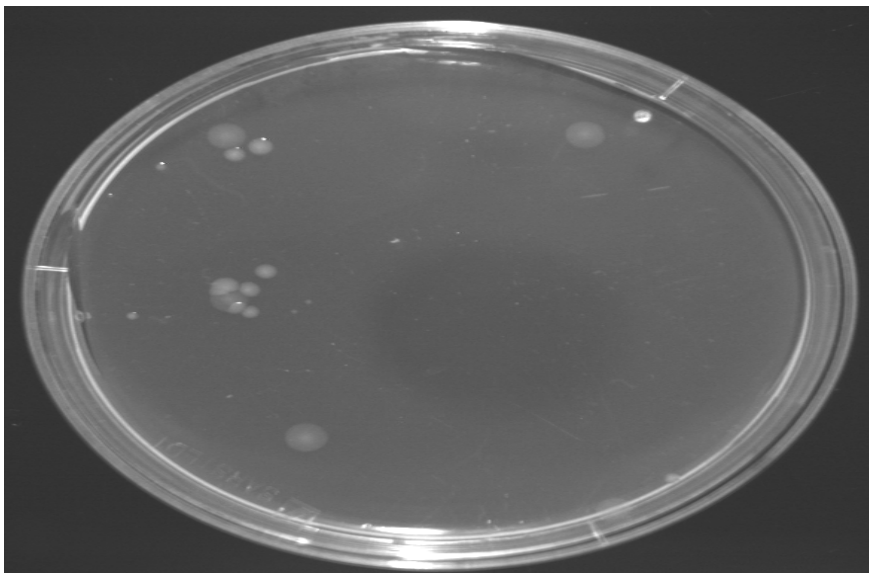

Fig 15: Colony growth of bacteria transformed with plasmids containing OR sequence grown overnight for the receptor attB-(92-a)-OR-a

3.2.3 Colony PCR

The result of the colony PCR shows no PCR product for OR-a and OR-c which are shown in fig. 16.

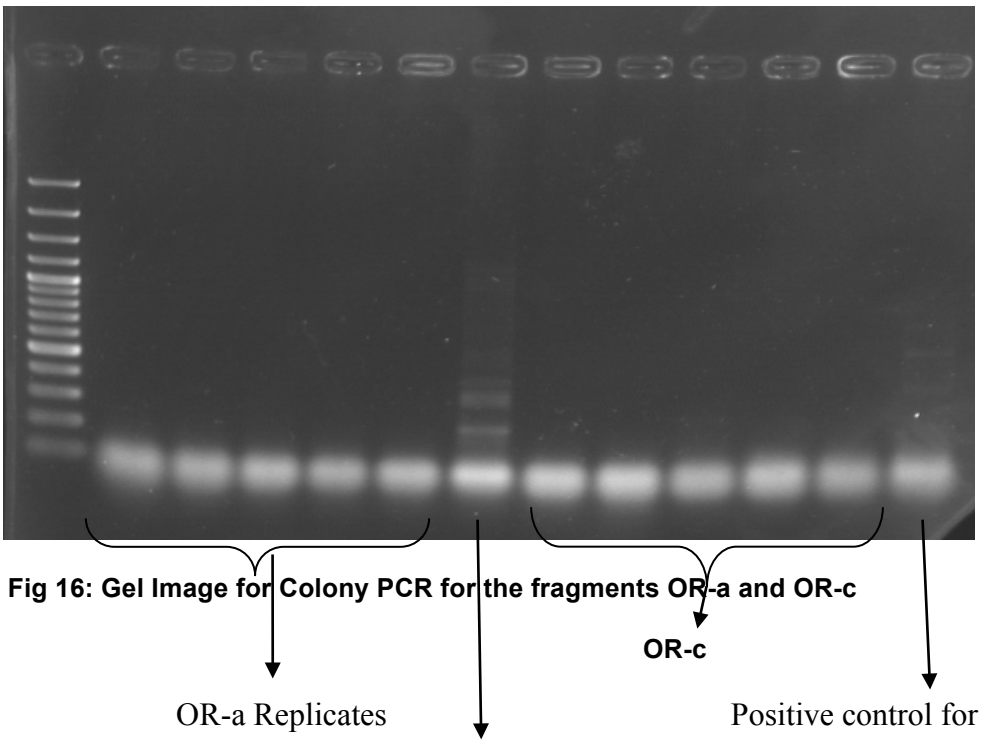

Fig 16: Gel Image for Colony PCR for the fragments OR-a and OR-c

There was no PCR product for the required fragments. All are the primer dimers.

3.3 Two step infusion method

3.3.1 Results for the PCR for the synthesis of OR-a and OR-b

The two step infusion method, in which I aimed to incorporate *Drosophila* promoter 92-a and the other SlitORs fragment separately into the plasmid, was unsuccessful. During PCR amplification the fragments OR-a and OR-c were successfully amplified while there was no product formation for Or92-a promoter. The gel image after PCR of all the fragments is shown below (fig.17).

