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Functional Imaging of Higher Olfactory Centres of the Brain in Tethered Fly Set-up

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Abbreviations

.seq	Sequence file
.tif	Tagged Image File
.txt	Text file
AL	Antennal Lobe
BEA	Benzaldehyde
CAR	Carboxen
cVA	11-cis-Vacenyl Acetate
DAQ	Data Acquisition
DVB	Divinylbenzene
ePN	Excitatory Projection Neuron
ETA	Ethyl Acetate
GC	Gas Chromatography
mACT	Medial Antenno-protocerebral Tract
iPN	Inhibitory Projection Neuron
LH	Lateral Horn
LN	Local interneuron
mlACT	Mediolateral Annteno-protocerebral Tract
MB	Mushroom Body
MOL	Mineral Oil
MS	Mass Spectrometry
MSC	Methyl Salicylate
lACT	Lateral Antenno-protocerebral Tract
OCT	1- Octanol
OR	Odourant Receptor
ORD	Odour Response Domain
OSN	Olfactory Sensory Neuron
PDMS	Polydimethylsiloxane
PID	Photoionization Detector
PN	Projection Neuron
ROI	Region of Interest
SPME	Solid-Phase Micro Extraction
VIN	Vinegar
vlPr	Ventrolateral Protocerebrum
WBA	Wing Beat Amplitude
WBF	Wing Beat Frequency
WT	Wild Type

Abstract

Insects have been on the earth for millions of years, are continuously evolving and adapting to surrounding environments by recognizing chemical cues from both the biotic and abiotic components. This process known as chemosensation is a widely studied subject in one of the best model organisms, *Drosophila melanogaster*, or vinegar fly. Some of the basic needs of the fly, such as the requirement to feed, reproduce and evade danger are fulfilled by one of the chemosensory modalities- olfaction. By the process of olfaction, the fly perceives the external environment through a series of electrical signals in the brain in response to the surrounding cues. This functioning of the fly brain manifests physiological behaviour. This behavioural response can also bear ecological significance to the fly.

The fly behaviour can be of positive, negative or neutral valence, indicating attraction, aversion or neutral behaviour to chemical cues for food and mating partners, or evading toxicity or predation, respectively. My Master's thesis was aimed at understanding the physiological process of olfaction and the ethological responses to odours in vinegar flies. Using the tethered fly setup to conduct behavioural analysis during flight, a definite readout was established as the response towards volatile chemicals. Also, the neuronal activities in the higher centres of the brain responsible for valence coding were studied during odour stimulation in the flies using the technique of optical imaging.

The outlook of my thesis lies in integrating the behavioural analysis with the physiological readout in the brain to obtain a clear idea on neuronal activity and how the valence coding in the brain affects the behaviour of the fly. The aim is to simultaneously conduct behavioural analysis in the fly and image the fly brain for future experiments.

Keywords: *Drosophila, Olfaction, Behaviour, Lateral Horn, Brain*

Zusammenfassung

Insekten, die seit Millionen von Jahren auf der Erde leben, entwickeln sich ständig weiter und passen sich an die Umgebung an, indem sie chemische Bestandteile sowohl von biotischen als auch von abiotischen Faktoren erkennen. Dieser als Chemosensorik bekannte Prozess ist ein gut untersuchtes Thema in einem der meist genutzten Modellorganismen, *Drosophila melanogaster* oder Essigfliege. Die Grundbedürfnisse der Fliege, Gefahren, zu meiden, Nahrung aufzunehmen und die Reproduktion, werden durch eine der chemosensorischen Modalitäten, den Geruchssinn, erfüllt. Durch das Riechen nimmt die Fliege die äußere Umgebung durch eine Reihe von elektrischen Signalen im Gehirn wahr, die eine Reaktion auf die umgebenden Signale darstellen. Diese Funktion des Fliegengehirns manifestiert sich als Verhalten. Diese Verhaltensreaktion kann auch für die Fliege von ökologischer Bedeutung sein

Das Flugverhalten kann von positiver sowie negativer oder auch neutraler Wertigkeit sein, was auf eine Anziehung oder Abneigung gegenüber der chemischen Information für Nahrung und Paarungspartner oder auf ein Ausweichen gegenüber einem Toxin bzw. Prädation hinweist. Ziel meiner Masterarbeit war es, den physiologischen Geruchsprozess und die ethologischen Reaktionen auf Gerüche in Essigfliegen zu verstehen. Unter Verwendung des tethered fly Aufbaus zur Durchführung einer Verhaltensanalyse während des Fluges wurde eine eindeutige physiologische Analyse für verschiedene Düfte erstellt. Auch die neuronalen Aktivitäten in den höheren Zentren des Gehirns, die für die Valenzkodierung verantwortlich sind, wurden während der Geruchswahrnehmung unter Verwendung optischer Bildgebungstechniken untersucht.

Die Perspektive meiner Masterarbeit liegt in der Integration der verhaltenstechnischen Analyse mit der physiologischen Analyse im Gehirn, um eine klare Vorstellung von der neuronalen Aktivität im Gehirn zu erhalten / wie die Valenz das Schicksal der Fliege beeinflusst. Ziel ist es, in zukünftigen Experimenten gleichzeitig Verhaltensanalysen im Flug durchzuführen und das Gehirn abzubilden.

Schlüsselwörter: *Drosophila, Olfaction, Verhalten, L*aterales *Horn, Gehirn*

1. Introduction

A species' constant interaction with the abiotic and biotic factors in its environment drives evolution in that species group (Dobzhansky 1956, Nosil et al. 2018). The basic needs of an organism to interact with its environment are to forage for edible food or to locate suitable mating partners, hospitable breeding substrates or to evade toxic, or harmful substances and predators (Hansson and Stensmyr 2011). The environment comprises a plethora of chemical molecules that can be beneficial or detrimental or neutral to a species. It is highly important that an animal is able to detect chemical cues in nature to survive. Almost all species are able to perceive different cues in their environment by chemosensation accomplished by the senses of smell and taste. As Darwin's theory on the process of natural selection says, the fittest survive in an environment and evolution is driven by nature and its components.

Having existed on the planet for over 400 million years, the insects have constantly adapted to their environment and stand as model organisms to study the process of evolution. Many insects undergo holometabolous development where the adult arises from the advantageous larval stage after complete metamorphosis. The completely different larvae and adult forms occupy different ecological niches and resources preventing competition amongst themselves and that is one of the reasons for their successful evolution. Responses to chemical cues of insects have played a pivotal role in enabling them to adapt to changing environments (Grimaldi et al. 2005). One of the widely studied models of chemosensation is *Drosophila melanogaster*, an organism used in many other fields such as cell biology, physiology, behaviour, evolution and ecology (Ashburner 1989, Lachaise et al. 1988).

1.1. Olfaction in *Drosophila melanogaster*- "Common Vinegar Fly"

Drosophila melanogaster commonly known as the "vinegar fly", is a species of the Drosophilidae family. It is a classic organism to study the chemosensory model of

olfaction along with the mouse and the nematode *C. elegans*. With studies indicating the similarity in mammalian and insect olfactory circuits (Hildebrand and Shepherd 1997, Kaupp 2010), the fly, having lesser number of cells as compared to vertebrates, not only is simpler to study but also offers powerful genetic tools to manipulate neural activity during olfaction (Holmes et al. 2007, Su et al. 2009). The process of olfaction begins with the peripheral olfactory organs detecting the chemical odours (Hildebrand and Shepherd 1997). These are the antennae and the maxillary palps in the adult *Drosophila* carry out olfaction (Shanbhag et al. 1999). Olfaction is initiated by the olfactory sensory neurons (OSN) enclosed in hair-like structures known as sensilla. Sensilla are of four morphological types- trichoid, basiconic, coeloconic and intermediate. All four types cover the antennae while the maxillary palp houses only the basiconic sensilla. Each sensillum can hold 1 to 4 OSNs.

The number of OSNs in the antennae is about 1200 and 120 in the maxillary palps as compared to a total of 2 million OSNs in mice (Couto et al. 2005, Shanbhag et al. 1999, Stocker 2004). An OSN is bipolar with the dendritic end exposed to bind the odours and the axonal end extends to transmit information to the central brain. These neurons express a protein with seven membrane spanning domains that is encoded by one of the 60 genes of the olfactory genes (Robertson et al. 2003, Vosshall et al. 2000). These are known as the odourant receptors (OR) which convey odour specificity. A total of 62 ORs are known to be transcribed from the 60 OR genes of which two ORs are got by alternate RNA splicing (Robertson et al. 2003). Odour binding to ORs at the neuron membrane generates an action potential and this signal is carried to the central olfactory system for processing (Bichão et al. 2005). The second group of receptors are the ionotropic receptors (IRs) that function as ligand-gated channels. IRs are signalling proteins that respond to external chemical changes when a ligand molecule binds to them and undergo conformational changes generating a signal in the OSNs. An IR is singly or co-expressed along with other co-receptor-IRs in subsets of the coeloconic sensillae. IRs belong to the family of the ionotropic glutamate receptors (Croset et al. 2010).

Axons of OSNs lead to the antennal lobe (AL), which is an equivalent brain structure to the olfactory bulb in vertebrates. The AL is the first odour processing centre with spherical structures called glomeruli, where signal processing occurs. OSN arbors in the glomeruli and synapse onto second-order neurons known as the projection neurons (PN) (Hildebrand and Shepherd 1997). PNs, being the output neurons of the AL, carry the information to the higher regions, such as the mushroom body (MB) and lateral horn (LH). Within the AL, the local interneurons (LNs) serve multiple functions such as control of information transfer between the OSNs and the PNs. The entire signal processing ultimately generates a behavioural response. **Figure 1** shows the olfactory pathway in a fly brain.

Going deeper into the properties of OSNs and receptors, scientists showed that a single OSN expresses only one receptor protein in vertebrates (Malnic et al. 1999). But in flies, most OSNs have a ligand-binding OR along with the universal coreceptor OR83b known as ORCO (Benton et al. 2006, Vosshall et al. 2000). ORCO is required in the heteromeric complex formation with most other ORs to function in both larval and adult stages of the fly (Benton et al. 2006). The three main variables attributed to odour recognition are the chemical identity, the odour concentration and the duration of exposure. In addition, the responses elicited by each OSN might be broadly tuned - responding to multiple chemical odourants or narrowly tuned - recognising specific odourants or single odourants. The response elicited by OSNs can be excitatory or inhibitory (Hallem and Carlson 2006). The AL aids the fly in assigning the odour a valence that is capable of generating specific behavioural patterns in the fly (Haddad et al. 2008). Evaluating the odour and valence coding helps the fly to assess if the substrate is beneficial or detrimental.

1.2. Spatial segregation and Valence Coding in the antennal lobe

The behavioural responses to odour stimuli in flies are mostly stereotypic as a result of the standardised olfactory circuit. It is important to know that odourants bear an ecological and ethological relevance to flies as their aim is to survive, feed and reproduce. Food odours, odours from substrates suitable for egg-laying,

pheromones, and attractive odours in general, elicit responses in a spatially segregated part of the AL, implying a specific region, namely the medial region in the AL (Datta et al. 2008, Karlson and Lüscher 1959, Knaden et al. 2012, Ruta et al. 2010). It is highly important for the flies to recognize and discriminate toxic odours in order to survive. Though the circuit coding for innate aversion is still not clear, extensive work has been carried out to show that the AL has dedicated glomeruli mediating aversion. The glomeruli responsive to aversive odours form a cluster in the lateral part of the AL (Knaden et al. 2012, Stensmyr et al. 2012).

Each glomerulus in the AL has inputs from OSNs with the same OR. Adding to the principle, each OSN projects onto a single glomerulus. Neighbouring OSNs in the same sensillum does not necessarily map to neighbouring glomeruli. The only exception to this is the set of OSNs in the basiconic sensillum type, where OSNs with related receptors map to glomeruli close to each other (Couto et al. 2005, Hallem and Carlson 2006). A combinatorial response pattern generated in the glomeruli to a chemical cue codes for the cue's valence.

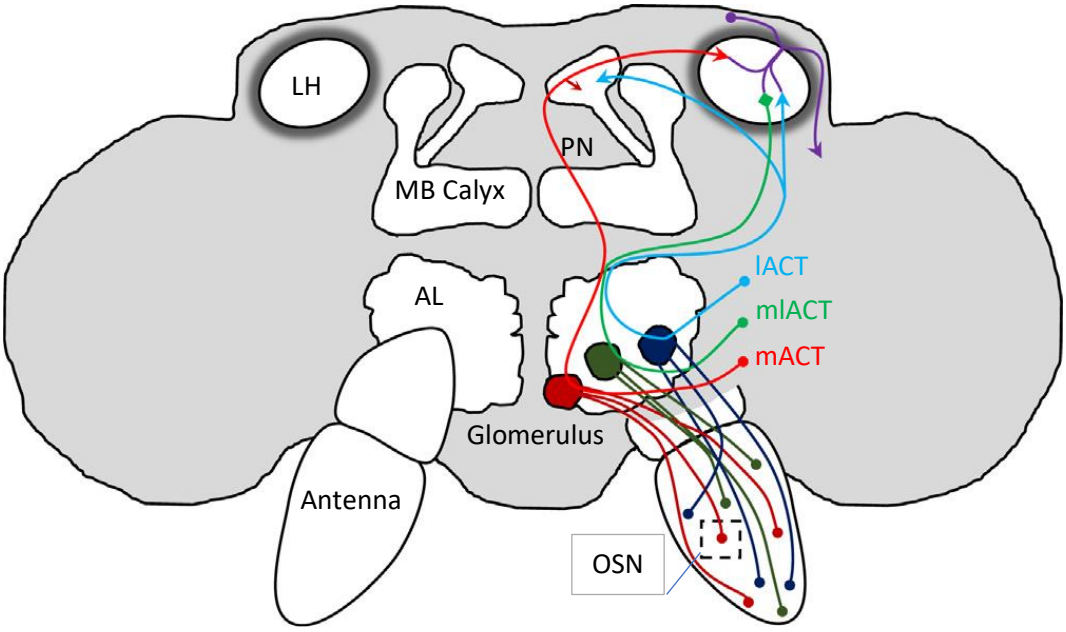


Figure 1. The olfactory circuit in *Drosophila* brain.
Olfactory sensory neurons (OSNs) housed within the antennae lead to the glomeruli in the antennal lobe (AL). Uniglomeruli projection neurons (PNs) from the AL reach the mushroom body (MB) and lateral horn (LH), the higher brain centres, via the medial antenno-protocerebral tract (mACT). Multiglomerular PNs innervate the LH alone through the mediolateral antenno-protocerebral tract (mlACT). Lateral antenno-protocerebral tract (lACT) carries PNs projecting to both MB and LH; Image Adapted from (Schultzhaus et al. 2017).

An important aspect is the spatial representation of odourant responses in the AL. The responses elicited by the OSNs in the glomeruli draw a valence representation at the level of the AL. Also, the responses observed at the level of the second-order neurons are generally not the same as those at the antennae due to the presynaptic inhibition of stimuli at the OSN levels (Couto et al. 2005, Hallem and Carlson 2006, Knaden et al. 2012, Olsen and Wilson 2008). For instance, studies show segregation of responses in glomeruli to different classes of chemicals such as aliphatic and aromatic odourants arising in the medial regions and ventrocentral regions of the AL respectively (Couto et al. 2005).

1.3. Second-order neurons and their role in olfaction

The PNs receive information directly from the OSNs within the glomeruli. On average, each glomerulus has dendrites from three innervating PNs (Couto et al.

2005, Grabe et al. 2016). PNs being broadly tuned retain the odour identity as OSNs do (Seki et al. 2017). Identical responses in both the OSN axons and PN dendrites have been imaged (Ng et al. 2002). PNs innervating the same glomerulus tend to have similar axonal arborization in the protocerebrum. However, PNs from neighbouring glomeruli do not show any similarity in their axonal projections. At the level of higher brain centres, it is seen that the PN axons arborize in an overlapping fashion, allowing simultaneous olfactory processing from multiple glomeruli. The topography of PN axonal projection is maintained in the higher centres but not the spatial patterns. The OSN axons synapse in the spherical glomeruli while the PN axons extensively diffuse into the higher regions of the brain. While doing so, PN axons from multiple glomeruli cross over enabling multiglomerular signal processing (Wong et al. 2002). PNs can be excitatory or inhibitory in nature. The excitatory PNs (ePNs) have dendrites innervating single glomeruli and their axons are responsible for excitatory responses in the higher brain centres and cholinergic in nature. These ePNs arborize into the MB calyx and LH via the medial antenno-protocerebral tract (mACT). The inhibitory PNs (iPNs) mostly being multiglomerular in the AL, exclusively enter the LH bypassing the MB through the mediolateral antenno-protocerebral tract (mlACT) (Lai et al. 2008, Seki et al. 2017, Strutz et al. 2014, Tanaka et al. 2012).

1.3.1. Local Interneurons-Connecting Glomeruli

The type of neurons communicating between the elements of the AL is the local interneurons (LNs). There are about 200 LNs in the fly brain (Chou et al. 2010). LNs being mostly inhibitory by releasing GABA or glutamate (Liu and Wilson 2013, Wilson and Laurent 2005), receive excitatory inputs from OSNs and PNs. Seven different types of LNs, exhibit various morphologies spanning the whole of the AL, innervate the glomeruli in a non-stereotyped, variable fashion (Chou et al. 2010). Odour responses of LNs to the same stimulus being diverse, and the differential rate of activation in the LNs, is one of the ways by which LNs modulate the

temporal response patterns in the PNs. The three functions of LNs and their inhibition are

1) to control the gain of odour responses in the AL,

2) to influence response patterns in the PNs to retain stimulus identity,

3) to synchronize responses in PNs (Chou et al. 2010, McGann et al. 2005, Nagel and Wilson 2016, Olsen et al. 2010, Olsen and Wilson 2008).

1.4. Higher Processing Centres

The higher centres in the brain i.e., MB and LH, which are vertebrate equivalents of the piriform cortex and the amygdala respectively, are involved in determining the behavioural response of the fly to a stimulus. The LH receives the majority of the input from glomerular projections and is associated with innate responses (de Belle and Heisenberg 1994). The MB is involved in learning based on experiences and memory, namely olfactory learning, locomotor activities, male courtship behaviour, and more (Aso et al. 2014, Heisenberg 2003, Joiner et al. 2006, Martin et al. 1998, Sakai and Kitamoto 2006).

1.4.1. Lateral Horn

One of the important olfactory centres is the LH, an analogous structure to the mammalian amygdala and a processing centre important for innate responses in the fly (Heimbeck et al. 2001). The LH apart from being involved in olfaction is subject to inputs from other sensory modalities such as mechanosensation, visual stimuli, etc. In addition, it has been shown in a recent study that the LH has separate regions to process various sensory modalities. The ventral region in the LH is known to receive inputs from all senses while the dorsal regions exclusively processes olfactory inputs (Dolan et al. 2019). Apart from sensory processing, the LH also happens to control locomotory actions such as flight and speed modulation, stopping, and steering action during flight. It does so by integrating inputs from the fly's environments, but also integrating other information such as

fly's internal state, inputs from other sensory modalities, et cetera (Dolan et al. 2019).

The LH receives olfactory stimuli from the PN axons extending from the AL and also the ventrolateral protocerebrum (vlPr). The LH displaying no distinct landmarks or structural boundaries can be defined by the axonal projections of the PNs (Schultzhaus et al. 2017). Both ePNs and iPNs coming from the AL innervate the LH in spatial zones termed as odour response domains (ORDs) (Lee and Seung 1999, Strutz et al. 2014).

The iPNs whose dendrites are known to innervate multiple glomeruli, carry information mostly coding attraction directly from the AL. They enter the LH spatially along the posterior-medial region and are necessary for attractive behaviour. A set of iPNs is also known to enter the LH along the anterior-medial region assisting the tuning towards different odour intensities. The iPNs responsive to pheromones too end in the LH regions along with iPNs coding attractive odours (Strutz et al. 2014). But further processing of odour cues and pheromonal cues in the LH is different. The interesting fact about iPNs is that these neurons selectively inhibit LH neurons, by inhibiting food odour sensory pathways alone, keeping the pheromone pathway insulated from inhibition (Liang et al. 2013). A set of third-order neurons (iPNs again) from the vlPr are known to elicit responses in the anterior-lateral region of the LH to aversive cues.

On the other hand, the ePNs being uniglomeruli, are known to innervate the LH in regions responding to only pheromones, food odours, also attractive amines, and aversive acids/CO_2. The LH is spatially segregated to incoming odours based on identity, intensity and also behavioural relevance (Sachse and Beshel 2016, Strutz et al. 2014).

Studies show classification of the iPNs into two morphological classes spatially segregating in the AL and retaining the segregation in the LH (Fisek and Wilson 2014, Knaden et al. 2012, Strutz et al. 2014). Studies show that the other set of iPNs in the vlPr are generally not activated by attractive odours, but by repellent odour signals. The vlPR region is also innervated by neurons from the optic lobe,

suggesting that the LH receives information from various sensory modalities (Parnas et al. 2013, Strutz et al. 2014, Tanaka et al. 2004).

Neurons relaying information in the LH are many and it has been difficult to identify all types due to the lack of resources and distinct physical structure in the LH. Recent work by Dolan et al., 2019 however has provided some valuable insights regarding the LH and third-order neurons in the LH. The LH input neurons (LHINs) that relay information from various modalities such as auditory, mechanosensory, or the visual system are known to be either cholinergic or GABAergic or putatively both. The LH local neurons (LHLNs) arborize within the various regions of the LH and are glutamatergic or GABAergic indicating the presence of lateral inhibition within the LH. The other type of neurons, LH output neurons (LHONs), were directly involved in the relay of information to the higher regions in the brain. These neurons are known to project onto the superior lateral protocerebrum (SLP) rather than the descending neurons in the ventral nerve cord. This indicates that olfactory sensory input undergoes processing by one more centre before any motor-related activities such as an increase in locomotory speed, turning, decrease in speed of flight, etc. occur. Also, the LH directly does not send output to the motor neurons but acts in tandem with the other brain centre, the MB (Dolan et al. 2019).

Several LHONs are known to control motor activities such as flight, irrespective of the valence coding in the brain. Valence coding is driven by all types of LH neurons. Aversion is coded by both the LHON and LHLN, attraction is coded by the LHONs. The LH not only codes innate behavioural responses, but also is a centre for multimodal sensory processing with simultaneous control over the motor functions (Dolan et al. 2019).

1.5. Preface to the Project

The entire process of olfaction finally ends with a decision made by the fly depending on the hedonic valence of the odour and its intensity. Odour processing occurs stepwise in the fly brain. The OSNs express receptors with response

patterns to odours of different classes/identities. The AL has a spatially segregated representation of odour valences where certain glomeruli code for aversion while certain others for attraction (Hallem and Carlson 2006, Knaden et al. 2012). The PNs maintain the specificity of odour identity and hedonic valence whilst carrying information to the higher centres in the brain (Couto et al. 2005, Nagel and Wilson 2016). The higher centres, MB and LH are involved in decoding the input information. The MB, required for memory and learning takes receives information as it comes with no separation-based odour identity (Li and Liberles 2015). The LH displays zonal segregation of sensory input and receives information from different sensory modalities just as the other centre, the MB. This region is the main centre for innate responses and valence coding. The odour identification and discrimination here gives rise to innate behavioural states of the fly (Heimbeck et al. 2001, Strutz et al. 2014):

a.) The LH receives olfactory input in a spatially segregated manner according to odour valence and odour intensity.

b.) The LH neurons have multiple roles in processing of olfactory cues simultaneously with other sensory cues.

The above stated facts lead to the aim of my master's Thesis.

1.5.1. Aim of the Project

The main intention is to understand chemosensation, the process of olfaction with regard to ethological and physiological significance. The project began with confirming the valence coding of odours (Hallem and Carlson 2006, Knaden et al. 2012) during flight using the tethered fly setup. The further steps involved measuring the neuronal activities and responses mainly in the LH of the brain to the same set of odours using the optical imaging technique. The project was conducted at the Max Planck Institute of Chemical Ecology, under the abled guidance of group leader Dr. Silke Sachse and group member and postdoctoral researcher Dr. Veit Grabe. The initial work of devising the behavioural tethered flight setup and test runs was conducted by Dr. Veit Grabe and the intern fellow

Julia Reinecke. My contribution to the project in the Department of Neuroethology was to troubleshoot and improve a functional tethered fly chamber set up. I also conducted behavioural analyses in tethered flies to a set of odours. With Dr. Grabe's mentoring and help, we functionally imaged fly brains to test activity in the LH to the set of odours in the tethered fly condition. The hypotheses of the project are:

a.) Odours of different chemical identity and based on ethological relevance to the fly elicit positive (attraction), or negative (aversion) or neutral (no response) behavioural responses.

b.) Behavioural responses are the result of neuronal activity in the higher centres of the brain in the fly.

The project will continue with experiments combining the behavioural assay and calcium imaging to obtain a clear understanding of how the internal state of the fly affects the neuronal activities during odour encounter and also try to understand the functioning of the LH during multisensory input stimulation.

2. Materials

2.1. Fly Stocks

Wild type female *Drosophila melanogaster* (Canton-S) species from Bloomington Stock Centre, USA, Indiana were used as test specimens for the behavioural assays. A cross of the transgenic line carrying the GH146-Gal4, UAS-GCaMP6s was used to image the excitatory projection neurons (ePNs).

2.2. Chemicals

Table 1, provides the list of chemicals used as odours during experiments, and chemicals used for disinfection and cleaning procedures.

Table 1. List of chemical reagents used

Reagent or Resource	Source	Identifier
11-cis-Vaccenyl acetate	Cayman Chemicals, USA, Michigan	CAS: 6186-98-7
Benzaldehyde	ACROS, France, Molinons	CAS: 100-52-7
Mineral oil	ROTH Germany, Karlsruhe / Sigma Aldrich, USA, St. Louis	CAS: 8020-83-5
Balsamic vinegar	Commercial	NA
1- Octanol	Sigma, Germany, Steinheim	CAS: 111-87-5
Methyl salicylate	Sigma, Germany, Steinheim	CAS: 119-36-8
Acetoin	Supelco, USA, Pennsylvania	CAS: 513-86-0
Ethyl acetate	Sigma Aldrich, Germany, Steinheim	CAS: 141-78-6
Ethanol	Merck, Germany, Darmstadt	CAS: 64-17-5
Labosol D	NeoLabLine, Germany, Heidelberg	NA
Acetone	ROTH Germany, Karlsruhe	CAS: 67-64-1

2.3. Consumables

The list of consumables made use for the project during the experiments is listed in **Table 2**.

Table 2. List of consumables

Item	Commercial Name	Manufacturer
Glass bottles with inward/ outward valves	Duran GL 32, 50 ml	SCHOTT, Germany, Mainz
Stainless steel pins	Austerlitz Insect pins-0.10 mm	ENTOMORAVIA, Czech Republic
3 Component glue	3M ESPE, Protemp II	Germany, Neuss
Gloves	TouchNTuff NBR 92-600	Ansell, USA, New Jersey
Insect pins	Kabourek Insect pins	Kabourek, Czech Republic, Zlín
UV Adhesive gel	Fotoplast Gel	Dreve Otoplastik GmbH, Germany, Unna

2.4. Software Used

The software used in order to conduct the experiments and later analyse the recorded data are provided in the table (**Table 3**) below.

Table 3. List of software used

Name	Provider
StreamPix (Version 7.0)	NorPix, Canada, Montreal
Fiji (Version 1.47f)	ImageJ, USA, Madison
R (Versions 0.99.903, 1.1.423)	R core team
ZEN 2010 BSP1 (Version 6.0)	Zeiss, Germany, Oberkochen
Data Analysis (Version F.01.03.2357)	Agilent ChemStation, USA, California

2.5. Instruments Used

Table 4 shows the various instruments that aided in conducting the experiments for the project and instruments used for miscellaneous activities (cleaning purposes, etc.).

Table 4. List of instruments

Instrument	**Model**	**Company**
Microscope	Stereomicroscope MZ16	LEICA, Germany, Wetzlar
Camera to record experiment	Mako U-130	Allied Vision, Germany, Stadtroda
Data Acquisition Tool (DAQ)	USB-6008	National instruments, USA, Austin
Portable spot system LED	LED-100	Electro-lite, USA, Connecticut
GC-MS (DB5)	TRACE GC 2000; TRACE MS	Thermo- Finnigan, USA, California
SPME fibre assembly (DBS/CAR/PDMS)	57328-U	Supelco Inc., USA, Pennsylvania
GC-MS (Wax)	7890B GC System; 5977A MSD	Agilent Technologies, USA, California
2-Photon confocal laser microscope	Zeiss Imager. Z2	Zeiss, Germany, Oberkochen
Heating oven	EUT 6130 Drying Oven	Heraeus Laboratory, Germany, Hanau
Ultrasonic Cleaner	USC100TH	VWR, USA, Pennsylvania
Temperature/ Humidity logger	EL-USB-2	Lascar Electronics, UK, Wiltshire
Fluorescence Stereo Zoom Microscope	Axio V16	Zeiss, Germany, Oberkochen

2.6. Recipe- Cornmeal Medium for *Drosophila* species

All flies used in the experiments were maintained on a cornmeal-agar-molasses medium. The ingredients and ratios of mixing are provided in **Table 5**.

Table 5. Ingredients for fly food

Reagent / Substrate	**Amount (g)/ Volume (ml) in 500 ml of food**
Treacle	59 g
Brewer´s yeast	5.4 g
Hot water	101 ml
Agar	2.1 g
Coldwater	135 ml
Polenta	47 g
Fill up with hot water	135 ml
Flush out with hot water	34 ml
Cold water	54 ml
Propionic acid	1.2 ml
Nipagin 30%	1.65 ml

3. Methods

3.1. Odour Preparation and Maintenance

Odours were prepared in 50 ml Duran GL 32 *(SCHOTT, Germany)* bottles. Concentrations of 10^{-2} were prepared using mineral oil as the solvent. Each odour bottle was filled with 4.5 ml of pure solvent and 0.5 ml of the odour stock of 10^{-1} concentration respectively using *Eppendorf* pipettes, making a total of 5 ml in the bottle. 5 ml mineral oil was used as a negative control. Initial stock solution of 10^{-1} was prepared using 100 µl pure chemical in 900 µl mineral oil as the solvent. The odour bottles were filled with the respective odour dilutions and labelled accordingly. The odour bottles were stored in the refrigerator.

Pure chemical compound concentration= 1 g/ml

Final volume of odour dilution in bottle (V2) = 5 ml

Volume of odour in each bottle (V1) = 500 µl.

$$V1 = \frac{C2 \times V2}{C1}; V2 = 5\ ml$$

Final concentration C2= 10^{-2} mol/l; Initial concentration C1=10^{-1} mol/l

Initial dilution preparation

10^{-1}: 100 µl pure odour + 900 µl mineral oil.

The final volume in each odour bottle= 500 µl of each dilution respectively + 4500 µl mineral oil.

The odours were prepared freshly every two weeks to prevent contamination. The process of washing the odour bottles involved four steps. Initially, after emptying the old odour solution, the bottles were rinsed with distilled water, filled with Labosol disinfectant and placed in the ultrasonic water bath for 30 min. After this step, the bottles were again rinsed with distilled water and with a small quantity of

pure ethanol rinsed thoroughly. This was followed by emptying out the ethanol and the bottles were given a quick acetone wash and washed again with distilled water for the last time. In the last step, the odour bottles were dried first using the blow-dry manually and the bottles and caps were kept separated in a drying oven at 50 °C overnight.

3.2. Fly Stock Maintenance and Fly Preparation

The fly stocks, both wild type and transgenic lines were maintained in 25° C incubators under a 12h light-dark cycle and 70% RH. The female flies used for behavioural experiments were 7 days old and the transgenic flies for imaging experiments were 6-8 days old.

3.2.1. Fly Preparation for Behavioural Experiments

The flies had to be glued to a needle in order to fix them while they were able to fly. The experimental female flies were starved 24 h prior to the experiment. The flies were starved to increase sensory neuronal responses and behavioural outputs, as shown previously that starvation in flies leads to increased food-seeking behaviour and brain responses (Root et al. 2011). Therefore, the flies were flipped into a vial with a water-soaked sponge. Before fly preparation, the flies were immobilized on ice for 15 min. A needle *(ENTOMORAVIA, Czech Republic)* was glued to a toothpick end with a dental glue mixture *(Protemp II, Germany)* and bent at the tip by 90°. This glue mixture was prepared by mixing the base paste and the two-catalyst components in the ratio of 1:2. The fly held by its legs using fine-tip forceps was tethered to the tip of a needle using a blob of dental glue mixture. Once the fly was tethered at the dorsal region between its head and thorax, it could rest in the humid chamber and recover at room temperature for about an hour. The fly preparation was performed with the aid of the dissecting stereomicroscope MZ16 *(LEICA, Germany)*.