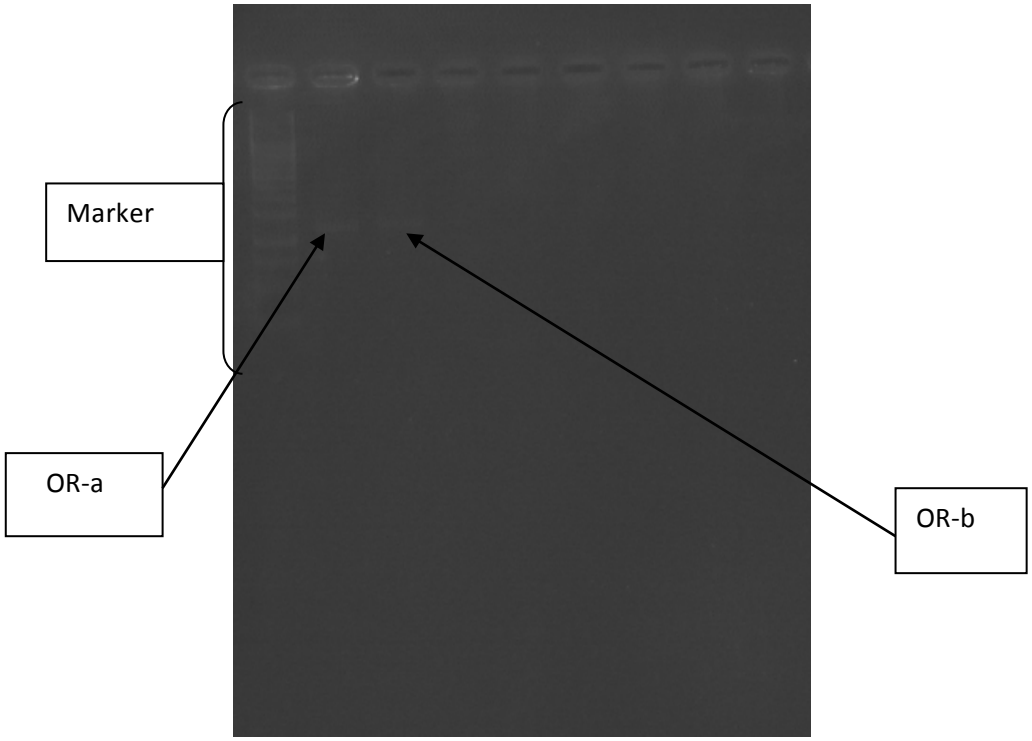

Fig17: Gel image for the PCR (purified) Or-a & Or-b

3.3.2Results for Cell culturing for the OR-a and OR-b

There was *E. Coli* growth after transformation with attB-Or-a and attB-Or-c overnight incubation at 37^{O}C.

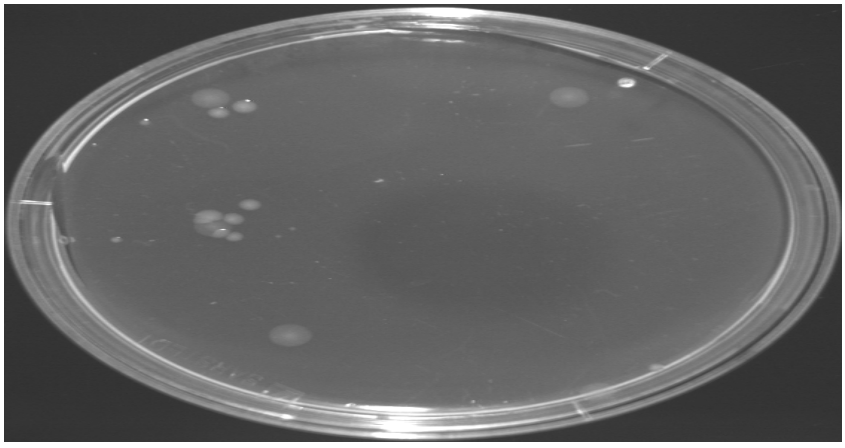

Fig18: Colonies for the Or-a and Or-c

3.3 Results for the Colony PCR for OR-a and OR-b

The bacterial growth of the required colonies attB-OR-a and attB-OR-b indicated that the fragment was taken up by the bacteria. The presence of the construct in the bacteria was further confirmed by diagnostic PCR. The gel image of the PCR is shown in the fig: 19.