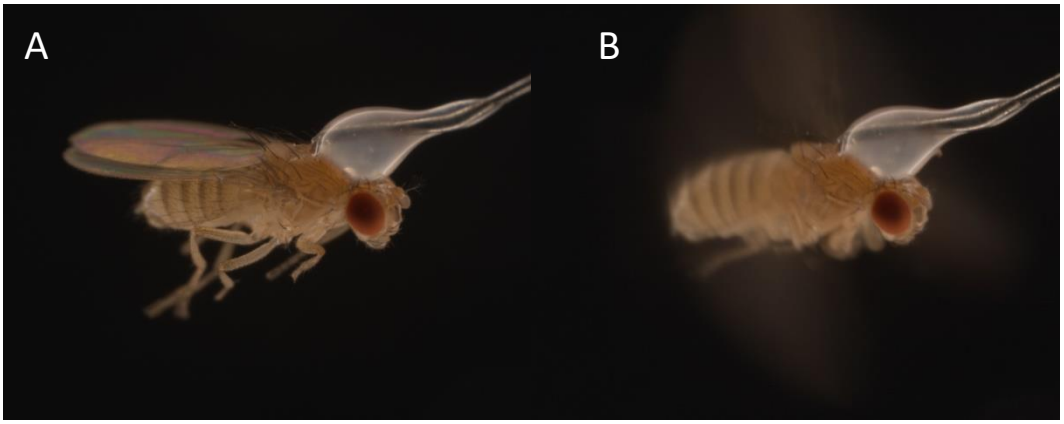


Figure 2. Tethered fly.
(A), (B) Tethered fly stationary and during flight. The region between head and thorax is glued to the needle keeping the antennae dry.

3.2.2. Fly Humidity Chamber

The humidity in the experimental room was measured using the temperature/humidity logger and compensated by using the humid chamber. The humid chamber was used to ensure a humidity of more than 30% RH for the flies. The humid chamber was a small box with a lid. The prepared flies could rest on a strip of clay provided as support and the box contained a tissue soaked in water to maintain the relative humidity.

3.3. Tethered Fly set up

3.3.1. Photoionization Detector measurements

The first step to allow the successful running of the experiment was to use the photoionization detector (PID) to monitor and verify the odour delivery. It was an important checkpoint to see if the fly encountered the odour stimulus during the experimental protocol. The PID, used for detecting vapour molecules, was used to measure the presence of ionizable particles during odour delivery. The nozzle was placed inside the chamber and the external record button was pressed. The experiment was recorded externally on the computer using the software LabView that detected the current from the mini PID control. The PID measurement as in

the experiment lasted 15 s. Sucked air molecules were guided on to the UV lamp for ionisation and were read as voltage sensory output. The PID parameters, namely offset was kept minimum, with a low pump rate and a gain of 10x.

3.3.2. Tethered Fly Setup- Instrumentation and Protocol

The tethered fly setup is an apparatus to conduct behavioural analysis during insect flight (Martí-Campoy et al. 2016). Our experimental apparatus installed for the behavioural analysis consisted of a hollow plastic chamber. A tethered fly was placed through the hole at the top right corner of the chamber. Two tube fittings at an angle of 45° were fitted along the axis of the hole. These tubes were connected to the odour bottles. An infrared LED light source was housed in the chamber illuminating the fly from the rear end. At the bottom left corner of the chamber was a suction tube to suck out residual air. It was maintained at 0.5 lpm. The fly specimen remained directly above a high-speed recording camera device, Mako U-130 *(Allied Vision, Germany)*. The camera controlled by the software was in series with the external computer to record the video streaming during the experiments. An Arduino chip, an integrated circuit chip aided by the USB-6008 data acquisition tool (DAQ) *(National Instruments, USA)* was attached to the main record button, which on pressing, initiated the experiment protocol. A main valve was provided outside of the chamber to enable odour delivery to the fly. The valve directly connected to the Arduino chip was the mediator for odour delivery to the fly specimen. The valve working in row of an efflux flowmeter at 0.5 lpm, provided the inlet into the odour bottle and the lateral tubes acted as the outlet respectively. The odour was delivered to the fly from one of the two lateral tubings in an alternate manner with an inter-trial-interval of 1 min between each trial. All odour puffs were maintained at the rate of 0.1 lpm with the help of a digital flowmeter.

Pressing the external record button initiated the protocol of 15 sec duration, with the camera capturing 750 frames per second (fps). The odour was delivered 2 sec after protocol initiation and lasted for 5 s. The experiment was streamed via a GigE connection and recorded by StreamPIX 7.0 *(NorPix, Canada)*. Each experimental trial

consisted of the negative control, mineral oil, delivered via the lateral tubings alternating between each of the two tubes. Simply put, the odour was delivered in two sets from each tube respectively or four times alternating. The fly behaviour was recorded to the stimulation in the same manner, via the lateral tubes in two sets. In between experimental trials, a continuous air stream of 2.0 lpm flushed out residual odour preventing contamination. The odours tested for a behavioural response were vinegar (VIN), cis-vaccenyl acetate (CVA), octanol (OCT), methyl salicylate (MSC), and ethyl acetate (ETA). These odours code attraction or aversion.

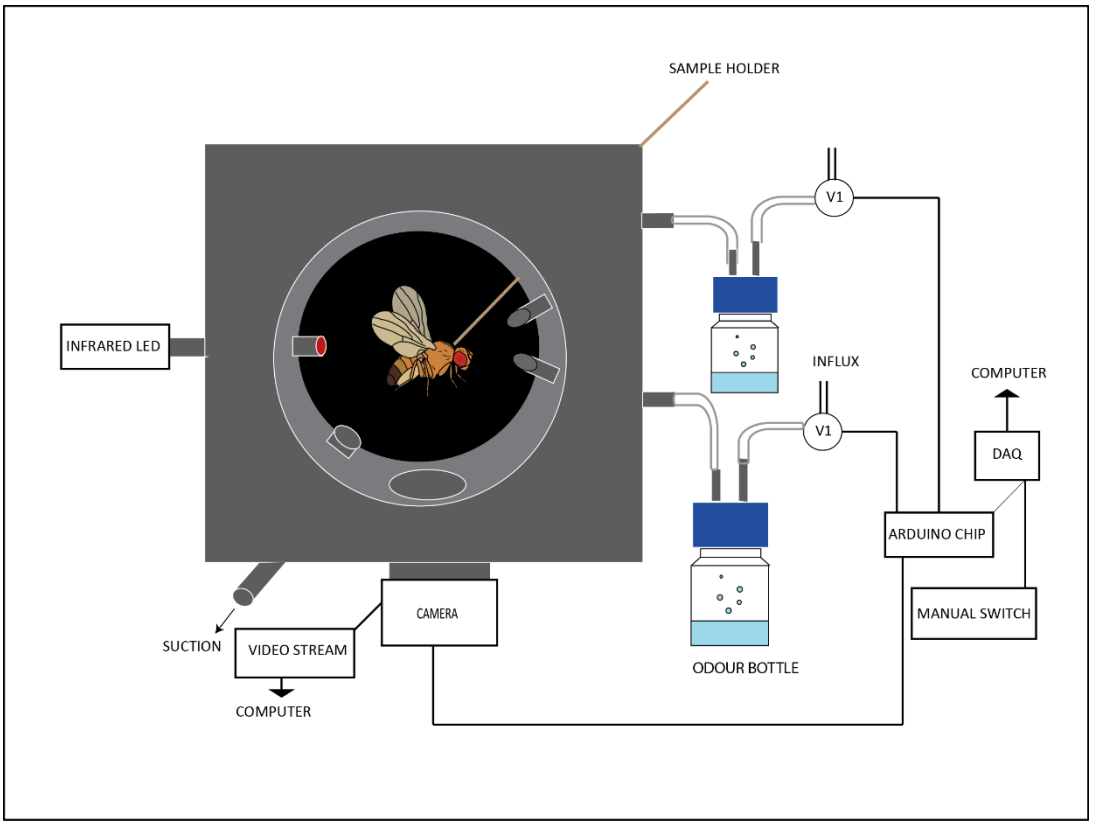


Figure 3. The tethered fly setup.
A chamber houses the tethered fly illuminated by an infrared LED and receives odour from the two odour tubings laterally. The tubings were controlled by the DAQ and Arduino chip. And below the chamber was the camera to record specimen flight externally connected on a working computer.

Figure 3 shows the setup optimized and used for the project and currently in use for other ongoing experiments in the laboratory. The project began with an initial setup consisting of a slightly different setting. The initial setup had a single odour delivery tube facing the specimen head. The odour delivery lasted for 2 sec in the

15 s window. A continuous supply of air was provided to the chamber to make the environment as realistic as possible for the fly. The odour was being delivered at the rate of 0.5 lpm and the setup was provided a compensatory stream to facilitate the switch from the continuous air stream supply to the odour bottle.

The initial setup was optimised to obtain a clearer behavioural readout in the flies. The odour was delivered from the sides of the tethered fly rather than head-on. It was delivered laterally from either side, the left or right side alternating during the experiment as seen in **Figure 3.** The flow rate of the odour was reduced to 0.1 lpm and lasted 5 s. The supply of continuous airstream was cut off. The compensatory switch was eventually removed. To obtain a clear behavioural response, the concentration of all odours being tested was maintained at 10^{-2} mol/l. **Appendix figure 2** shows the temperature and humidity readings inside the tethered fly setup and the outer environment before the use of the humid chamber to house the flies prior to the experiment. The temperature was 20 °C and humidity was 30% both inside the setup and outside in the room. Preferable humidity for flies being 50% was ensured using the humid chamber. The temperature could range between 20-25 °C.

3.3.3. Data Analysis

The tethered fly apparatus used in our project was an open-loop system where the output was not controlled by the input. The behavioural response or the output of the experimental specimen was recorded as a video sequence during the experiment. The software that recorded the 15 sec experimental protocol was StreamPix 7.0. The two parameters considered to analyse the behaviour in flies were the wingbeat frequency (WBF) and wing beat amplitude (WBA). WBF is the representation of flight speed. WBA measured the steering action of the fly towards or away from an odour. The difference between the left- and right-wing amplitude values were calculated in comparison to the baseline values of WBA and averaged over the total number of specimens tested for. As the odour was delivered to the specimen alternating between the left and right tube during each

trial, for simplicity, the direction of the odour was positioned to be from the left side of the fly.

In order to extract the WBF/WBA values from the .tif files, the specimen trial for each measurement and was subjected to a set of defined regions of interests (ROIs) in the software ImageJ's Fiji, 1.47f *(USA)*. Two ROIs, each one on either side of the fly body axis and placed in front of each wing, were defined for the WBF measurements to capture the intensity values each time the fly's wings passed through it. And for WBA measurements, 40 ROIs defined were divided equally between the two wings to measure the angle each wing made. The intensity values measured using ImageJ were fed as input into an R script written in editor RStudio *(USA)* for further calculation. The R script created vector matrices of the intensity values and compared each of the intensity value captured by the respective ROI at that time point with its subsequent value in the matrix. Two matrices each for the right and left-wing were created to hold the differences between each intensity value and the subsequent value recorded by the two ROIs respectively. Each vector in the two matrices, each for the left and right-wing were compared to a threshold value of 30 and averaged as ΔWBF values for the corresponding odour. For WBA calculations with 40 ROIs, values of intensities recorded for a single ROI in time was put into two separate vector matrices for each wing. Intensity values recorded at the rate of 750 fps and 15 sec duration, the values were divided into groups of three and averaged. The averaged 3,750 values in number were put in a new vector for each wing. The maximum value in each row of the vector matrices was fed into a new vector. The difference between the two set of values namely the left- and right-wing maximum intensity values provided the ΔWBA for that odour. This difference in values provided us with insight regarding the steering effect of the wings.

The values obtained in R was then opened in an Excel sheet for further analysis. The base frequency/amplitude for each odour puff either from the right- or the left- side was calculated by averaging the values of intensities before odour stimulus. And each of the intensity values at that time point was standardised to the baseline. The differences were averaged for deltaWBF values. For deltaWBA

values, median values of the differences in groups of 25 were averaged. And the values representing the right-wing were negated, keeping the odour delivery to the leftside of the fly. For each odour, including the negative control, the deltaWBF/deltaWBA plots were plotted on the Y-axis against the duration of the experimental protocol in seconds on the X-axis.

ΔWBF plots showing a change in frequency for an odour was followed by plotting the ΔWBA plots. An increase in the frequency indicated the behavioural response in the fly on odour encounter. To confirm if the behavioural readout was positive/negative, the amplitude values were plotted. The odour delivery was fixed to the left side, amplitude values with a positive value during/or after odour delivery was attributed to a negative steering action of the wings or aversion to the odour. And negative values in the plot indicated attraction.

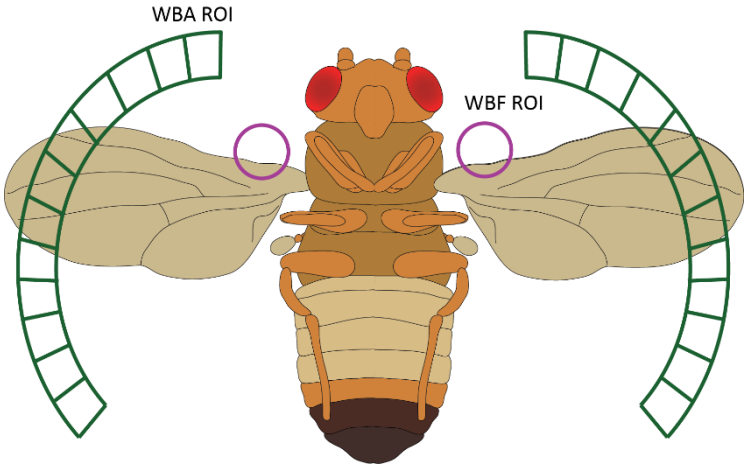
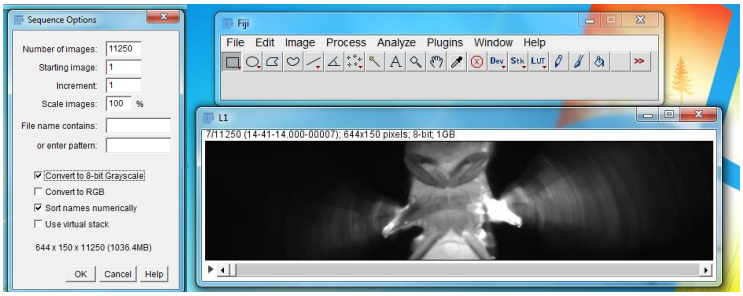


Figure 4. WBF/WBA analysis.
Two parameters to analyse fly behaviour are the wingbeat frequency and amplitude (WBF/WBA). Two ROIs (purple) for WBF analysis capturing intensity values each time the wing passes through it was positioned in front of each wing. Forty ROIs (green) were positioned at different angular positions along the wing to calculate the angular movement of each wing for WBA analysis.

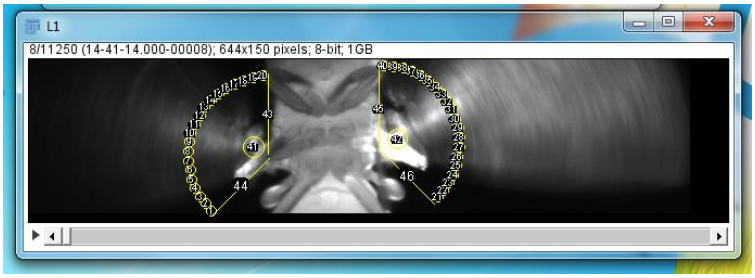
A **StreamPix** → **Quick Export** → **Save .tiff file**

B **Fiji** → **Import** → **Image Sequence**



C **Analyze** → **Tools** → **ROI Manager**

D **Image** → **Transform** → **Rotate/Translate**



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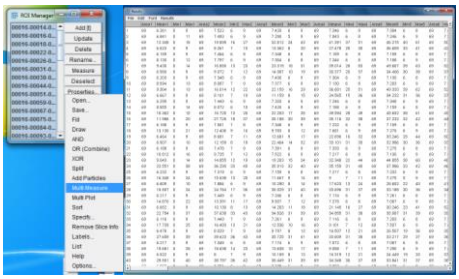

Figure 5. Analysis steps.
Flight behaviour was analysed using StreamPix, Fiji and RStudio, lastly Excel. **(A)** Exportation of .seq file to .tiff file. **(B)** Image analysis in Fiji by importing exported file. **(C)** Placement of ROIs for further measurement. **(D)** Fitting of fly within ROIs for WBA analysis. **(E)** Measurement of all ROIs; Results were further analysed in RStudio and Excel.

3.4. Gas Chromatography and Mass Spectrometry

Odour bottles were susceptible to contamination despite repeated washing steps and fresh preparation of odours during the piloting of the project using the initial setup. During apparatus optimisation, we ensured a routine of preparing fresh odour dilutions every two weeks in thoroughly washed bottles and provided a strong puff of continuous air supply in the chamber in between specimen trials. As a confirmatory step of odour purity, we conducted an analytical testing of one of the odours and the negative control. The odour we tested was vinegar and this served the dual purpose of enabling us to test odour purity and breakdown the complex mixture into its individual components that were responsible for producing a behavioural readout in the flies.

3.4.1. Instrumentation and GC conditions

The GC-MS consisted of the 7890B GC System and the 5977A MSD *(Agilent Technologies, USA)*. This device was fitted with a Wax column (30 m, 0.25 mm I.D., 0.25 µm film thickness) in connection to an 80 cm guard column. Helium, being the carrier gas remained at a constant flow rate of 1.15 ml/min. Initial conditions for the GC oven started at 40 °C held for 2 min. The temperature was ramped to 260 °C at the rate of 20 °C/min. The liner's temperature was 250 °C, and the splitless method was used. Conditions for the MS measurements were as follows- the transfer line was at 260 °C, the ion source at 230 °C with 70 eV as the electron impact ionization. The grey 57328-U fibre *(Supelco Inc., USA)* was used for sample collection. It was fitted into its corresponding holder.

3.4.2. Sample Preparation

We followed the SPME method of volatile extraction to analyse balsamic vinegar. The grey fibre (50/30 µm DVB/CAR/PDMS, composition of the grey fibre) was inserted into the sample bottle covered by an aluminium foil. The grey fibre made up of the extraction phase was held a few seconds inside the vinegar bottle of 10^{-2}

mol/l dilution, and mineral oil bottle as control, for analytes collection before injection into the instrument. Depending on chemical properties of odours and the high intensities of the standards, collection time varied from 10 s for vinegar to just a couple of seconds for the standards respectively. **Figure 12** shows the plots obtained for the odour, control and the standards tested.

3.4.3 Analysis

The GC plots and Mass spectra obtained for the analysed samples were obtained on Agilent's MSD Chem Station Data Analysis *(version F.01.03.2357, Agilent ChemStation, USA)*. The GC plots were copied on Adobe Illustrator for refinement.

3.5. Optical Imaging

Having confirmed a valence for odours tested during the behavioural analysis of *Drosophila melanogaster*, we were interested in understanding the underlying neuronal activity in the higher centres of the brain. The outlook is to simultaneously conduct imaging and behavioural assays in the flies as represented in the schematic **Figure 13**. We first wanted to ensure that the LH responded to the odour set. We started with the technique of imaging the LH in the transgenic line GH146-Gal4, UAS-GCaMP6s, expressing a reporter GCaMP, a GFP protein under the influence of the Gal4 driver line in the ePNs leading to the LH. These flies were subject to the different test odours/control and activity in the LH was imaged using a Zeiss Imager. Z2, 2-photon confocal laser scanning microscope *(Zeiss, Germany)*. A laser beam of 925 nm wavelength was used to excite the GCaMP expressed in the ePNs. A Chameleon UltraTM diode-pumped laser *(Coherent, USA, California)* was used to illuminate the specimen with a resolution of 1024×1024 square pixels. The emitted wavelength light ranging between 500-560 nm filtered using the green filter. Each section of the brain was at a frame rate of 4 Hz. The change in fluorescence detected by the photomultiplier tube in the detector could build a live image of the brain region. Odours tested were vinegar (VIN), cis-vaccenyl acetate (CVA), octanol (OCT), methyl salicylate (MSC), benzaldehyde (BEA), and

ethyl acetate (ETA) and control MOL. The odours were tested during frame 9-29, 2 sec after initiating experiment protocol and lasted 5 sec. A continuous airstream was provided via a peek tube as were the odours. Odour delivery was controlled by the trigger software in LabVIEW. Reconstruction of the image and odour delivery was controlled by the software Zen2010 V.6.0 *(Zeiss, Germany)*.

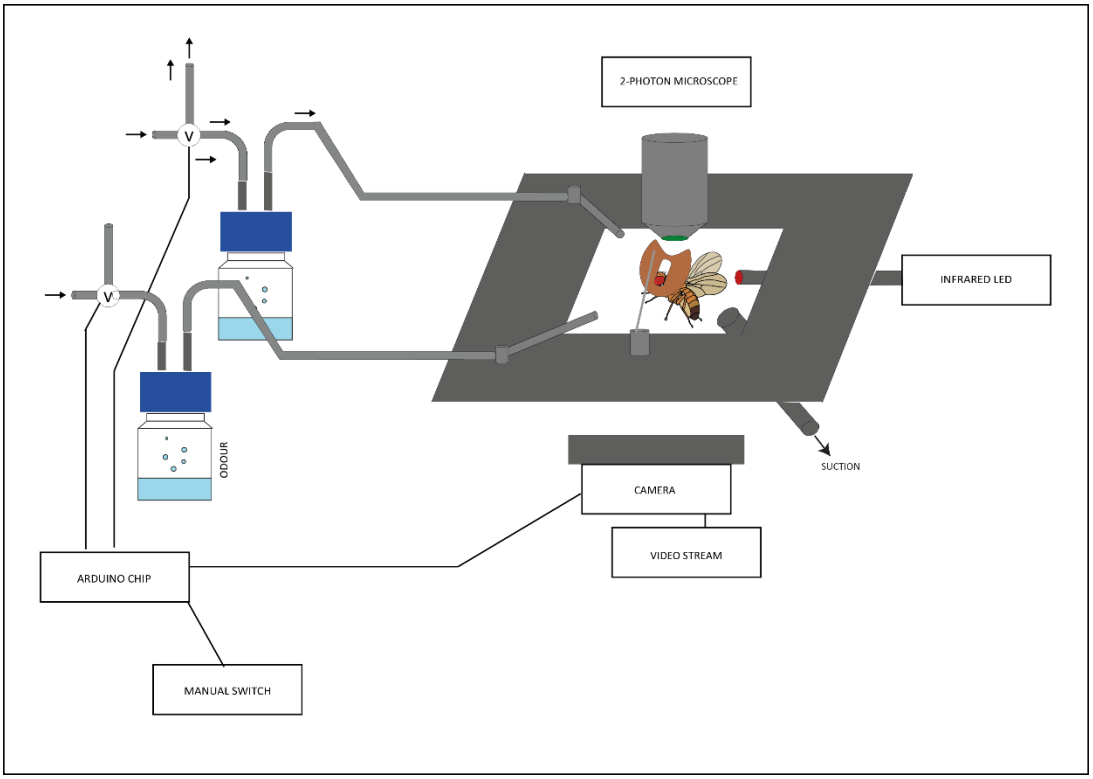

Figure 6. Optical imaging setup.
The schematic representation of the setup to simultaneously image the brain and conduct behavioural experiments in the fly specimen. Seen in the diagram is a camera to record the flight of the specimen below the stage area and the microscope to image the fly brain from above. Odour is presented to the fly laterally.

3.5.1. Fly Dissection

Flies aged 6-7 days post eclosion were used for the imaging experiments. The flies were briefly anaesthetized before mounting them on a 1GN42S nickel plate (*Plano Gmbh, Germany)* extending from an insect pin *(Kabourek Insect pins, Czech Republic).* The nickel plate was folded on its shiny side and the slit was applied with a fine layer of the Fotoplast UV gel *(Dreve Otoplastik GmbH, Germany)*. With the aid of the dissecting

microscope, the fly was held by its forelegs and glued onto the slit. The fly was glued by its head region only, parallel to the slit, keeping the antennae free and the thorax/wings free. The bent nickel plate provided space for the specimen to fly.

Having glued the fly to the nickel plate, a drop of Ringer's solution (NaCl: 130 mM, KCl: 5 mM, MgCl2: 2 mM, CaCl2: 2 mM, Sucrose: 36 mM, HEPES-NaOH (pH 7.3): 5 mM) was added on top of the slit. Using a scalpel, a slit was made in the head of the fly, the region in between the two compound eyes. And finally, using scalpel/forceps, the cuticle was removed from the head, followed by carefully removing the fat, air sacs and the trachea.

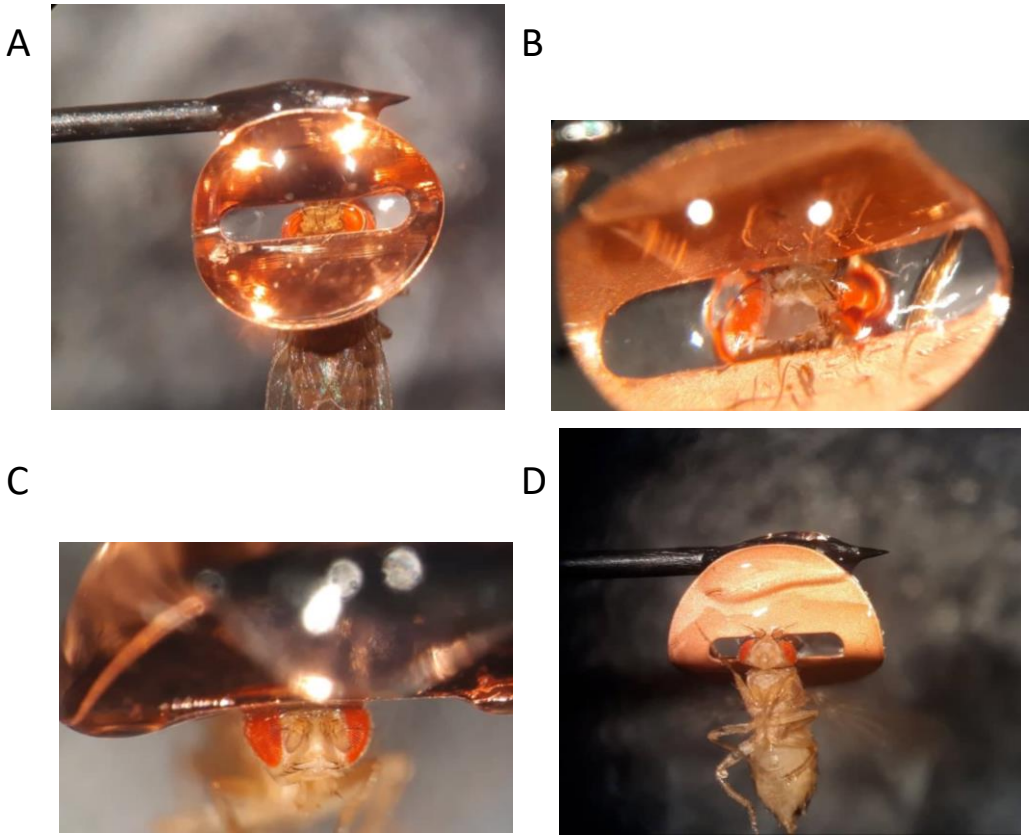

Figure 7. Dissection of tethered fly for optical imaging.
(A) Fly glued to folded nickel plate. **(B)** Brain dissected for imaging. **(C)** Frontal view of the fly with antennae free. **(D)** Dorsal view of the fly in flight.

3.5.2. Data Analysis

The confocal time series of individual odour measurements were processed using a plugin in ImageJ (Fiji) for movement corrections (StackReg). Each specimen was defined with a set of ROIs in the LH that served to measure the changes in fluorescence. Changes in fluorescence over time was calculated as a ratio of the base fluorescence. The base fluorescence, F0 corresponded to the averaged values of fluorescence 2 s before odour delivery (0-8 frames). Temporal responses to an odour in a specimen were obtained by plotting the absolute values of ΔF/F0 against time in seconds.

4. Results

The main goal of the project was understanding the functioning of the neurons in the LH of the fly brain during stimulation with behaviourally relevant odours. In order to do so, it was necessary to confirmed that the odour set induced a behavioural response in the flies. The tethered fly setup gave us the scope to confirm the valence coded for the different set of odours we tested. Once we confirmed the valence coding, we set out to analyse the neuronal activity in the LH to the set of odours tested using the optical imaging technique. The outlook of the project lies in obtaining a behavioural readout while we perform functional imaging of the neuronal activity in the higher regions of the brain. We propose to simultaneously carry out both the behavioural and imaging techniques in future experiments.

4.1. Wingbeat Frequency and Amplitude read-outs

4.1.1. Initial Set-up read-outs

For the behavioural analysis, an open-loop tethered fly setup, was used. The initial apparatus was used during the piloting of the project with odour delivered to fly via a single tubing facing the fly head directly and lasted 2 sec during the 15 sec experiment protocol. Parameters WBF/WBA gave insights regarding flight behaviour. Only if the frequency values showed a change, an amplitude analysis carried out as a velocity change would indicate the fly's attempt to fly towards or away from the odour. **Figure 8** shows the frequency and amplitude plots of specimen flight before/during/after odour encounter when tested with the initial tethered fly set-up. The frequency changes in the wing beat movements showed an increase from baseline (**Figure 8A**). The increase in frequency represented the change in the speed of flight implying the need to go towards or away from the odour and that the fly was able to perceive the odour when delivered. This change in frequency was enough to suggest that the fly responded to the odour being

tested. But the variations in the amplitude values (**Figure 8B**) was questionable as the values remain within the baseline. Without a clear indication in the steering effect of the fly wings, a valence could not be associated with the odour being tested. The question if the odour elicited an attractive or aversive or a neutral behaviour could not be inferred. The odours tested were BEA, ETA and MSC of concentration 10^{-2} mol/l were tested. Also, BEA is known to be aversive from previous work (Knaden et al. 2012) never seemed to produce a consistent frequency change. The negative control tested MOL however produced a constant frequency plot indicating a neutral response to it.

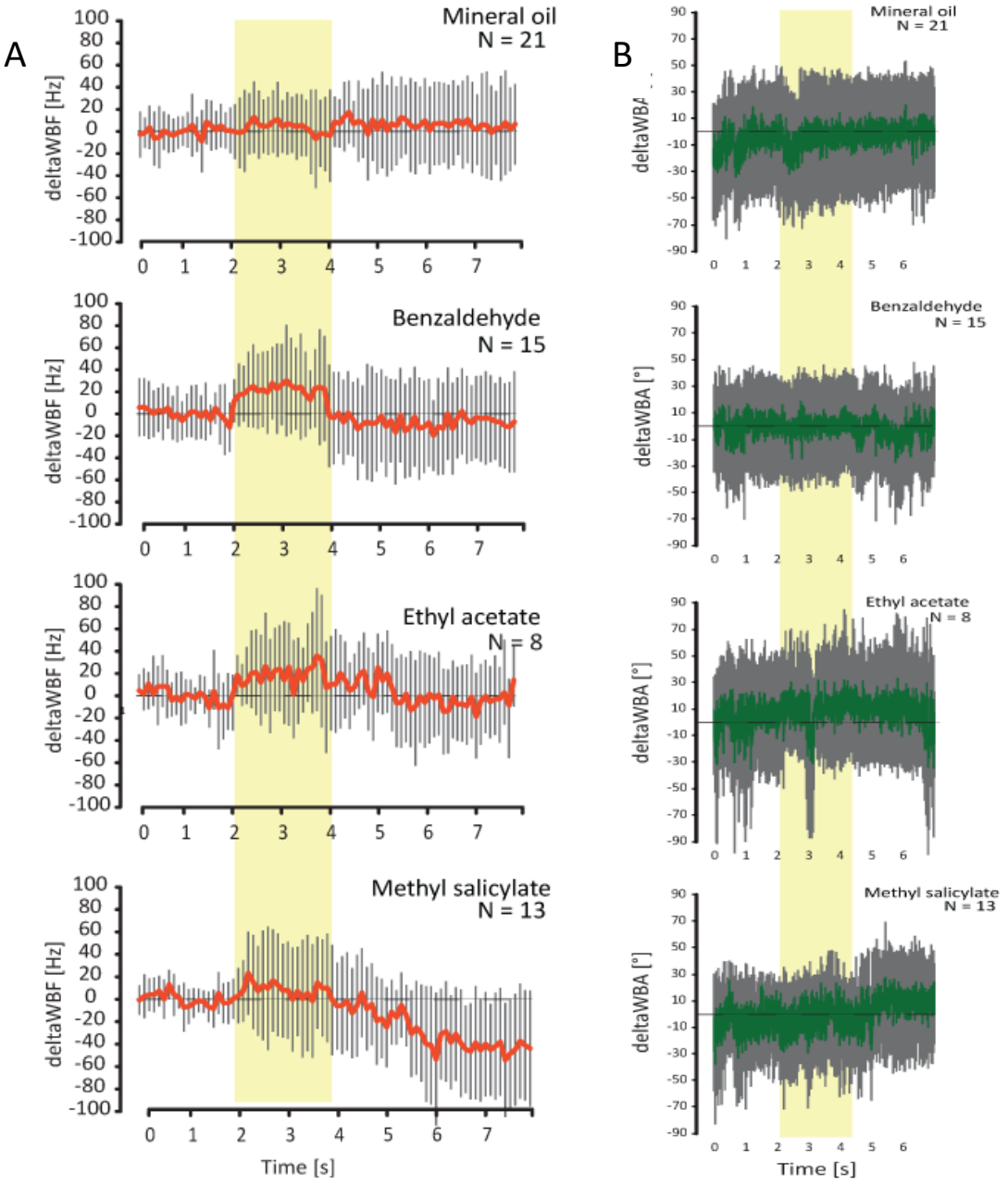

Figure 8. WBF/WBA plots with the old setup.
(A) shows the WBF plots for odours of concentration 10^{-2}. Increase in frequency from 2-4 s of odour delivery is clearly seen. The increase stays as long as odour is delivered (BEA and ETA). **(B)** shows amplitude plots for odours BEA, ETA and MSC. The plots lie well within the baseline with not much deviation indicating no clear valence. **N** represents the number of specimens tested with that odour respectively.