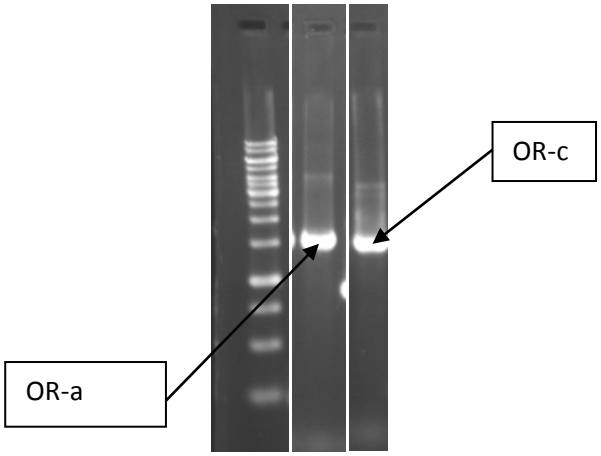

Fig: 19 Gel image of colony PCR for the OR-a and OR-c

I successfully incorporated fragments OR-a and OR-c in the plasmid. However, I repeatedly failed to amplify the OR-6, OR-b and *Drosophila* Promoter 92-a fragments. Without the *Drosophila* Promoter 92-a fragment, which was intended to be incorporated, together with the SlitOrs within the attB plasmids, I could not proceed further with this construct within the time frame of the project.

4. Discussion

My aim was to investigate the olfactory behaviour response of the *Drosophila* to different odours, having olfactory receptors from the moth *S. littoralis* together with their native receptors. Preparation of transgenic constructs with direct fusions of *Drosophila* promoter and *Spodoptera* olfactory receptor genes in order to obtain constitutive expression of selected *Spodoptera* receptors in *Drosophila*. There was no significant effect of co-expressing SlitORs in OR92-a neuron on the olfactory behaviour of *D. melanogaster.*

Most strains were significantly attracted to acetoin, as expected (Stensmyr et al., 2003). In contrast, very few were attracted to the corresponding ligands for the Slit-ORs, regardless of whether the *Spodoptera* OR was expressed or not. Neither of the experimental lines displayed any significant attraction to linalool, pheromone or eugenol.

In some cases there was attraction towards the test compound by the experimental and control lines of the same genotype, possibly suggesting a general contribution from the genetic background of the ORs parental line rather than specifically from ORs expression in Ab1B (Or92a) neurons. There was only one case where Or-b-expressing line Or-b x 23140 displayed significant attraction to beta-ocimene. From these results it can be concluded that there is no unambiguous effect on the olfaction of *D. melanogester* when ORs from *S. littoralis* is incorporated. It could also be due to the lack of other signalling components that have been shown to be important for reception of the tested odour in the environment of the 92a sensilla such as odour binding proteins (Hildebrand and Shepherd, 1997). Another factor may be sensillum environment (Trichoid to basiconic) that may lack certain components of the transduction cascade: Odorant/Pheromone binding proteins and other pheromone-related transduction components (Syed et al., 2006; Benton et al., 2007).

In this case it seems that a single neuron ab1B type is not sufficient for the behavioural response to acetoin. Activation of a single neuron ab1B by the tested compound is not sufficient to replicate the behavioural response to acetoin. This indicates either that acetoin needs to be detected by more than one neuron or the non-specific activation of other neurons by the tested compound that may inhibit the olfactory response towards acetoin. There does indeed seem to be receptors and ORNs functioning as so-called labelled lines, mediating information about single compounds to the olfactory system as in CO_2-sensitive pathway (de Bruyne et al., 2001, Suh et al., 2004). Detection of CO_2 is dependent on specific receptors detecting this unique stimulus (Jones et al., 2007). Another example is the strong avoidance of

the fungal odour geosmin in *Drosophila*, which is mediated by a specific receptor (Stensmyr et al., 2012).
Most odours are to a greater or lesser extent detected by combinations of ORN types rather than individual types as in the case of both moths and flies (de Bruyne et al. 2001;Carlsson et al., 2002; Wang et al., 2003; Hallem et al. 2004, 2006; Binyameen et al., 2012;)

The reason for ambiguous behavioural responses to our alternative compounds could be that the acetoin attraction is dependent on responses from more than one ORN type. Acetoin sensory neuron responses are shown sometimes in some large-scale screens of neurons/receptors of *Drosophila*, and the two most similar compounds: 2,3-butanedione (detected by Or92-a, Or42-a) and 2,3-butanediol (detected by Or9-a) also appear to elicit responses from some other receptor(s) (Hallem et al., 2004).

Another option could be that our alternative compounds elicit responses in other types of sensory neurons than the Or92a neurons (based on native fly receptors). The presence of these responses (if any) along with the Or92a response could be enough to shift the overall impression of the olfactory system away from the acetoin perception.

According to the fly data base, 92-a also responds to eugenol (a very weak response) and linalool is detected by Or9-a. (http://neuro.uni-konstanz.de/DoOR/content/DoOR.php#).

Various concentrations of the tested compounds can be tried for behaviour studies in-order to overcome any effect due to the concentration of the compound if there is any. Another possible strategy may be to test these compounds as a blend of different common plant volatiles as the insects are also known to respond to the blends of volatiles (Bruce et al., 2005; Riffell et al., 2009).

Regarding the preparation of constructs, in the first methodology, there was no PCR amplification for fragments attB-(92a)-OR6 and attB-(92a)-OR-b. In the second methodology I successfully managed to PCR amplify attB-OR-a and attB-OR-c while no amplification formation of promotor92-a. The possible reason for this may be I was unable to find the optimum running conditions for PCR or primers are not compatible.

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