The initial setup was used during the piloting of the behavioural studies. Although there were frequency changes observed, the results in the amplitude was not

convincing to designate a valence to the odour. Therefore, a few optimisations were introduced in the setup.

4.1.2. Optimized Set-up read-outs

With a few modifications in the setup, the odour valences could be attributed to the corresponding odours being tested. As seen in **Figure 3** above**,** the odour was delivered laterally in two tubes alternatively and odour lasted 5 sec during the experiment protocol. **Figure 9** and **Figure 10** show the frequency and amplitude readouts, respectively. The frequency and amplitude plots for the negative control mineral oil (**Figure 9A** and **10A**) remained constant with minimal divergence from the baseline, indicative of no response or neutral response. The frequency values for all odours tested, namely VIN, CVA, OCT, MSC, and ETA all of concentration 10^{-2} mol/l showed an increase on odour encounter. The values remained high until the end of odour delivery (**Figure 9**). The prestimulus frequency averaged over the specimens was observed to be ~200 Hz (**Figure 9G**), in accordance with previous works (Fry et al. 2005, Zanker 1990). The number of specimens tested for each odour is represented by **N** in the graphs. An increase in frequency indicated a response in the flies by a modulation in the flight speed.

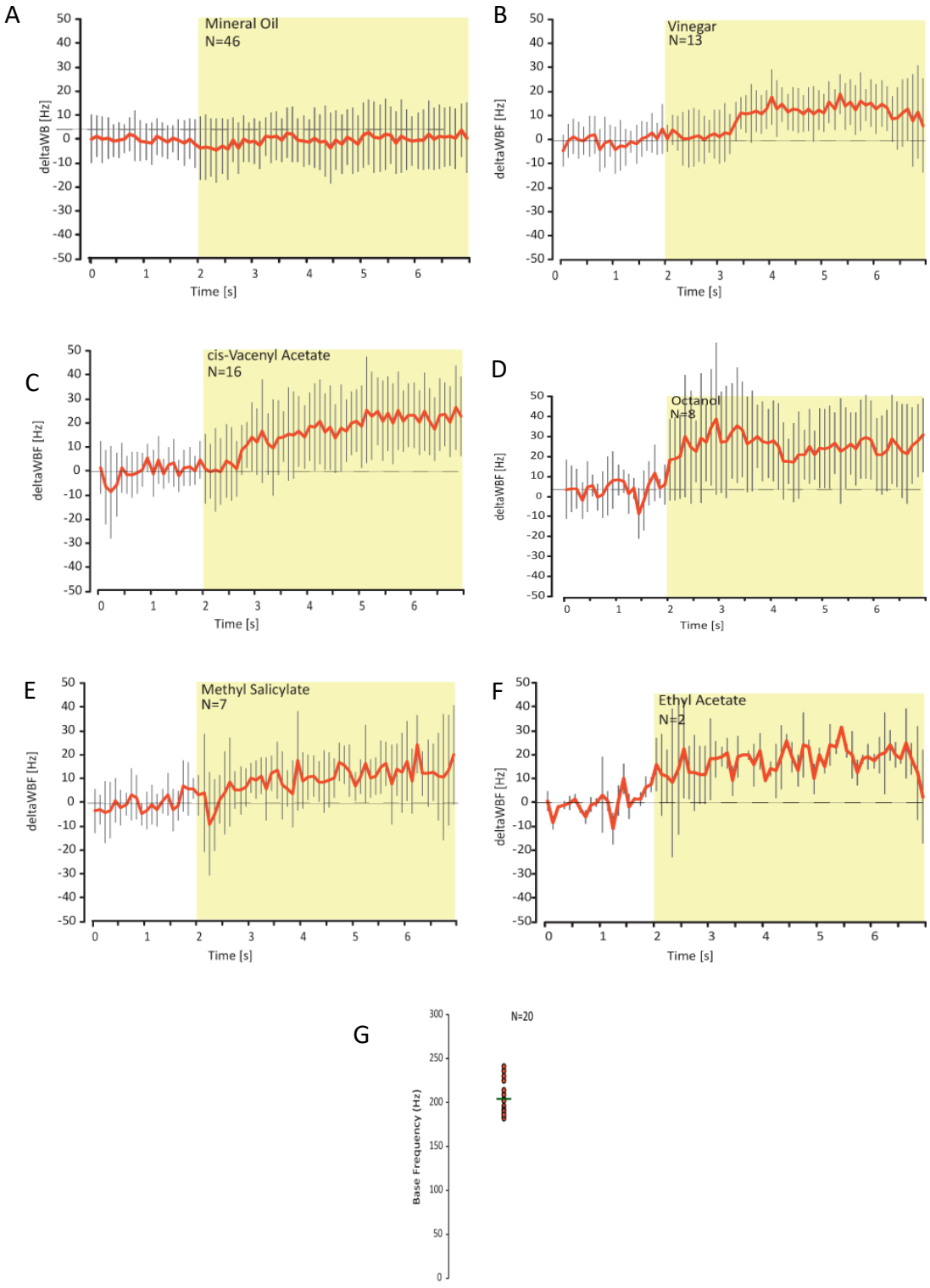

Figure 9. WBF readouts with optimized setup.
(A) Frequency plot for negative control (MOL) resulted in a constant frequency readout. **(B), (C), (D), (E), (F)** Frequency plots for VIN, CVA, OCT, MSC and ETA indicating an increase in the frequency response on odour delivery. The yellow box indicates the duration of odour delivery; **N** represents the number of specimens tested with that odour respectively; grey bars indicate standard deviation from baseline (represented as black dashed line). **(G)** The base frequency of ~200 Hz observed in specimens before odour stimulus.

Amplitude changes were analysed after obtaining a definitive change in the frequency. The increased frequency implied that flies responded to the odours in an attempt to fly towards or away from the odour plume. The amplitude plots, **Figure 10** indicated a clear divergence from the baseline on odour onset. Keeping the direction of odour delivery constant from the left side of the fly, a positive divergence from baseline corresponded to aversion while the negative deflection meant attraction and within the baseline indicated neutrality. In **Figure 10B, C** for odours VIN and CVA, the values shift below the baseline after the onset of odour indicated the specimen's attempt to steer towards the odour implying approach behaviour. For the odours OCT, MSC, and ETA the values shift above the baseline as shown in **Figure 10D, E, F** indicated a repulsive behaviour of the fly specimen trying to steer away from the odour source. Though frequency change was not as high as observed with the initial setup, the clear amplitude shifts from the baseline enabled us to attribute a behavioural valence to the odours tested.

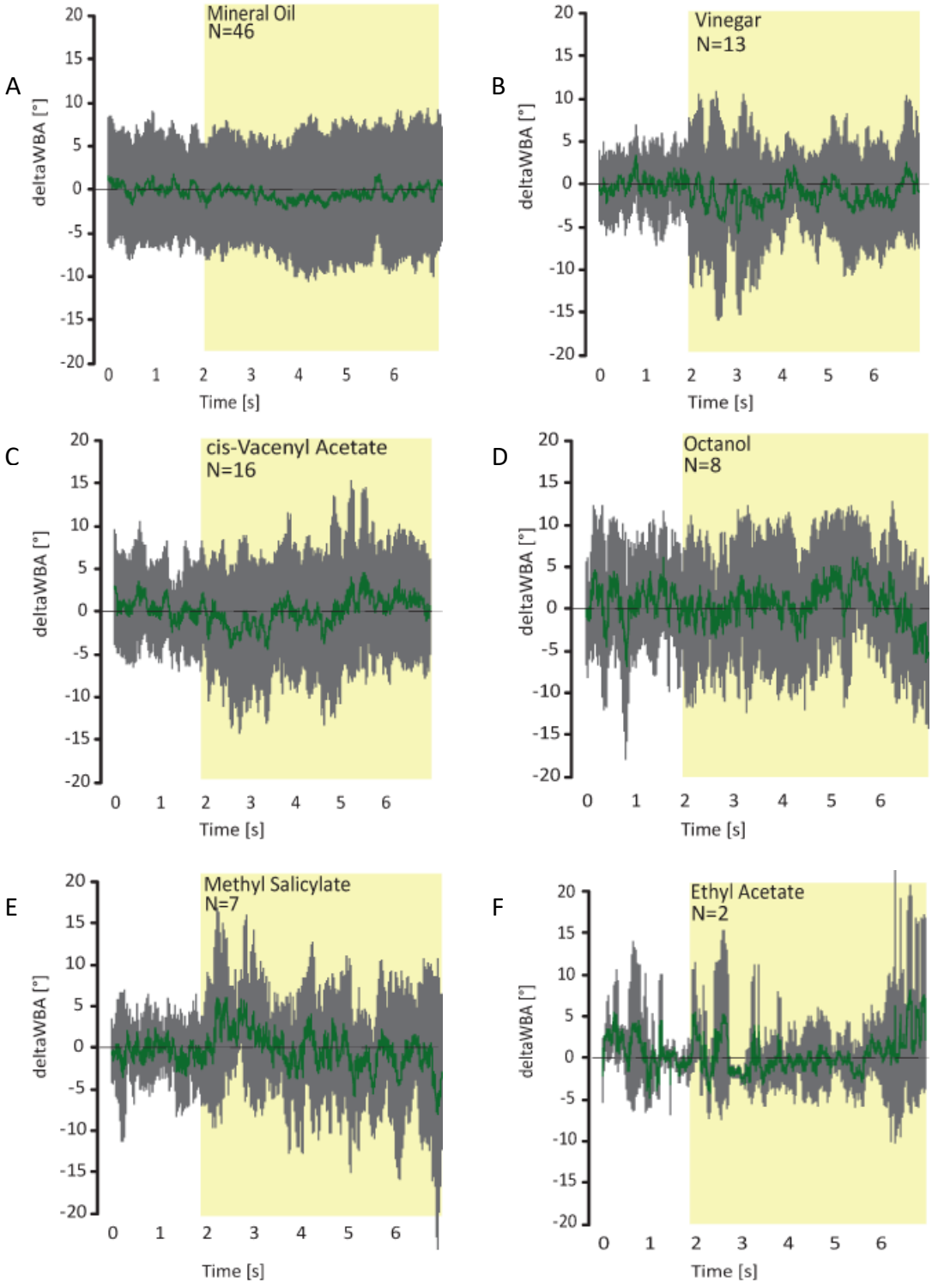

Figure 10. WBA readouts with optimized setup.
(A) Amplitude plot for negative control (MOL) resulted in a constant amplitude readout. **(B), (C),** Amplitude plots for VIN, CVA indicating a decrease in the amplitude values on odour delivery. **(D), (E), (F)** Amplitude plots for OCT, MSC and ETA showing an increase in amplitude values on odour delivery. The yellow box indicates the duration of odour delivery; **N** represents the number of specimens tested with that odour respectively; grey bars indicate standard deviation from baseline (represented as black dashed line).

We observed a clear attractive/aversive valence for the odours VIN, CVA, OCT and MSC. We began to test specimens with ETA and observed a repulsive behaviour in the two specimens. This confirmation of a solid behavioural output in terms of the increased flight speed and changes in the steering movements of the fly wings was necessary to proceed with further imaging experiments in the fly brain.

4.1.3. Photoionization Detector measurements

It was important for a conditional test run to ensure the tethered fly setup worked alright and the specimen's behavioural output was a result of odour encounter. Thus, we used the PID for measuring the presence of odour vapours inside the chamber during the experiment protocol. A rise in voltage to around 1.3 eV was observed for the time duration of 2-7 s during the PID measurement indicating the presence of ionisable molecules in the chamber space during odour delivery. The PID measurement confirmed the presence of odour molecules as vapour inside the chamber. By this, it confirmed that the odour delivery system was functioning appropriately, ensuring the odour molecules remained in the chamber for 5 s during the 15 s protocol. This window of 5 s allowed the fly specimen to perceive and respond to the odour during the experiment.

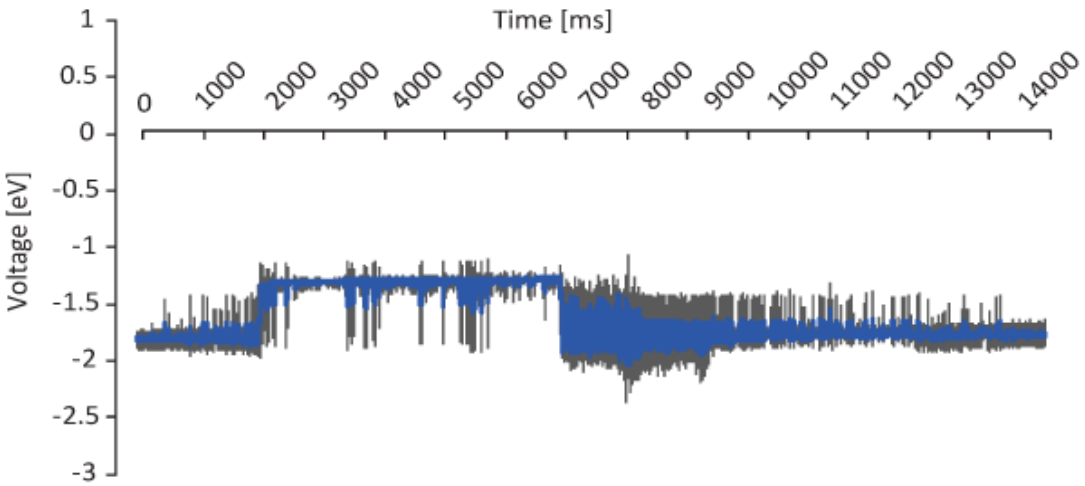

Figure 11. PID measurement.
PID measurements for the odours ETA, indicating the presence of ionizable molecules during 2-7 s of the experiment protocol. A Voltage of 1.3 eV was obtained. Grey bars indicate error bars.

4.2. Gas Chromatography Results

The two purposes served by conducting the analytical technique of GC-MS, was to 1.) check the purity of odours being used in the experiment, and 2.) analyse the natural blend vinegar for its individual components responsible for eliciting behavioural responses in flies. The GC plot obtained by SPME analysis of vinegar showed 4 abundance peaks for ethanol, ethyl acetate, acetic acid, and acetoin namely. And the mass spectra fit according to the mass to charge ratio for the standards tested (**Figure 12B**). As seen in **Figure 12A**, the peaks in the GC plot fit the standards tested. The retention time for the peaks obtained at 2.7 min, 3.1 min, 6.4 min corresponded to the peaks of the standards tested for ethanol, ethyl acetate and acetoin respectively. The largest peak at 7.7 min analysed was acetic acid, the main component of vinegar. The plot for the negative control mineral oil showed peaks for saturated hydrocarbons. From **Figure 12,** the peaks indicated no signs of impurity/contamination for the control/odour. The individual components of vinegar obtained by GC-MS are shown to elicit approach behaviour and vinegar itself is highly attractive to the flies (Frank et al. 2015, Steck et al. 2012)

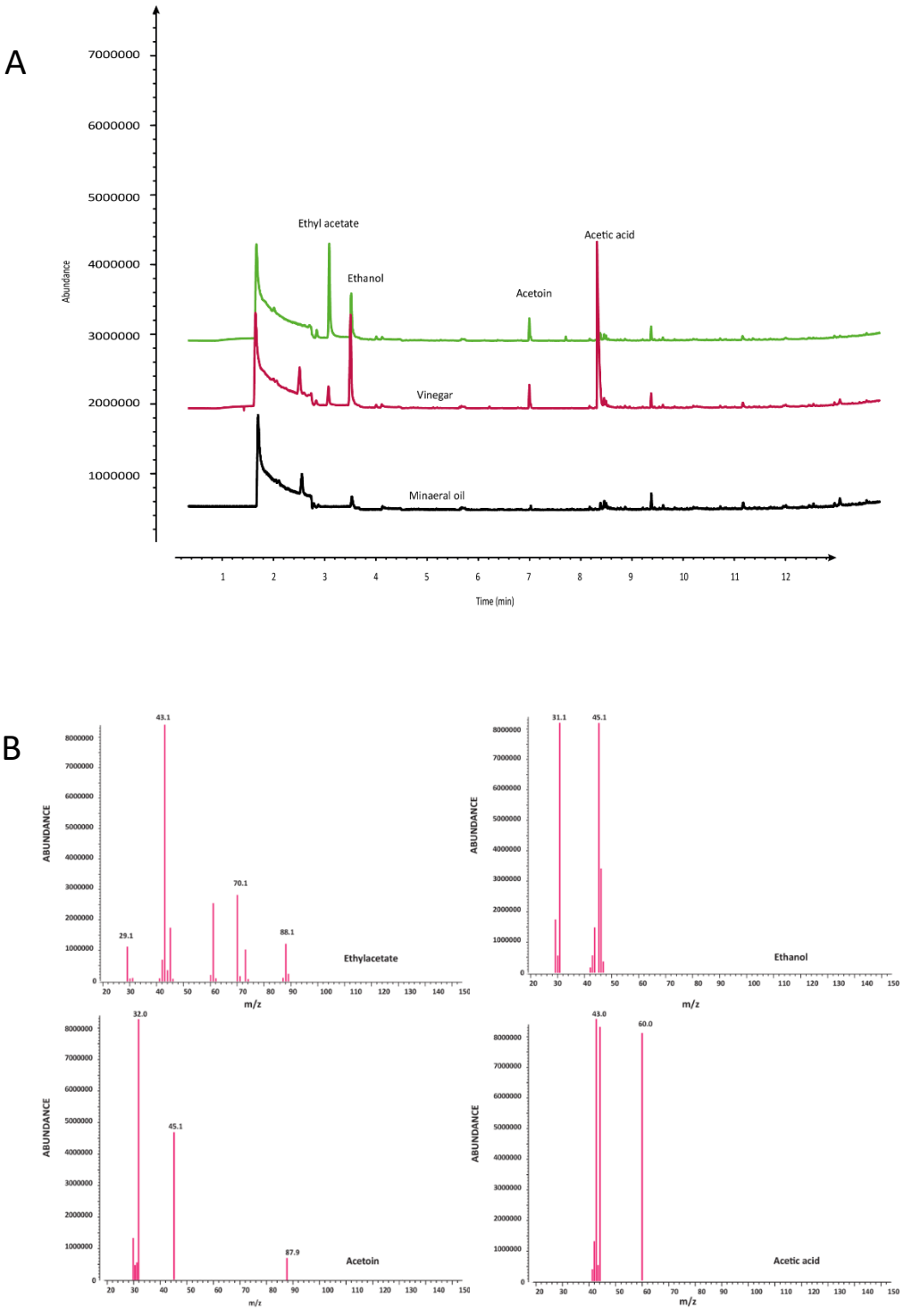

Figure 12. GC and MS plots for vinegar.
(A) The plot in green represents the peaks obtained when respective standards were tested. Peaks for ethyl acetate, ethanol, acetoin and acetic acid were obtained on analysis of commercial balsamic vinegar (pink plot). Control tested was mineral oil (black plot). **(B)** Mass spectra plots for ethyl acetate, ethanol, acetoin and acetic acid**.**

4.3. Optical Imaging Results

It was necessary to confirm a behavioural response in flies to relevant odours before studying the flies' neuronal activity during odour encounter. Having obtained a valence coding for the attractive odours namely vinegar (VIN), 11-cis-vaccenyl acetate (CVA), and aversive 1-octanol (OCT), methyl salicylate (MSC), benzaldehyde (BEA), and ethyl acetate (ETA), we were interested to see how the higher region in the LH responded to these odours. Live imaging of the GH146-Gal4, UAS-GCaMP6s transgenic lines displayed the neuronal circuit followed by the ePNs to the LH and corresponding responses to the odour set/control tested- VIN, CVA, OCT, MSC, BEA, ETA, and control mineral oil (MOL). **Figure 13B shows** the innervation pattern of the ePNs in the LH of the right lobe in one of the specimens before odour encounter. And subsequent panel **Figure 13B'** displays the spatial response pattern seen in the LH during the odour delivery to the staged fly. Here, the response to OCT is seen. And the last panel, **Figure 13B"** indicates the set of ROIs used to analyse the response in terms of change in fluorescence expressed as a per cent.

The concentration of odours used was 10^{-2} mol/l and MOL was the control. And **Figure 13A** indicates the averaged temporal responses of the ePNs in the LH. **Figure 13C** shows the spatial responses to all odours tested. **Figure 14** is the box-plot representing the relative fluorescence observed in different marked regions in the LH to the odours tested.

As in the work by Strutz et al. 2014, regions in the LH, known as the Odour Response Domains (ORDs) responding to different classes of odours were assigned. Three domains- ORD1, ORD2, ORD3 were assigned according to their positions in the LH. ORD1 (coded purple) was assigned to the posterior-medial region in the LH, ORD2 (coded pink) to the anterior-lateral, and ORD3 (coded green) to the anterior-medial region. And the corresponding (averaged) temporal patterns arising in the various ORDs are seen in **Figure 13**.

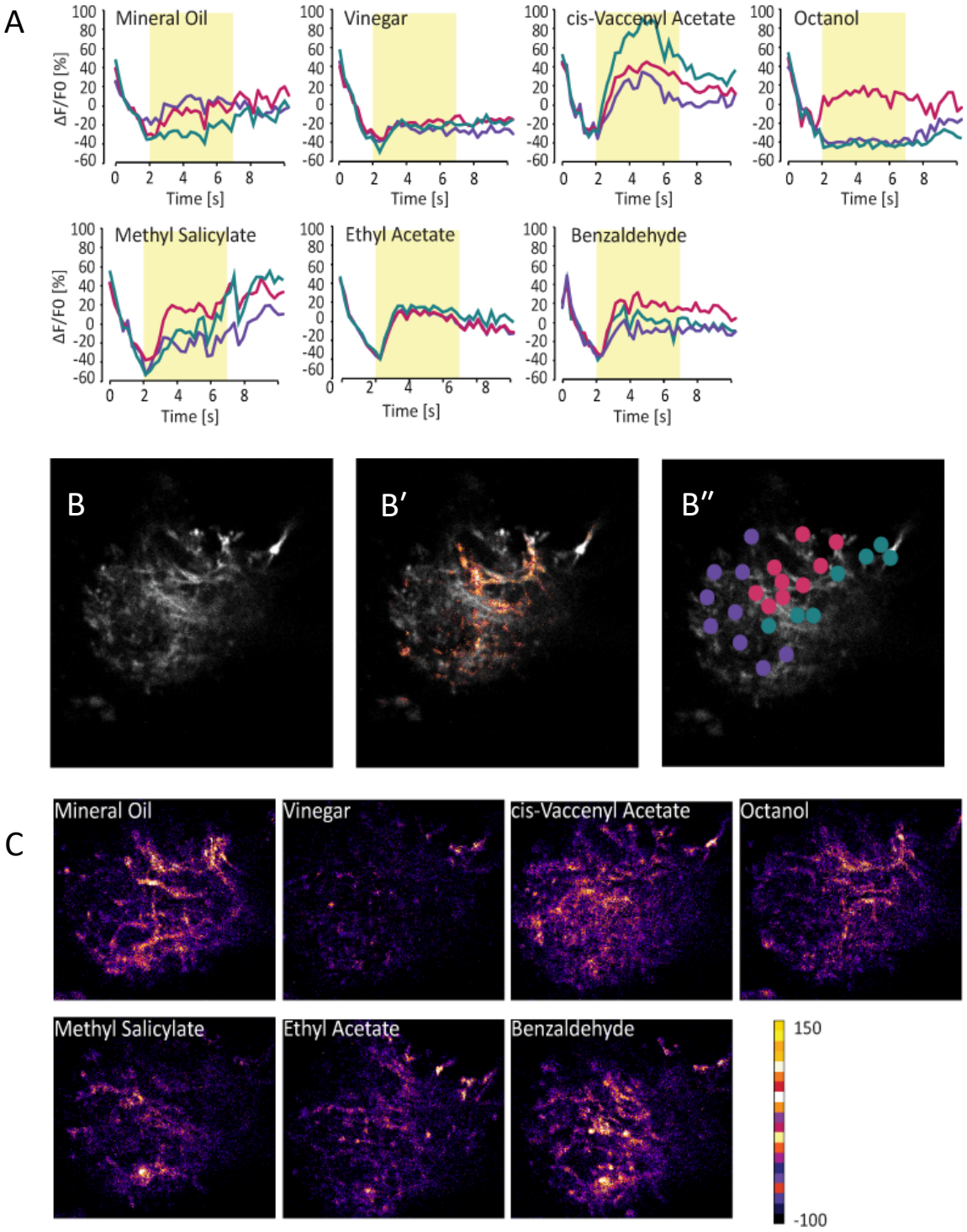

Figure 13. Response in **LH**.
(A) Temporal responses (averaged) observed in the ROIs set in the LH of the fly brain to control MOL, VIN, CVA, OCT, MSC, ETA, and BEA respectively. Y-axis is the absolute value of change in fluorescence (ΔF) to the base fluorescence (F0) expressed as a per cent. **(B), (B'), (B")** (Left to right) Expression pattern of PNs in the lateral horn before odour stimulation in GH146-Gal4 line expressing GCamp6; response pattern in LH during odour stimulation (OCT); ROI set used to measure response during odour stimulation. **(C)** Spatial responses observed in the LH when different odours and control MOL were tested. All odours were diluted 10^{-2} mol/l

in mineral oil (control). Bright colours (orange) represent an increase in calcium levels and darker shades (purple) indicate a decrease in calcium levels.

Figure 14 indicates the differential response patterns in the three ORDs for various odours. ETA, CVA, VIN induce excitation in all three ORDs. MSC, BEA show a slightly increased activity in ORD2 as compared to the other odours. OCT was tested in lesser specimens than the other odours and hence a definitive increase was not observed. The total number of specimens imaged were four.

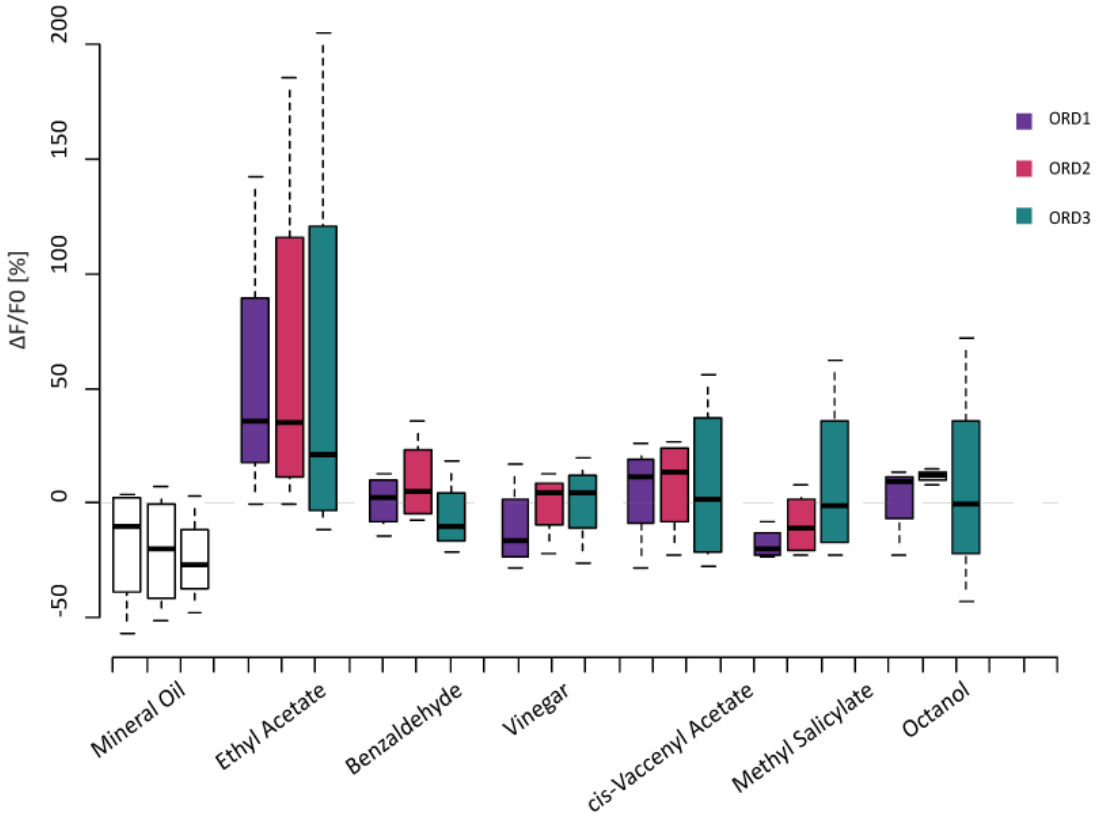

Figure 14. Box-plot of responses in the ORDs of the LH.
Coloured boxes indicate a significant difference in response patterns during odour stimulation as compared to pre-stimulus condition.

5. Discussion

The results obtained during the behavioural experiments confirmed the first hypothesis that stated that different classes of odours based on ethological relevance to the fly elicits positive, or negative, or a neutral behaviour. The optical imaging experiments in stationary flies confirmed the fact that different odours elicit responses in specific regions of the LH, one of the higher centres in the brain. This partly adhered to the second hypothesis that stated neuronal activity in the higher brain centres resulted in a behavioural response.

5.1. Tethered fly setup

The sense of smell, touch, taste, vision and hearing are the main forces aiding an organism in maintaining body homeostasis (Li and Liberles 2015). It is important to understand the relevance of the physiological outputs as a result of sensory perception of the environment in an organism as they point towards ecological being in terms of evolutionary divergence and isolation (Devaud 2003, Dobzhansky 1956, Hoffmann et al. 1984). Chemosensation, the perception of smell and taste, is an essential process for the fly's survival. It has been well understood and studied in *Drosophila melanogaster* at both genetic and behavioural levels (Carlson 1996, Devaud 2003). *Drosophila melanogaster* displays different behavioural responses to stimuli such as chemicals, light, gravity, temperature, humidity, and sound (Borst 2009, Katz and Minke 2009, Vang et al. 2012). The first set of behavioural experiments in my master's thesis has dealt with the process of olfaction in the vinegar flies and their flight responses to different odour chemicals.

Behavioural assays quantifying a fly's response to an odourant molecule is an area of relevance as physiological readouts are variable and obtaining robust results is tedious (Knaden et al. 2012). The tethered fly setup, used for various behavioural studies and migratory studies, provides real-time effect with no requirement of expensive and specialized tools and allows conduction of experiments at regular room environment (Chapman et al. 2011, Martí-Campoy et al. 2016). The tethered

fly setup allows us to study fly behaviour and change in the behaviour as a result of sensory modulation. The tethered fly setup was used to study physiological responses to odour stimuli. Odour delivery in the tethered fly setup head-on was not very suitable to obtain a clear behavioural output in terms of amplitude readouts (Frye and Dickinson 2004). It has been shown in *Drosophila* larvae that a difference in odour intensity is detected by lateral, side to side head movements indicating the differential odour recognition pattern by the lateral hemispheres of the olfactory sensing units (organs or in the brain; Slater et al. 2015). In adult flies capable of long-distance odour recognition and quick easy manoeuvres during flight, the asymmetrical gradients of chemicals during flight is recognized and analysed by the antennal segments. This is required for the fly to evaluate its surroundings and decide by integrating differential input from both the antennae (Gomez-Marin et al. 2010). Hence, the odour was presented laterally in our setup to observe the steering effect in the fly.

The tethered fly setup we devised as an open-loop system enabled us to moderately quantify the fly behaviour when odours were tested. An open-loop system, where the system's functioning is independent of the output's control, had the tethered fly fixed and unable to rotate about its axis. Our setup can be made closed loop where the output/behavioural response drives the input/odour delivery, by providing the odour stimuli to the flies along a moving axis and studying the fly response correspondingly. The advantages of the closed-loop system help us understand how the fly responds to changing stimuli by capturing immediate responses arising from self-generated motion to the stimuli. But also, one must keep in mind that there are possibilities that the responses might be exaggerated in case of closed-loop systems as the responses are generated to fluctuating input stimuli. Both open-loop and closed-loop systems are useful during experiments and the ability to switch between the two systems with the setup devised for our experiments adds to the researcher's benefits.

5.3. Behavioural Responses and Valences of *Drosophila melanogaster*

The ethological responses seen in the flies enabled us to attribute each tested odour with a hedonic valence. Attractive odours are easy to categorize as a behavioural approach response is definite. The aversive odours eliciting a behaviour response is seldom easy to observe as the fly would choose not to respond (Knaden et al. 2012) rather than respond as for attractive odours. As seen during fly walk experiments conducted by Knaden et al., 2012 the flies walked towards attractive odours but would freeze/stop during delivery of repellent odours. The other category of odours is those that elicit no response, or neutral odours. Our experiments studied flight behaviour in flies. During the flight, a fly would steer towards an attractive odour while steering away or evade a repellent cue. To test the fly behaviour during flight, wing amplitude/frequency were the parameters analysed. As seen in the WBF/WBA plots for mineral oil (**Figure 9A, 10A**), a constant plot indicated no response to it making it a neutral odour. With the initial tethered setup (**Figure 8**), though odours caused an increase in frequency, there was no apparent amplitude change making it hard to associate a positive/negative valence to the odours. It was rather important to see a change in the steering movement of the fly to associate an odour with its valence.

It could happen that a flying specimen decides no more to fly after odour encounter or during the encounter or even before odour encounter. It has been shown in previous studies that flight is the response generated reflexively as a result of the OSN action reaching its threshold. Irrespective of the total OSN activity, it is important that the odour activates its corresponding OSN to induce flight modulations in the specimen (Bhandawat et al. 2010). One of the anatomical reasons for some of the test flies to stop flying midway during the experiment could be attributed to the above statement, the required OSN was probably not activated during the encounter with an odour. It is true that a slight movement artefact, for instance a slight movement of the cables/tubings in the setup, is capable of inducing flight in the specimen. But it is necessary for the right OSNs to be active for an odour to induce a behavioural response.

Figure 9 shows the increase in frequency. Seen in **9B, E** is a gradual increase in the frequency over time. And in **9C, D, F** the increase in frequency is almost immediate. The differential response rate could point towards how fast the neuronal signals travel to the brain and how long the fly takes to make a decision and respond correspondingly. Depending on the nature of the chemical and its ethological relevance to the fly, response to it in the fly brain is decided and translated as a gradual or an immediate increase in frequency.

From previous reviews and studies, we are aware that the vinegar flies find acetic acid highly attractive (Semmelhack and Wang 2009). It is seen in the experimental results, **Figure 10B**, that a negative amplitude mean indicates the flies' attempt to go towards the odour source. This indicates an approach to behaviour. Vinegar, a common component during alcoholic fermentation by yeasts in fruits, is useful for flies to detect overripe substrates for feeding. Acetic acid, a major component in vinegar is beneficial to flies in locating substrates for oviposition and only females in the process of laying eggs are attracted to acetic acid (Semmelhack and Wang 2009). This was also observed in some of the flies tested. Some flies that were in the process of laying eggs, responded instantly to the odours and required no motivation by puffing air at the start of the experiment. Mentioned in the review by Mansourian et al., 2015 an enlarged reproductive tract induces this attraction to acetic acid which otherwise is repellent even in walking flies. From the observations made, it can be said that the need to lay eggs makes an odour such as vinegar attractive to the flies. The fly's motivation results in its behavioural responses in any situation. As tested using the technique of GC-MS (**Figure 12**), vinegar is a complex mixture containing mostly acetic acid, and traces of acetoin, ethyl acetate and ethanol. From previous work, acetic acid, ethyl acetate and ethanol are attractive in nature to *Drosophila melanogaster* (Hallem and Carlson 2006, Knaden et al. 2012). Also, acetoin in combination with acetic acid has been proven to be attractive to the flies (Ishii et al. 2015). This makes balsamic vinegar an attractive blend.

The pheromone cis-vaccenyl acetate is attractive to the female flies tested and was characterised by a negative mean amplitude value in **Figure 10C**. This odour plays a

significant role in nature acting as a guide to finding suitable mating partners and pheromone rich oviposition sites for females. CVA, one of the sex pheromones, plays an important role in complementing the cuticular hydrocarbons in flies enabling them to take part in social recognition and interaction. This pheromone is known to have opposite effects on the two sexes, attractive to females and unattractive to males and also induces aggregation specifically in the presence of food (Billeter and Levine 2013). It would be interesting for further studies to compare behavioural responses between mated and virgin females and between male and female flies. It would give us the scope to see how the same odour affects fly behaviour in a sex-specific manner and how an internal state of the fly can alter ethological responses in flies.

1-Octanol and methyl salicylate elicited an increase in frequency accompanied with a mean positive amplitude value suggesting an aversive behaviour. With previous works on different behavioural assays indicating, averseness is hard to detect (Knaden et al. 2012), it was an interesting challenge to have a concrete readout with the tethered fly setup. In the results obtained (**Figure 10D, E**), there is a neat indication of repellence in the flies tested. A positive amplitude value was indicative of averseness in the behavioural setup we devised. In nature, most odours exist as a blend in different ratios rather as single molecules making it hard for the fly to categorise the aversive odours. Though certain odours such as geosmin and other toxic chemicals are easy for the fly to recognize and evade due to an already present aversive olfactory circuit in the brain, the other common odours still might be overlooked as they may be present in a blend with an attractive odour making it hard to recognize. Thus, the single odour testing using the tethered fly setup enabled us to categorize odours as aversive when presented singly. Methyl salicylate, a fruit volatile with a benzene ring is useful in locating ripe fruits (De Bruyne et al. 2001). *Drosophila* species generally prefer overripe fruits, but certain fruity smells indicate unfavourable fruit features for feeding/oviposition (Keesey et al. 2015, Mansourian and Stensmyr 2015). Methyl salicylate though from the plant tissue, is indicative of “fruitiness” or overripe condition with rotting, harmful bacteria/yeast and hence avoided. Octanol, an

alcohol has been shown to induce aversion behaviourally (Knaden et al. 2012). Octanol, falling into the alcohol group, is a component of the green leaf volatiles indicating unripe fruits and thus repellent. Benzaldehyde is aversive but the reason behind why it is aversive to the flies remains a mystery still (Mansourian and Stensmyr 2015). As seen in **Figure 8B**, the amplitude response shows no change making it hard to classify benzaldehyde.

In total, from the results obtained with the behavioural analysis, it strengthens the fact that a behavioural response in the fly depends not just on the odour identity, but also the intensity, and if it exists as a blend. In nature, the fly hardly encounters a single odourant molecule. It is subjected to multiple odours in different ratios that can have a different response when compared to individual chemical odourant (Ibba et al. 2010). The positive/negative valence coding in the brain can be understood as a reward or punishment system or the substrate would be beneficial or detrimental, where a reward would indicate a food source/mating partner and punishment would be a toxic substrate/pathogen (Li and Liberles 2015).

The different behavioural responses boil down to the neuronal activity in the brain. Not just the immediate responses to the odours play an important role in the behavioural output, but also the internal state of the fly too should be considered for further experiments. States like hunger, mating, the need to oviposit, and presence of food odours do play a role in the fly's behavioural decision (Hussain et al. 2016, Reisenman and Scott 2019, Sayin et al. 2018). This we observed with the behavioural responses due to longer starvation (24 h), and responses to vinegar in egg-laying flies. Pathways leading to aversion or attraction have been shown to be hardwired and spatially segregated both in the AL and the higher centres of the brain (Marin et al. 2002, Wong et al. 2002). Food-related odours elicit responses in one or more glomeruli in the AL and pheromones like cis-vaccenyl acetate excite one specific glomerulus, some chemicals like methyl salicylate/CO_2/geosmin are wired to specific glomeruli (Semmelhack and Wang 2009, Wasserman et al. 2013). In the LH, PNs it has been shown that food odours elicit a response in the LH at specific sites and pheromone related PNs innervate the LH at different other regions, showing spatial segregation (Strutz et al. 2014).

5.4. Activity in the Lateral Horn

The LH in flies, responsible for innate responses was imaged during our project during odour encounter. It was observed that changes in neuronal activity to attractive/aversive odours occurred in certain regions demarcated as odour responding domains. Odour responding domain (ORDs) have been introduced in the LH as the regions consisting of neuronal cell types that take part in the making of final behavioural decisions (Lee and Seung , Strutz et al. 2014).

The box-plot shown in **Graph 6**, shows the response patterns in each ORD to the corresponding odour and indicates that ORD 2 responds to aversive cues while the other two ORDs to attractive cues. It would be interesting to further study regions in the LH responsive to sexual cues and chemical odours of different concentrations. The further work we plan to conduct includes observing the third-order neurons in the LH receiving input from these ePNs. It would be interesting to see the pathway of behavioural decision/output in these neurons as they receive information from various sensory inputs. The LH is where inputs are provided not just from the olfactory senses, but also the visual senses, and mechano-/thermo-sensory inputs. How the LH is able to integrate multimodal information simultaneously and how olfactory processing in the LH ePNs is modulated due to multimodal processing would be a further topic of research.

A recent work conducted in mice indicated that when one sense was diminished, the responsiveness of other senses was heightened. Attenuation of the auditory senses caused an increase in the responsiveness of the visual cortex to simultaneous auditory and visual cues, suggesting an intermodal communication to compensate the loss of one senses (Teichert et al. 2018). It would be interesting to see how cross-modal interactions work in LH as it is known to receive input from various sensory pathways and how olfaction would be affected if another sensory pathway were to be attenuated. The interesting question would be, if activity in the AL and further the LH increase if vision is impaired? The other question would be if the LH shows increased activity not just from olfactory input but also mechano-/thermo- sensory stimuli when vision is impaired? Another recent work

in *Drosophila* by fellow lab member, Mohamed et al., 2019 has shown that by surgically ablating one antenna during olfactory processing, the ipsilateral responses arising in the third-order neurons in the ventrolateral protocerebrum (VLP) was increased in the hemisphere with the intact antenna whilst the contralateral responses reduced in the VLP in the contralateral hemisphere. It was shown that LH inhibitory neurons were responsible for the inhibition in the VLP neurons in the contralateral hemisphere at the presynaptic spaces in the LH. There is further scope to investigate inter/intra sensory modal communication in the higher centres of the brain.

The other study we propose to conduct is simultaneous behavioural analysis in the fly during brain imaging. By this, we will try to study how the behavioural state of the fly is coded in the brain whilst the internal state holds true. And we can observe a concrete translation of the valence coding in the brain into actions as a behavioural readout.

5.5. Evolutionary and Ecological Implications in the Olfactory Process and Behavioural Responses

Innate attraction and aversion have been extensively studied and the conclusions drawn so far point out that at the level of the AL, certain olfactory pathways are hard-wired such as for CO_2, cis-vaccenyl acetate, geosmin, methyl salicylate (Jefferis et al. 2007, Stensmyr et al. 2012). Looking at *Drosophila melanogaster*'s olfactory system and process in an evolutionary perspective, one cannot deny that these vinegar flies are one of nature's standing model organisms to study evolution. These flies are attracted to various substrates emitting different compounds such as ammonia, amines, fruity-smelling acetate esters, terpenes, etc. Similarly, they are repelled by certain other chemicals such as pyrazines, geosmin, acids such as octanoic acid, etc. The behavioural responses to different odours are a reflex to either feed, mate or to oviposit, or evade toxic substrates and prevent predation. The interesting facts to look at are the adaptations of the olfactory pathways. At both high levels and low levels of vinegar, the flies avoid going

towards that substrate (Frank et al. 2015). Avoidance behaviour is hard to categorize as was the case in the fly walk experiments conducted previously (Knaden et al. 2012). The flies encountering repulsive odours would tend to stop walking or freeze. This behaviour did not tell much about the repulsive behaviour. But in the experiments, we conducted using the tethered fly setup, the steering away from the odour delivery is a clear sign of repulsive behaviour.

Ethyl acetate we tested showed to be aversive. Though the number of specimens tested was low, the behavioural response shows aversion. It is contrary to the finding that ETA is attractive to walking flies (Bhandawat et al. 2010). The imaging experiments show activation of regions corresponding to both attraction and aversion as seen in the temporal responses in **Figure 13A**. This can be further studied to understand how the same odour leads to different behaviours as a result of the fly's internal state.

The vinegar flies in nature coexists with inter-/intra-species. It is interesting to study how behavioural responses, for instance the feeding pattern, can affect other organisms. To elaborate, the vinegar flies are saprophagous, feeding on rotten plant material and while doing so become vectors to many types of bacteria and yeast. The microorganisms can be deadly in nature. Thus, as the flies move on from plant to plant, they facilitate pollination on one hand but can also be vectors of deadly diseases in plants, wiping out an entire section of plants in that area (Buda et al. 2009). The question of how vinegar flies cope with such harmful microbes is intriguing area of research. Coping with harmful chemicals like octanoic acid is seen in *D. sechellia* that is facilitated by two macro glomeruli in the AL (Ibba et al. 2010). Inter-/intra-species communication is thus a very promising area of research.

5.6 Conclusion

The vinegar flies, holding ecological and evolutionary implications are one of the widely studied organisms in the research field of chemosensation. Olfaction in the flies mediated by the antennae and maxillary palps generate through electrical signals, a response to an odour manifested as behaviour. This physiological response to the odour coded by the valence of attraction or aversion is confirmed by the behavioural analysis conducted using the tethered fly setup. The response to a neutral odour is also captured. The neuronal activities leading to a behavioural response was also studied using the technique of optical imaging. With an ethological readout to an odour and corresponding neuronal activity in the brain, it paves the way to further our scope of research and perform behavioural and imaging experiments simultaneously.

Outlook

The future of the project comprises of two main aims:

1) To study the multisensory processing in the LH (to study the third-order neurons and their output pathway);

2) To get a behavioural readout whilst observing the brain activity (to perform behavioural analysis using the tethered fly setup and optical imaging simultaneously).

We propose to test odour blends and analyse behaviour responses. The experiments can be extended to compare behavioural patterns between the two sexes (male/female) and within same-sex (mated/virgin females) flies. For a variation in the tethered fly set up we devised, we propose to incorporate a movable odour delivery system, making our setup a closed loop.

The vinegar flies are shown to have preferential liking towards citrus fruits for egg-laying (Dweck et al. 2013), but if provided a choice with ancestral morula fruit would prefer the marula. This clearly points towards host specialization in the insects. Looking at the olfactory circuit linked to this in different species of the *Drosophila* genera, the Or22a circuit was studied (Dekker et al. 2006, Linz et al. 2013). Or22a, a receptor in the OSN in *D. melanogaster* targeting DM2 glomerulus, was involved in recognising the ester ethyl isovalerate from the marula fruit. But it was also confirmed that this was required only for locating the fruit from distances and the oviposition behaviour was coded by a different pathway (Mansourian et al. 2018). This instigates us to plan choice-based experiments using the tethered fly assay, where the flies can be provided with two odours or a blend of odours and the change in WBA/WBF can be analysed. This choice-based assay can be incorporated in an open-loop system, where two different odours being presented from two different directions, and the fly reaction could be analysed.

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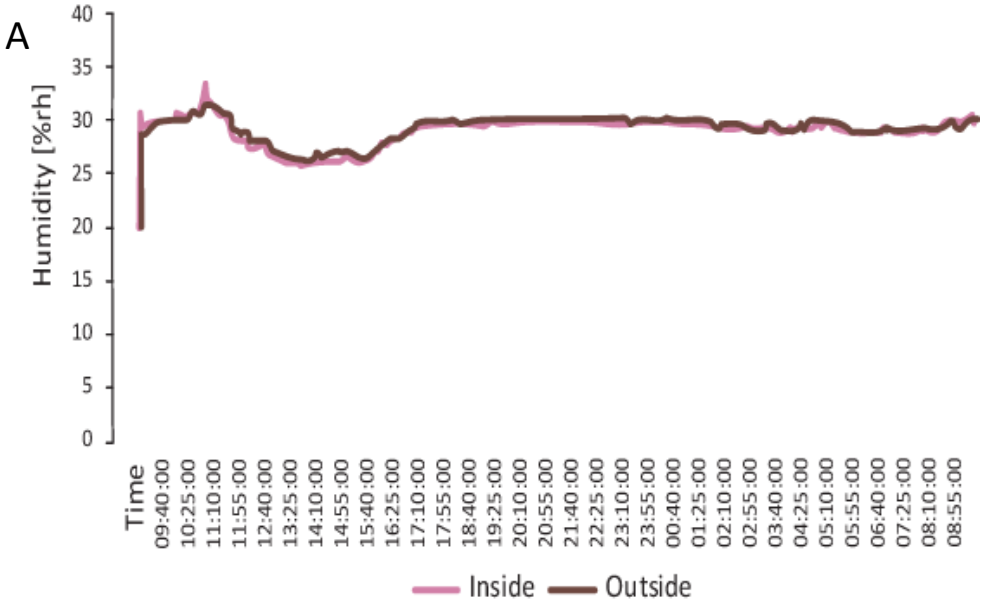
7. Appendix

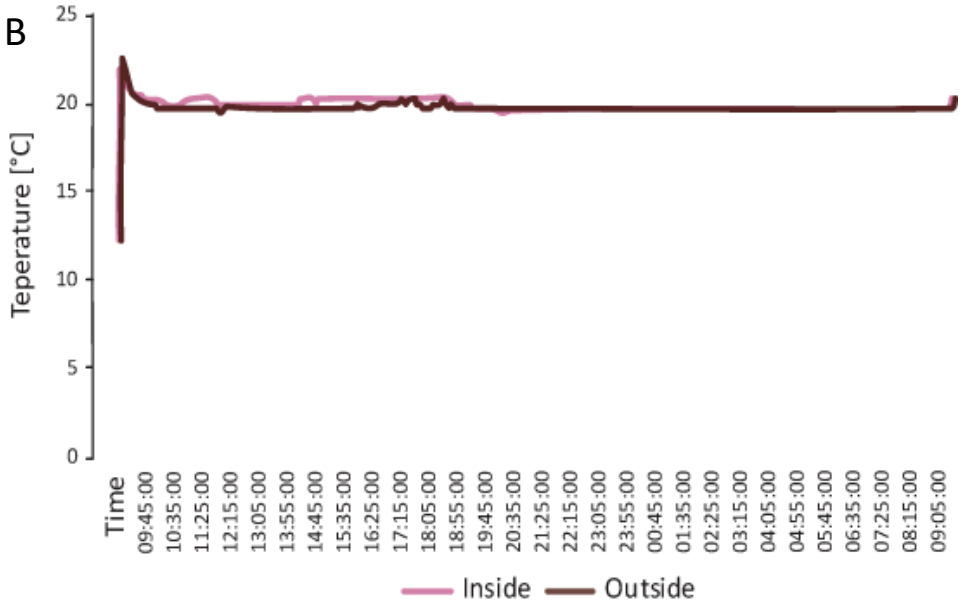
A

B

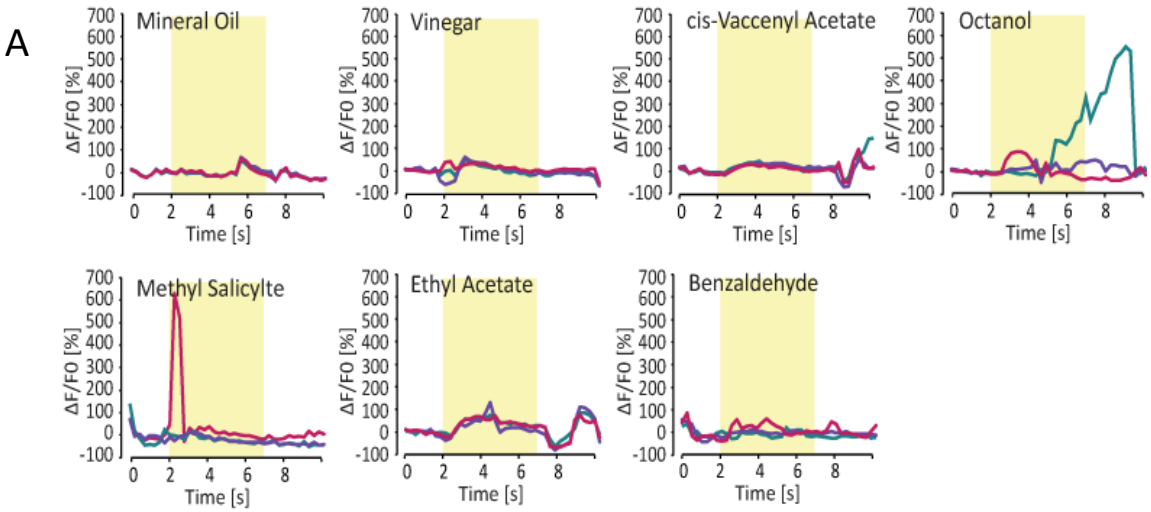
Appendix figure 1. Fly starvation and Preparation.
(A) Flies transferred to vials with water-soaked sponges during starvation. **(B)** Humid chamber to house the dissected flies before housing them in the tethered fly setup.

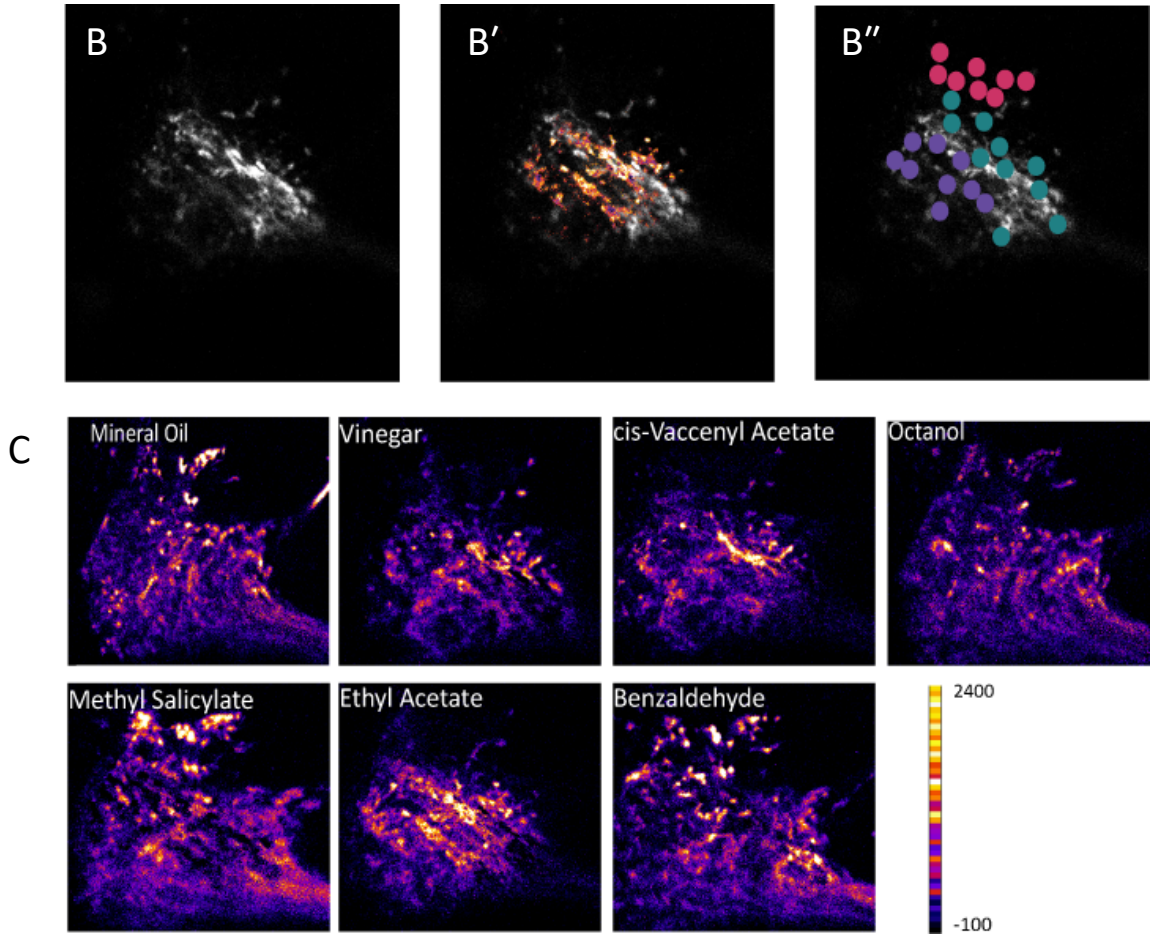
A



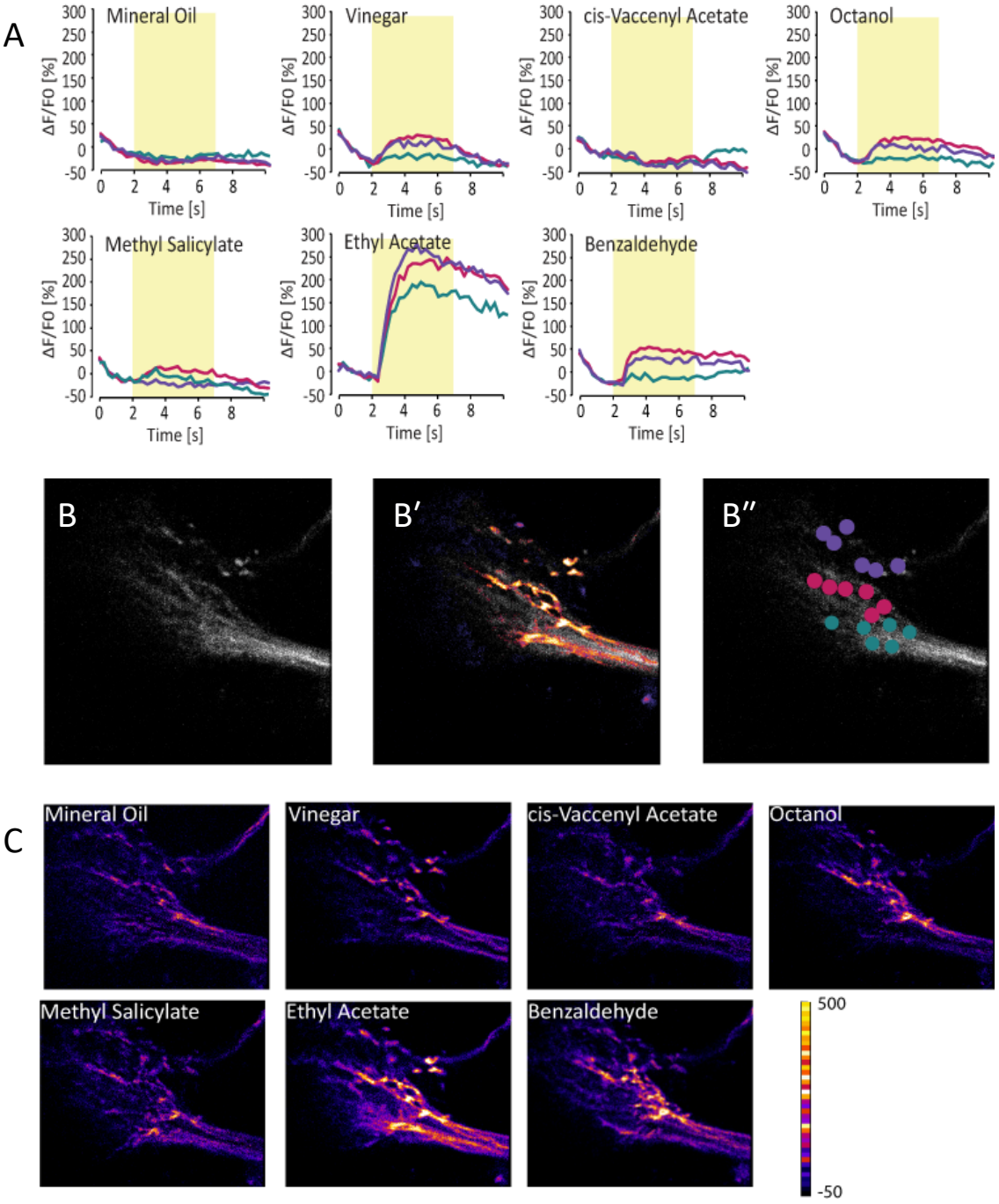


Appendix figure 2. Humidity and Temperature readouts from Data logger.
(A) Humidity plots showing a relativity of 30% RH outside and inside the tethered fly setup. **(B)** Temperature plots showing 20° C inside/outside tethered fly setup.

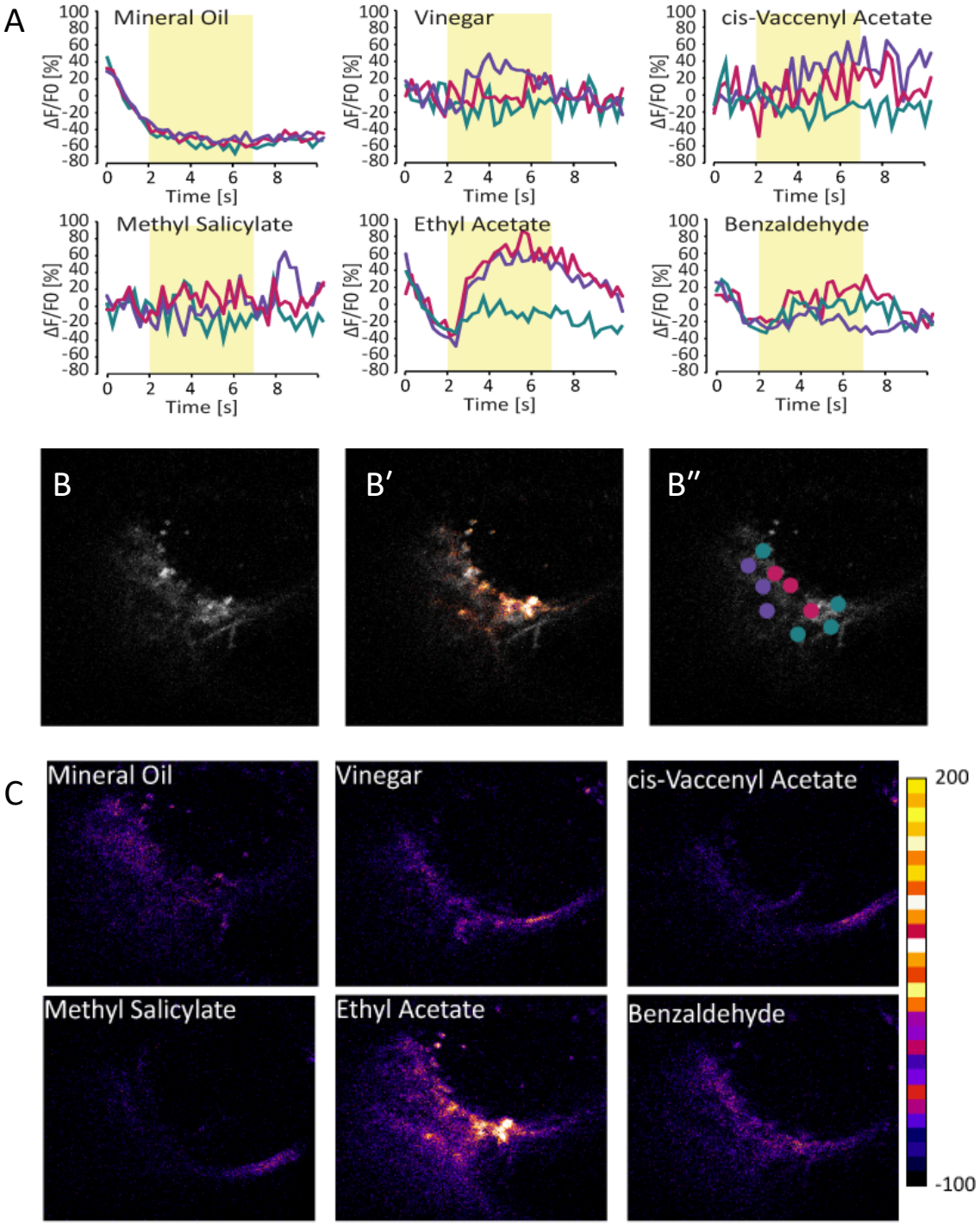




Appendix figure 3. Response in **LH (Specimen 2)**.
(A) Temporal responses (averaged) observed in the ROIs set in the LH of the fly brain to control MOL, VIN, CVA, OCT, MSC, ETA, and BEA respectively. Y-axis is the absolute value of change in fluorescence (ΔF) to the base fluorescence (F0) expressed as a percent. **(B), (B'), (B")** (Left to right) Expression pattern of PNs in the lateral horn before odour stimulation in GH146-Gal4 line expressing GCamp6; response pattern in LH during odour stimulation (ETA); ROI set used to measure response during odour stimulation. **(C)** Spatial responses observed in the LH when different odours and control MOL were tested. All odours were diluted to 10^{-2} in mineral oil (control). Bright colours (orange) represent increase in calcium levels and darker shades (purple) indicate decrease in calcium levels.



Appendix figure 4. Response in **LH (Specimen 3)**.
(A) Temporal responses (averaged) observed in the ROIs set in the LH of the fly brain to control MOL, VIN, CVA, OCT, MSC, ETA, and BEA respectively. Y-axis is the absolute value of change in fluorescence (ΔF) to the base fluorescence (F0) expressed as a percent. **(B), (B'), (B")** (Left to right) Expression pattern of PNs in the lateral horn before odour stimulation in GH146-Gal4 line expressing GCamp6; response pattern in LH during odour stimulation (ETA); ROI set used to measure response during odour stimulation. **(C)** Spatial responses observed in the LH when different odours and control MOL were tested. All odours were diluted 10^{-2} in mineral oil (control). Bright colours (orange) represent increase in calcium levels and darker shades (purple) indicate decrease in calcium levels.



Appendix figure 5. . Response in **LH (Specimen 4)**.
(A) Temporal responses (averaged) observed in the ROIs set in the LH of the fly brain to control MOL, VIN, CVA, OCT, MSC, ETA, and BEA respectively. Y-axis is the absolute value of change in fluorescence (ΔF) to the base fluorescence (F0) expressed as a percent. **(B), (B'), (B")** (Left to right) Expression pattern of PNs in the lateral horn before odour stimulation in GH146-Gal4 line expressing GCamp6; response pattern in LH during odour stimulation (ETA); ROI set used to measure response during odour stimulation. **(C)** Spatial responses observed in the LH when different odours and control MOL were tested. All odours were diluted 10^{-2} in mineral oil (control). Bright colours (orange) represent increase in calcium levels and darker shades (purple) indicate decrease in calcium levels.

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Thanking you all!!!

9. Declaration of Authorship

I hereby declare that I have written my thesis on my own and have dutifully acknowledged every idea or statement provided by anyone other than me. I DO NOT give consent to make my thesis work public on Library websites or for any third party to use my thesis for public viewing.

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